



Second International Phytoplasma Working Group Meeting

Neustadt an der Weinstraße, Germany
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Edited by Assunta BERTACCINI and Stefano MAINI

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INTERNATIONAL PHYTOPLASMOLOGIST WORKING GROUP



Welcome by the organizers

We are pleased to welcome you to the “Second International Phytoplasma Working Group Meeting” in Neustadt an der Weinstraße.

After the great success of the first meeting which took place in Bologna, Italy, in 2007 it is a great honour for us to organize the second meeting. We like to continue the tradition established by the first IPWG meeting to bring together researchers from entomology, molecular biology, and plant pathology to increase and expand knowledge about phytoplasma diseases worldwide. This second meeting provides an overview over the ongoing phytoplasma research and, therefore, all topics dealing with new advances in phytoplasma research will be treated. Knowledge about phytoplasmas and their interaction with their plant and insect host has increased dramatically in the recent years. Despite this, phytoplasma diseases continue to cause important damage to agriculture worldwide and control of these diseases remains a challenge. Therefore, this meeting offers the great opportunity to bring together newest basic research data with the needs of applied research and we hope this will be of benefit for both sides.

The need for the transfer of research data to practical application will be emphasised by the integration of working group meetings of the COST action FA0807 “Integrated Management of Phytoplasma Epidemics in Different Crop Systems” into this second IPWG meeting.

We thank all participants for their numerous contributions and we wish them a pleasant and fruitful time in Neustadt an der Weinstraße

On behalf of the local organizers

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Preface

We are delighted to edit the extended abstract book of the “Second International Phytoplasma Working Group Meeting” that will be held in Germany next September 2011.

The group born in Bologna in 2007 is actively working in entomology, molecular biology, and plant pathology to increase and expand knowledge about phytoplasma diseases worldwide. The meeting is the forum for sharing information and strength and/or built new and more intense interactions among participants.

Many exciting findings since the last meeting in the phytoplasma field: pathogenicity factors are starting to be unveiled, insect vector-phytoplasma interaction mechanisms are being discovered and some of the phytoplasma effectors are reported to positively interact with insect vector increasing their fitness, plants infected can be more attractive for insect vector, resistance to phytoplasma diseases is feasible, and more ‘news’ will be reported during this meeting.

We acknowledge that the ‘mutualistic symbiosis’ started four years ago among entomologists, plant pathologists and molecular biologists is fundamental to try to know more about phytoplasma-associated diseases affecting the worldwide agriculture!

The project COST FA0807 ‘Integrated Management of Phytoplasma Epidemics in Different Crop Systems’ founded by the European Union two years ago is also helping the realization of phytoplasma disease of ‘management’; the only way to control the insect vectors populations and diseases is a sustainable and integrated approach. The project is grouping 28 European Countries including Turkey and Israel, and several Countries worldwide mainly among those in which phytoplasma disease knowledge is more present, covering Americas (Canada, Brazil and Chile), Australia and New Zealand, Africa (South Africa and Egypt), Middle East (Lebanon, Iran), and last but not least Mauritius and India and it is open to all other Countries in which there is interest in phytoplasma research. The efforts to mitigate the incidence of phytoplasma diseases in plants is reaching a coordinated international level increasing co-operation between specialist both on basic and applied research.

This meeting is a great opportunity for us, as both organizers and participants, to share mutual experience in such important field to agriculture.

It is a pleasure for the editorial board of the Bulletin of Insectology to publish researches carried out by leading experts in Phytoplasma, a science that may be considered, as emerging in the agriculture field.

Over the four days of the main conference, there will be 120 presentations submitted to the scientific committee before the end of April 2011 plus 19 contributions enclosed in the four Working Group workshops of the COST Action FA0807.

Papers and posters are distributed over 6 sessions; the meeting will be opened by two invited presentations from prominent scientists in the field: the senior member Prof. Karl Maramorosch and the fellow Dr. Michael Kube – representing respectively the History and the Future of Phytoplasma.

All the extended abstracts published in this issue have been reviewed and accepted by the IPWG Scientific Committee. We want to thank the contributors for their diligence in preparing their manuscripts.

Modifications in the layout of abstracts received from authors have been made to fit with the publication format of Bulletin of Insectology. We apologize for errors that could have arisen during the editing process despite our careful attention.

As editors we would like to extend our gratitude to all of the Scientific Committee members. Many thanks also to Davide Montanari for the great assistance during the preparation of the extended abstracts of the second IPWG meeting published as a Supplement of the Bulletin of Insectology 2011 volume 64. Special thanks also to Fabio Montanari for the help in revising the graphic part of the book. Many thanks also to all the members of the local organizing committee that allowed the realization of this second IPWG meeting.

The scientific committee members and the editors discharged their refereeing responsibilities. Any errors could be indicated, till October 31, 2011, by authors sending e-mail to the Bulletin of Insectology secretary. Unintentional substantial mistakes will be corrected adding an errata corripse, as a last page of the ‘electronic reprint’, on the online version of the second IPWG meeting extended abstract book.

Online information concerning the IPWG proceedings is available at www.bulletinofinsectology.org

The Editors, Assunta BERTACCINI and Stefano MAINI

Historical reminiscences of phytoplasma discovery

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Abstract

Many plant diseases, earlier described as virus diseases, have been recognized as phytoplasma or spiroplasma diseases during the past 44 years. The breakthrough discovery came in 1967, when Japanese plant pathologists and entomologists reported detection of mycoplasma-resembling microorganisms in diseased plants and insect vectors, and the temporary recovery of diseased plants, treated with tetracycline antibiotics. For many years no credit was given to the crucial role played by the veterinarian Kaoru Koshimizu, who first recognized phytoplasmas in electron micrographs of thin sections from mulberry dwarf diseased plants, prepared by Y. Doi. In 1967, at the same plant pathology conference in Japan, entomologists S. Nasu and associates reported the detection of phytoplasmas in rice yellow dwarf disease and in the insect vector, but this report was hardly mentioned. Attempts to culture the fastidious phytoplasmas did not succeed, while spiroplasmas, first recognized by R. Davis, have been cultured. Several careers have been made by phytoplasma researchers, but some were destroyed by erroneous reports and one ended tragically through political involvement. The striking progress in the study of phytoplasmas, demonstrated by the First, and the current, Second IPWG meeting, clearly illustrates the benefits derived from collaboration between experts working in different countries, from free exchanges of information, and from participating in symposia and congresses.

Key words: phytoplasma, tetracycline antibiotics, yellows-type diseases.

Introduction

The first phytoplasma disease has been described 1000 years ago in China (Wang and Maramorosch 1998). During the Song dynasty, 960-1227, the “Yao-yellow kind” peonies, hailed as the most beautiful tree peonies, with a delicate green color, were annually presented to the imperial court. The pale-green flowers were widely acclaimed in China for centuries, although the peony trees that produced them were less vigorous and the green flowers produced no seed. It took more than 800 years before the cause of tree peony greening and the beneficial effect of phytoplasmas could be documented. Is it proper to call these phytoplasma infected peony trees “diseased”? The phytoplasmas made the peony trees more desirable and the phytoplasma infection, in this instance, became beneficial to tree peonies. Currently, our interest in phytoplasmas is primarily directed to the serious diseases, caused by them all over the world. Phytoplasmas have destroyed pears and apples in Europe and in the United States, coconut and other palms in tropical and subtropical areas, food crops, lumber, shade trees and ornamental flowers all over the world. Since 2004, when the first phytoplasma genomic sequence was published, the genomic sequencing of phytoplasmas has progressed, promising the creation of novel measures to stop the devastating infections of crop and fiber plants.

In my historical recollection I shall focus not only on the published findings but also on the scientists involved in the early phytoplasma and spiroplasma research. Before 1967, many plant pathologists and entomologists, working with yellows-type diseased plants, tried to detect particles resembling known viruses of plants, animals, or bacteria. I shall describe my own failure to find the pathogens of aster yellows disease, the failed attempts to culture phytoplasmas, the background of the 1967 recognition of phytoplasmas in Japan, and the er-

rors that occurred in my own and in other laboratories.

Early criteria, applied to viruses, were inadequate to distinguish between viruses and other filterable disease agents. When electron microscopy of thin sections was introduced, no virus-like particles could be detected in thin sections of many diseased plants or in purified plant or insect vector extracts. Errors made before and after 1967 by me and others demonstrated how failure to collaborate with colleagues, working in different fields, resulted in missed opportunities. I shall stress the concept that progress in phytoplasma research can best be achieved by collaboration with colleagues in other laboratories and in other fields.

Missed opportunities

In 1924 the mystery transmission of the aster yellows disease was solved by L. O. Kunkel at the Boyce Thompson Institute in Yonkers, New York, when he found that a leafhopper, *Macrostelus fascifrons*, transmitted the infectious agent from plant to plant (Kunkel, 1926). Since no bacteria or fungi were found in diseased plants, Kunkel concluded that the causative pathogen was a virus. He suspected that this virus multiplied in leafhopper vectors and this assumption was confirmed by my serial passage technique, using needle inoculation of leafhopper vectors (Maramorosch, 1952).

In the summer of 1957 I was working at the Cold Spring Harbor Laboratory on Long Island, New York, where Barbara McClintock, who 30 years later received the Nobel Prize for her discovery of “jumping genes”, permitted me to use her greenhouses for maintaining leafhopper vectors of aster yellows. I prepared extracts from diseased plants and from leafhopper vectors, adding measured amounts of penicillin, streptomycin, and tetracycline, and injecting small amounts into the bodies of leafhoppers. I was convinced that the antibiotics

would have no effect and that the injected leafhoppers would continue to infect aster seedlings. As expected, this happened with the insects that were injected with penicillin and streptomycin. However, the tetracycline injected leafhoppers failed to infect plants. I was convinced that the failure to transmit was meaningless, because it was well known that viruses were not affected by tetracycline antibiotics. Instead of repeating the experiment during the fall, I published the results, concluding that the lack of transmission was, most likely, caused by the heat in the green houses (Maramorosch 1958). Had I repeated the tests, perhaps the correct conclusion would have been reached and I would have made the discovery of phytoplasmas 10 years before my Japanese colleagues announced their findings. I missed the opportunity because I was convinced that I was working with a plant virus.

In 1966, together with my associate Hiroyuki Hirumi, I visited in Philadelphia Werner Henle, the discoverer of the mononucleosis virus. Henle's electron microscopist Hummeler examined our electron micrographs and remarked: I see that your cultures are contaminated with mycoplasmas. I had never before heard the word mycoplasma, but, instead of inquiring what the word meant, I said that the pictures were not of cell cultures, but of thin sections of leafhopper salivary glands, made by Hirumi (1969). I was not familiar with the work of Chanock *et al.* (1962), who successfully cultured the infectious agent of "atypical virus pneumonia", named by Hayflick *Mycoplasma pneumoniae*. Apparently, Hummeler recognized phytoplasmas in our electron micrographs of leafhopper vector salivary glands. It was another missed opportunity.

The 1967 breakthrough

In November 1967 the Japanese Plant Pathology Society was holding their annual meeting in Sapporo. My former associate, Eishiro Shikata, at Hokkaido University, was the secretary of the society, receiving abstracts of papers a few weeks earlier. Shikata wrote to me, requesting 6 negatives of electron micrographs made by him in 1964. Several hundred glass negatives were stored in my laboratory and since Shikata mentioned that the requested plates contained the aster yellows pathogen, I checked the numbered negatives, but found no virus particles on the pictures and did not mail the requested plates. Several weeks later I found out, that Shikata wanted to take part in the discussion of Doi's paper and show phytoplasmas in his own electron micrographs, that he could not identify earlier. I wondered how Doi was able to recognize the "MLOs"? At the same meeting the entomologist S. Nasu from Tsukuba submitted an abstract, reporting MLOs in thin sections of rice yellow dwarf diseased plants, and in the leafhopper vector *Nephotettix apicalis*. (Nasu *et al.*, 1967). Why was this important contribution ignored in subsequent papers and review articles, not only in Europe, but, surprisingly, also in Japan? It took several years before I solved this puzzle. Japanese plant pathologists omitted Nasu *et al.* because they knew that Nasu submitted his

abstract only after reading the tentative draft of the program. He rushed to his laboratory, prepared thin sections of diseased rice plants and leafhopper vectors, and submitted the results in time to be presented orally, and printed, in the same issue of the journal as the two reports from Asuyama's plant pathology department, by Doi *et al.* (1967) and Ishiie *et al.* (1967). Asuyama knew that Nasu found out about MLOs only after being tipped off by the abstracts of the Tokyo plant pathologists. Asuyama felt that Nasu's findings were not an original idea and the work was not worth mentioning. In the meantime I was told that the MLOs were not the original idea of Doi either and that there was someone, who tipped Doi off. Intrigued, I wrote to Asuyama, but he did not reply. After my third letter, Asuyama replied, that Doi was familiar with all earlier mycoplasma publications and that he was the sole discoverer. Was this really so?

In 1974, in Tokyo, I finally met the mysterious person who was responsible for recognizing mycoplasma resembling structures in electron micrographs made by Doi. It was Kaoru Koshimizu, a veterinarian from the Poultry Department of Tokyo University. In 1967 he saw the electron micrographs made by Doi, and asked whether Doi was working with mycoplasmas. Doi, who never heard about mycoplasmas, immediately noticed the similarity of the structures in Koshimizu's and his own electron micrographs. He replied that he was searching for virus particles in sections of mulberry dwarf diseased plants. Koshimizu then asked whether Doi tried to cure diseased plants with tetracyclines. Doi replied that tetracyclines have no effect on viruses. "Not on viruses, but they are used to cure turkeys suffering from mycoplasma infection" stated Koshimizu. Doi repeated the conversation to Asuyama, who then requested Ishii to obtain tetracycline samples from the poultry department. He requested Ishiie to apply tetracycline to leaves and the soil around potted mulberry seedlings, infected with mulberry dwarf "virus". When the treated mulberry seedlings began to recover, other diseased plants were similarly tested and the two seminal papers were submitted to the forthcoming annual meeting in Sapporo.

I felt that the crucial role of Koshimizu should have been acknowledged by Asuyama and his associates. This did not diminish their achievement, but it demonstrated the advantage of communicating with scientists from other departments, other universities and institutes, as well as other countries.

In January 1968, in the program of lectures, to be presented at the New York Academy of Sciences, appeared the title and short abstract of my lecture on "MLOs" in aster yellows and corn stunt diseased plants. The abstracts were distributed to more than 20,000 Academy members. Among them was C. Vago in St. Cristol les Ales, France. My abstract did not mention the three 1967 Japanese papers, but my paper, published a few weeks later, presented the complete story, including details of the Japanese breakthrough (1968). In May 1968 in the *Compte Rendu* appeared the first French paper on the detection of "MLOs", by Giannotti *et al.* (1968), submitted by C. Vago. Not only was there no mention

about the Japanese findings or work in the United States, but the work in France was presented as one of the greatest discoveries of the XX Century, comparable to Pasteur's work in the XIX century.

I was well acquainted with Vago. Years earlier, he was very helpful in my attempts to culture invertebrate cells, we communicated frequently, and jointly organized the First Invertebrate Cell Culture Conference in Montpellier in 1962. Therefore I send to Vago the published full text of my lecture, with the references of the Japanese papers. I asked him why the work, carried out by others, had not been mentioned. Vago replied that there were strikes at French universities and the library in Montpellier did not receive current literature. Despite this explanation, a whole series of papers by Giannotti *et al.* was published during the following months, constantly omitting the work published in 1967, and the subsequent extensive work, carried out by several Japanese experiment stations. The claim, that the finding of phytoplasmas was a French discovery, was repeated several times and its great importance for world's science repeated. Then came Giannotti's claim, that he succeeded in culturing phytoplasmas. Attempts to confirm this in other laboratories failed. Giannotti was invited to Bové's laboratory to demonstrate how he cultured phytoplasmas in cell-free media. He brought his material to Bordeaux, and presented his technique. When he was ready to leave for the airport, he wanted to take back his media and everything else that he brought from St. Christol. Robert Davis was spending his sabbatical in Bové's laboratory at that time. He and Bové wanted to repeat the experiments with Giannotti's original material. On the morning of his departure Giannotti was told that his material was locked in a greenhouse by a gardener, who became ill and could not come that morning. Giannotti had to depart, leaving his media and plants in Bordeaux. Bové and Davis, unable to confirm the phytoplasma cultivation, notified Giannotti. Nevertheless, he did not recant his results. He came to a meeting in Florida several months later and stated again that he successfully cultured phytoplasmas.

Failed cultivation attempts in my laboratory

Attempts to culture phytoplasmas were also made in my laboratory. One of my postdoctoral associates, a Fulbright scholar from Yugoslavia, Biljana Plavsic, used horse serum in her media and after a few days observed what appeared like colony growth. Fortunately, before rushing to submit the results to a scientific journal, I mailed the photographs of the presumptive colonies to Ruth G. Wittler, a mycoplasma expert at Walter Reed Army Institute in Washington, D.C. She immediately replied, identifying the growth as "pseudo colonies", that were known to appear when high concentrations of horse or rabbit serum were used in culture media. The reply saved me the embarrassment of publishing the presumptive successful cultivation of phytoplasmas. We published a short abstract about the pseudo colonies (1971).

Although cultivation of phytoplasmas has not yet been achieved, I hope that collaborations between phytoplasma-mologists and other microbiologists will eventually result in the cultivation of the fastidious microorganisms.

Incompatibility of phytoplasma research with politics

In 1972 my postdoctoral associate, Biljana Plavsic, made her most important discovery, recognizing phytoplasmas in inflorescences of coconut palms affected by lethal yellowing disease (1972). Earlier reports listed the palm disease as a virus disease. The devastation caused by it on several Caribbean islands and in southern Florida was of great concern. The findings by Plavsic *et al.* were soon confirmed in Great Britain and in Germany. Plavsic published 7 additional papers during her 18 months in my laboratory and she continued her phytoplasma research after returning to her university in Sarajevo, former Yugoslavia.

When Yugoslavia fell apart into 7 republics, she decided to become a politician. At first, she was very successful, becoming the only woman elected president of the newly created Republic of Bosnia. She was hailed as the ablest politician in former Yugoslavia, solving many problems and achieving great popularity. Unfortunately, when war broke out between Croats, Serbs, and Bosnian Muslims, Plavsic became the supporter of the campaign of persecution, and in 1992 tens of thousands of Bosnians were killed and ethnic killing was being carried out under her presidency. When Plavsic became vice-president under Radovan Karadzic, she inspired the Serbs to take up arms against their Croat and Muslim neighbors, proclaiming the cultural and racial superiority of Serbs over Muslims.

In 2002 Plavsic travelled voluntarily to The Hague, to face the United Nations International War Tribunal. She was promptly arrested and presented with the evidence of the horrendous war crimes. She pleaded guilty and was sentenced to 11 years in jail. Two years ago she was released. Biljana Plavsic lives now in retirement in Beograd. Very few people know that the discoverer of the cause of lethal yellowing is the same person who became first famous, and later infamous, as president of Bosnia. Had she remained a virologist and phytoplasma-mologist, instead of turning to politics, she would have been a very prominent scientist today.

Conclusions

Phytoplasma research has greatly progressed during the passed 44 years. Nearly 1,000 plant diseases and many insect vectors have been identified and collaboration between researchers from different countries and different disciplines accounted for the rapid progress achieved in recent years. Science recognizes no political, religious, ethnic, or geographic borders, and we, as scientists, speak the same language of science, collaborating with each other, irrespective of background and

political believes. Currently molecular biologists, plant pathologists entomologists and microbiologists from different countries collaborate, increasing and expanding our knowledge of phytoplasma pathogens, vectors, and phytoplasma diseases worldwide. Sequencing of phytoplasma genomes is yielding new knowledge, leading to novel approaches to the control of phytoplasma diseases and control of insect vectors. The historical events of the passed century provided the basis for the current molecular biology study of phytoplasmas.

Acknowledgements

I want to express my gratitude to Assunta Bertaccini for inviting me to the Second IPWG conference, and to my postdoctoral associates who worked with me in the United States and abroad during the past years. To them I express my sincere thanks and best wishes for their continuous successful research and happy life.

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Insights in host dependency encoded within phytoplasma genomes

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Abstract

A strong community of scientists has been established within the last 40 years in phytoplasma research. Hereby, knowledge of these phytopathogenic bacteria increased dramatically in all fields catalysed by the introduction of molecular techniques and bioinformatics. The determination and analysis of genome data provided insights in the genome organization, content, evolution but also an excellent database for experiments studying putative virulence factors and for the development of diagnostics tools. In contrast to this progress, the understanding of the metabolism appears still weak. Its interpretation is essential for the understanding of the pathogen-host and host-pathogen interaction. An overview about the genetic information in the 'Candidatus Phytoplasma asteris' strains OY (line OY-M), AY-WB, 'Ca. P. australiense', and 'Ca. P. mali' is provided with respect to the encoded pathways and the resulting general requirements on the host.

Key words: comparative genomics, sieve cell sap, metabolism.

Introduction

Phytoplasma research blooms since the assignment of these fascinating phytopathogenic organisms as bacteria in 1967 (Doi *et al.*, 1967). In contrast to many other *Mollicutes*, it was not possible to cultivate phytoplasmas in cell-free media so far. This fact is severely handicapping phytoplasma research and in particular the experimental analysis of the metabolic capacities. Molecular analysis of phytoplasma genes and genomes is thus an essential means to improve our knowledge of diagnosis, evolution, pathogenicity and metabolism of these bacteria.

Genomes of phytoplasmas

The complete determination of four phytoplasma genomes including those of 'Ca. Phytoplasma asteris' strains OY (line OY-M) and AY-WB (Oshima *et al.*, 2004; Bai *et al.*, 2006), 'Ca. P. australiense' (Tran-Nguyen *et al.*, 2008) and 'Ca. P. mali' (Kube *et al.*, 2008) provided valuable insights into the genetic environment of these bacteria. These strains represent only a relatively small part of phytoplasma diversity as we know it today. Sequence information provided significant insights in chromosome organization, such as the circular and linear forms and the variation in genome size (Neimark *et al.*, 1993). Within the last years it has become clear that this variation is mainly based on integration and duplication events. The integration of complex transposons (PMUs) (Bai *et al.*, 2006; Toruno *et al.*, 2010), prophage-related elements (Wei *et al.*, 2008), and plasmid sequences (Bai *et al.*, 2006; Liefting *et al.*, 2006) results in genome instability (Bai *et al.*, 2006), an increase of the genome size (Bai *et al.*, 2006; Kube *et al.*, 2008), and stands in contrast to genome condensation processes. The importance of these events for virulence remains unclear due to the limited number of viru-

lence-related genes identified so far. An indirect effect is probably given for the extrachromosomal elements because it was shown that their genetic material influences vector transmissibility at least of some strains (Ishii *et al.*, 2009). Examples of integration events can be identified in each phytoplasma genome examined so far. The genome data suggest that the integrated genetic material is, on one hand, of significant importance for the phytoplasmas but, on the other hand, probably also an accident resulting from a high pressure of integration events.

Metabolism and requirements on the host

The impact of these genetic elements on the limited chromosomal metabolic capabilities of phytoplasmas appears to be low. The metabolic repertoire deduced from the four phytoplasma chromosomes is characterized by the loss of pathways and obviously corresponds to an adaptation to plant and insect hosts. Genome condensation resulted in the absence of the F₀F₁ ATPase system in all four phytoplasma genomes. A complete gene set encoding all proteins needed for the glycolysis was identified in phytoplasmas except for 'Ca. P. mali', which is lacking the energy yielding part. This finding raises the question, which alternative pathway is encoded in the phytoplasmas to gain ATP (Kube *et al.*, 2008). Phytoplasmas lack the gene sets for the sterol biosynthesis, tricarboxylic acid cycle, phosphotransferase system, nucleotide *de novo* synthesis and amino acid synthesis. These deficiencies result in strong requirements on the plant host and the colonized environment of the sieve tubes for example. The sieve tube sap must and can provide a wide variety of metabolites needed in consequence. Its composition varies depending on the plant species, but always contains large amounts of carbohydrates. The most abundant is sucrose beside other sugars, minerals, proteins, amino acids,

ATP (van Helden *et al.*, 1994; van Bel and Hess, 2008) and usually low amounts of bicarbonate and malate. Malate and citrate represent the predominant organic acids in phloem and xylem sap (Ziegler, 1975). Phytoplasmas share a common genetic repertoire for the uptake of the components which they are incapable to synthesize and for the cofactors needed for their functional protein machinery. The complete pathways are limited to replication, repair, transcription, translation and carbohydrate metabolism. In particular, the metabolism differs with respect to the phylogenetic branch and still appears incomplete with respect to our current knowledge. However, besides the differences in the genome size and the number of paralogs, all phytoplasmas share a remarkable common set of protein functions.

Membrane proteins and secretion

Immunodominant membrane proteins represent the major portion of the total cellular membrane proteins in most phytoplasmas (Kakizawa *et al.*, 2006a) and probably interact with the insect host at least (Suzuki *et al.*, 2006; Kakizawa *et al.*, 2006b). The percentage of predicted proteins by Phobius (Käll *et al.*, 2004) carrying transmembrane helices ranges from 26-31 % of the annotated genes. ATPases involved in cation exchange and mechanosensitive channel proteins were embedded in the membrane of all four phytoplasmas in addition to

the ABC transporters and symporter required to supply the phytoplasma cytosol with the essential biochemical modules. A functional active sec-dependent secretion system works in the opposite direction and allows the export of signal peptide carrying proteins (figure 1). Around 5% of all proteins belongs to this group including prominent members such as SAP11 (Bai *et al.*, 2009) and the protein 'tengu' of OY-M (Hoshi *et al.*, 2009), which induces witches' broom and dwarfism.

Outlook

Other proteins involved in manipulation of the plant and/or insect host will be identified by genetic manipulation, genome comparison and expression studies within the next years. Impact in phytoplasma research will also be provided by studies of the analysis of metabolism and the corresponding release of metabolic products and their so far unknown impact in virulence. These analyses will be supported by experimental studies of the genome, transcriptome and proteome. New high throughput techniques within these research areas, such as deep sequencing by next generation sequencing, allow the determination of phytoplasma and the discrimination or analysis of host derived data. First projects are completed (Ji *et al.*, 2009) and several others are in progress worldwide. Results are needed for deeper insights in these parasitic bacteria.

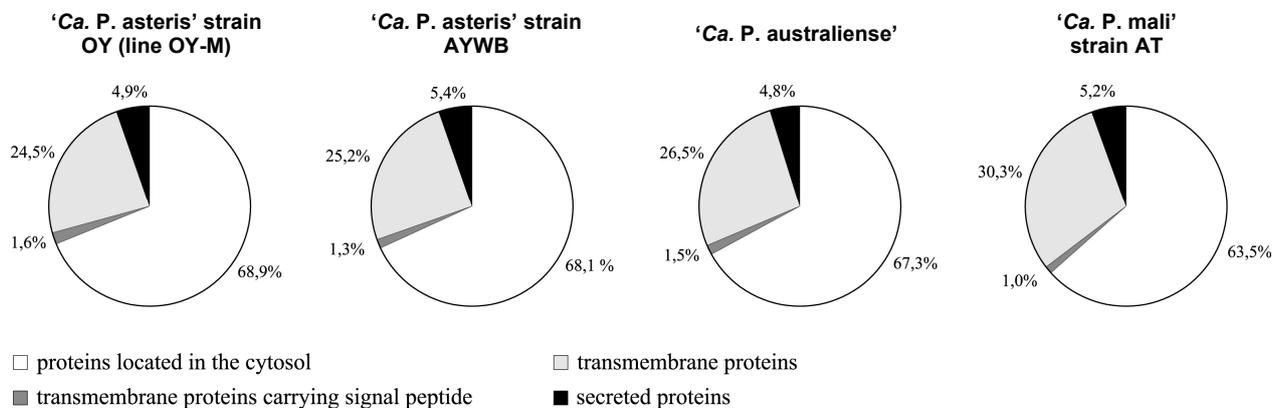


Figure 1. Percentage of the putative secreted proteins calculated on the deduced proteins of the chromosomes (AP006628.2, CP000061, AM422018 and CU469464).

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'Flavescence dorée' phytoplasma genome: a metabolism oriented towards glycolysis and protein degradation

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Abstract

The 670 kbp chromosome of 'flavescence dorée' phytoplasma line FD92 was partially sequenced by pyrosequencing and SOLEXA. More than 94% of the chromosome could be assembled and the 22 largest contigs representing 85% of the chromosome were annotated under the semi-automatic annotation platform iANT. Out of 464 chromosomal coding sequences (CDS), 174 CDS (38%) were involved in information transfer (DNA replication, protein production, RNA modification and regulation), 88 CDS (19%) were encoding metabolic enzymes, 40 CDS (9%) corresponded to transporters, 8 CDS (1%) corresponded to cellular processes, whereas 145 CDS (31%) remained cryptic. At this stage of incomplete assembly, repeated sequences were underestimated and transposon and phage-related CDS (2%) could not yet be precisely evaluated. FD92 phytoplasma possesses a complete glycolytic pathway and has a prominent system for proteolysis possibly resulting from the adaptation to its woody hosts.

Key words: Phloem-restricted bacteria, plant pathology, bacterial genomics, ATP-dependent zinc protease, HflB.

Introduction

Vineyards of southern Europe are affected by the 'flavescence dorée' phytoplasma (FD), inducing an epidemic and quarantine disease (Boudon-Padiou, 2002). Whereas important progress has been made in phytoplasma classification and ecology, little is known about mechanisms of phytoplasma physiology, phytopathogenicity and transmission by insects. These research areas should benefit from the comparative analysis of phytoplasma genomes. A physical and genetic map of the FD92 line chromosome (670 kbp) was established (Malembic-Maher *et al.*, 2008). To decipher FD phytoplasma genome, enriched DNA of FD line FD92 was prepared from broad beans by repeated bis-benzamide-CsCl density gradient centrifugations. A preliminary set of shotgun sequences were homologous to known phytoplasma sequences in 27% of the cases. The sequencing of the FD phytoplasma genome could therefore be undertaken.

Materials and methods

FD92 line was transmitted to *Vicia faba* var. Aqua Dulce using *Scaphoideus titanus* collected in infected vineyards of South-West France in 1992, and maintained since then by transmission to *V. faba* by *Euscelidius variegatus*. Total nucleic acids were extracted from stems and leaf midribs by the CTAB procedure (Murray *et al.*, 1980) followed by 4 repeated bis-benzamide Cesium chloride gradients (Kollar *et al.*, 1989).

Pyrosequencing of 8 µg of FD92 DNA enriched fraction was performed on a 454 GLX (Roche) by GATC BIOTECH (Germany). This produced 528,000 flowgrams totalling 122 Mb with an average of 230 bp read length. After assembly with Newbler (Roche), 1,067

contigs containing at least ten reads were compared to the genome of 'Candidatus Phytoplasma asteris' line OY-M and to the non redundant database (nr) with BLASTX and BLASTN considering significant alignments below a cut-off E value of 10⁻¹⁰. Out of the 1,067 contigs, positive contigs were therefore selected as they showed homology to phytoplasmas or other bacteria. Plant DNA was screened out from the assembly. The 72 selected contigs were composed of 107,000 reads with an average coverage of 39 X. Additional contigs showing no homology but having coverage in the range of 20-100X were also selected after PCR performed on healthy and FD-infected *V. faba*. Phytoplasma contigs were further extended by genome walking (BD Bioscience, USA). Finally, 85 contigs totalling 629 kbp (94% of the chromosome) resulted from the final assembly with Phred-Phap-Consed Package. To overcome the errors on homopolymers falsely introduced by 454GFLX reads, Solexa sequencing was performed by GATC Biotech (Germany). About 100 Mb of 35 bp reads allowed to correct the homopolymers errors.

The 22 largest contigs, ranging from 11 to 58 kbp (85% of the chromosome), were annotated under the semi-automatic annotation platform iANT developed by LIPM at INRA Toulouse.

Results

The FD92 chromosome has 21.1% G+C, possesses 2 rRNA operons, 27 tRNAs as well as 464 coding sequences (CDS) (table 1). Most of the sequenced CDS (38%) participate to the information transfer, *i.e.* replication, transcription and production of the proteins. Regarding to DNA rearrangement, FD92 should be capable of homologous recombination as it has a complete *recA* gene, *ruvA* and *ruvB* genes encoding DNA

helicases and RuvX, a putative holiday junction resolvase. In addition, 5 putative phage recombinases of *xerC* family and phage-related integrases could be evidenced among the 464 CDS of FD92 chromosome.

Table 1. Structural RNAs and functional distribution CDS from partial FD92 chromosomal sequences.

Functional classification	Number of CDS
16S-23S-5S rRNA operons	2
tRNAs	27
CDS	464
Information transfer	174
Protein translation	98
DNA replication and modification	43
RNA modification	30
Regulation	3
Metabolism	88
Protein degradation	24
Glycolysis and energy metabolism	10
Nucleic acid metabolism	10
Lipids and phospholipids metabolism	9
Cofactors	3
Amino acids metabolism	2
Others	30
Transport	40
ABC transporters	25
Protein secretion	6
Cation dependent P-ATPase	4
2-hydroxycarboxylate transporters	2
Others	3
Cellular processes	8
Phage related	8
Transposon related	1
Cryptic	145

Metabolic genes account for 19% of the sequenced CDS out of which 27% participate to protein degradation. It comprises 15 copies often truncated of *HffB/FtsH* encoding ATP-dependent zinc proteases as well as various other proteases. ‘*Ca. P. asteris*’ line OY-M possesses 20 copies of *HffB/FtsH* genes obviously not all functional (Oshima *et al.*, 2004). As most of phytoplasma genomes sequenced to date, FD92 chromosome encodes the complete glycolytic pathways. Nine CDS are controlling lipids and phospholipids metabolism, whilst 10 CDS are involved in amino acid synthesis. Finally, the precise role of 30 other CDS metabolic pathway could not precisely determined.

Most of the transporters were ABC transporters (62%) aimed to transport maltose, spermidin/putrescine, oligopeptides, methionine, cobalt and manganese/zinc. Other transporters were assigned to protein secretion (sec system), to cation dependent P-ATPase and two 2-hydroxycarboxylate (malate/citrate) cation transporters.

As long as the remaining 15% repeated part of the genome are not sequenced and annotated, it is too early to state about the presence of complete potential mobile units homologous to ‘*Ca. P. asteris*’ PMU (Bai *et al.*,

2006). However, *dnaD*, *insK* (transposase), *ssb* and *hup* (putative bacterial nucleoid binding protein) were scarcely and randomly distributed into contigs. *HffB*, were not adjacent to CDS homologous to PMU genes.

As virulence and defence factors, FD92 has a type III haemolysin and a superoxide dismutase (*sod*) that allow phytoplasma to resist to oxidative stress.

Discussion

FD phytoplasmas complete genome deciphering is not yet achieved. However, guidelines can already be drawn: FD phytoplasma metabolism is oriented towards protein degradation and transport. Its carbon, oxydo-reductive and energetic metabolism seems to rely exclusively on glycolysis, maltose and 2-hydroxycarboxylate import and degradation, and P-ATPases. These findings will help a better understanding of FD phytoplasma specialisation in woody hosts.

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Development of specific *secA*-based diagnostics for the 16SrXI and 16SrXIV phytoplasmas of the Gramineae

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Abstract

Phytoplasmas are responsible for a range of diseases in the Gramineae in Sub-Saharan Africa and Asia including sugarcane grassy shoot (white leaf), rice yellow dwarf, napier grass stunt and Bermuda grass white leaf. In previous work, we have designed universal nested PCR primers based on the *secA* gene for amplification of DNA from phytoplasmas in most 16Sr groups. However, these primers did not appear to work well on phytoplasmas in the 16SrXI and XIV groups. In this work, we have designed specific sets of primers based on the *secA* gene that can be used in a nested PCR assay to amplify either the napier grass stunt phytoplasma or the sugarcane phytoplasma or the rice/bermudagrass phytoplasmas. These assays and *secA* sequences have also been used to redefine the taxonomic relationships between these phytoplasmas.

Key words: diagnostics, Gramineae, phylogenetics, phytoplasmas, *secA* gene.

Introduction

Phytoplasmas are responsible for many serious plant diseases in Sub-Saharan Africa and South Asia including in major cereals, fodder crops and weeds (Arocha and Jones, 2010). In maize (Americas and Europe), wheat (China), barley and oats (Europe), phytoplasmas of the 16SrI aster yellows type are associated with diseases such as maize bushy stunt and wheat blue dwarf, and these can be found in many plant species, not just the Gramineae. By contrast, the phytoplasmas of the Gramineae in Africa and Asia appear to be specifically adapted to the grasses. These include the 16SrXI sugar cane grassy shoot / white leaf diseases and the rice yellow dwarf disease (Arocha and Jones, 2010). In Africa, there is the 16SrXI napier grass stunt phytoplasma which was first recorded on napier grass (*Pennisetum purpureum*) only 11 years ago and for which the vector has been identified as *Mastomys natalensis* (Obura *et al.*, 2009). In addition, there is the closely related 16SrXIV bermuda grass white leaf phytoplasma of *Cynodon dactylon* and other grasses, that is widespread throughout Africa and Asia, vectored by *Exitianus capicola*.

To improve detection and taxonomic classifications of phytoplasmas, we have designed primers for PCR based on the *secA* gene (Hodgetts *et al.*, 2008; Bekele *et al.*, 2011), along with assays based on the plant *cox* gene to confirm that DNA extracts support PCR/LAMP amplification. In this work we describe the development of specific assays for the phytoplasmas of the Gramineae.

Materials and methods

Samples were obtained from 3 napier grass stunt infected plants (originally from Kenya but maintained at

the University of Nottingham), 20 sugarcane plants exhibiting grassy shoot (10) and white leaf (10) symptoms from Sri Lanka, 1 rice plant exhibiting yellow dwarf symptoms from Sri Lanka, 3 bermuda grass plants exhibiting white leaf symptoms from Sri Lanka, 8 sugarcane samples exhibiting grassy shoot symptoms and 1 rice yellow dwarf sample from Nghe An Province, central Viet Nam and 1 bermudagrass, 1 setaria grass and 5 digitaria grass samples exhibiting yellowing/white leaf symptoms from Ethiopia. DNA was extracted using the CTAB DNA preparation method (Doyle and Doyle, 1990), and the presence of PCR amplifiable DNA confirmed using primers based on the *cox* gene (Tomlinson *et al.*, 2010). The presence of phytoplasma in these samples was confirmed using 16S rRNA universal primers, and samples were then used to develop and validate the *secA* primers as outlined in the results.

Results

Initial tests using the *secA* nested primers as described in Bekele *et al.* (2011) failed to amplify from the grass samples even though these had proven to be positive for the presence of phytoplasma using 16S rRNA primers. However, by reducing the annealing temperature to 45°C, PCR products were obtained using certain combinations of the different nested primers. These PCR products were subsequently sequenced and used to design more specific primers that could then be used to amplify reliably from all the samples at 53°C annealing temperature. These new specific primers are listed in table 1, defined by the organism they are specific for.

Following amplification with these primers, a phylogenetic analysis was conducted on the *secA* sequences obtained (figure 1). This analysis indicated that all the

Table 1. Sequences of the *secA* gene primers developed and used in this and previous studies.

Primer name	Sequence 5'-3'	Reference
SecAF1	GARATGAAAACCTGGRGAAGG	Hodgetts <i>et al.</i> (2008)
SecAR3	GTTTTRGCAGTTCCTGTCATNCC	Hodgetts <i>et al.</i> (2008)
SecAF5	ASTCGTGAAGCTGAAGG + AGCTAAAAGAGA- ATTTGAAGG	Bekele <i>et al.</i> (2011)
SecAR2	CCNTRCCTAAATTGNCGTCC	Bekele <i>et al.</i> (2011)
NGSsecfor1	TATACWACWAATAGTGAATWGG	Abeyasinghe <i>et al.</i> submitted
NGSsecrev1	GATAAGTAATAGTAGCAGCAATTTTCAG	Abeyasinghe <i>et al.</i> submitted
NGSsecfor2	CGATGAAGTGWATTCTGTC	Abeyasinghe <i>et al.</i> submitted
NGSsecrev2	AGCTTCTAAAGCTTGATGTAATCC	Abeyasinghe <i>et al.</i> submitted
Sugarsecfor2	GATTCTGTCTTAATAGACGAAGCTAG	Abeyasinghe <i>et al.</i> submitted
Sugarsecrev2	GTAAATTGATCTATTATCAAAACATTATTT	Abeyasinghe <i>et al.</i> submitted
Ricesecfor2	GATTCTGTCTTGATAGATGAAGCAAG	Abeyasinghe <i>et al.</i> submitted
Rice secrev2	GTAAATTGGTCTATAATTAATCTGATTA	Abeyasinghe <i>et al.</i> submitted

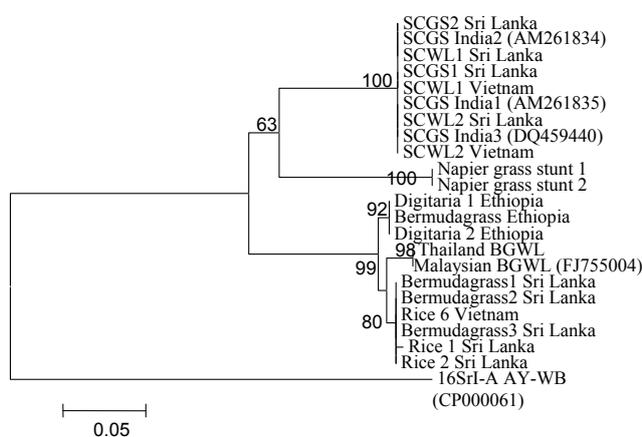


Figure 1. Phylogenetic tree based on the *secA* sequences obtained in this study and some obtained from GenBank (for which accession numbers are shown in parenthesis).

sugarcane phytoplasmas from Viet Nam and Sri Lanka were identical, and the same as the sequences that had been deposited in GenBank from India. These sequences were distinct from the Napier grass stunt *secA* sequences. There was then a separate cluster that contained the rice, bermudagrass and other grass sequences. Interestingly, these sequences appeared to be separated from each other on the basis of Country of origin rather than plant host, with the Ethiopian strains being slightly different from the Thailand/Malaysian strains which were in turn different from the India/Sri Lanka/Viet Nam strains.

Discussion

Primers have been designed in this study that can be used to differentiate between the phytoplasmas in the Gramineae in Africa and Asia based on the *secA* gene. This gene is more powerful than the 16S rDNA since it gives better resolution between the different groups, and is more reliable than use of the 16S-23S rRNA region, since there is only one copy of the *secA* gene, so less

likely to be any problems due to heterogeneity between copies. The results based on the *secA* gene clearly differentiate the sugarcane, napier grass and rice/bermuda grass clusters from each other and also provide evidence that the rice/bermudagrass cluster can be separated on the basis of regional origin.

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The use of groEL gene for characterisation of aster yellows phytoplasmas in field collected samples

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Abstract

Amplification of fragments containing phytoplasma groEL gene sequence with a newly designed nested PCR system allowed to specifically detect the presence of 'Candidatus Phytoplasma asteris' in reference strains as well as in samples field collected and maintained as dry/freeze dried nucleic acids. After RFLP analyses it was possible to confirm further finer differentiation among strains enclosed in subgroup 16SrI-B by previous ribosomal gene classification.

Key words: phytoplasmas, PCR/RFLP analyses, groEL gene, molecular characterization, epidemiology.

Introduction

Phytoplasma classification established using 16S ribosomal groups and 'Candidatus Phytoplasma' taxon is mainly based on 16S rDNA properties and do not always provide molecular distinction of the closely related strains such as those in the aster yellows group (16SrI or 'Candidatus Phytoplasma asteris' - related strains). More variable single copy genes, such as ribosomal protein (rpl22 and rpS3), secY, tuf, and groEL were employed for finer aster yellows phytoplasma differentiation (Marcone *et al.*, 2000; Lee *et al.*, 2004; 2006; Martini *et al.*, 2007; Mitrović *et al.*, 2011). A nested PCR system was developed on groEL gene and it was tested on aster yellows phytoplasma strains from field infected samples from different plant hosts and different geographical locations toward epidemiological purposes.

Materials and methods

Nucleic acid samples extracted from 1992 to 2010 from 36 symptomatic plants of different species identified as 16SrI phytoplasmas by PCR/RFLP analyses (Lee *et al.*, 1998), and maintained as dry or freeze dry pellet at 4°C or -20°C respectively, were employed. The 36 nucleic acid samples (table 1) were employed for amplification in direct PCR with newly designed primers AYgroesF (ATCAGAAAAGAAAATCCT) and AYampR (GCAACAGCAGCAAATAAAAC) that amplify a region of about 2,100 bp external to AYgroelF/AYgroelR (Mitrović *et al.*, 2011). Nested PCR was then carried out on amplicons diluted 1: 30. Reference strains employed for preliminary specificity assays were aster yellows from France (AY-J, 16SrI-B), primrose virescence from Germany (PRIVA, 16SrI-B) stolbur from pepper from Serbia (STOL, 16SrXII-A), and tomato big bud from

Australia (TBB, 16SrII-D) in periwinkle, and on 'Ca. P. japonicum' in *Hydrangea* sp. (16SrXII), 'flavescence dorée' in grapevine from Serbia (FD, 16SrV-C), and European stone fruit yellows in peach from Serbia (ESFY, 16SrX-B). Each 25 µl PCR reaction mix contained 20 ng template DNA, 5 U of Sigma-REDTaq® DNA Polymerase (Sigma-Aldrich Co.), 2.5 µl of its 10X buffer, 2 µl d-NTPs (Fermentas, Vilnius, Lithuania) and 0.4 µM of each primer. Samples lacking DNA were employed as negative controls. Thirty-five PCR cycles were performed for both primer pairs as previously described (Mitrović *et al.*, 2011). The groELI (groEL gene RFLP group I) RFLP subgrouping was performed with *AluI* and *TruI* restriction enzymes (Fermentas, Vilnius, Lithuania). Restriction products were separated in 5% polyacrylamide gel and profiles were compared with those obtained from positive reference strains.

Results and discussion

Expected length amplicons (about 1.4 kb) of partial groEL gene were obtained only with reference strains belonging to aster yellows group (16SrI) confirming specificity of the nested PCR system employed to 16SrI phytoplasma group. All 36 field collected samples were amplified producing the expected length amplicons and RFLP analyses with *TruI* and *AluI* restriction enzymes yielded six and seven different profiles respectively according to Mitrović *et al.* (2011) (table 1) allowing differentiation of AY phytoplasmas to eight groELI RFLP subgroups, of which two were newly identified: one in cabbage and one in periwinkle. The developed method allows detection of aster yellows strains from field collected samples allowing discrimination that can be of epidemiological relevance such as in the cases of phytoplasmas in carrot, onion, oil palm and corn.

Table 1. Results from AYgroelF/AYgroelR nested PCR amplification and RFLP analyses for fine characterization of aster yellows phytoplasmas from field collected samples.

Phytoplasma disease	Geographical location	Number of tested samples (year)	RFLP literature	Grouping			
				TruI	AhI	groELI	16SrI
Papaver virescence	Forli (Italy)	1 (2009)	This paper	1	1	III	-B
Oil seed rape phyllody	Padova (Italy)	6 (2009)	Mori <i>et al.</i> , 2010	1	1	III	-B
Onion phyllody and virescence	Potenza (Italy)	4 (1994)	Vibio <i>et al.</i> , 1995	1	1	III	-B
				2	2	IV	-B
Cabbage phyllody	Ravenna (Italy)	6 (1994)	Bertaccini <i>et al.</i> , 1998	1	1	XI	-B
				7	1		
Lettuce yellows	Florence (Italy)	1 (2000)	This paper	1	1	III	-B
Lettuce yellows	Imperia (Italy)	5 (1992)	Vibio <i>et al.</i> , 1994	1	1	III	-B
Oil palm 'machite lethal'	Colombia	1 (2004)	-	2	8	V	-B
Corn stunt	Palmira (Colombia)	2 (2007)	Duduk <i>et al.</i> , 2008	2	8	V	-B
Periwinkle virescence	Belgrade (Serbia)	2 (2010)	This paper	4	4	VI	-C
Periwinkle virescence	Torino (Italy)	1 (1998)	This paper	1	1	III	-B
Periwinkle virescence	Saint Louis, USA	1 (1994)	This paper	1	9	X	-B
Periwinkle virescence	Ferrara (Italy)	1 (1998)	This paper	1	1	III	-B
				1	1	III	-B
Carrot proliferation	Begeč (Serbia)	3 (2006)	Duduk <i>et al.</i> , 2009	2	2	IV	?
				3	3	I	-A
Populus decline	Belgrade (Serbia)	1 (2009)	Mitrović <i>et al.</i> , 2011	6	7	IX	-P
Grindelia virescence	Ravenna (Italy)	1 (2008)	Bertaccini <i>et al.</i> , 2011	2	2	IV	-B

Acknowledgements

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Phylogenetic position of 'bois noir' phytoplasma based on analyses of *rpsJ-rplC-rplD-rplW-rplB* gene sequences

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Abstract

In the present work, a 2,738 bp DNA segment of the operon S10, including the genes *rpsJ*, *rplC*, *rplD*, *rplW* and *rplB*, from an Italian 'bois noir' phytoplasma strain detected in grapevine was amplified. Nucleotide and amino acid sequence analyses allowed to clarify the phylogenetic position of this 'bois noir' phytoplasma strain within 16S rDNA RFLP group 16SrXII and to identify molecular markers useful for specific phytoplasma identification. The results stimulate further work aimed at obtaining the nucleotide sequence of the entire superoperon S10-*spc-alpha* for a more detailed characterization of 'bois noir' phytoplasma.

Key words: S10 operon, ribosomal proteins, single nucleotide polymorphism, RFLP.

Introduction

Phytoplasma strains associated with 'bois noir' (BN) disease of grapevine have been tentatively classified in the species '*Candidatus* Phytoplasma solani'. (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004; according to rule 28b of the Bacteriological Code, '*Candidatus* Phytoplasma solani' is an incidental citation and does not constitute prior citation). Most '*Ca. P. solani*'-related strains belong to 16S rDNA RFLP subgroup 16SrXII-A (stolbur subgroup). Stolbur phytoplasmas infect not only grapevine, but also a wide range of wild and cultivated plants in several areas worldwide.

Stolbur phytoplasma 16S rDNA is 97.6% identical to the 16S rDNA of '*Ca. P. australiense*', its closest known relative. Because this value is above 97.5%, the designation of stolbur phytoplasma as a '*Candidatus*' species would be possible only considering its specific biological properties and comparing nucleotide sequences of multiple genes (Quaglino *et al.*, 2010).

Analyses of concatenated amino acidic sequences of housekeeping proteins have previously been carried out to investigate evolutionary relationships among phytopathogenic mollicutes (Zhao *et al.*, 2005). In the present study, we sequenced five ribosomal protein genes of the superoperon S10-*spc-alpha* amplified from two stolbur phytoplasma strains. Sequence analyses allowed us to clarify the phylogenetic position of BN phytoplasma within the taxonomic group 16SrXII and to identify molecular markers useful for specific phytoplasma identification.

Materials and methods

Nucleic acids were extracted from 1 g of leaf tissues of a periwinkle plant, infected by grafting with stolbur phytoplasma strain StolC, and from 1 g of leaf veins of a grapevine plant naturally infected by BN phytoplasma

strain BNFc6. A segment of S10 operon, including the genes *rpsJ*, *rplC*, *rplD*, *rplW* and *rplB*, was PCR-amplified using primer pairs designed on the basis of phytoplasmal sequences deposited in the GenBank by Cimerman *et al.* (2006). Nucleotide sequences were compiled in FASTA format. Closest phytoplasmal and bacterial gene sequences were retrieved from the GenBank (table 1). Amino acid sequences were deduced from nucleotide sequences by use of the software Expasy (<http://expasy.org/>).

Concatenated sequences were aligned by using the program ClustalX. Output alignment was trimmed using the program GBLOCKS to eliminate poorly aligned positions; the trimmed alignment was converted to MEGA format for phylogenetic NJ analyses.

Further, concatenated nucleotide sequences were searched for the presence of stolbur phytoplasma-specific restriction patterns by virtual RFLP assays carried out by using the program pDRAW (<http://www.acaclone.com/>). Digestions were performed by using both frequent cutting and rare cutting enzymes: *AluI*, *FauI*, *HinfI*, *HpaII*, *MboII*, *MseI*, *TaqI*, and *Tsp509I*.

Table 1. Phytoplasmas, other prokaryotes, and GenBank accession numbers of DNAs used for phylogenetic analyses.

Species-strain	Abbreviation	Acc. No.
Stolbur - StolC	StolC	Unpublished
Stolbur - BNFc6	BNFc6	Unpublished
' <i>Ca. P. australiense</i> '	Cpaus	NC_010544
' <i>Ca. P. asteris</i> ' - OYM	OYM	NC_005303
' <i>Ca. P. asteris</i> ' - AYWB	AYWB	NC_007716
' <i>Ca. P. mali</i> ' - AP	AP	NC_011047
<i>Acholeplasma laidlawii</i>	Achl	NC_010163
<i>Bacillus cereus</i>	Bacc	NC_011969
<i>Clostridium botulinum</i>	Clob	NC_010520
<i>Mycoplasma mycoides</i>	Mycm	NC_005364
<i>Spiroplasma kunkelii</i>	Spik	AY198133

Results

PCR-amplified S10 operon segments from stolbur phytoplasma-infected periwinkle and grapevine plants were sequenced. Genes (open reading frames) were identified, and their positions were recorded (table 2). The sequences from strains StolC and BNFC6 were identical.

Table 2. Genes from the S10 operon of stolbur phytoplasma strains infecting periwinkle and grapevine.

Gene	Position (bp)	Encoded Protein	Amino acids
<i>rpsJ</i>	1-318	S10	105
<i>rplC</i>	319-984	L3	221
<i>rplD</i>	985-1616	L4	207
<i>rplW</i>	1617-1902	L23	96
<i>rplB</i>	1903-2738	L2	276

Phylogenetic analyses of both nucleotide and amino acid concatenated sequences evidenced that stolbur phytoplasma strains clustered together with ‘*Ca. P. australiense*’, but formed a clearly distinct subclade within the cluster of taxonomic group XII (figure 1).

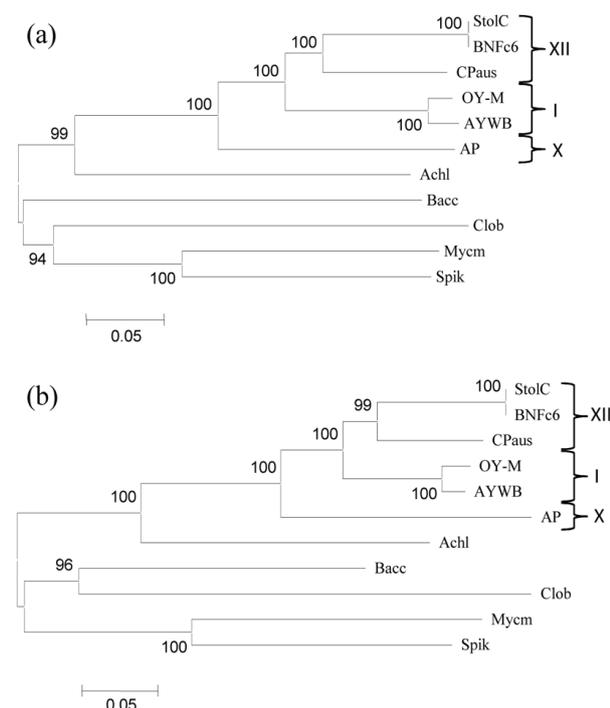


Figure 1. Phylogenetic tree constructed using NJ method of analysis of concatenated (a) rp gene nucleotide sequences and (b) amino acid sequences of rp proteins.

Further, virtual RFLP evidenced significantly different patterns distinguishing stolbur phytoplasma strains from ‘*Ca. P. australiense*’ (figure 2).

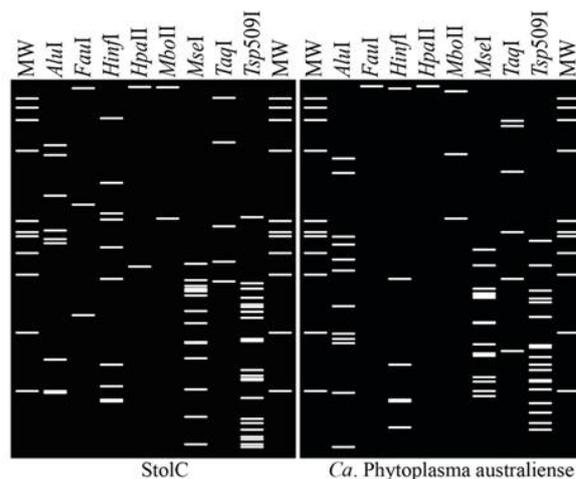


Figure 2. Virtual RFLP patterns of S10 operon segments from phytoplasmas in subgroups XII-A (strain StolC) and XII-B (‘*Ca. P. australiense*’).

Discussion

Preliminary results from analyses of a partial superoperon S10-*spc-alpha* and encoded proteins emphasized that stolbur phytoplasma strains cluster together in a distinct subclade within the taxonomic group 16SrXII. Further, virtual RFLP analyses confirmed reported sequence divergence between stolbur phytoplasma strains and ‘*Ca. P. australiense*’. These findings indicate the usefulness of ribosomal protein genes for differentiation among distinct phytoplasmas, encouraging further work aimed at defining more accurately the phylogeny of stolbur phytoplasma strains.

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StAMP encoding the antigenic membrane protein of stolbur phytoplasma is useful for molecular epidemiology

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Abstract

The antigenic membrane protein of stolbur phytoplasma has been cloned and characterized. The expression of StAMP in *Escherichia coli* produced a 16 kDa peptide recognized by an anti-stolbur monoclonal antibody. *Stamp* is submitted to a positive diversifying selection pressure (Fabre *et al.*, 2011). The genetic diversity of *stamp* was evaluated among a collection of stolbur phytoplasma strains representative of the *tuf* and *secY* genetic diversity of stolbur phytoplasmas in the Euro-Mediterranean basin. Most of the French, Italian and Croatian strains clustered on the same phylogenetic branch (*tuf*-type b cluster I). A second branch of the phylogenetic tree corresponded to strains of central and Eastern Europe (*tuf*-type b cluster II), while a third branch grouped strains of the east of the Mediterranean basin (Greece, Serbia, Lebanon, and Azerbaijan). Strains of the *tuf*-type a genotype clustered together in an independent monophyletic branch of the *stamp* phylogenetic tree. In conclusion, *stamp* variability seems to be correlated to geographical origin in the case of the *tuf*-type b strains.

Key words: 'Bois noir' disease, molecular epidemiology, bacterial surface protein, positive selection.

Introduction

'Stolbur' phytoplasma (StoIP) affects a wide range of crops and wild plants in the Euro-Mediterranean area including solanaceous crops, grapevine, lavender, strawberry, sugar beet, maize, stinging nettles and bindweed. It is transmitted by three *Fulgoroomorpha* planthoppers of the family *Cixiidae*.

Analysis of stoIP *tuf*-type b variability led to the discovery that different *tuf* genotypes can be associated with infection of bindweed and nettle (Langer and Maixner, 2004). Due to its complex ecology, StoIP is difficult to trace without the help of variable genetic markers. We recently described the striking genetic diversity of *vmp1* encoding a variable membrane protein specific to StoIP (Cimerman *et al.*, 2009). Variability of *vmp1* combined with that of *tuf* or *secY* proved to be efficient to differentiate StoIP strains (Fialová *et al.*, 2009; Murolo *et al.*, 2010; Pacifico *et al.*, 2009). We report here the isolation and characterization of *stamp*, the gene encoding the antigenic membrane protein of stolbur phytoplasma. Due to the synteny of the *groL-amp-nadE* locus between phytoplasma in the 16SrI and 16SrXII groups, the cloning of *stamp*, a StoIP homolog of '*Ca. P. asteris*' *amp* was undertaken. Its usefulness as a genetic marker possibly correlated to StoIP geographical origin or to association with insect vector species or ecotypes is currently evaluated.

Materials and methods

StoIP-infected periwinkles were maintained by graft inoculation. Grapevines, bindweeds, nettles, lavenders,

potatoes, tomatoes, peppers, eggplant, cherry, and common medlar, were collected in France, Italy, Germany, Hungary, Croatia, Serbia, Greece, Bulgaria, Lebanon, Azerbaijan and Egypt. *Hyalesthes obsoletus* (Signoret, 1865) insect vectors were collected in Germany, Italy, Slovenia and Croatia. Nucleic acids were extracted as previously described (Maixner *et al.*, 1995)

For fluorescence microscopy with 2A10 anti stoIP mAB, fresh periwinkle midribs were processed as previously described (Garnier *et al.*, 1990). Methods for amplification of *groL-stamp-nadE* and its cloning in *E. coli* as well as the detection of StAMP expression in *E. coli* was recently published (Fabre *et al.*, 2011).

Nested-PCR amplification and sequencing of *stamp* (Fabre *et al.*, 2011) produced chromatograms that were assembled and edited using GAP4. ClustalW multiple alignments and maximum of parsimony phylogenetic analyses were performed by MEGA 4. Inference of positive selection and determination of dN/dS was according to PARRIS method (Scheffler *et al.*, 2006).

Results

The gene *stamp* could be amplified and cloned in *E. coli*. It encodes a 157 amino acid-long protein with a predicted signal peptide and a C-terminal hydrophobic alpha helix. StAMP was 26%-40% identical to AMP of '*Ca. P. asteris*' strains and 40% identical to AMP of '*Ca. P. japonicum*'. The expression of StAMP in *E. coli* produced a 16 kDa peptide recognized by 2A10 mAB. *Stamp* was more variable than the house-keeping gene *secY* and the ratio between non synonymous over synonymous mutations (dN/dS) was 2.78 for *stamp* as

compared to 0.64 for *secY*. This indicates that *stamp* is submitted to a positive diversifying selection pressure.

Stamp genetic diversity was evaluated among a collection of StolP strains representative of the genetic diversity in the Euro-Mediterranean basin. Most of the French, Italian and Croatian *tuf*-type b strains as well as Egyptian potato strains clustered on the same phylogenetic branch (*tuf*B cluster I) (figure 1). Another branch corresponded to strains of Central and Eastern Europe including German, Slovenian, Hungarian, Bulgarian and Romanian strains (*tuf*-type b cluster II). A third branch grouped strains of the east of the Mediterranean basin, collected in Lebanon, Greece, Serbia and Azerbaijan (*tuf*-type b cluster III). Strains of the *tuf*-type b genotype clustered together on a monophyletic branch.

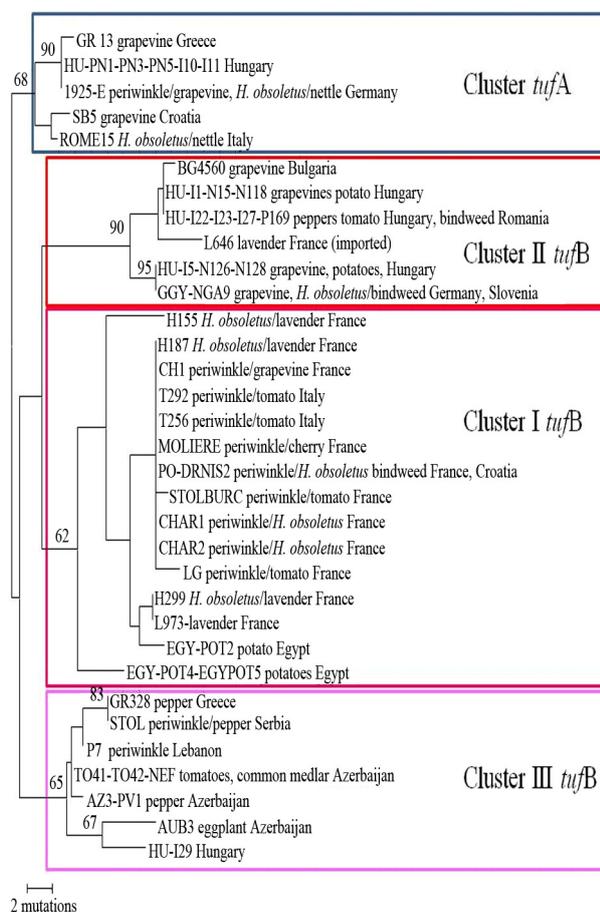


Figure 1. Phylogenetic consensus tree of StolP *stamp* sequences analysed by maximum of parsimony. Numbers above branches indicate the bootstrap values (500 replicates). Plant, insect and geographical origin are indicated on the right of StolP isolate names.

Discussion

Stamp encodes the antigenic membrane protein of StolP. Its variability is to be correlated to geographical origin of *tuf*-type b strains. Does this correlation correspond to insect vectors geographical distribution or to association with different insect species or ecotypes? To answer this

question more samples will need to be analysed improving the genetic investigation by a multilocus and integrated approach with variable and house-keeping genes as well as insect population genetics.

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Use of quantitative real time PCR for a genome-wide study of AYWB phytoplasma gene expression in plant and insect hosts

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Abstract

Phytoplasmas are obligate parasites of plants and insects and cause significant crop yield losses worldwide. A number of microarray gene expression studies have been performed to understand better the effects of phytoplasma infection on plant physiology. However, little effort has been made for the study of changes in gene expression patterns of the pathogen itself. Quantitative real time PCR in combination with the delta delta Ct method has been shown to be a relatively inexpensive and simple alternative to microarrays. We employed this method to explore whether it is possible to apply this technique for reliable gene expression quantification of phytoplasmas on a large scale. In our experimental setup, 242 genes of aster yellows phytoplasma strain witches' broom (AY-WB) were tested for differences in expression in plant and insect host environments, and were shown to be predominantly expressed in the plant or insect hosts. *In silico* operon prediction corroborated the experimental data. Our findings suggest that the delta delta Ct method can be used to study the physiology of this pathogen.

Key words: AY-WB, plant, insect vector, gene expression, relative quantification.

Introduction

Phytoplasmas are plant pathogenic bacteria that are transmitted by sap-feeding leafhopper vectors. Due to the economic impact phytoplasmas incur via crop loss, there is an increased interest in studying the effects of the pathogen on its plant hosts. A number of gene expression and protein profiling studies were performed on agronomically important crops severely affected by phytoplasma diseases, including grapevine (Margaria and Palmano, 2011). However, there are few reports of gene expression profiling of the pathogen itself (Ji *et al.*, 2010), reflecting the major difficulties in molecular characterization of this intracellular pathogen. Indeed, phytoplasmas are obligate parasites, which means that RNA preparations contain large portions of host nucleic acids, and hence it is required to utilize highly sensitive methods to quantify phytoplasma transcript levels.

Reverse transcription coupled with real-time PCR (quantitative real-time PCR, qRT-PCR) has been shown to be the most sensitive technique for quantification of mRNA, especially for low-abundance transcripts or tissues with low RNA concentrations (Pfaffl *et al.*, 2002). There are two major approaches to gene expression quantification. The absolute quantification method is considered to be a "golden standard" of gene quantification, however, it requires use of calibration curves with a template of known concentration, which is both laborious and costly. The relative quantification method allows avoidance of the use of standards, as it relies on the relative quantification of a target gene versus normalization genes, in which the latter are constitutively expressed genes involved in the general metabolism of the organism.

Here we report that the relative delta delta (dd)Ct method can overcome many problems encountered in

phytoplasma gene expression quantification. Considering that the phytoplasma genomes are relatively small, the ddCt method may be successfully applied for studying genome-wide gene expression of phytoplasmas in various hosts and over time.

Materials and methods

Aster yellows phytoplasma strain witches' broom (AY-WB) – infected *Macrosteles quadrilineatus* and *Arabidopsis thaliana* were used for this experiment. Healthy *M. quadrilineatus* and *A. thaliana* were used as negative controls. Total RNA was extracted with TRIzol reagent (Carlsbad, CA, USA), treated with DNaseI (Invitrogen) and used for cDNA synthesis (High Capacity RNA-to-cDNA Kit, Applied Biosystems) according to the manufacturers' protocols. To check for efficiency of genomic DNA removal, we included a control in which reverse transcription was omitted. Power SYBR Green Master Mix (Applied Biosystems) and ABI PRISM 7900HT sequence detection system (Applied Biosystems) were used for running qRT-PCR reactions. Primers were designed with Primer Express software (Applied Biosystems).

Two technical replicates were run for each biological sample (5 *Arabidopsis* and 3 populations of *M. quadrilineatus*) and results were expressed as threshold cycle (Ct) values. Replicative DNA helicase *dnaB*, DNA gyrase subunit A *gyrA*, pyruvate kinase *pykF*, 6-phosphofructokinase *pfkA* and docking protein *FtsY* were used as controls for normalization. The ddCt method (Applied Biosystems, User Bulletin 2, 1997) implemented in the REST 2009 software (QIAGEN and Pfaffl) was used for data analysis. OperonDB online tool (Ermolaeva *et al.*, 2001) was used for operon prediction.

Results

To study whether a relative gene expression quantification method could be applied to phytoplasmas in a high throughput manner, an AY-WB pathosystem was used. AY-WB phytoplasma has a unique life cycle that includes both plant and insect hosts. We hypothesised that the adaptation to such different habitats would be reflected in gene expression in the respective hosts, which in turn could be detected by the ddCt method.

Hence, 242 annotated AY-WB genes belonging to different COG groups, and representing 36% of the total number of AY-WB phytoplasma ORFs were selected for the study. These genes also included 56 effector and 20 secreted membrane proteins, which were believed to be potentially involved in phytoplasma – host interactions (Bai *et al.*, 2009). To ensure that each primer pair would amplify only one target we performed a search for homologs for each gene and designed the primers in a gene-specific manner. No amplification was found in healthy samples. Dissociation curves were run after completion of PCR and presence of a single product for each gene assay was confirmed.

Additionally, to increase reliability of the data analysis, amplification of serial dilutions for each gene was performed. REST (Pfaffl *et al.*, 2002) software was used for statistical data analysis. This tool performs sophisticated data analysis, which allows the use of multiple normalization genes and can correct for differences in amplifications efficiencies if standard curve data are available.

The majority of the 242 AY-WB genes tested yielded relative expression values in plant versus insects or insects versus plants between >1 and 3 (not considered significantly regulated), while 47 genes (or 19% of all genes tested) had a significant level of up-regulation, ranging from relative expression values of 3 to 29. These highly expressed genes included members of the COG functional groups: energy production and conversion; nucleotide transport and metabolism; transcription; and posttranslational modification. Several effector and secreted membrane protein genes were also highly differentially expressed.

Discussion

Phytoplasmas are intracellular parasites, unable to grow on an artificial media. This limitation has prevented a rapid progress in understanding the pathogen's biology. However, recent advances in sequencing technology allowed the completion of genome sequencing projects of four phytoplasmas, including AY-WB (Bai *et al.*, 2006). This prepared a framework for functional large-scale studies of this particular phytoplasma. However, there are a few obvious problems encountered during phytoplasma gene expression quantification. Inability to determine pathogen's titre in a high host RNA background makes it impossible to use absolute RT-PCR quantifica-

tion and the normalization of microarray data is difficult. Relative quantification (ddCt method) overcomes these problems. Our results show that there are differences in expression of AYWB phytoplasma genes in plant and insect hosts, suggesting that this method can be robustly used for study phytoplasma physiology and host adaptation. The experimental data were corroborated by *in silico* operon prediction, as genes predicted to belong to the same operon exhibited similar levels of expression. This method of gene expression analysis opens new possibilities for studying biology of this pathogen on a large scale, for example by comparing phytoplasma gene expression between different plant hosts, different tissues of the same plant or by performing genome-wide gene expression studies.

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Universal primers for plasmid detection and method for their relative quantification in phytoplasma-infected plants

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Abstract

Rep gene occurring in the majority of plasmids associated with phytoplasmas was selected as a target for real-time PCR together with single-copy *Tuf* gene from the phytoplasma genome. *Rep*-specific primers amplified products from host plants infected with phytoplasmas of groups 16SrI, -II, -III, -V, -X, and -XII. Relative concentrations of plasmids ranged depending on strain from 1-2 copies to several hundred copies per each phytoplasma studied. Primers targeting the *rep* gene are proposed for direct PCR detection of phytoplasma plasmids from experimental periwinkle host as well as from natural plant hosts.

Key words: plasmid, universal primers, *rep* gene, phytoplasma.

Introduction

In addition to the relative small genome of 530 to 1,350 kbp (Marcone *et al.*, 1999), circular extrachromosomal DNAs of various sizes have been detected on phytoplasmas 'Candidatus Phytoplasma asteris', 'Ca. P. trifolii', 'Ca. P. pruni' and 'Ca. P. australiense'. The origin of these molecules has not been clearly elucidated, but similar A+T total content to that of the host phytoplasma implies a putative phytoplasma origin. The *repA* or *dnaG* proteins accompanied by a single-strand DNA-binding protein (*ssb*) involved in self-replication have been identified by sequence comparison in all of these plasmids.

Observed sequence variability and the occurrence or absence of distinct open reading frames (ORFs) in various beet leafhopper-transmitted virescence phytoplasma strains suggest that the genes carried are not essential to phytoplasma viability and replication, but they may encode other factors that are required at various levels or during different phases of phytoplasmas life cycle (Liefing *et al.*, 2004).

It has been documented that several plasmids are present in distinct phytoplasma strains: four plasmids from 1.7 to 5.2 (or 7.4) kbp in size have been detected in Western aster yellows phytoplasma and tulalake aster yellows phytoplasma, respectively, in China aster, celery, or periwinkle hosts (Kuske and Kirkpatrick, 1990). Another set of four plasmids 3.8 to 5.1 kbp in size was identified in lettuce infected with aster yellows witches' broom phytoplasma (Bai *et al.*, 2006). Different strains of onion yellows phytoplasma contained two or three small plasmids in garland chrysanthemum (Nishigawa *et al.*, 2003). No relationship has been observed between number of plasmids and distinct host and/or phytoplasma species or plasmid influence on severity of disease symptoms, but, in fact, no detailed evaluation has yet been performed.

Here we present a PCR amplification method for plasmid screening in phytoplasma infected plants and evaluation of their relative quantification.

Materials and methods

Periwinkle plants infected with phytoplasmas from ribosomal groups 16SrI, -II, -III, -V, -X, -XII as well as collected samples of apple, pear, red clover, red currant, peach, poplar, oak, grapevine, willow, and many herbaceous plants were tested. Total DNA was isolated from 0.1 g of leaf tissue with the NucleoSpin PlantII kit (Macherey-Nagel, Germany) according to the manufacturer's instructions and eluted with 50 µl of water. The single copy *tuf* gene from the phytoplasma genome was amplified as a reference gene with primers fTufu/rTufu (Schneider *et al.*, 1997) in parallel with amplification of the *rep* gene localized on plasmid with Rep200/Rep750 primers (table 1). The 20 µl reactions were performed on an iCycler (Bio-Rad) in the presence of 0.3 µM of each primer, 2.5 mM MgCl₂, 200 µM of each dNTP, 1X PCR buffer, 1.25 U of DreamTaq DNA polymerase (Fermentas), 1x SYBR Green I, and 1µl of DNA. Cycling conditions were as follows: 95°C for 2 minutes, 40 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute. The amplification products were electrophoresed on agarose gel (figure 1), excised, extracted with NucleoSpin Extract II kit (Macherey-Nagel, Germany), and sequenced with *rep* 750 primer using a BigDye sequencing kit (Applied Biosystems).

Results

Amplification products of about 550 bp size were obtained using the Rep220/Rep750 primers in all infected periwinkle plants and also in samples from original host plants. Healthy periwinkle did not produce such amplicon. Nucleotide sequence comparison revealed onion yellows phytoplasma as the most similar sequence.

The threshold value (C_T) for the reference *Tuf* gene product was around 23, the C_T of *Rep* ranged from 14 to 23, depending on the sample (figure 2). The calculated plasmid copy number was from 1 to about 500 per single phytoplasma genome.

Table 1. Primers used in this work.

Primer name	5'-3' sequence	Reference
Rep200	TATATTTAAGATTTAATTATGC	this work
Rep750	ACGTAGGTCATCTAAAATAATAC	this work
fTufu	CCTGAAGAAAGAGAACGTGG	Schneider <i>et al.</i> , 1997
rTufu	CGGAAATAGAATTGAGGACG	Schneider <i>et al.</i> , 1997

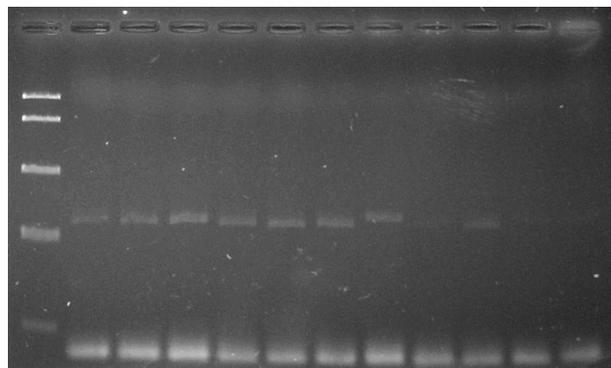


Figure 1. PCR amplification product of grapevine yellows samples with Rep200/Rep750 primers.

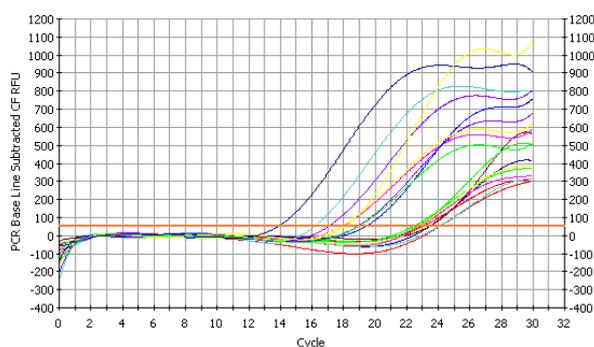


Figure 2. Real-time Amp/Cycle graph of 9 phytoplasma infected samples with Rep and Tuf primers. (In colour at www.bulletinofinsectology.org)

Discussion

Two different *rep* genes could drive the replication of phytoplasma plasmids. The Rep200/Rep750 primers amplify efficiently the plasmid related to pOYNIM (Nishigawa *et al.*, 2003), but not the one related to pBLTVA or to pPaWB_{Ny} (Liefiting *et al.*, 2004; Lin *et al.*, 2009). However, both types of plasmid were detected also in one host (Bai *et al.*, 2006). Assuming that presence of phytoplasma plasmids is inseparable from phytoplasma itself, the Rep200/Rep750 primers are recommended for routine screening of phytoplasmas in natural hosts.

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'Flavescence dorée' phytoplasma strain differentiation in the translocase (secY) gene

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Abstract

'Flavescence dorée' (FD) associated phytoplasmas are molecularly distinguished in strains belonging to 16SrV-C and -D subgroups that are geographically separated in the majority of the European countries where the disease was reported. While the subgroup differentiation on the 16S ribosomal gene is quite stable, RFLP analyses on the translocase gene allow differentiation of subtypes in both subgroups confirming ability of FD phytoplasmas to rapidly differentiate lineages with possibly diverse aggressiveness.

Key words: 'Flavescence dorée', secY gene, strain differentiation, epidemiology.

Introduction

'Flavescence dorée' (FD) is a quarantine phytoplasma disease in Europe still expanding its geographical distribution despite all the quarantine measures applied in many countries over the last 20 years. The major problem viticulturists are facing is the great ability of phytoplasmas associated with this disease to differentiate new strains in short periods of time. The molecular differentiation of FD strains present in diverse grape growing areas where the disease is present, is therefore of major relevance towards a correct disease management.

Several molecular markers were employed to differentiate FD strains after the first identification of two subgroups in 16S ribosomal gene (Bertaccini *et al.*, 1997; Martini *et al.*, 1999). One of the most informative and widely employed was shown to be the translocase gene (SecY) (Martini *et al.*, 2002; Botti and Bertaccini, 2007; Arnould *et al.*, 2007) therefore a molecular comparison among several FD strains from various locations in Italy and in Serbia, collected in different years was carried out.

Materials and methods

Selected grapevine samples collected in Italy and in Serbia in the period 2002-2010 were employed to verify presence of variability in the secY gene. After total nucleic acid extraction PCR/RFLP analyses on 16S ribosomal gene were carried out to distinguish between 16S ribosomal subgroups 16SrV-C and 16SrV-D. The strains were further examined by PCR/RFLP analyses on SecY (translocase) genes (Martini *et al.*, 2002; Angelini *et al.*, 2001). To further evaluate variability two strategies (I and II) were employed. In (I) selected FD9f3/r2 amplicons from 16SrV-C strains were purified and cloned by InsT/Aclone PCR Product Cloning Kit

(Fermentas, Vilnius, Lithuania) specific for PCR fragments. Recombinant clone selection was carried out by PCR amplification using universal M13f/r primers on the colonies and/or nested PCR on M13f/r amplicons diluted 1:30 in sterile water with primers used to generate the PCR fragments cloned. Amplicons obtained from 4 to 20 colonies per cloned fragment were then subjected to RFLP analyses with *AluI*, *TruI*, *TaqI*, and *Tsp509I* restriction enzymes (Fermentas, Vilnius, Lithuania) to verify consistence of restriction profiles with those derived from the non-cloned amplicons. In strategy (II) selected FD9f3/r2 amplicons from 16SrV-D strains were produced with nested-PCR amplification carried out at least 3 times from each amplicon obtained from direct PCR and then RFLP analyses were carried out on all amplicons with the above listed enzymes in order to verify profile consistency.

Results

The examined strains belonging to subgroup 16SrV-C showed RFLP polymorphisms with *TruI* and *TaqI* restriction enzymes on SecY gene that resulted partially related to their geographic origin. In particular strain differentiation was achieved for samples from Serbia and Italy and their tentative grouping showed identity between strains from Aleksandrovac (Serbia) and Tuscany (Italy). RFLP profiles identity was present also among samples from Niš, Irig (Serbia) and Veneto (mainly Treviso province) (Italy). Further RFLP profiles differentiable from each other, and from all the previous ones were also identified in samples from Niš and Irig.

The RFLP analyses on SecY gene from 16SrV-D strains collected in Veneto region show identical profiles with *TruI*, *Tsp509I* and *TaqI* restriction enzymes with reference strain FD-88 from France (kindly provided by E. Boudon-Padieu). This profile was clearly

differentiable from the one identified in the majority of samples from Emilia (Lambrusco varieties) (Italy).

Some strains from Italy (VR32 and PC4, collected in 2002 in Veneto and Emilia, respectively) showed a 16SrV-C profile in 16S ribosomal gene, but the profiles on SecY gene were either consistent with 16SrV-C or -D, according with the enzyme used. Cloning of strains PC4 and VR32, showed RFLP profiles not always confirming those detected in the non cloned amplicons, although the PCR products were of the expected length, however the most frequent profile was identical to the one of the not cloned amplicons (figure 1). The profile comparison allow to verify that one of the profiles obtained from cloning of sample PC4 is undistinguishable from profiles obtained with the same restriction enzyme from samples of Lambrusco varieties in 2009 and 2010 samples (figure 1). On the other hand the use of strategy II allow the identification of polymorphic profiles in different PCR assays from the same direct amplicons in both samples from Emilia and from Veneto (data not shown).

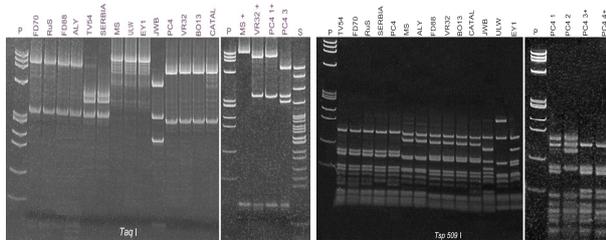


Figure 1. RFLP profiles obtained from FD strains and controls on secY compared with those on cloned amplicons. +, most common profile in 2002 samples that was retrieved in 2009-2010 samples from Emilia.

Discussion

The RFLP analyses on nested amplicons and on clones or on repeated amplicons allow to verify the presence of variability on SecY gene from 16SrV-C strains from Italy and Serbia and on 16SrV-D strains collected in Veneto and in Emilia. The results not only confirm the strain differentiation in SecY gene of FD-D type phytoplasmas according with geographic distribution and variety (Bertaccini *et al.*, 2009) but also the temporal differentiation of strain population in different geographic areas of Italy. The presence of heterogeneous profiles in the different colonies obtained after cloning and in amplicons from the same template suggests the presence of further variability not observed in 'regular' amplicon RFLP analyses.

These results can be explained with the presence of a population of distinguishable phytoplasmas infecting the same plant (Angelini *et al.*, 2004). The repeated finding of profiles only detected in one clone of a FD-C strain from 2002 in FD-D strains from 2009 and 2010 in the same region indicates the increase in a few years of a phytoplasma population barely detectable about 10 years ago. The results indicate that now this phytoplasma is prevalent in some of the areas where the

disease is spread. The presence of phytoplasma population is not very easy to be detected in routine sample analyses, but it is clear that a mixture of heterogeneous phytoplasmas colonizing new environments, or the residues of old phytoplasma populations, that lost transmissibility or virulence characteristics can play important roles in FD epidemiology.

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Molecular diversity of 'flavescence dorée' phytoplasma strains in Slovenia

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Abstract

The 'flavescence dorée' (FD) phytoplasma is associated with the most devastating grapevine yellows disease 'flavescence dorée'. It belongs to the ribosomal group 16SrV in which a high 16SrDNA identity among subgroup members exists. To investigate the genetic diversity of this phytoplasma in Slovenia a large analysis of strains in known and possible hosts and vectors was performed. The genotyping has revealed the occurrence of FD strains FD1, FD2 and FD3. Although isolates of FD2 and FD3 were detected in grapevines, those of FD2 prevailed. On the other hand, symptomatic as well as asymptomatic clematis (*Clematis vitalba*) plants were exclusively infected with FD3. Strains undistinguishable from FD1, FD2, FD3 and alder yellows were also detected in *Alnus glutinosa* and *A. incana*. In some alder samples mixed infections with different strain combinations were also demonstrated. FD2 strain was shown in the leafhopper vector of the disease *Scaphoideus titanus*. The FD1 strain was detected in the mosaic leafhopper *Orientus ishidae*. In addition, various specimens of this leafhopper were positive in PCR with a high presence of mixed infections.

Key words: 'Flavescence dorée', genetic clusters, *Vitis vinifera*, *Clematis vitalba*, *Alnus glutinosa*, *Alnus incana*, *Scaphoideus titanus*, *Orientus ishidae*, *Oncopis alni*.

Introduction

Europe is the world's major producer and exporter of grapevine (*Vitis vinifera*) cuttings and wine. This important economic segment is currently facing an epidemic threat associated with the 'flavescence dorée' disease, which has been known in France since the middle of the 1950s (Boudon-Padieu, 2002). The disease spread to other European countries and was detected for the first time in Slovenia in 2005. Since then the number of foci of the disease in Slovenia has been growing rapidly.

The agent associated with 'flavescence dorée' is FD phytoplasma, which is transmitted among grapevines by insects. The only known natural vector of FD is the leafhopper *Scaphoideus titanus*. However, some other leafhoppers e.g. *Orientus ishidae* (Mehle *et al.*, 2010) and *Dictyopara europa* (Filippin *et al.*, 2009) have been shown to harbour FD phytoplasmas. The latter was also shown to transmit FD from clematis (*Clematis vitalba*) to grapevine. The alder yellows phytoplasma (AldY), which is genetically related to FD, has been detected in more than 85% of alder trees in SW France (Malembic-Maher *et al.*, 2009). These findings support the hypothesis that these plants might be a natural source of FD. FD belongs to the ribosomal group 16SrV with high 16SrDNA identity among the subgroups (Lee *et al.*, 2004). The phylogenetic analysis has shown the existence of three genetic clusters FD1, FD2 and FD3 (Arnaud *et al.*, 2007, Filippin *et al.*, 2009). AldY strains do not form a homogenous phylogenetic group but are distributed in every cluster, except FD3 (Malembic-Maher *et al.*, 2009). In order to study the prevalence and distribution of FD strains in Slovenia a molecular comparison of FD strains involved in former outbreaks in Slovenian vineyards, and strains from clematis and alder plants as well as from *Scaphoideus titanus*, *Orientus ishidae*, and *Oncopis alni* was carried out.

Materials and methods

Under the official survey of the Phytosanitary Administration of the Republic of Slovenia, 1,679 symptomatic grapevine (*V. vinifera*) samples were collected from 2002 to 2010. They were sampled in the vineyards from the three Slovenian winegrowing regions - SW Primorska, SE Posavje and NE Podravje. In addition, clematis, *Alnus glutinosa* and *A. incana*, as well as insect specimens of *S. titanus*, *O. ishidae* and *O. alni* were also collected. All samples were analyzed for the presence of FD with a real-time PCR procedure (Hren *et al.*, 2007). Further molecular characterization was than performed on the positive samples by PCR with FD9R1/FD9F1 primers followed by nested PCR with FD9F3b/ FD9R2 primers and RFLP. The purified nested PCR products were cloned into pGEM-T vector and sequenced.

Results

From tested grapevine samples, 123 were proved to be FD positive and 86 of them were further analyzed. Amplicon digestion with the restriction enzymes *HpaII*, *TaqI* or *AluI* revealed that the genetic cluster FD2 represented the 66.3% of all analyzed grapevine samples and that FD3 occurred in the 31.4% of the samples. We did not detect strains in the FD1 cluster in any grapevine sample. However, 13 dubious samples are still under investigation. From 69 tested clematis samples, 49 were FD positive and all of them had the FD3 profile. The incidence of FD infection in alder trees was very high and mixed infections were frequent. In alders all three genetic clusters were detected, as well as the AldY one (table 1).

The RFLP pattern of sample from *S. titanus* was identical to those of the reference FD92 from the genetic cluster FD2. Mixed infection in various samples from

Table 1. Distribution of FD clusters in plants and insects in Slovenia. Frequency of occurrence (%) in brackets; OWG, out of wine growing region.

	FD1 (%)	FD2 (FD-D) (%)	FD3 (FD-C) (%)	AldY (%)	Winegrowing region
<i>Vitis vinifera</i>	0	57 (66.3)	16 (31.4)	0	SW, SE, NE
<i>Clematis vitalba</i>	0	0	49 (100)	0	SW, SE, NE, OWG
<i>Scaphoideus titanus</i>	0	1 (100)	0	0	SE
<i>Alnus glutinosa</i> and <i>A. incana</i>	3 (16.7)	7 (38.9)	7 (38.9)	1 (5.6)	OWG
<i>Orientus ishidae</i>	3 (25.0)	6 (50.0)	3 (25.0)	0	OWG
<i>Oncopsis alni</i>	1 (14.2)	0	3 (42.9)	3 (42.9)	OWG

O. ishidae and *O. alni* were common. However, the sequence analysis from the former demonstrates the presence of FD1, FD2 and FD3. In the latter the FD2 cluster has not been confirmed, but AldY was present (table 1).

Discussion

The molecular analysis of FD strains in Slovenia showed a high diversity of genetic clusters and their distribution among host plants, and established, as well as putative, insect vectors. As in France (Salar *et al.*, 2009) the genetic cluster FD2 was the prevailing one in grapevine and was not related to any specific winegrowing region. The strains belonging to FD3 were detected in about one-third of the grapevine samples and were exclusively present in FD infected clematis plants. Clematis samples were collected in the vicinity of the FD infected vineyards, but also in those where FD had been never confirmed or even outside the winegrowing regions. Some of the infected clematis plants showed the symptoms, but the frequency of totally asymptomatic but infected plants was also very high. The collective evidence of FD3 distribution among clematis plants in Italy, Croatia and Macedonia (Filippin *et al.*, 2009) as well as in Slovenia suggests that this plant species might constitute a wild reservoir of FD3 strains. High incidence of FD strains in alder trees in an agreement with the situation in France (Malembic-Maher *et al.*, 2009) was detected. Alders hosted phytoplasmas from clusters FD1, FD2 and FD3 and AldY. Phytoplasmas from the same clusters with the exception of FD2 were detected in *O. alni*, the insect which has been confirmed as vector of Palatinate grapevine yellows (Maixner *et al.*, 2000). FD was only detected in one out of 57 tested samples of *Scaphoideus titanus*. However, the strain belonged to the FD2 cluster. On the other hand, in the polyphagous leafhopper *O. ishidae*, the presence of both FD1 and FD2 was confirmed (Mehle *et al.*, 2010). To shed light on the role of *O. ishidae* in the possible transmission of FD from reservoir host plants, or from grapevine to grapevine research is in progress.

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Discriminating 16Sr groups of phytoplasmas by an oligonucleotide microarray targeting 16S-23S spacer region

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Abstract

A microarray detecting phytoplasma 16Sr groups -I, -II, -III, -V, -VI, -X and -XII was developed based on probes targeting 16S-23S spacer region. Despite that it is considered as more suitable than 16S rDNA for the fine differentiation of phytoplasmas, the microarray did not demonstrate discriminatory potential higher than the only phytoplasma microarray published so far based on 16S rDNA. Nevertheless the method used was cheaper and faster bringing the microarray towards routine analysis.

Key words: phytoplasmas; detection; microarray; 16Sr groups; 16S-23S ribosomal spacer region.

Introduction

For phytoplasma classification the RFLP analysis of 16S rRNA gene is traditionally used, dividing phytoplasmas into ribosomal groups. However, this technique is laborious, sensitive to point-mutations, and only one sample can be analyzed per reaction. Therefore, new techniques have been searched for enabling reliable determination and broader, high-throughput screening of phytoplasmas. Such a technique is represented by microarrays (Hadidi *et al.*, 2004), allowing detection of many different sequences in parallel. In contrast to several microarrays discriminating plant viruses (Boonham *et al.*, 2008), and bacteria (Huyghe *et al.*, 2009), only one microarray detecting phytoplasmas (Nicolaisen and Bertaccini, 2007) was developed so far. Furthermore, not all 16Sr groups reported so far were fully distinguished by this microarray based on 16S rDNA. The authors suggested in accordance with the literature, that probes targeting 16S-23S spacer region may be more appropriate for phytoplasma identification. Therefore, the aim of this study was to develop a microarray to test

phytoplasma discriminatory potential of 16S-23S spacer region.

Materials and methods

Phytoplasmas under study were identified strains belonging to 16Sr groups -I, -II, -III, -V, -VI, -X and -XII obtained from collection maintained by A. Bertaccini, Italy. Total DNA was extracted from each sample by NucleoSpin Plant II kit (Macherey-Nagel), diluted 1: 50 in distilled water and 1 µl was used in subsequent PCR. PCR was performed using PCR Master Mix (Fermentas, Vilnius, Lithuania), the 5'-GGGATGGATCACCTCCT TTC-3' was the forward, and the Cy3-5'-ACAAACCC CGAGAACGTATTC-3' was the reverse primer; 35 cycles of 93°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute were carried out to achieve the reaction. Amplified labeled fragments (3 µl) were checked by gel-electrophoresis (1% agar; 0.5xTBE) and purified (17 µl) using GenElute PCR CleanUp kit (Sigma-Aldrich) into 70 µl of final volume.

Table 1. Probes designed according to the alignment – position; accession number are listed for orientation only; lower case in probe sequence: random nucleotides.

16Sr group	probe name	probe sequence (5' – 3')	position	accession number
I	1a	AAGAAAGTTTTTCATTGTAACCTTGCTTGCAAATTG	1527-1561	HM590616
I	1b	agtccgTATTTGCAACATTTTAATCTTTTTAAactaaag	1601-1626	HM561990
II	2	ttaAAATAATAGTTATTTATCCGGAAACATTAAAGtcc	212-243	DQ471318
III	3a	tcgcaagcgTTCTTTTTTAAGGCATTAAGGaatgcatcc	1558-1578	HQ589207
III	3b	CAGTTTATTTTAAAAGTTATAAGCACTGTCTTAAAAA	1678-1714	HQ221554
III	3c	CCAGTTTATTTTAAAAGTTATAAGCACTGTCTTAAA	1677-1712	HQ221554
V	5	tgteCAATTTTATATCAGGAAATTATTTACTTCGAAGctga	1712-1744	HQ199312
VI, VII	6/7	cggctaactCAAAAAAAGGTCTGCTTAAAcgctacttga	1582-1603	HQ589189
IX	9	agtccgGTTTTTCTGATTTATTTTGTTTTTTtgctaaag	1551-1575	AF515637
X	10	TTTTTTATTTTAAAGATAAAAATCAATAATGGCTTGGGC	1556-1595	AJ575106
XII, XIII	12a	aacatCAAAAATAGGTCACATCTTAAAAAAGCTCgcattc	1642-1670	HQ589193
XII, XIII	12b	catCAAATAAAAATAGGTCACATCTTAAAAAAGCTCgca	1668-1701	FJ943262

Two microlitres of DNA targets were sequenced directly (BigDye Terminator kit, Applied Biosystems).

Capture probes (30 - 40 nt) were designed according to the alignment of 16S-23S spacer sequences (GenBank). Shorter probes were extended by random sequences on both ends (table 1). Hybridization of targets (15 µl), microarray washing, scanning and evaluation were done as described previously (Lenz *et al.*, 2010). Only spots with signal-to-noise ratio ≥ 3 were considered as positive.

Results

Twelve capture probes targeting ribosomal spacer of phytoplasma 16Sr groups -I, -II, -III, -V, -VI, -IX, -X and -XII, -XIII were designed. Two of the probes were specific for more than one group (table 1). Sequences of the other groups were not available in GenBank at the time of design. Two double-dots of each probe were printed per microarray. Three replicate samples were hybridized from each of the eight groups tested. All hybridization patterns including cross-hybridization observed (figure 1) correspond to target-to-probe homology revealed by sequencing.

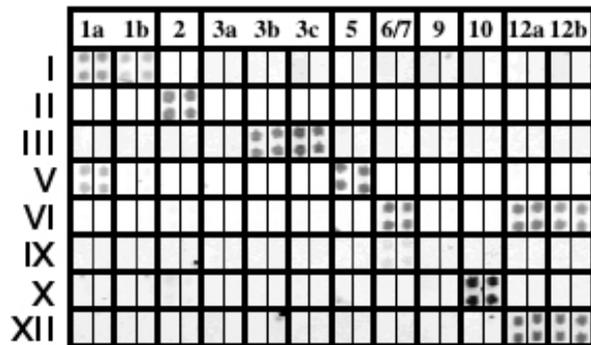


Figure 1. Hybridization results (bleached, inverted colors, contrast adjusted). Rows: 16Sr group of phytoplasma hybridized; columns: name of probes.

Targets of each 16Sr group hybridized to relevant group-specific probes except for probes 3a and 9. For both of these, hybridized groups contained different sequence variant in the probe-targeted site. Furthermore, targets of group 16SrV and 16SrVI hybridized also to the probes designed for groups 16SrI and 16SrXII, respectively. Below-threshold cross-hybridization of 16SrIX group to the probe 6/7 also appeared.

Discussion

In the literature, the usability of 16S-23S ribosomal spacer for better phytoplasma discrimination has been suggested several times. To test this possibility, the microarray targeting different 16Sr groups was developed. Not all 16Sr groups possess unique regions of sufficient

length and three of the probes (6/7, 12a, 12b) were not specific for one group only. Furthermore, probe 1a showed significant similarity to 16SrV group in hybridization experiments, and targets of 16SrVI group hybridized to probes 12a and 12b, also. Despite these unexpected cross-hybridizations, all targets hybridized to group-specific probes except of group 16SrIX, variability of which need to be targeted by additional probes.

Compared to the only microarray published so far detecting phytoplasmas based on 16S rDNA (Nicolaisen and Bertaccini, 2007), ribosomal spacer does not provide more discriminating potential. Given that 23S rDNA is much more conserved than both of these, microarray for proper phytoplasma determination has to be based on the other less-conserved genes.

By contrast to the two-step Cy3-dNTPs labelling technique and overnight hybridization used in the previous mentioned paper, the microarray developed here used single PCR Cy3-primer labelling and one-hour-only hybridization, making the process of phytoplasma determination cheaper and faster, thus shifting it more towards a real application.

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Survey and genetic diversity of phytoplasmas from the 16SrV-C and -D subgroups in Hungary

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Abstract

In order to evaluate the risk represented by the wild reservoir as a possible source of 'flavescence dorée' outbreaks in Hungary, diverse wild perennial plants growing in vineyard areas were tested for the presence of 16SrV-C and D subgroup phytoplasmas. 16SrV phytoplasmas were detected by nested PCR-RFLP on the 16SrDNA in alders (86% infected) and in clematis (71% infected). Further characterisation by sequencing of the *map* gene revealed in both plants strains having the same *map* gene sequence as 'flavescence dorée' strains.

Key words: alder yellows, grapevine yellows, clematis, molecular typing, plant reservoir, *Mollicutes*.

Introduction

'Flavescence dorée' (FD) is a quarantine disease of grapevine spreading in south European vineyards. Although not reported in Hungary, it is present in the bordering country of Serbia (Duduk *et al.*, 2004). Because *Scaphoideus titanus* Ball, the vector of the phytoplasma, has been described in the south and in the centre of Hungary (Der *et al.*, 2007), there is an important risk of introduction of the disease in the country by infectious insects.

Another source origin for outbreaks could be the transfer of phytoplasmas from wild plants to grapevine. Indeed, phytoplasmas of the 16SrV-C and -D subgroups, genetically close to FD phytoplasmas, have been detected in alder (AldY phytoplasma) (Arnaud *et al.*, 2007; Malembic-Maher *et al.*, 2011) and in clematis (Filippin *et al.*, 2009). It has been shown that they can be transmitted to grapevine by occasional feeding of insect vectors living on these wild plants surrounding vineyards (Maixner *et al.*, 2000; Filippin *et al.*, 2009). With the presence of *S. titanus* in the vineyards, the phytoplasma could be spread epidemically.

In order to evaluate the risk originating from wild reservoirs, wild perennial plants collected in different parts of Hungary were screened for the presence of phytoplasmas of the 16SrV-C and -D subgroups. Phytoplasmas detected were further characterized by sequencing of the *map* gene.

Materials and methods

Between 2007 and 2009, 62 plant samples were collected in the Hungarian counties of Győr-Sopron, Veszprém, Zala, Somogy, Pest and Heves (figure 1). Samples collected were asymptomatic *Alnus glutinosa*, *Clematis vitalba* and other wild perennial plants showing symptoms of yellowing, reddening and, in some cases, dwarfism and small leaves. After

total DNA extraction, samples were tested by nested PCR-RFLP on the 16SrDNA with the primers P1/P7 (Schneider *et al.* 1995) and R16F2n/R2 (Gundersen and Lee, 1996) followed by *TruI* digestion of the amplification products. Restriction profiles were compared with those of reference strains from the 16SrV and 16SrXII group. Samples detected positive for phytoplasmas of the 16SrV group were further characterized by nested-PCR followed by sequencing of the *map* gene as described in Arnaud *et al.* (2007). Sequence analyses, multiple alignment and phylogenetic tree (method of parsimony) were performed including reference *map* gene sequences of the 16SrV group previously described in Arnaud *et al.* (2007) and in Malembic-Maher *et al.* (2011).

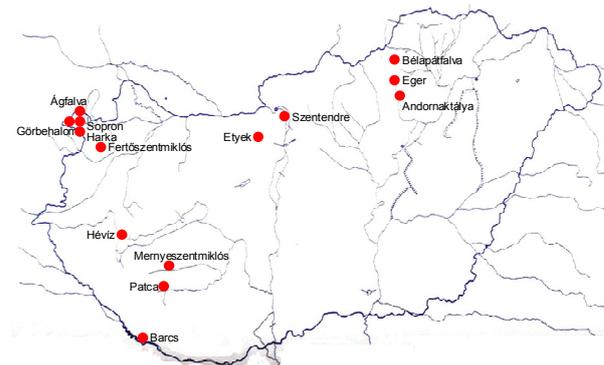


Figure 1. Localisation of the sampling sites in Hungary.

Results

Sequence typing and phylogenetic analysis based on *map* gene are presented in figure 2. Phytoplasmas of the 16SrV group were identified in 31 of the 36 alders tested. Among these, 22 samples were characterized on the *map* gene. Nine were found infected with a mix of AldY strains. Twelve were infected with

strains belonging to Map-AldY genetic clusters, some having the same *map* gene sequence as Palatinate grapevine yellows phytoplasmas from France and Germany (AM384890 and AM384892). One, B38, was infected by a strain belonging to the Map-FD1 cluster with a *map* genotype identical to FD and AIY strains from south-west France (AM238512). Ten of the 14 clematis were positive for 16SrV group phytoplasmas. The 6 strains genotyped all belonged to the Map-FD3 cluster and had the same *map* gene sequence as a FD strain from Italy (FN811141). None of the other 12 perennial wild plants were infected by 16SrV phytoplasmas, but one (*Fragaria sp.*) was infected by a 16SrXII group phytoplasma.

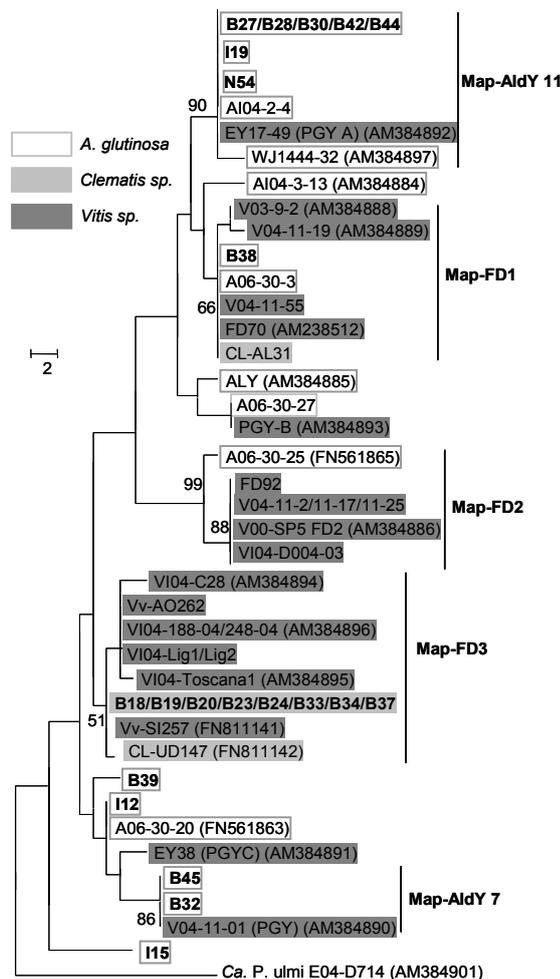


Figure 2. Phylogenetic tree constructed by *map* gene sequence analysis of the 16SrV group phytoplasma strains collected in Hungary. Hungarian strains are in bold and begin by the letters B, I and N. Reference strains are described in Arnaud *et al.* (2007) and Malembic-Maher *et al.* (2011).

Discussion

Genotyping studies have shown that phytoplasmas genetically closed to FD phytoplasma epidemic strains are present in Hungarian alders and clematis.

A recent survey conducted in 2010 has identified the presence of insect vectors *Oncopsis alni* and *Dictyophara europaea* which were shown to transmit these phytoplasmas from alders and clematis respectively, to grapevine (Maixner *et al.* 2000; Filippin *et al.* 2009). Characterisation of the strains present in the vectors is in progress. These findings show that the wild reservoirs such as alders and clematis constitute a risk for FD outbreaks in Hungary if *S. titanus* continues to colonize the vineyard.

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Deep amplicon sequencing reveals mixed phytoplasma infection within single grapevine plants

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Abstract

The diversity of phytoplasmas within single plants has not yet been fully investigated. In this project, deep amplicon sequencing was used to generate 50,926 phytoplasma sequences from 11 phytoplasma-infected grapevine samples from a PCR amplicon in the 5' end of the 16S region. After clustering and alignment to phytoplasma reference sequences it was shown that the phytoplasmas in the 11 plants belonged to diverse 16Sr groups and that a high number of single nucleotide polymorphisms were present.

Key words: pyrosequencing, phytoplasma, population, molecular identification.

Introduction

Phytoplasmas within single plants can be considered as populations of individuals, and as phytoplasmas are obligate parasites of plants, there are no possibilities to know unless after obtaining single clones. For identification of phytoplasmas, PCR is usually followed by RFLP or by sequencing the PCR product directly or by sequencing cloned PCR products. This procedure will, however, not show the diversity of the population, as only the most frequent genotypes will be detected and identified, unless many clones are sequenced.

Deep amplicon sequencing has been used to analyse complex microbial communities in a number of environments (Huse *et al.*, 2008), but never applied to phytoplasma detection. In this project, we selected a number of grapevine samples (figure 1), in which mixed phytoplasma infections was preliminary detected by nested-PCR technique, for deep amplicon sequencing on the Roche Genome Sequencer FLX system preliminary experiments.

Materials and methods

Samples employed were selected from those that are routinely processed every year during surveys for phytoplasma detection and identification in Northern Italy 'flavescence dorée' infected areas (Veneto region). Total DNA of 30 samples was extracted from 1 g of mid-vein leaf tissue following the procedure of Prince *et al.* (1993) and 10 more samples were extracted following the procedure of Angelini *et al.*, (2001). Phytoplasma detection was carried out by direct PCR on ribosomal gene and spacer region (Martini *et al.*, 2002) followed by nested amplification with R16(I)F1/R1 (Lee *et al.*, 1994) and 16R_{758F}/V₁₇₃₀ (Martini *et al.*, 1999) primer pairs. RFLP analyses with *TruI* on the first amplicons and *TaqI* on the second one allow identification of 16SrXII (stolbur) and 16SrVC/D ('flavescence dorée') phytoplasmas after 5%

polyacrilamide gel electrophoresis.

Among samples showing mixed phytoplasma infection 11 were chosen for deep amplicon sequencing. Tagged primers (forward primer: primer A – TAG – 16F2n; reverse primer: primer B – ACTTAYTAAACC GCCTACR-CACC) were used for generating pyrosequencing samples. PCR cycles were: 94°C for 3 minutes followed by 35 cycles at 94°C for 15 seconds, 64°C for 30 seconds, and 72°C for 1 minute. PCR products were pooled in equimolar amounts, run on an agarose gel and a band of the correct size was excised from the gel and purified using QIAquick gel extraction kit from QIAGEN. This pool of 11 samples was sequenced on a GS FLX plate at Eurofins MWG.

Tag-sorted sequences were quality filtered using CLOTU software at the Bioportal webportal (<http://www.bioportal.uio.no/>). To minimize sequencing errors, only the first ~200 nucleotides of each sequence were used. Accepted sequences were clustered using CD-HIT with a 99% similarity threshold, and singleton sequences were discarded. To identify sequences, these were aligned together with reference sequences from GenBank using MEGA and phylogenetic trees were constructed.

Results

A total of 74,817 sequences were generated from the 11 field collected samples of phytoplasma infected grapevine. After quality filtering, 50,926 sequences remained, varying from 1,914 to 9,868 sequences per sample among the 11 samples. After clustering at 99% similarity threshold, sequences were aligned to a reference set of sequences of known identity. This showed that 11,730 sequences belonged to phytoplasma 16SrV group, 38,456 sequences were belonging to 16SrXII group, 576 sequences were related to 'Candidatus Phytoplasma prunorum' whereas the last 164 sequences could not be assigned to a single phytoplasmas group.



Figure 1. Grapevine cv Chardonnay showing symptoms of phytoplasma infection.
(In colour at www.bulletinofinsectology.org)

There was a large variation in the number of sequences within each sample: 16SrV group ranged between 33 to 3,009 sequences; 16SrXII from 0 to 6,823 sequences; ‘*Ca. P. prunorum*’ from 0 to 278 sequences. Apart for the overall grouping of phytoplasmas, a high number of single nucleotide polymorphisms were detected in the sequences.

Discussion

Phytoplasmas are unable to survive on an artificial growth media available so far, therefore identification to 16Sr group or ‘*Candidatus* species’ must be carried out on infected plant material. This means that identification is carried out on populations of phytoplasmas rather than on individual clones, which is the case for most microorganisms. To investigate the variation of phytoplasma sequences within single plants, a number of samples were selected that showed mixed phytoplasma presence in nested-PCR, however the detection system employed was only target to the detection of 16SrV-C/D and 16SrXII phytoplasmas since these are known to be associated with the majority of the phytoplasma diseases in grapevine in Europe (Botti and Bertaccini, 2007).

These samples were subjected to deep amplicon sequencing in which the sequence of individual PCR

products is determined without the need of cloning.

From the 11 samples examined a total of 50,926 good quality sequences were analysed. Several 16Sr groups/‘*Candidatus*’ species were identified, but also a high number of single nucleotide polymorphisms was found. This experiment demonstrates that phytoplasmas in individual grapevine plants are composed of highly diverse populations of individuals and that there are possibility to employ pyrosequencing techniques to verify presence of mixed phytoplasmas populations in naturally infected samples.

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Development of a duplex TaqMan real-time PCR for the general detection of phytoplasmas and 18S rRNA host genes in fruit trees and other plants

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Abstract

The detection of phytoplasmas in fruit trees and other important crops is demanded from nurseries and certification agencies. We have therefore developed an efficient assay for general detection of phytoplasmas. This duplex assay combines the phytoplasma detection with an internal control, detecting host gene 18S rRNA in the same reaction. The internal control enables the confirmation of an efficient DNA extraction and the recognition of eventual inhibition of the PCR. In contrast to a cytochrome oxidase (COX) assay, which did not work in fruit trees, 18S rRNA host gene detection was shown to be usable in a broader range of plants. The sensitivity and robustness of the duplex qPCR was evaluated with different dilutions of samples and compared with traditional nested PCR. The duplex qPCR was at least as sensitive as nested PCR and less susceptible to inhibition due to impurities in DNA extracts. The method was successfully employed for the screening of phytoplasmas in samples of fruit tree, raspberry, and grapevine. Typically, phytoplasma positive samples were tested with Ct values of 22-30, whereas Ct values for the internal control were in the range of 13-23.

Key words: TaqMan, real-time PCR, apple proliferation, pear decline, European stone fruit yellows, *Rubus* stunt, 'bois noir', 'flavescence dorée'.

Introduction

For phytoplasma detection real-time fluorogenic PCR (TaqMan) offers considerable advantage in terms of time and sensitivity compared to traditional or nested PCR. Furthermore, real-time PCR machines equipped with different filters allow the multiplex detection of different DNA targets simultaneously in the same reaction. For routine analysis, an internal control enables the confirmation of efficient DNA extraction and the recognition of inhibition of the PCR. Hence, false negative results can be excluded already in the first round of screening when the amplification of the host gene shows sigmoid curves of fluorescence with typical Ct values for the kind of plant tissue analyzed.

Materials and methods

DNA was extracted from phloem tissue of roots of apple and pear trees, leaves and petioles of raspberry and grapevine with 0.5 g of plant tissue ground 1:10 (w/v) with the HOMEX 6 in extraction bags «Universal» (both from BIOREBA) using the CTAB extraction method according to Angelini *et al.* (2001) and finally resuspended in 0.25 ml of 10 mM Tris pH 7.6.

Duplex real-time fluorogenic PCR was performed with the following primers and probes: for general detection of phytoplasmas, the two forward primers JH-F1 and JH-Fall and the reverse primer JH-R were used at a final concentration of 0.3 μ M each, the probe JH-Puni labelled with FAM-MGB-NFQ was used at a final concentration of 0.1 μ M (Hodgetts *et al.*, 2009). For 18S rDNA detection, the sequences of the following primers (forward: 5'-AGAGGGAGCCTGAGAAACGG-3', reverse:

5'-CAGACTCATAGAGCCCGGTATTG-3') and of the probe (5'-ROX-CCACATCCAAGGAAGGCAGCAGGCG-BHQ2-3') were generated with the software Beacon Designer (Version 7.2, Premier Biosoft International) based on the 18S rRNA gene from *Malus domestica* (Accession No. DQ341382) and used at a final concentration of 0.05 μ M each. These primers/probes were run in 20 μ l reactions employing the hot start Absolute QPCR Mix (ABgene) and the real-time cycler iQ5 (Biorad) with an initial denaturation and enzyme activation of 12 min at 95°C, followed by 40 cycles with 15 sec denaturation at 95°C and 60 sec annealing/elongation at 60°C. FAM and ROX signals were recorded in real-time during each annealing/elongation step.

Results

The general detection of phytoplasmas by real-time PCR according to Hodgetts *et al.* (2009) was evaluated successfully for detection of phytoplasmas in root, leaf or petiole tissue of different plants. These pathogens were equally well detected in both, simplex or duplex assay formats. In table 1, the evaluation of the duplex assay is presented for roots of healthy and with apple proliferation infected apple trees. The simultaneous detection of the 18S rRNA host gene had no effect on the amplification signal of the specific phytoplasma reaction both in terms of Ct value and sigmoid shape and height of the fluorescence curves (not shown) when compared to the simplex reaction with different dilutions of DNA extracts. Often, however, DNA extracts contained inhibitors retarding or inhibiting the amplification and had to be diluted 10-100 X in order to obtain positive reactions (table 1).

Table 1. Sensitivity comparison of simplex and duplex real-time fluorogenic PCR: DNA extracts from root samples of healthy and infected apple trees were tested in different dilutions with the primers/probe for general phytoplasma detection only (FAM signal; simplex reaction), and as duplex reaction in combination with primers/probe for 18S rRNA detection (FAM and ROX signal). Data represent Ct mean values of two repetitions per treatment.

Root sample	Dilution of (DNA extract)	Phytoplasma general simplex real-time PCR	Phytoplasma general + 18S rRNA duplex real-time PCR	
		FAM Ct	FAM Ct	ROX Ct
Healthy	undiluted	>40.00	>40.00	19.97
Healthy	1:100	>40.00	>40.00	20.43
Healthy	1:1000	>40.00	>40.00	23.52
infected/healthy mixed (1:10)	undiluted	25.30	26.06	22.31
infected/healthy mixed (1:10)	1:100	30.03	29.98	21.31
infected/healthy mixed (1:10)	1:1000	32.86	32.77	24.16
infected/healthy mixed (1:10)	1:10000	36.34	35.89	27.55
infected	undiluted	>40.00	>40.00	>40.00
infected	1:100	27.31	27.21	20.00
infected	1:1000	29.19	29.22	22.31
infected	1:10000	33.30	32.76	26.03

In routine analysis, the Ct values ranged from 22 - 30 when detecting *Rubus* stunt in raspberry, ‘flavescence dorée’ (FD) and ‘bois noir’ (BN) in grapevine, and apple proliferation (AP), pear decline (PD), and European stone fruit yellows (ESFY) in apple, pear and peach, respectively. Detection limit was between Ct values of 38 - 40. The duplex assay was shown to be sensitive enough for detecting one positive sample in a pool of 10 root samples (table 1).

The duplex TaqMan assay of grapevine phytoplasma FD and BN was shown to be at least as sensitive as nested PCR (Smart *et al.*, 1996), but less susceptible to inhibitors. Hence, DNA extracts containing inhibitors could be assayed less diluted in the TaqMan assay, resulting in higher detection sensitivity (data not shown).

Discussion

The duplex real-time fluorogenic assay presented here allows in one reaction the reliable general detection of phytoplasmas simultaneously with the host gene in different plants.

First, the host gene cytochrome oxidase (COX) according to Weller *et al.* (2000) was chosen as the target for the internal control. This worked well for grapevine, potato and raspberry but surprisingly not for fruit trees. Therefore, we have developed the internal control based on the 18S rRNA gene of apple that worked also with grapevine and potato (data not shown) and has therefore the potential as a universal host gene control.

The general detection method of phytoplasmas according to Hodgetts *et al.* (2009), detects phytoplasmas

with almost the same sensitivity when compared to specific detection of FD, BN, or *Rubus* stunt (data not shown). This system is therefore reliable for a first screening of diverse plant samples. Positive samples might then be further differentiated or confirmed with specific detection if required.

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Variability of stolbur phytoplasma strains infecting Croatian grapevine by multilocus sequence typing

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Abstract

In order to investigate the genetic variability among selected grapevine strains belonging to 16SrXII-A subgroup (stolbur) as a case study, multilocus sequence typing (MLST) approach was used. Three non-ribosomal house-keeping genes were analyzed: *tufB*, *secY* and *vmp1* gene, encoding a putative membrane protein of stolbur phytoplasma. Restriction analysis of *tufB* gene amplicons revealed the presence of both *tuf*-type a (VK-I) and *tuf*-type b (VK-II), with the latter being prevalent among the stolbur strains from Croatian grapevines tested. In genotyping of *vmp1* gene, five different RFLP profiles were obtained including a mixed pattern observed in one sample. Phylogenetic analyses of *secY* gene sequences were in accordance with the results of *tufB* and *vmp1* typing; however in some of the analyzed strains incongruences were observed. The presence of considerable genetic variability among relatively small number of 10 selected stolbur strains from grapevine was observed and the importance of MLST application in differentiation of closely related strains was verified.

Key words: MLST, *tufB*, *vmp1*, *secY*, phytoplasmas, grapevine.

Introduction

Main agents associated with grapevine yellows (GY) in Croatia are phytoplasmas belonging to the 16SrXII-A subgroup (stolbur) which are widespread and detected in most of the grapevine growing regions of the country (Šeruga Musić *et al.*, 2009).

Natural life-cycle of stolbur phytoplasma infecting grapevine may be associated with different herbaceous plants and vector populations (Langer and Maixner, 2004). In order to clarify the epidemiology of the disease, variability of non-ribosomal house-keeping genes such as *tufB* and *secY* is usually studied. Recently, *vmp1* gene encoding a putative membrane protein and potentially involved in phytoplasma-host interactions was shown to be a valuable molecular marker in differentiation of stolbur strains and assessment of genetic variability (Cimerman *et al.*, 2009).

The aim of this study was to examine the variability among selected stolbur phytoplasma strains infecting Croatian grapevine by using multilocus sequence typing (MLST).

Materials and methods

Ten phytoplasma strains from Croatian grapevine collected in different grapevine growing regions, previously characterized as members of 16SrXII-A subgroup (Šeruga Musić *et al.*, 2009; unpublished) were chosen for MLST. Abbreviations of samples are listed in table 1.

The *tufB* gene fragments of approximately 940 bp were amplified in a nested PCR using fTufu/rTufu primer pair followed by ftufAY/rtufSTOL primers (Schneider *et al.*, 1997; X. Foissac and A. Fabre, personal communication). Amplicons were digested with *HpaII* (Langer and Maixner, 2004) and analyzed by

electrophoresis in 2.5% agarose gel. The *vmp1* gene fragments were amplified in a nested PCR assay using StolH10F1/R1 primers, followed by TYPH10F/R primer pair, as described by Fialova *et al.* (2009). Fragments of approximately 1.1 to 1.5 kbp were digested with *RsaI* and separated by electrophoresis in 2.5% agarose gel. Amplification of *secY* gene fragment of 998 bp was done in a nested PCR with PosecF1/R1 primers, followed by PosecF3/R3 (Fialova *et al.*, 2009). Sequencing of both strands of *secY* gene fragments was done by MacroGen Inc. (Seoul, Republic of Korea), using PosecF3/R3 primer pair. Obtained sequences were edited and assembled using Sequencher™ 4.10. demo version (<http://www.genecodes.com/>). Multiple alignments were done using ClustalX 2.0 (Thompson *et al.*, 1997) and subsequent phylogenetic analyses performed using MEGA 4 (Tamura *et al.*, 2007).

Results

Amplicons of *tufB* gene were successfully obtained from all samples. Restriction analysis with *HpaII* enzyme indicated that samples SB1, SB5 and 21OS belong to *tuf*-type a (VKI), while all the other samples were typed as *tuf*-type b (VKII) according to Langer and Maixner (2004) (data not shown).

In *vmp1* gene typing, restriction analysis with *RsaI* of fragments obtained in all samples showed the presence of 5 different profiles (figure 1) in the 10 analyzed samples. Restriction patterns of fragments from samples 8TO, 11VZ and 15PO were identical (V14 type). Another pattern (V3 type) was detected in samples SB1, SB5 and 21OS, while samples SI2 and VU6 showed a third type of *RsaI/vmp1*-profile (V4 type). A unique profile was observed in sample 23DB (V5 type), while sample VU7 revealed a mixed restriction pattern of V2 and V18 types.

Phylogenetic analyses of *secY* gene sequences showed that the strains having the same *tuf*- and/or *vmp1*-profile grouped together in the same branch of the tree. Non-congruence was observed in samples 23DB and VU7 that were typed as *tuf*-type b, but grouped with the *secY* sequences of *tuf*-type a samples (data not shown).

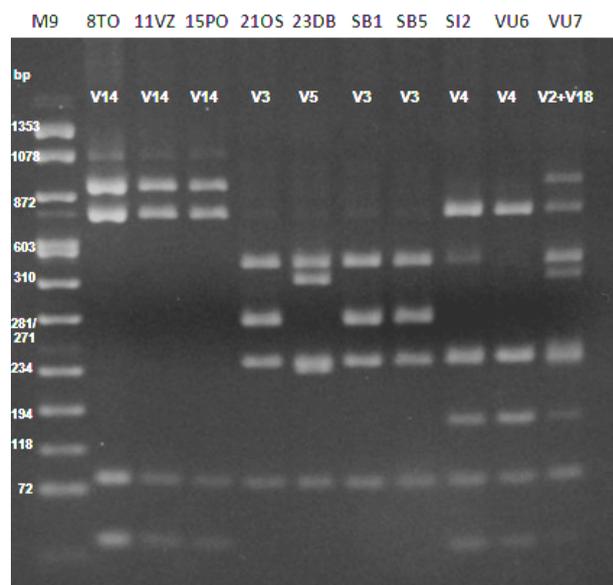


Figure 1. RFLP analysis of phytoplasma *vmp1* gene fragments amplified from Croatian grapevine extracts in a nested PCR assay using StolH10F1/R1, followed by TYPH10F/R primers and digested with *RsaI*. Electrophoresis was performed in 2.5% agarose gel. Abbreviations of samples are same as in table 1. M9 - Marker 9; Φ X174 DNA/*BsuRI*(*HaeIII*) digested (Fermentas, Lithuania). Different *vmp1*-restriction profiles are marked as V-types.

Table 1. List of samples analyzed in this study.

Sample	Year	Cultivar	Location
SB1	2000	Chardonnay	Brodski Stupnik
SB5	2000	Chardonnay	Brodski Stupnik
SI2	2001	Debit	Drniš
VU6	2007	Chardonnay	Ilok
VU7	2007	Blaufränkisch	Ilok
8TO	2008	Chardonnay	Ilok
11VZ	2008	Chardonnay	Železna gora
15PO	2008	Chardonnay	Dajla
21OS	2008	Chardonnay	Erdut
23DB	2008	Chardonnay	Kneževi vinogradi

Discussion

Restriction analysis of *tufB* gene showed the presence of polymorphism with a prevalence of the *tuf*-type b, which is also the most diversified elsewhere in France

and Italy (Pacífico *et al.*, 2009). Genotyping of *vmp1* revealed more variability with five different RFLP profiles observed in this study. Comparisons of phylogenetic analyses of *secY* sequences and RFLP analyses of *tufB* and *vmp1* genes proved that *vmp1* and *secY* possess greater variability than *tufB* hence are more informative markers for finer differentiation of closely related strains. Nevertheless, MLST has shown the presence of considerable genetic variability among the relatively small number of analyzed stolbur strains from Croatian grapevine. These results present a step forward in a better understanding and clarifying GY disease epidemiology in Croatia.

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Development of rapid in-field loop-mediated isothermal amplification (LAMP) assays for phytoplasmas

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Abstract

Loop-mediated isothermal amplification (LAMP) is an isothermal amplification technique that can be undertaken with minimal equipment to obtain amplification of target DNA within 30 minutes. A range of assays for specific 16Sr phytoplasma groups, which when combined with rapid DNA extraction techniques can result in detection of the phytoplasma in plant material within 1 hour of sampling was developed. A range of alternative methods are available for detection of the amplification product including incorporation of hydroxyl naphthol blue into the reagent mix, agarose gel electrophoresis and real-time detection systems. The advantage of the real-time method is that tubes do not have to be opened, minimising the risk of contamination of samples.

Key words: DNA extraction, LAMP assays, field detection, phytoplasmas.

Introduction

Numerous techniques have been developed for phytoplasma diagnostics, in particular the use of PCR-based methods. These may either be generic, with the use of RFLP analysis or sequencing to assign the phytoplasma to a 16Sr group, or they may involve the use of group-specific primers (Smart *et al.*, 1996; Firrao *et al.*, 2005). However, it is also important to guard against false negatives during such detection by building an internal control into the diagnostic test to confirm that a negative result is due to a lack of phytoplasmas and not PCR inhibition. More recently, real-time PCR assays have also been developed for both generic and specific phytoplasma detection, and these assays have the advantage of being more easily automated and less labour intensive than conventional PCR, such that appropriate controls can be conducted more easily (Christensen *et al.*, 2004; Hodgetts *et al.*, 2009; Hren *et al.*, 2007).

However, these assays are also relatively slow compared to Loop-Mediated Isothermal Amplification (LAMP) assays, and require bulky equipment such that they generally have to be conducted in laboratories. Our goal has been to develop a more rapid diagnostic assay for phytoplasmas that can be used to produce a diagnosis within an hour of sampling in the field. Whilst techniques such as lateral flow devices have been used to achieve this for some viral plants diseases, antibody-based techniques have had limited success for phytoplasma diagnostics because of a lack of sensitivity. We have therefore been developing the use of LAMP which, when combined with rapid DNA extraction techniques, has the potential to provide rapid in-field analyses. Here we report some of the specific tests that we have developed to date along with a protocol that we are piloting for rapid in-field diagnostics of phytoplasmas.

Materials and methods

Plant material (healthy and infected) for testing the DNA extraction techniques and LAMP assays were obtained from the University of Nottingham and the Food and Environment Research Agency phytoplasma collections, along with samples of coconut trunk borings from Ghana (kindly provided by J. Nipah), and papaya and wild grass plant material from Ethiopia (kindly provided by B. Bekele). Two methods were used for DNA extraction; an LFD extraction method (Tomlinson *et al.*, 2010) and an alkaline polyethylene glycol DNA extraction method (Chomczynski and Rymaszewski 2006). Primers for the LAMP assays were designed as described in Tomlinson *et al.* (2010) and Bekele *et al.* (2011) based on the 16S-23S intergenic spacer region, and LAMP reactions and detection methods were as previously described (Tomlinson *et al.*, 2010; Bekele *et al.*, 2011). In addition, *cox* gene primers were used to confirm that all DNA extractions supported LAMP (Bekele *et al.*, 2011).

Results

Primers for LAMP assays were designed against a range of ribosomal groups (16SrI, 16SrII, 16SrIII, 16SrV, 16SrXI, 16SrXII and 16SrXXII) and these primer sequences are listed in previous papers and/or are available from the authors. The different primer sets were tested on a range of DNA strains from our collection, and with the exception of the 16SrXI assay, the primers were group specific i.e. they only amplified DNA samples from the group they were designed to detect. The 16SrXI primers were different in that they also detected 16SrXIV isolates, but did not detect sugarcane whiteleaf/grassy shoot or rice yellow dwarf samples, even though these are also designated as 16SrXI group

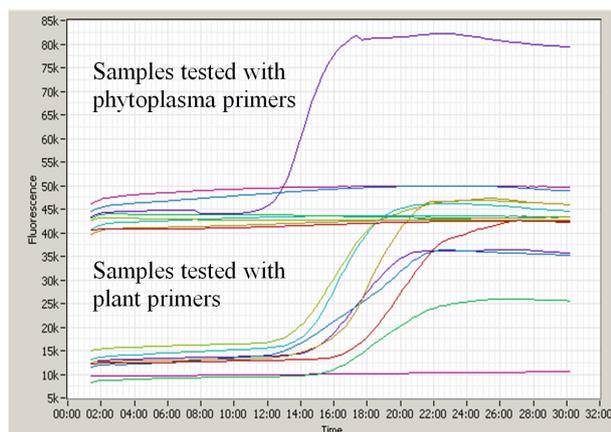


Figure 1. Real-time LAMP profile for seven infected plant material samples and a negative (water) control. The top panel used the 16SrXXII group-specific primers and the bottom panel used the *cox* gene primers. Amplification can be detected as the increase in fluorescence at 12-20 minutes.

(In colour at www.bulletinofinsectology.org)

phytoplasmas. Figure 1 shows the typical results of a LAMP assay using the real-time detection system developed by OptiGene (Horsham, UK), in which seven samples plus a negative (water) control have been tested with the 16SrXXII group phytoplasmas (upper panel) and the same samples tested with the *cox* gene primers (lower panel). The results show that all seven plant samples were amplified within 20 minutes by the plant primers but only the positive control sample was amplified with the 16SrXXII primers.

To compare the DNA extraction methods, samples of periwinkle (*Catharanthus roseus*) leaves, papaya and wild grass leaves and coconut trunk borings were tested using the LFD-based method and the alkaline PEG method. Both methods reliably produced DNA able to support LAMP, though the DNA from the PEG method started to show reduced reliability after storage for more than a week, whilst the LFD DNA was stable for many months.

Discussion

We have developed a protocol for LAMP-based diagnostics for a range of phytoplasmas that can be conducted in the field and used to provide a diagnosis within 1-hour of DNA extraction. The simplest method of DNA extraction is to use the alkaline-PEG method, which involves gently macerating a small amount of plant tissue in the buffer and then using this in the LAMP reaction. The disadvantage of this method is that the DNA cannot be stored reliably long-term, but it is quicker and requires less equipment than the LFD-based method, reducing the likelihood of sample contamination. Having isolated the DNA, the LAMP reactions take only a few minutes to set-up, particularly when field stable lyophilised reagents are used.

Currently we are using the Genie II real-time LAMP reader (Optisense), which is portable and battery operated, enabling amplification within 30 minutes and a visual display of results. Whilst removing the need to open tubes following amplification, it does not integrate DNA extraction. The VITISENS project seeks to develop a hand held device capable of performing extraction, set-up and real-time detection for grapevine phytoplasmas. The device will make a single step homogeneous system from sampling to results, further reducing the risk of sample-to-sample contamination and enabling testing by non-specialists in the field.

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Use of a recombinant protein for development of a DAS-ELISA serological kit for sensitive detection of witches' broom disease of lime

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Abstract

Witches' broom disease of lime (WBDL), associated with '*Candidatus* Phytoplasma aurantifolia' is the most devastating disease of acid lime in Southern Iran. Lack of an efficient approach for control of the disease has resulted in application of quarantine measures for protection of healthy plants and to limit the spread of disease to uninfected areas. Toward this aim, development of a rapid and efficient method for detection of infected plants is a major focus. The present study introduces application of recombinant DNA technology for development of a sensitive serological technique (DAS-ELISA) for detection of infected plants.

The immunodominant membrane protein (IMP), as a major protein present on the surface of phytoplasma cells, was selected as a target for generating specific antibody molecules. The gene encoding IMP of '*Ca. P. aurantifolia*' was obtained from infected plants. The region encoding the IMP fragment was isolated by PCR amplification followed by insertion into the pZ57R/T cloning vector. Intact clones containing the right sequences were selected and sub-cloned into the pET28a bacterial expression vector. Large scale expression of recombinant protein was performed in *E. coli* and purification was carried out through affinity chromatography in Ni-agarose columns.

To obtain specific polyclonal antibodies against WBDL, the purified recombinant IMP was used for rabbit immunization. The antisera titer was determined after each boosting via indirect ELISA. When the titer reached 1:100,000, the animal was sacrificed, blood was collected and serum was separated from blood cells. The IgG molecules were purified from serum content by affinity chromatography using protein A columns followed by conjugation to alkaline phosphatase (AP) enzyme. The purified specific antibodies and conjugate were used for detection of the corresponding antigen, IMP in infected plants in DAS-ELISA and dot-blot methods. The results confirmed the capability of this technique for efficient detection of infected plants, while no reaction was observed in negative controls. The detection limit of the DAS-ELISA method was determined at 70 µg IMP/ml leaf extract.

Key words: Witches' broom disease of lime, DAS-ELISA, IMP, phytoplasmas.

Introduction

The witches' broom disease of lime (WBDL) was observed in Iran in 1997 (Bové *et al.*, 2000). The disease destroys thousands of trees yearly throughout these regions and it is associated with '*Candidatus* Phytoplasma aurantifolia' (Zreik *et al.*, 1995).

The IMP proteins are predominant proteins located on the external surface of the cell membranes of phytoplasmas and could be used as a target for efficient detection of infected plants. They play important roles in the attachment of the bacteria to their host cell surface, and are involved in phytoplasma-host interactions (Kakizawa *et al.*, 2010).

There are several diagnostic techniques to detect phytoplasmas. Molecular methods, especially the polymerase chain reaction (PCR), are specific and sensitive (Namba *et al.*, 1993). Serological methods applying specific antibodies are convenient and economic in that many samples could be analyzed in a short time in a lab with minimal equipment.

This paper describes application of the recombinant IMP protein for production of polyclonal antibodies with high binding ability and development of a specific DAS-ELISA serological kit for efficient detection of WBDL.

Materials and methods

Total DNA was extracted from the midribs of healthy and infected lime plants as described by Zhang *et al.* (1998). The gene encoding the IMP protein of the phytoplasma was obtained by PCR amplification using specific primers and inserted into a pTZ57R/T vector.

The IMP coding region previously cloned in pTZ57R/T was digested by using *SalI/NotI* restriction enzymes and sub-cloned into the pET-28a bacterial expression vector. The plasmids obtained from the recombinant clones were used to transform *E. coli* BL21(DE3) competent cells. The expression and purification of recombinant IMP from bacterial cultures was accomplished. Recombinant proteins were purified by a Ni-agarose column. Protein concentration was measured using comparative analysis with a known concentration of BSA protein.

Two white inbred rabbits were used for immunization. Five injections were given at intervals of 2 weeks. Each injection containing about 100 µg was carried out intramuscularly in the hind legs with an emulsion of equal volumes of IMP protein and Freund's complete or incomplete adjuvants. Animals were bled 4-5 times from the marginal ear vein at 14 day intervals for estimation of antibody titer by indirect ELISA.

Finally, blood was collected from rabbit hearts 14 days after the last immunization. The blood was clotted at room temperature and then the serum was separated by centrifugation.

Purification of polyclonal antibodies was performed by using protein A columns in an affinity chromatography method. Purity and concentration of polyclonal antibodies was measured by SDS-PAGE electrophoresis. Conjugation of purified immunoglobulin to alkaline phosphatase was accomplished using a conjugation kit (AbD serotec, UK).

The purified polyclonal antibodies and conjugate were used for detection of infected plants by DAS-ELISA, western blotting and Dot immunobinding assay (DIBA) methods.

In order to determine the detection limit of the constructed kit, a real time PCR analysis using specific primer pairs amplifying a 158-bp DNA fragment of the IMP gene was performed.

Results and discussion

The IMP proteins are predominant on outer membranes of phytoplasma cells and therefore are ideal targets for development of serological techniques for efficient detection of phytoplasma infected plants. The IMP gene of 540 bp encoding the immunogenic protein of the phytoplasma agent was amplified using specific primers. Database alignment of the sequences obtained using the universal M13 primer revealed a 100% homology with the accession number GU339497 in the NCBI database associated with '*Ca. P. aurantifolia*'. The digested IMP gene was then cloned into the pET-28a bacterial expression vector, transferred into the competent cells resulting in the successful expression and production of the recombinant IMP fused to a 6-His tag. The IMP produced by the clones was purified and its integrity was measured on an SDS PAGE gel at 35 kDa. The concentration of purified IMP was measured at around 6 mg/ml obtained from one liter culture medium. Western blot analysis using specific anti-His tag monoclonal antibodies confirmed the presence of recombinant fusion protein in the right position.

The specific polyclonal antibodies were obtained by intramuscular immunization of rabbits by the purified recombinant IMP protein. The antibody titer was determined after each immunization by indirect ELISA. When the antibody titer exceeded 1:100,000, the full serum including the whole antibodies was obtained from rabbits. Purification of IgG was performed by affinity chromatography using staphylococcus protein A columns. The purity and integrity of purified antibodies were evaluated by SDS-PAGE analysis. These results revealed the presence of two bands on the gel around 25 and 50 kDa belong to light and heavy chains of antibodies, respectively. The comparative analysis using BSA

as standard protein showed the concentration of purified IgG to be about 0.5 mg/ml.

To make conjugate, purified antibody was fused to the alkaline phosphatase enzyme. The conjugate and purified antibodies were used in DAS-ELISA, western blotting and DIBA serological analyses by using plant extracts and purified IMP as controls. These results confirmed the ability of the antibodies to efficiently detect infected plants. The sensitivity of the constructed kit was found to be as high as 100%. The optimum dilution of conjugate and purified antibodies were measured around 1:1,000. In order to quantify the detection limit of the constructed DAS-ELISA kit for the phytoplasma agents in the samples, real time PCR was used and it was determined around 70 µg IMP/ml leaf extract. To determine cross reactivity of the polyclonal antibody with other phytoplasma agents, DAS-ELISA and DIBA analyses were performed by using infected sesame, alfalfa and almond. These results showed no reaction when the leaf extracts obtained from the phytoplasma-infected sesame and almond samples were used. However, there was a positive reaction against the phytoplasma from alfalfa.

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Use of a fragment of the *tuf* gene for phytoplasma 16Sr group/subgroup differentiation

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Abstract

The usefulness of RFLP analyses on a 435 bp fragment of the *tuf* gene for preliminary identification of phytoplasmas from a number of phytoplasma ribosomal groups and/or 'Candidatus Phytoplasma' was verified. The strains employed belong to thirteen 16Sr DNA groups and 22 different subgroups and were obtained from both experimentally and naturally infected plants. The combined RFLP patterns obtained with the three restriction enzymes employed allow the distinction of a total of 18 different profiles, however no discrimination was provided for some of the ribosomal groups for which sequencing remains the main tool for phytoplasma identification.

Key words: phytoplasma identification, 16Sr RNA groups, PCR/RFLP, molecular identification, *tuf* gene.

Introduction

The increase of international trade of plant material and of phytoplasma-associated diseases worldwide demands a quick and handy system for phytoplasma identification. In the frame of a DNA barcoding project funded by EU FP7 to create a barcode database for all quarantine plant pathogens, phytoplasma barcoding is in progress and one of the selected genes is the elongation factor Tu (*Tuf*).

For amplification of short barcoding sequences a cocktail primer system was developed amplifying 435 bp of *tuf* gene for subsequent sequencing (M. Nicolaisen *et al.*, unpublished data; Contaldo *et al.*, 2010). The usefulness of this fragment for RFLP analyses for preliminary identity screening of phytoplasmas from a number of phytoplasma ribosomal groups and/or 'Candidatus Phytoplasma' was verified in this study.

Materials and methods

Phytoplasma strains maintained in periwinkle (Bertaccini, 2010), in tomato or field collected were used for PCR amplification with newly developed primers for *Tuf* in a cocktail reaction (M. Nicolaisen *et al.*, unpublished). The strains belong to thirteen 16S rDNA groups and to 22 different subgroups (table 1) and were both from periwinkle infected plants (Bertaccini, 2010) and from naturally infected plants such as grapevine from Italy (FD-VE 210/06), and erigeron and turnera from Brazil (Montano *et al.*, 2011). Nucleic acid extraction was performed with a chloroform/phenol procedure (Prince *et al.*, 1993) and 20-60 ng of DNA were used as template per reaction.

RFLP analyses were carried out on 200 ng of DNA of each amplicons with *TruI*, *Tsp509I*, *TaqI* restriction endonucleases (Fermentas, Vilnius, Lithuania) following the instruction of the manufacturer. Restriction profiles

were analyzed in both 5% polyacrilammide, and 3% agarose gels in TBE buffer.

Results

PCR products of the expected length were obtained from all strain tested. Amplicons were not observed in the negative controls devoid of DNA and in samples from healthy plants. RFLP analyses allow relevant differentiation among phytoplasmas. Both polyacrylamide and agarose gels provided discriminating profiles. The use of *Tsp509I* allowed differentiation at the 16Sr DNA group level of the majority of the strains and at subgroup level of strains in groups 16SrI, 16SrII, and 16SrIII (figure 1).

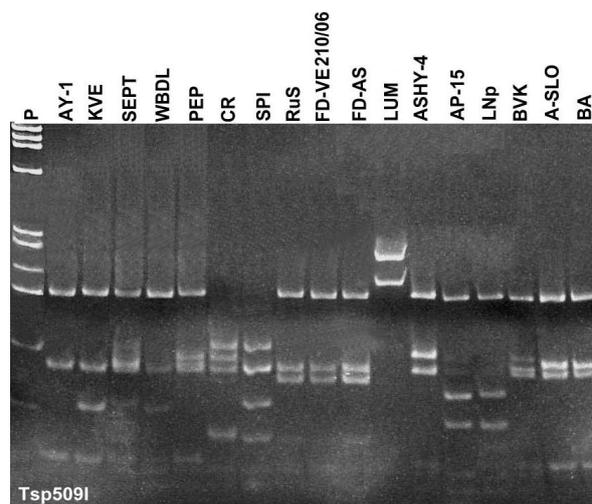


Figure 1. Polyacrylamide gel in which the different profiles distinguished by RFLP are in agreement with 16Sr DNA group/subgroup classification. Acronyms as in table 1. P, marker Φ X174 *Hae*III digested.

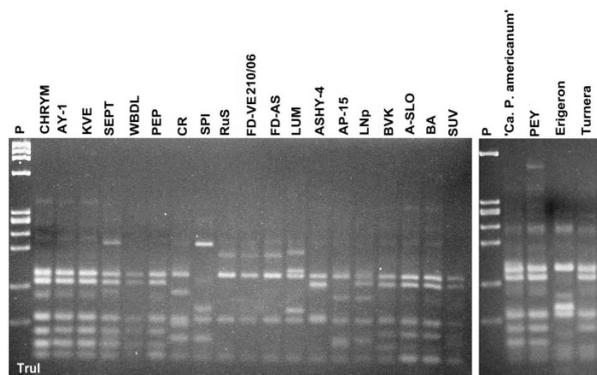


Figure 2. Agarose gel in which the different profiles distinguished by RFLP are in agreement with 16S rDNA group/subgroup classification. Acronyms as in table 1. P, marker Φ X174 *Hae*III digested.

Table 1. Results of phytoplasma strain differentiation by RFLP analyses on *tuf* gene amplicons (identical letter indicates identical profiles).

Strain acronyms	Tuf profiles			Grouping	
	<i>Tru</i> I	<i>Tsp</i> 509I	<i>Taq</i> I	16Sr	<i>tuf</i>
CHRYM	A	A	A	I-A	1
AY1	A	A	A	I-B	1
KVE	A	B	A	I-C	2
SEPT	A	A	B	II-A	3
WBDL	A	B	A	II-B	2
PEP	A	C	C	II-F	4
CR	B	D	B	III-B	5
SPI	C	E	B	III-E	6
RuS	D	F	D	V-E	7
FD-VE 210/06*	D	F	D	V-D	7
FD-AS	D	F	D	V-C	7
LUM	E	G	C	VI	8
ASHY-4	F	H	E	VII-A	9
Erigeron*	M	M	C	VII-B	10
PEY	A	A	C	IX-C	11
AP-15	G	I	C	X-A	12
LNp	H	I	C	X-B	13
BVK	A	L	F	XI-C	14
A-SLO	I	C	C	XII	15
BA	I	C	C	XII	15
Turnera*	I	A	C	XIII	16
SUV	L	C	C	XV	17
'Ca. P. americanum'***	A	A	D	XVIII-A	18

* From naturally infected species.

** Inoculated in *Solanum lycopersicum*.

The use of *Tru*I differentiated less among groups, however allowed distinguishing subgroups in strains belonging to groups 16SrIII and 16SrX (figure 2). The use of *Taq*I differentiated mainly the strains at the group level. The combined RFLP patterns obtained with the three restriction enzymes allowed the distinction of a total of 18 different profiles. No RFLP discrimination was found between 16SrI-A and I-B strains, as well as 16SrV-C, V-D and V-E strains (table 1).

Discussion

RFLP analyses of *tuf* amplicons allow group and subgroup discrimination that is useful for screening a large number of samples. This discrimination is in agreement with published phytoplasma groupings based on the 16S rDNA (Lee *et al.*, 1998; Montano *et al.*, 2001). The visualization of restriction profiles in agarose is a handy tool for large number of sample processing without missing identification ability. In case of phytoplasmas relevant for quarantine, sequencing may be necessary for confirmation and as the main tool for discriminating among 16SrV group strains.

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Improving molecular diagnostics for the detection of lethal disease phytoplasma of coconut in Ghana

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Abstract

Accurate and timely detection is important for the control of lethal disease of coconut in Ghana. To improve on the detection of the phytoplasmas involved, multiplex PCR with an in-built internal control and a real-time loop mediated isothermal amplification (LAMP) were used to eliminate false negative results and minimise cross-over contamination. Real-time LAMP provided a fast and reliable means of diagnosis.

Key words: Coconut, LAMP assay, multiplex PCR, Ghana, phytoplasma.

Introduction

Lethal disease (LD) of coconut locally called Cape Saint Paul Wilt disease (CSPWD) is the most important disease of coconut in Ghana and is associated with a phytoplasma. Early and accurate detection of the disease is an important trigger for the initiation of containment measures which includes felling of infected palms. Routine detection of the disease is carried out using PCR with ribosomal and non ribosomal primers such as P1 and P7 (Deng and Hiruki, 1991; Smart *et al.*, 1996) and primers based on the *secA* gene (Hodgetts *et al.*, 2008).

The loop mediated isothermal amplification (LAMP) technique is fast becoming a popular diagnostic tool for plant pathogens and assays for detecting the CSPWD phytoplasma have been developed (Tomlinson *et al.*, 2010). The technique uses *Bst* polymerase which has a strand displacement activity in conjunction with 4-6 specially designed primers that recognise 6-8 regions of the target DNA respectively, thereby making it very specific. Many methods have been developed for detecting LAMP reaction products including real-time methods (Bekele *et al.*, 2011). A multiplex PCR involving primers for amplifying both pathogen and plant DNA was used to ascertain the presence of inhibitors in the plant materials and to concurrently test the efficiency of extracting DNA from woody coconut tissues. A real-time LAMP assay that reduces the risk of cross-over contamination was used to amplify and identify the CSPWD phytoplasma in infected samples.

Materials and methods

Coconut trunk borings collected from symptomless and CSPWD infected West African Tall (WAT) ecotype and from symptomless hybrids of the Malayan Yellow Dwarf (MYD) and Vanuatu Tall varieties (VTT) (i.e MYD x VTT) located in diseased fields in the Western

region of Ghana were the sources of coconut and phytoplasma DNA used in the study. DNA was extracted with a modified protocol of Daire *et al.* (1997) using CTAB buffer.

PCR was carried out by multiplexing primers for amplifying the *sec A* gene from phytoplasma DNA and primers for amplifying a microsatellite marker CncirF3 from coconut DNA (Lebrun *et al.*, 2001). For the *SecA* gene the primers CSPWDSecAFor2 (CGAGATGCA GATCGTTTTG) and CSPWDSecARev2 (CCATCACC AAATTGACGTCC) were used. Since the proportion of pathogen DNA was expected to be significantly lower than that of the plant DNA, the volumes of the pathogen primers used were twice as much as those of the plant primers.

The LAMP primers used are described in Tomlinson *et al.* (2010) and amplicons were detected in real-time following the protocol of Bekele *et al.* (2011). The LAMP products were analysed in terms of the time taken to provide positive result and the melting temperatures of the amplicons (T_m) used to validate the results.

Results

A 380 kb fragment (approximately) from the coconut DNA and a 290 kb amplicon (approximately) from the DNA of CSPWD infected samples were amplified with the plant and pathogen primers, respectively, in the multiplex PCR. Positive results from infected plant samples appeared either as double bands on the gel representing the plant and pathogen fragments or as single bands with sizes corresponding to the expected pathogen fragment size (figure 1). Single bands amplified with the plant primers indicated that the palms were likely to be uninfected. Absence of bands for both plant and pathogen DNA indicated either a lack of DNA or PCR inhibition. LAMP amplicons were observed to begin to form as early as in 12 minutes and results of the LAMP assay were also comparable to those from the PCR analysis (table 1).

Table 1. Comparison of PCR and LAMP assays results.

Variety	Sample number	No. Infected*	Positives samples	
			(PCR)	(LAMP)
WAT	98	34	29**	29**
MYD x VTT	10	0	1	1

* Infected palms from field sampling including diseased palms and palms which had died from infection.

** 28 of the samples were the same assays. A sample each from the dead palms was tested by two assays.

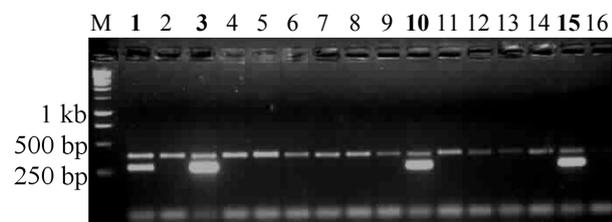


Figure 1. PCR amplification of plant and pathogen DNA. Lanes 1, 3, 10 and 15: Amplification of plant and pathogen DNA. Lanes 2, 4-9, 11-14: Amplification of plant DNA. Lane 16: No template control.

Discussion

Several PCR primers have been developed for amplifying the CSPWD phytoplasma, however, these assays do not have an internal control to guard against false negatives resulting from PCR inhibition or a lack of phytoplasma DNA. Multiplexing primers for amplifying both plant and phytoplasma DNA ensured that samples which gave negative reactions, particularly those from infected palms, were re-extracted to ascertain the effectiveness of the extraction protocol. The results showed that it is difficult to obtain DNA from tissues of palms that had died from the disease as repeated extractions and analyses for most of such samples continually produced negative results.

Real-time LAMP, which, is performed in a closed system and does not require post-amplification manipulations ensures that false positive results arising from cross over contamination are reduced if not eliminated. The technique apart from producing similar results as PCR, has the advantage of detecting amplicons in a relatively short time as compared to PCR. The LAMP technique is currently being trialled for in-field use.

Acknowledgements

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Nucleotide sequencing of *imp* gene in phytoplasmas associated to 'flavescence dorée' from *Ailanthus altissima*

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Abstract

The molecular characterization of *imp* phytoplasma gene from 15 *Ailanthus altissima* plants infected by 'flavescence dorée' type 16SrV-C phytoplasmas revealed a high polymorphism of the pathogen in this tree in Northern Italy. Eleven different 'flavescence dorée' phytoplasma strains were identified in *A. altissima*, two of them having *imp* nucleotide sequence 100% identical to 'flavescence dorée' phytoplasmas found in plants of *Clematis vitalba* collected closely. This finding suggests a phytoplasma exchange between the two plant species.

Key words: clematis, epidemiology, grapevine.

Introduction

Ailanthus altissima (Mill.) Swingle (tree of heaven) is an invasive tree species, originally introduced from China, which arrived in Italy two centuries ago (Celesti-Grapow *et al.*, 2009). Nowadays it is found ubiquitously in urban and rural areas not only in Italy, but also in the entire world, due to its efficient spreading.

Previous research works showed that in Italy it can be infected by phytoplasmas related to 'flavescence dorée' (FD) (Filippin *et al.*, 2008; 2010). FD is an epidemic disease of grapevine, mainly spread in Southern Europe, where it causes serious damages in vineyards and the quantitative and qualitative decrease of grape and wine production. FD and other related phytoplasmas have been identified also in alder (*Alnus glutinosa*) and in clematis (*Clematis vitalba*) (Angelini *et al.*, 2001; 2004; Filippin *et al.*, 2009).

The aim of this work was to understand the possible role of the tree of heaven in FD epidemics in Italy by means of molecular characterization of the FD strains in the *imp* gene, which is very polymorphic in the 16SrV subgroup phytoplasmas.

Materials and methods

One hundred and three leaf samples from *A. altissima* trees were collected in 2007-2010, mainly in Northern Italy (table 1). Each sample was made by a pool of leaves collected from different plants in the same restricted area. *C. vitalba* and *Vitis vinifera* plants growing close to the trees and showing phytoplasmas symptoms were sampled too.

DNA was extracted according to previously reported protocols (Angelini *et al.*, 2001). Real time and nested PCR/RFLP analyses on ribosomal genes were carried out in order to identify and characterize the phytoplasmas (Angelini *et al.*, 2001; 2007).

Phytoplasma *imp* gene from samples which tested positive was amplified by specific nested PCR (Da Rold *et al.*, 2010) and amplicons were double strand sequenced. Nucleotide sequences were compared with *imp* sequences from other FD phytoplasmas obtained in previous studies (Da Rold *et al.*, 2010).

Table 1. *A. altissima* plants collected in the different geographical regions in Italy and number of samples found positive and negative for FD presence by molecular test.

Region	Province	PCR results	
		Positive samples	Negative samples
Friuli Venezia Giulia	Trieste	0	1
Friuli Venezia Giulia	Udine	3	9
Friuli Venezia Giulia	Pordenone	1	13
Venetia	Treviso	8	39
Venetia	Venice	0	4
Venetia	Vicenza	2	2
Venetia	Verona	0	1
Venetia	Rovigo	0	1
Lombardy	Milan	0	1
Lombardy	Brescia	0	1
Piedmont	Asti	0	1
Piedmont	Alessandria	1	0
Piedmont	Cuneo	0	1
Tuscany	Florence	0	1
Tuscany	Siena	0	2
Marche	Ancona	0	3
Latium	Rome	0	3
Latium	Frosinone	0	2
Apulia	Bari	0	3
Total		15	88

Results

Molecular analyses showed that 15 *A. altissima* samples out of 103 (about 15%) were infected with an FD-related phytoplasma (table 1). All infected trees were from Northern Italy, while the few plants collected in Central and Southern Italy were always FD-related phytoplasma-free. RFLP analyses on ribosomal genes showed that all detected phytoplasmas belonged to the ribosomal subgroup 16SrV-C, confirming previous preliminary data (Filippin *et al.*, 2010). No clear association between symptom and phytoplasma occurrence was observed.

The nucleotide sequencing of phytoplasma *imp* gene from *A. altissima* infected plants showed 11 different strains to be present, with a high degree of polymorphism among variants. The nucleotide identity among the most different strains was 69.4%, while the lowest aminoacid identity in the *imp* protein was 51.9%. None of the *imp* genetic variants found in *A. altissima* was 100% identical to other phytoplasma strains identified in alder and grapevine samples from the same geographic areas. In contrast, two FD-related phytoplasma strains from *A. altissima* collected in Friuli-Venezia-Giulia and Piedmont, respectively, showed the very same *imp* sequence found in FD-related phytoplasma infected clematis from the same areas.

Discussion

The finding of this study pointed out that phytoplasma exchange between clematis and the tree of heaven does exist. In contrast, the *imp* variants identified in *A. altissima* were similar but not identical to those found in grapevine. Further studies on a higher number of grapevine samples are needed to confirm these preliminary data.

The *imp* gene is demonstrated to be very useful as a molecular marker for epidemiology and traceability studies of FD-related phytoplasmas, due to its high polymorphism. Indeed, the sequencing of other less variable phytoplasma genes would have furnished less information useful for this study.

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Detection and characterization of phytoplasmas infecting apple trees in Czech Republic during 2010

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Abstract

Apple trees showing proliferation disease symptoms were found at different locations in Czech Republic during 2010. Direct and nested PCR with primers specific for phytoplasmas, and the subsequent RFLP analyses were used for phytoplasma detection and in order to differentiate ribosomal subgroups and apple proliferation phytoplasma subtypes. Out of 74 apple trees examined, 63 plants revealed positive reaction for phytoplasma presence. The majority of apple trees was infected by phytoplasmas belonging to apple proliferation ribosomal group (16SrX). In the 16S plus spacer region two apple proliferation phytoplasma profiles P-I and P-II were determined as well as the presence of two phytoplasma genetic lineages designated as pattern '1' and '2'. Apple proliferation phytoplasmas belonging to ribosomal protein rpX-A subgroup were detected in the majority of apple trees, while phytoplasmas belonging to subgroup rpX-B were detected sporadically. Apple proliferation phytoplasma subtypes AP, AT-1 and AT-2 singly or in different combination were also found in samples collected from apple trees.

Key words: phytoplasma, apple proliferation, PCR/RFLP, subgroups differentiation.

Introduction

Phytoplasmas are prokaryote organisms of the *Mollicutes* class. In apple trees, phytoplasmas belonging to different ribosomal groups and subgroups have been described till this time. However, apple proliferation phytoplasma (ribosomal subgroup 16SrX-A, '*Candidatus* Phytoplasma mali') seems to be the most frequent phytoplasmas associated with apple proliferation disease. As revealed by RFLP analysis, '*Ca. P. mali*' is homogeneous at the level of 16S rRNA gene: however, differences were observed when more specific tools were used for characterization (Casati *et al.*, 2010; Martini *et al.*, 2008; Paltrinieri *et al.*, 2010). The results presented here confirm that phytoplasmas classified in different ribosomal subgroups affect apple trees in Czech Republic, and, especially, showed a high degree of genetic diversity among '*Ca. P. mali*' population in samples collected from apple trees all over the country during 2010.

Materials and methods

Apple trees showing shoot proliferation, enlarged stipules, reduced growth habit, abnormal growth of shoots in the autumn and fruit malformation were found at different locations in Czech Republic during 2010.

DNA was extracted from phloem tissues of 82 samples from 74 apple trees. The polymerase chain reaction (PCR) was carried out to amplify region that includes the 16S-23S rRNA gene of the phytoplasma genome. The primer pairs P1/P7 and P1A/P7A were used in direct PCR. PCR products were diluted with sterile distilled water (1:29) prior to amplification by nested PCR using P1A/P7A, F1/B6, R16F2n/R2 and F1/B6, F2n/R2 primer pairs, respectively. Double nested PCR was carried out by two ways with subsequent primer pairs combinations: P1/P7 – P1A/P7A – F2n/R2 and P1/P7 –

F1/B6 – F2n/R2. About 20 ng of each DNA preparation in water were added to the PCR mix (Schaff *et al.*, 1992) in a final reaction mixture volume of 25 µl. Approximately 200 ng of DNA of each positive PCR product were separately digested from R16F2n/R2, F1/B6 and P1A/P7A amplicons. Digestions were carried out with 2.5 U of *Mse*I and *Hpa*II restriction enzymes after R16F2n/R2 and F1/B6, P1A/P7A amplification, respectively. Restriction patterns obtained were compared with those described in the literature (Paltrinieri *et al.*, 2010; Casati *et al.*, 2010) after electrophoresis in 8% polyacrylamide gel in 1x TBE buffer followed by staining with GelRed and visualization under an UV transilluminator.

Non-ribosomal region (nitroreductase-like gene) was amplified using primer pair AP13/AP10, followed by primer pair AP14/AP15. PCR reactions were carried out as previously described by Casati *et al.* (2010). Products obtained by means of primer pair AP14/AP15 were separately digested with *Hinc*II and *Pag*I endonucleases, according to the manufacturer's instruction. The restriction patterns were then observed and compared with literature as described above.

For PCR amplification based on *rpl22* and *rps3* genes of S10 ribosomal protein (rp) operon primer pair rpAP15f/rpAP15r was used in direct PCR according to Martini *et al.* (2008). PCR products were digested using *Alu*I restriction enzyme.

Results

Although all 74 apple trees examined showed apple proliferation disease symptoms, 63 plants (e.g. 85%) were positive for phytoplasma infection using at least one of the PCR/RFLP methods. The best results were obtained using nested PCR with primer combination P1A/P7A – R16F2n/R2 (32 samples were positive out of 36 samples

tested, e.g. 88.9%), nested PCR using primers AP13/AP10 – AP14/AP15 (59 samples were positive out of 82 tested, e.g. 72.0%) and direct PCR by primers rpAP15f/rpAP15r (57 samples positive out of 82 examined, e.g. 69.5%). RFLP with *Mse*I endonuclease of the DNA sequences amplified by PCR with primers R16F2n/R2 showed that apple phytoplasma strains from 51 trees had a pattern identical to each other and to the apple proliferation phytoplasma (ribosomal group 16SrX, AP). Mixed infection of AP and phytoplasmas belonging to 16SrI-C subgroup (aster yellows group) was detected in five plants. Phytoplasmas belonging to 16SrI-C subgroup (which type strain is clover phyllody) alone were detected in two apple trees.

Primers F1/B6 amplified DNA out of 50 trees (67.6%) in nested PCR assay. RFLP analyses (*Hpa*II) on these amplicons distinguished AP phytoplasma profile P-I in 39 plants, P-II in four plants and both P-I and P-II profiles were observed in six trees. Moreover, unusual pattern, previously unpublished, was observed in one sample originated from AP infected tree.

Primers P1A/P7A amplified DNA out of 42 plants (56.8%) in direct and/or nested PCR assay. RFLP analyses of 1,759 bp fragment, revealed the presence of two phytoplasma genetic lineages designated as pattern '1' in five apple trees, pattern '2' in 32 trees and both together in five plants.

In parallel, the PCR/RFLP method based on ribosomal protein gene sequences as well as on a non-ribosomal DNA fragment recently established were used in order to differentiate ribosomal subgroups (rpX-A, -B, -C, -D) and AP phytoplasma subtypes AP, AT-1 and AT-2, respectively. In the majority of apple trees examined (53 trees out of 74 tested), revealed the presence of AP phytoplasmas belonging to subgroups rpX-A, while phytoplasmas of rpX-B subgroup were detected in three plants. All three phytoplasma subtypes (AP: 18 plants, AT-1: 7 plants, AT-2: 19 plants) singly (44 plants) or in different combination (10 plants) were found in examined samples.

Discussion

The first biological proof of apple proliferation disease was carried out in Czech Republic by Seidl (1965). Subsequently phytoplasma bodies were observed on ultrathin sections of roots and abnormal underground sprouts of apple trees artificially infected with the proliferation disease (Brčák *et al.*, 1972). Using molecular tools, phytoplasmas belonging to different ribosomal groups and subgroups were described in limited number of apple and pear trees (Bertaccini *et al.*, 2001; Fránová, 2005) in our country. Firstly, we report here the genetic diversity of '*Ca. P. mali*' population in Czech Republic. The majority of apple trees examined was infected by '*Ca. P. mali*' belonging to ribosomal subgroup rpX-A, subtypes AP and AT-2. Diversity in 16S-23S rDNA was also

observed. Most frequently, pattern designated as '2' by Casati *et al.* (2010) and profile P-I according to Paltrinieri *et al.* (2010) were determined in specimens collected from apple trees during 2010. Our data demonstrated high degree of genetic diversity of AP in Czech Republic. Sequencing of different strains on the various genes is in progress.

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Phytoplasma and spiroplasma diseases in open-field crops in Israel

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Abstract

During 2009 - 2011, symptoms of curling, yellow and purple discoloration of leaves, stunting of shoots, and formation of bunchy, fibrous secondary roots were observed in several ornamental crops, carrot, celery, and parsley fields located in several production areas of Israel. Incidence of disease was almost 20-80% in individual affected fields. Moreover, the observed symptoms resembled those caused by *Spiroplasma citri* in carrots affected by the carrot purple leaf disease, recently reported in United States and in Spain. Analyses revealed that high percentage of symptomatic plants tested positive for *S. citri*. Some of the plants were double infected by *S. citri* and a phytoplasma. Leafhopper species known to vector phytoplasmas and/or spiroplasmas, have been trapped in several locations in Israel. To our knowledge, this is the first report of *S. citri* associated with the 'yellow disease' syndrome in open-field crops in Israel.

Key words: carrots, field crops, spiroplasma, phytoplasma, leafhopper, vectors.

Introduction

Phytoplasma diseases have been identified in Israel in cultivated ornamentals and fruit trees (Gera *et al.*, 2004, 2007; Weintraub *et al.*, 2007). Carrot is a major crop worldwide and in Israel, about 245,000 tons are produced annually. Carrot (*Daucus carota* L.) have been known to be susceptible to phytoplasma for more than 50 years, however the disease was first reported in 1995 (Orenstein *et al.*, 1999). A number of leafhopper species known to be vectors of phytoplasmas and spiroplasma occur in Israel (Orenstein *et al.*, 2003; Weintraub and Orenstein, 2004). The present paper reports outbreak of phytoplasma and spiroplasma associated diseases in carrots and other commercial field crops in Israel.



Figure 1. Symptoms of the yellows disease in carrots.
(In colour at www.bulletinofinsectology.org)

Materials and methods

Sample collection

Carrot, parsley (*Petroselinum crispum*) and celery (*Apium graveolens*) plants grown in commercial fields and carrying symptoms typical of the yellow disease infection were sampled.

Polymerase chain reaction and sequence analysis

Total DNA was extracted from symptomatic and asymptomatic plants as described by (Gera *et al.*, 2004). DNA samples were analyzed by PCR assays according to Lee *et al.* (2006).

Leafhopper survey

Commercial carrot fields at Kibbutz Sa'ad were monitored for two consecutive years. Yellow sticky traps (14 x 20 cm) were placed at plant height evenly spaced around the field, and replaced weekly throughout the year.

Results

Symptoms

Symptoms typical of a phytoplasma infection were observed on a large number of carrot plants. Symptoms included: curling, yellow and purple discoloration of leaves, stunting of shoots, and formation of bunchy, fibrous secondary roots (figure 1). Incidence of this disease was 20-80% in individual affected fields.

Molecular identification

Analyses revealed that high percentage of symptomatic plants tested positive for *Spiroplasma citri* (56%). Some of the plants were infected by phytoplasmas (20%). Among these, 12% were found to be double infected by *S. citri* and a phytoplasma while others (24%) were spiroplasma- and phytoplasma-free. Spiroplasma was also detected in celery, parsley and several weeds grown in proximity to infected carrot fields.

Leafhopper survey

Leafhopper vectors of phytoplasmas and spiroplasma (*Orosius orientalis*, *Circulifer spp.*, *Exitianus capicola*, *Neolaliturus fenestratus*, and *Hyalesthes obsoletus*), were captured on sticky traps during the survey.

Discussion

During 2009 - 2011, symptoms of curling, yellow and purple discoloration of leaves, stunting of shoots, and formation of bunched, fibrous secondary roots were observed in carrot, celery and parsley grown in commercial fields located in several production areas of Israel. Incidence of this disease was cyclic and ranged 20-80% in individual affected fields. Furthermore, the disease in Israel is highly sporadic and cyclic.

PCR analyses revealed that high percentage of symptomatic plants tested positive for *S. citri*. Some of the plants were double infected by *S. citri* and a phytoplasma. Although all 50 carrot samples analysed by PCR for the presence of phytoplasma DNA showed full symptoms of infections, only 76% of them were positive for spiroplasma, phytoplasma or both.

Plants showing typical phytoplasma symptoms, but having negative PCR results, are not unique to Israel; similar results have been reported from Italy (Marzachi *et al.*, 1999) and Australia (Gibb *et al.*, 1995). Symptoms similar to phytoplasmas have been shown to be associated also with other prokaryotes such as rickettsia or rickettsia-like-organisms; or by '*Candidatus Liberibacter solanacearum*' transmitted by the carrot psyllid (*Trioza apicalis*) (Munyaneza *et al.*, 2010).

Phytoplasmas and spiroplasma are transmitted by leafhoppers and planthoppers. In our survey, leafhoppers and planthoppers were trapped throughout the year. Since potential leafhopper vectors (*O. orientalis*, *Circulifer haematocephus*, *C. tenellus* and *Exitianus exitiosus*) were already present in the carrot growing areas, it is not surprising that the disease rapidly spread in carrots and other open-field crops.

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Detection of phytoplasmas in mixed infection with begomoviruses: a case study of tomato and pepper in Mexico

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Abstract

Scanning electron microscopy (SEM) and molecular techniques were employed to investigate the possible causal agents of yellow-type diseases in tomato and pepper in Baja California Sur (BCS) state of Mexico. Mixed infection of phytoplasmas (16SrIII, X-disease group) and two different begomoviruses (TYLCV and ToChLPV) was identified in pepper. Phytoplasma infection was also confirmed in tomato by SEM and nested PCR assays and along with mixed infection by two begomoviruses (TYLCV and PepGMV).

Key words: phytoplasma, begomoviruses, SEM, nested-PCR, RFLP, tomato, pepper, BCS.

Introduction

In the Mexican state of BCS the occurrence of phytoplasmas associated with yellow type diseases of tomatoes and peppers was obtained by SEM analysis during 2005-2008 in field and greenhouse-growing crops from principal agricultural areas (Lebsky and Poghosyan, 2007, Poghosyan *et al.*, 2008). Some symptoms in pepper and tomato plants associated with phytoplasma infection are similar to those provoked by begomovirus group (*Geminiviridae*). Thus and accurate disease diagnosis is necessary to prove the disease aetiology and the possible presence of both pathogens.

The study was initiated in 2008 to determine the origin of yellow type disease in tomato and pepper in two experimental plots of CIBNOR in El Comitan (tomato of Japanese selection) and in El Carrizal (chile ancho), where the plantings were established. A very high incidence of disease (up to 80-90%), was recorded on both crops. The yellows-type symptoms may be indicative of both phytoplasma and begomovirus presence and the presence of large whiteflies populations in the crops also confirm the possibility of mixed infection. To verify this hypothesis scanning electron microscopy (SEM) and molecular techniques were applied.

Materials and methods

Symptoms in diseased plants were strong reduction, chlorosis and foliar malformations in apical and internodal leaves, more differently and extensively expressed in tomato plants (spoon-like lamina and claw-like apex), discoloration and necrotic lesions on old leaves, shortened internodes, distortion and thickness of leafstalks and stems, dried and aborted flowers and fruits, deformed and reduced in size and quantity.

The symptoms of disease were transmitted by grafting from field plants to tomato and pepper test plants in greenhouse conditions. Samples from both field and test

plants were processed for SEM (S-3000N Hitachi) analysis of phytoplasmas as reported earlier (Lebsky and Poghosyan, 2007), and for molecular probes. Micropropagated plants (phytoplasma-free) were used as controls. For molecular detection of phytoplasmas total DNA was extracted from the same plants by Zhang *et al.* (1998). Nested-PCR assay was performed with phytoplasma-specific primer pairs P1/P7 and R16F2n/R16R2 (Gundersen and Lee, 1996). PCR products were analysed as described by Lee *et al.*, (1998), cloned in vector pGEM-T-Easy vector, sequenced (Genwiz, USA).

To detect virus, total DNA from symptomatic samples was obtained by a modified Dellaporta method and analyzed by nested PCR using the begomovirus universal primers (Mauricio-Castillo *et al.*, 2007, Wyatt and Brown, 1996). Amplicons of ~1.4 kb were cloned and digested by enzymes *EcoRI* and *HinfI*. Sequence of genomic component A of viruses was determined by PCR amplification of viral DNA with degenerate primers (Mauricio Castillo *et al.*, 2007). Cloning, sequencing and subsequent analysis using GenBank database (BlastN and ClustalV alignment method) were employed.

Results

Observations of field and greenhouse indexed samples by SEM revealed the presence of phytoplasma cells ranging from 400 to 1500 nm in the phloem of diseased plants: in leafstalks, leaf midribs, stems floral parts and roots (figure 1). Some asymptomatic field samples also had a low concentration of phytoplasmas in their phloem tissue.

No pathogen was detected in healthy micropropagated plants. In phloem tissue of some samples along with phytoplasmas some rod-shaped bacteria and groups of twinned particles characteristic of geminiviruses (*Geminiviridae*) were detected (figure 2).

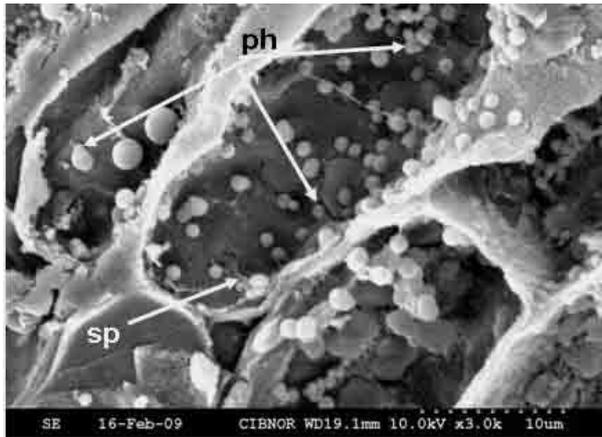


Figure 1. Phytosmas (ph) in sieve tubes of tomato. Sp: sieve pore.

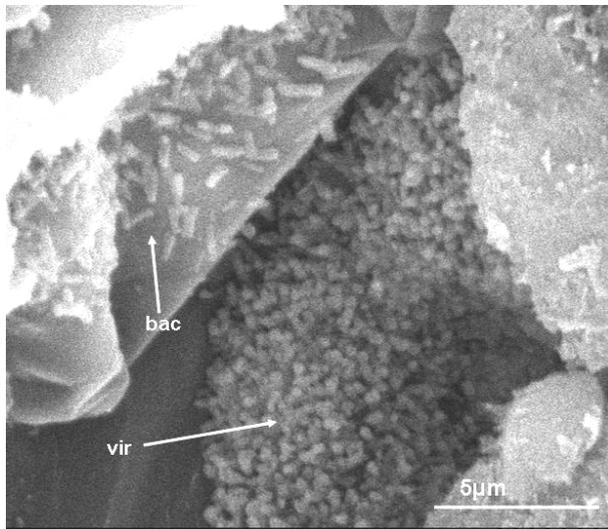


Figure 2. Groups of geminate virus particles (vir) in phloem parenchyma of the same tomato. Bac: rod shaped bacteria.

Nested PCR analysis of both pepper and tomato samples from the plants tested by SEM, revealed a 1,200 bp amplified product, thus supporting the presence of phytosmas in plants with yellows symptoms.

Phytosmas were not detected in micropropagated plants. Sequence analysis of amplified products proved that the phytosmas detected in pepper belonged to 16SrIII X-disease group. Sequence analysis of amplified DNA products of tomato demonstrated only 80% of similarity with known phytosmas. The taxonomic grouping of this phytosmas needs further confirmation.

The results of PCR assays indicated association of two different begomoviruses in pepper: *Tomato yellow leaf curl virus* (TYLCV), reported first time in Baja California Peninsula, and *Tomato chino La Paz virus* (first report of ToChLPV in pepper, Cardenas-Conejo *et al.*, 2010). Virus analysis of tomato symptomatic samples by nested PCR and subsequent procedures also revealed a co-infection with two begomoviruses: TYLCV and *Pepper golden mosaic virus* (PepGMV).

Discussion

The presence of phytosmas and two different begomoviruses in tomato and pepper samples in Mexico was verified by SEM and PCR assays. This is the first evidence of mixed phytosmas-begomovirus infection in tomato and pepper plants. In the last decade the complex phytosmas-virus associations with plant diseases are discussed more extensively (Arocha *et al.*, 2009). Such mixed infections need to be analysed from both epidemiological and pathogens interaction point of view. The work is in progress to define the phytosmas identity in tomato and the phylogenetic position of phytosmas detected in tomato and pepper plants showing yellows symptoms in Mexico.

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Occurrence of two 'Candidatus Phytoplasma asteris'-related phytoplasmas in poplar trees in Serbia

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Abstract

During a survey carried out in Serbia in black poplar trees, typical symptoms of phytoplasma presence were observed such as yellowing and undersize of the leaves, witches' broom and decline. Survey for phytoplasma presence in Belgrade and in its surrounding allow to identify phytoplasmas belonging to 'Candidatus Phytoplasma asteris' group, in particular to two 16SrI subgroups 16SrI-P and 16SrI-A. Blast search of obtained strain in subgroup 16SrI-P of the 16SrDNA sequence showed 100% homology with aster yellows phytoplasma strain from poplar from Croatia.

Key words: 'Candidatus Phytoplasma asteris', black poplar, PCR-RFLP.

Introduction

Populus nigra L. 'Italica' is one of several populus species that are known to be affected by phytoplasma diseases (Berges *et al.*, 1997). The witches' broom (PopWB) disease of black poplar (*Populus nigra* L. 'Italica') and *P. canadensis* was observed for the first time in 1973 in Bulgaria by Atanasoff. Van der Meer (1980, 1981) observed the disease on white poplar (*P. alba*), grey poplar (*P. canescens*) and black poplar in the Netherlands. The electron microscopic detection of phytoplasmas in white poplar was reported in France (Sharma and Cousin, 1986) and in *P. tremula* in Germany (Seemüller and Lederer, 1988). It was then demonstrated using PCR/RFLP analyses, that the PopWB phytoplasma in France and Germany belongs to the aster yellows group, AY, (Maurer *et al.*, 1994; Cousin, 1997). Later AY phytoplasmas were reported in black poplar also in Croatia (Šeruga *et al.*, 2002).

Black poplar, like other poplar species, is one of the common ornamental trees grown in Serbia: they are grown in city parks, street avenues, and in other horticultural areas, and are often present by the river coasts. In previous reports, the presence of phytoplasmas in black poplar was associated with witches' broom symptoms although, in some cases, less specific syndromes were reported such as undersized leaves, yellowing, sparse foliage, stunting and dieback (Sharma and Cousin 1986; Cousin 1996; Berges *et al.*, 1997).

On black poplar trees, on Belgrade street avenue, typical symptoms of phytoplasma presence were observed: yellowing and undersize of the leaves, witches' broom and decline. Survey for phytoplasma presence in poplar trees in Belgrade (Serbia) and in its surrounding was therefore performed.

Materials and methods

To verify the presence and determine the identity of phytoplasma, molecular assays were performed on 10 DNA extracts from symptomatic black poplar leaf mid-ribs. Total DNA extraction was performed using CTAB protocol described by Angelini *et al.*, 2001. Polymerase chain reaction (PCR) was performed for amplification of phytoplasma 16S rRNA gene, spacer region and part of 23S rRNA gene, using phytoplasma-universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995).

For identification, nested PCR on P1/P7 amplicons was performed using R16F2/R2 primer pair under reported conditions (Lee *et al.*, 1995). Products amplified by PCR assays were visualised and positive samples were subjected to the restriction fragment length polymorphism (RFLP) analysis. Three restriction endonucleases were used, *Tru*II, *Taq*I and *Hha*I (Fermentas, Vilnius, Lithuania), according to the manufacturer's instructions. Reference phytoplasma strain used were: chrysanthemum yellows 16SrI-A (CHRYM), primula green yellows 16SrI-B (PrG) and carrot yellows 16SrI-C (CA). The P1/P7-amplified product of a poplar sample, was purified using Qiagen PCR purification kit (Qiagen GmbH, Hilden, Germany) and sequenced in both directions with two forward primers P1 and R16F2 (Lee *et al.*, 1995) and one reverse primer P7, using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The sequence was assembled using Pregap4 from the Staden program package (Staden *et al.*, 2000) and compared with 16S ribosomal sequences of phytoplasmas in the GenBank database using blast (version Blast N 2.2.18) at the National Center for Biotechnology Information.

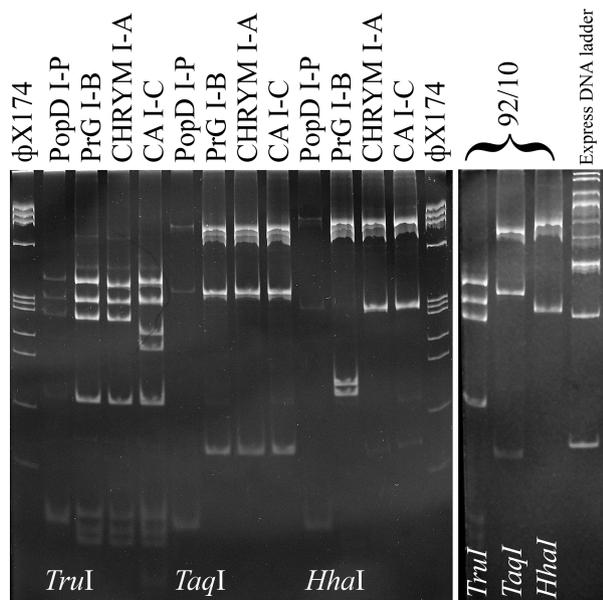


Figure 1. Differential RFLP profiles obtained from R16F2/R2 amplicons of two phytoplasmas from poplar trees and reference strains with different restriction enzymes.

Results and discussion

Among the 10 plants tested only two (PopD and 92/10) showed phytoplasma presence, both resulting positive also in direct PCR with P1/P7. Collectively, restriction profiles on R16F2/R2 amplicons for phytoplasma identification showed that phytoplasmas from Serbian black poplar trees belong to the 'Candidatus Phytoplasma asteris' group and to two diverse 16SrI subgroups (figure 1). The strain PopD belongs to 16SrI-P, while strain 92/10 belongs to 16SrI-A subgroup. Blast search of obtained PopD phytoplasma 16SrDNA sequence (1,494 bp) showed 100% homology with aster yellows phytoplasma strain from poplar from Croatia (AF503568). This is the first report of this phytoplasma in black poplar in Serbia, and indicates that this strain is present in larger areas than previously reported (Šeruga *et al.*, 2002). On the other hand the presence of 16SrI-A phytoplasma in poplar tree was never reported before.

Acknowledgements

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Polyclonal antibodies for the detection and identification of Fars alfalfa witches' broom phytoplasma

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Abstract

Alfalfa witches' broom (AIWB) is a destructive phytoplasma disease in Iranian southern and central provinces. Antiserum was raised by injection of partially purified Fars AIWB phytoplasma (FAIWB) from 20 g infected alfalfa tissue into a rabbit. The specificity of the polyclonal antibody against phytoplasmas was shown by its reaction with diseased, but not with healthy alfalfa upon dot immunobinding assay (DIBA) tests. Phytoplasmas associated with Borazjan fababeen phyllody and Chahbahar periwinkle phyllody were also shown to be serologically related to FAWBP. No serological relationship was found between FAIWB and phytoplasmas associated with Yazd AIWB, witches' broom disease of lime, Khafr almond witches' broom, carrot witches' broom, sesame phyllody, eggplant big bud and tomato big bud.

Key words: phytoplasma purification, dot immunobinding assay, Iran.

Introduction

Iranian alfalfa witches' broom (AIWB) disease has been found in economically significant amounts in central and southern provinces (Salehi *et al.*, 1995; Salehi *et al.*, 2000). Fars (Iran) AIWB phytoplasma (FAIWB) has been characterized using both biological and molecular methods (Salehi *et al.*, 1995, Salehi *et al.*, 2005). On the basis of full length 16S rRNA gene and SR sequences, FAIWB is classified in the pigeon pea witches' broom (16SrII) group and is not distinguishable from agents of witches' broom disease of lime (WBDL) in Iran (Salehi *et al.*, 1998), and of the pigeon pea witches' broom related phytoplasma in Iran (Salehi *et al.*, 2005). The objectives of the present study were to partially purify the FAIWB, to produce antiserum, to achieve serological detection of FAIWB and to investigate serological relationships among FAIWB and a selection of other phytoplasmas including WBDL phytoplasma using dot immunobinding assay (DIBA) tests.

Materials and methods

Infected alfalfa from Juyom (about 250 km south east of Shiraz, Fars province) was used for partial purification of the FAIWB phytoplasma. The nine following strains maintained and propagated in a red line of periwinkle (*Catharanthus roseus*) were also included in this study: Borazgan fababeen phyllody (BFBP), carrot witches' broom (CWB), Chahbahar periwinkle phyllody (CPP), Khafr almond witches' broom (KLWB) sesame phyllody (SeP), witches' broom disease of lime (WBDL), Yazd AIWB (YAIWB), Zarghan eggplant big bud (ZEBB), and Zarghan tomato big bud (ZTBB).

The procedure of Clark *et al.* (1983) with modifications of Saeed *et al.* (1993) was used for partial purification and preparation of an antiserum. Infected alfalfa from Juyom (about 250 km south east of Shiraz,) was used for partial purification of FAIWB. Twenty grams of infected tissue (stem and leaf) were homogenized in cold GMS at a ratio of 1 g tissue to 5 ml GMS. The extract was filtered and then centrifuged at 2000 g for 15 min. This was followed by high-speed centrifugation at 39000 g for 1 h and resuspension of the pellet in 1.5 ml GMS buffer. The resuspended pellet was then incubated for 1 h at 4°C with 4 ml of undiluted antiserum prepared against healthy alfalfa extract. After another low and high speed centrifugation as described above, the final pellet was resuspended in 1 ml of PBS buffer (0.01 M phosphate buffer saline) and the resuspended pellet was used for immunization and production of polyclonal antiserum against FAIWB.

A white rabbit was injected intramuscularly in legs and subcutaneously in the neck with an emulsion of equal volumes of partially purified FAWBP and Freund's incomplete adjuvant. Four injections were given at intervals of 7 days. The rabbit was bled 4 times at daily intervals beginning 5 week after primary immunization. This antiserum after absorption with healthy plant sap was used for serological detection and identification.

Dot immunobinding assays (DIBA) were used for serological detection of FAIWB and for investigation of possible serological relationships between FAIWB and a selection of other phytoplasmas using the Hibi and Satio (1985) procedure. Appearance of well defined purple spots on nitrocellulose sheets were regarded as positive reactions.

Results and discussion

After absorption with healthy plant sap, the antiserum prepared against partially purified FAIfWB distinguished healthy and FAIfWB infected plants using DIBA tests. All symptomatic and 6 of 15 symptomless alfalfa plants from an infected alfalfa field in Juyom showed positive reactions. BFBP and CPP strains were shown to be serologically related to the FAIfWB phytoplasma. No serological relationship was found between FAIfWB and phytoplasmas associated with CWB, KALWB, SeP, WBDL, YAIWB, ZEBB and ZTBB (figure 1).

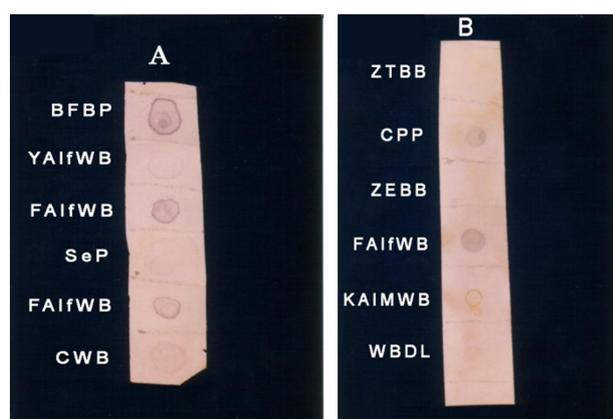


Figure 1. (A and B). Reaction of several Iranian phytoplasma strains to Fars alfalfa witches' broom antiserum in dot immunobinding assay. Phytoplasma abbreviations: BFBP, Borazgan fababeen phyllody; CWB, carrot witches' broom; CPP, Chahbahar periwinkle phyllody; FAIfWB, Fars alfalfa witches' broom; KALWB, Khafr almond witches' broom; SeP, sesame phyllody; WBDL, witches' broom disease of lime; YAIWB, Yazd alfalfa witches' broom; ZEBB, Zarghan eggplant big bud; ZTBB, Zarghan tomato big bud.

A polyclonal antibody against AlfWB was raised for the first time. Although the antibody was prepared by injection of partially purified phytoplasma, it exhibited a good degree of specificity and the background of non-specific reaction was considerably low. Use of this antiserum proved that BFBP and CPP phytoplasmas were related to FAIfWB phytoplasma, while YAIWB phytoplasma was not serologically related to FAIfWB

phytoplasma despite their common host and similar symptoms. Likewise, CWB, KALWB, SeP, WBDL, YAIWB, ZEBB and ZTBB phytoplasmas were serologically not related to FAIfWB phytoplasma. On the basis of 16S rRNA gene and SR sequence analysis CPP, BFBP, CWB, WBDL, YAIWB, ZEBB and ZTBB are related to 16SrII but KALWB and SeP phytoplasmas to 16SrIX Salehi *et al.*, 2005). This study showed that sequence-based related phytoplasmas can be differentiated using polyclonal antibodies and serology is a useful method for separating different 'Ca. Phytoplasma' species when they have over 97.5% similarity on 16S ribosomal gene as reported previously (IRPCM, 2004).

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Detection of 'Candidatus Phytoplasma brasiliense' in a new geographic region and existence of two genetically distinct *dnaK* genotypes

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Abstract

In September 2007, a peach tree (*Prunus persica*) displaying yellowing symptoms indicative of phytoplasma infection was sampled in Quba region of Azerbaijan. A phytoplasma was detected in the diseased peach tree by nested PCR amplification of its 16Sr DNA with universal primers for phytoplasmas. Phylogenetic analyses of the amplified 16S rDNA showed that the phytoplasma infecting peach tree corresponded to 'Candidatus Phytoplasma brasiliense', a phytoplasma not previously reported in the Euro-Mediterranean area. To set up a detection assay, cloning of a 'Ca. P. brasiliense' DNA fragment was undertaken by comparative RAPD. The amplified *dnaK-dnaJ* genetic locus was used to design a nested PCR assay able to amplify all 'Ca. P. brasiliense' strains of the subgroup 16SrXV-A without amplifying the related members of the group 16SrII. The use of this assay also confirmed detection for the first time of 'Ca. P. brasiliense' in diseased basil collected in south Lebanon.

Key words: 'Ca. P. brasiliense', hibiscus witches' broom disease, *dnaK* gene, molecular characterization.

Introduction

Phytoplasmas are plant pathogenic bacteria belonging to the class *Mollicutes*, a group of wall-less microorganisms having low G+C content, Gram-positive bacteria. They cause hundreds of diseases worldwide and are transmitted from plant to plant by sap-feeding hemipteran insects (Lee *et al.*, 2000; Weintraub and Beanland, 2006). Phytoplasmas have been classified into 30 phylogenetic groups and 28 'Candidatus Phytoplasma' species according to 16S rDNA phylogeny and RFLP profiles (Zhao *et al.*, 2010). Among these, 'Ca. P. brasiliense' has been described as the agent of hibiscus witches' broom in Brazil. During a survey of temperate fruit tree orchards of the North of Azerbaijan, a phytoplasma could be detected by 16S rDNA PCR of a chlorotic peach tree (*Prunus persica*). We report in this paper its identification as a strain of 'Ca. P. brasiliense' and the development of a specific PCR detection test developed from a 'Ca. P. brasiliense' sequence cloned after comparative RAPD analyses.

Materials and methods

Yellowing peach tree (*Prunus persica*) samples indicative of phytoplasma infection were collected in September 2007 in Quba region. The DNAs were extracted following the CTAB extraction protocol of Maixner *et al.* (1995). Detection of phytoplasma infection was performed by nested PCR with the 16S rDNA universal primers for phytoplasmas as described by Gundersen and Lee (1996). The PCR product obtained from one of the peach trees (PEACH19) was sequenced. The raw sequences were assembled and edited using GAP4 and the consensus

sequence deposited at EMBL (FR717540). ClustalW multiple alignments and maximum of parsimony phylogenetic analyses were performed by MEGA 4 (Tamura *et al.*, 2007).

To investigate genetic variability of the detected phytoplasmas non ribosomal *dnaK* isolated by comparative RAPDs, was amplified by group specific primers recently developed (Balakishiyeva *et al.*, in press) Nested PCR products were digested with *TaqI* (Promega) according to the manufacturer's instructions.

Results

The 16S rRNA sequence of the PCR product obtained from PEACH19 phytoplasma shared 100% identity with the 16S rRNA sequence of 'Ca. P. brasiliense' group 16SrXV (hibiscus witches' broom) (Montano *et al.*, 2001). Both sequences clustered together on the same phylogenetic branch supported by a bootstrap value of 100 (data not shown) indicating phytoplasma affiliation to subgroup 16SrXV-A, 'Ca. P. brasiliense'.

No specific detection tool was available for 'Ca. P. brasiliense' mainly because except for the 16S rRNA gene, no 'Ca. P. brasiliense' gene had been sequenced. Therefore, the characterization of a 'Ca. P. brasiliense' non ribosomal genetic locus was undertaken using random-PCR that allowed the *dnaF-dnaJ* locus to be cloned and sequenced. A PCR test amplifying the non ribosomal *dnaK* gene was developed with the aim of specifically detecting phytoplasmas of subgroup 16SrXV-A ('Ca. P. brasiliense') (Balakishiyeva *et al.*, unpublished). To verify the specificity of the *dnaK* gene primers (Bra-dnaKF1/R1 and Bra-dnaKF2/R2), nested-PCR

was performed on DNAs extracted from diseased peach, healthy peach, Suriname virescence infected periwinkle, 'Ca. P. brasiliense'-infected basil (Choueiri *et al.*, unpublished), three 'Ca. P. brasiliense'-infected *Hibiscus rosa-sinensis* plants, and ten phytoplasma strains belonging to the 16SrII group. The Bra-dnaK nested-PCR amplified phytoplasmas of the subgroup 16SrXV-A but did not amplify the related phytoplasmas of the group 16SrII. For further characterization of phytoplasmas of group 16SrXV-A, the PCR products were submitted to restriction fragment length polymorphism (RFLP) analysis with restriction enzyme *TaqI*. Results of RFLP analysis (figure 1A) showed that the restriction of the amplicons using *TaqI* revealed two restriction profiles among the different 'Ca. P. brasiliense' strains. All hibiscus witches' broom phytoplasmas gave the same pattern characterized by a DNA band at 0.45 kbp, whereas PEACH19, Suriname virescence and basil (figure 1A) had no 0.45 kbp DNA band but an additional band at 0.3 kbp and a brighter band at 0.15 kbp. These results indicate the existence of two genetically different strains of 'Ca. P. brasiliense'. This was confirmed by two sequence types after sequencing the six amplicons. The first sequence type (accession number FR775800), that of hibiscus witches' broom 121, 122 and CB2 exhibited 19 mutations when compared to the sequence of Suriname virescence, basil and PEACH19 which were totally identical and constitute a second sequence type (accession number FR717541). A guanine to thymine mutation at position 689 (figure 1B) in the bra-dnaK PCR product of hibiscus witches' broom strains eliminated a *TaqI* restriction site, responsible for the difference in restriction profiles with the Suriname virescence, basil and PEACH19 strains.

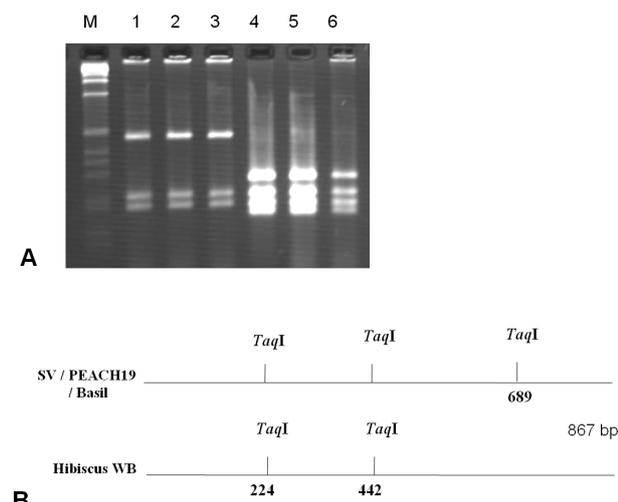


Figure 1. A: RFLP analysis with *TaqI*. Digested DNAs were analyzed on 3% agarose gel in 1X TBE. B: *TaqI* restriction map of bradnaK PCR products amplified from 'Ca. P. brasiliense' strains. Lane M, 1kb DNA ladder (Invitrogen), lane 1-Hib121, lane2-Hib122, lane3-Hib CB02, lane 4-SV, Suriname virescence infected periwinkle, lane 5- Bas, Basil from Lebanon, lane 6-PEACH19, diseased peach tree from Azerbaijan.

Discussion

Reported geographical distribution and host range of 'Ca. P. brasiliense' include *Sida rhombifolia* in Brazil (Eckstein *et al.*, 2011), *Guazuma ulmifolia* in Costa Rica (Villalobos *et al.*, 2010), and *Gliricidia sepium* in Ethiopia (unpublished GenBank accession AF361018). This phytoplasma, new for the old world was detected in a peach tree in the Quba region of Azerbaijan and in basil from Lebanon. Its presence not only in a woody host but also in an annual host (basil) indicates its possible local transmission by insects.

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Detection and identification of aster yellows and stolbur phytoplasmas in various crops in Spain

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Abstract

In this study '*Candidatus* Phytoplasma asteris' (subgroups 16SrI-B) and stolbur phytoplasma (subgroup 16SrXII-A) were sporadically identified in several horticultural crops in Spain by nested-PCR and RFLP analyses. One parsnip sample was infected with stolbur, and '*Ca. P. asteris*' was detected in lettuce and chicory plants. However, these two phytoplasmas were able to infect also carrot, celery and radish. This work extends the knowledge of phytoplasma diversity affecting horticultural crops in Spain.

Key words: phytoplasma, nested-PCR, horticultural crops, RFLP, 16S rDNA.

Introduction

Phytoplasmas are a group of pathogenic phloem-restricted plant wall-less prokaryotes (class Mollicutes) naturally transmitted by phloem-sap feeding insects specifically leafhoppers, planthoppers and psyllids (Lee *et al.*, 2000). Plants infected by phytoplasmas exhibit symptoms that suggest profound disturbances in the normal balance of plant hormones or growth regulators (Lee *et al.*, 2000; Bertaccini and Duduk, 2009). The development of polymerase chain reaction (PCR) techniques with universal primers pairs for general detection of various phytoplasmas and restriction fragment length polymorphism (RFLP) analysis of the 16S rDNA fragment enables phytoplasma detection, differentiation and classification (Lee *et al.*, 1993; Lee *et al.*, 1998; Marzachi, 2004). To date, 19 RFLP groups and more than 40 sub-groups have been identified based on 16S rDNA sequences (Bertaccini and Duduk, 2009). The main objective of the present work was to determine phytoplasmas presence in some affected horticultural Spanish crops which showed symptoms referable to phytoplasma presence, and identify the specific phytoplasmas groups and strains present in those samples.

Materials and methods

Samples of different horticultural crops with phytoplasmas-like symptoms were collected in different years (table 1). Healthy samples of each plant species and positive samples to stolbur and '*Ca. P. asteris*' were also included in the assay as negative and positive controls, respectively. Total DNA was extracted as described Green and Thompson, (1999). A nested-PCR assay was performed using the universal phytoplasma primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) in the first amplification followed by R16F2n/R16R2 (Gundersen and Lee, 1996) in the second amplification to detect phytoplasmas in the affected plants. The PCR

products were analysed on 1.2% agarose in TAE buffer gels, stained in ethidium bromide and visualized with a UV transilluminator. Restriction fragment length polymorphism (RFLP) analysis of the nested-PCR products (1.2 kb 16S rDNA fragments) was used for identification of the phytoplasmas detected (Lee *et al.*, 1998) with *MseI* and *HhaI* endonucleases (MBI Fermentas, Vilnius, Lithuania).

Results and Discussion

Fragments of the expected size (1.2 kb) were only amplified from the symptomatic samples and positive controls, but were not produced from healthy samples or water used as negative controls. The RFLP profiles when compared with control phytoplasma profile and with profiles of other phytoplasma 16S rRNA groups described by Lee *et al.* (1998) indicated that the phytoplasmas present in the different plant species belong to the 16SrI-B ('*Ca. P. asteris*') and 16SrXII-A (stolbur) subgroups (table 1). In some horticultural crops only one phytoplasma was detected, as for example in lettuce where just '*Ca. P. asteris*' was identified. However, in carrot, celery or radish both phytoplasmas were found. Phytoplasmas have been described in all the analyzed hosts in previous works worldwide. In some cases the phytoplasmas reported were the same as in this study such as in lettuce (Zhang *et al.*, 2004). However in other crops the phytoplasmas detected were different, as for example, celery which was reported to be infected by tomato big bud phytoplasma (subgroups 16SrI-A) in Australia (Tran-Nguyen *et al.*, 2003) or stolbur (subgroups 16SrXII-A) in Czech Republic (Navratil *et al.*, 2009) and parsnip which was infected by aster yellows phytoplasma (subgroups 16SrI) in Canada (Kadhair and Evans, 2000). To our knowledge, this work represents the first report of phytoplasmas detected in chicory, radish, parsnip and celery in Spain.

Table 1. Samples of different horticultural crops collected in different regions of Spain showing phytoplasma-like symptoms and characterization of the phytoplasmas by RFLP analyses with endonucleases *MseI* and *HhaI*.

Host	Sample code	Year of Collection	Region	Phytoplasmas group	Phytoplasmas subgroup
Carrot	Car-97	1997	Canarias	Stolbur	16SrXII-A
	Car-05	2005	La Rioja	Stolbur	16SrXII-A
	Car-09-1	2009	Alicante	' <i>Ca. P. asteris</i> '	16SrI-B
	Car-09-2	2009	Alicante	' <i>Ca. P. asteris</i> '	16SrI-B
	Car-10-1	2010	Albacete	' <i>Ca. P. asteris</i> '	16SrI-B
	Car-10-2	2010	Alicante	' <i>Ca. P. asteris</i> '	16SrI-B
	Car-10-3	2010	Valladolid	' <i>Ca. P. asteris</i> '	16SrI-B
Celery	Cel-07	2007	Alicante	Stolbur	16SrXII-A
	Cel-08	2008	Alicante	' <i>Ca. P. asteris</i> '	16SrI-B
	Cel-09	2009	Alicante	' <i>Ca. P. asteris</i> '	16SrI-B
	Cel-10-1	2010	Alicante	' <i>Ca. P. asteris</i> '	16SrI-B
	Cel-10-2	2010	Alicante	' <i>Ca. P. asteris</i> '	16SrI-B
Radish	Rad-10-1	2010	Valencia	' <i>Ca. P. asteris</i> '	16SrI-B
	Rad-10-2	2010	Valencia	Stolbur	16SrXII-A
Lettuce	Let-08-1	2008	Castellón	' <i>Ca. P. asteris</i> '	16SrI-B
	Let-08-2	2008	Castellón	' <i>Ca. P. asteris</i> '	16SrI-B
	Let-09	2010	Castellón	' <i>Ca. P. asteris</i> '	16SrI-B
Parsnip	Par-09	2009	Alicante	Stolbur	16SrXII-A
Chicory	Chi-99	1999	Murcia	' <i>Ca. P. asteris</i> '	16SrI-B

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Association of aster yellows subgroup 16SrI-C phytoplasmas with a disease of *Ribes rubrum*

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Abstract

A *Ribes rubrum* plant showing malformation and twisting of branches was found in a private garden in South Bohemia. Observation of ultrathin sections of tissues from symptomatic shoots revealed the presence of phytoplasma-like bodies. Different primer sets were used for amplification of the 16S-23S ribosomal gene segment. RFLP analysis and sequencing for phytoplasma identification classified the detected phytoplasma in the aster yellows group, subgroup 16SrI-C. Successful transmission of detected phytoplasma by dodder (*Cuscuta campestris* Yuncker) to periwinkle (*Catharanthus roseus* L.) was confirmed by detection of numerous phytoplasma bodies in ultrathin sections of *C. roseus* and by PCR from dodder and periwinkle. RFLP analyses of PCR products as well as nucleotide sequences of the currant plant and symptomatic periwinkles were identical. Sequenced data obtained from both currant and indicator plant, were aligned and sequences of 1,613 bp were found to be identical. Transmissions of phytoplasma by grafting to healthy currant rootstocks were unsuccessful.

Key words: PCR, RFLP analysis, sequencing, transmission, red currant.

Introduction

Many fruit species including small fruits are affected by phytoplasma infection. The most common symptoms in these hosts are proliferation, color changes of leaves, leaf roll, and flower malformations. There are only a few conclusive data concerning the phytoplasma occurrence in *Ribes* sp. For the first time, phytoplasma bodies were localized by electron microscopy in the phloem tissue of fruit stalks of red currant that exhibited full blossom symptoms by Rakús *et al.* (1974). Navrátil *et al.* (2004) detected phytoplasmas of aster yellows and apple proliferation groups in *Ribes rubrum* using nested PCR and RFLP analysis. Špak *et al.* (2004) firstly detected the presence of phytoplasma in black currant with symptoms of the severe form of blackcurrant reversion disease.

Materials and methods

A currant bush showing severe twisting of shoots (figure 1) and producing poor yield of berries was found in a private garden in southern Bohemia. The symptoms appeared on new growing shoots and persisted throughout the life of the plant.

For transmission electron microscopy (TEM) ultrathin sections from phloem tissue of affected currant shoots and from petioles of symptomatic periwinkles were used. Samples were prepared as described (Navrátil *et al.*, 2004).

Cuttings from infected shoots were rooted and maintained in an insect proof glasshouse for symptom observation. In subsequent years, dodder transmission by *Cuscuta campestris* of the possibly infectious agent to 2 plants of *Catharanthus roseus* (all plants grown from seed) was carried out. Some cuttings were grafted onto healthy currant rootstock material, 3 plants cv. Jonkheer van Tets and 3 plants cv. Baldwin, all raised from seeds.

DNA was extracted from phloem tissues of currant symptomatic shoots, leaves of symptomatic periwinkles and dodder plants after transmission tests, leaf midribs of rooted planted cuttings and grafted rootstock plants according to Lee *et al.* (1991). The primer pairs: P1 (Deng and Hiruki, 1991), P7 (Schneider *et al.*, 1995); R16F2n/R2 (Gundersen and Lee, 1996) and R16(I)F1/R1 (Lee *et al.*, 1994) were used to amplify 16S-23S rRNA genes of the phytoplasma genome. PCR reactions were carried out according to Špak *et al.* (2004). About 6 µl of positive nested-PCR products were digested from R16F2/R2 amplicons obtained from the original currant and symptomatic vinca. Digestions were carried out with 2.5 U of *AluI*, *HhaI*, *MseI*, *KpnI*, *RsaI* restriction enzymes for at least 16 h. The restriction patterns were compared after electrophoresis on a 10% polyacrylamide gel.



Figure 1. Symptoms of twisting and deformation of shoots from affected currant plant.

Results

The same twisted branches and shoot malformations were observed on planted rooted cuttings in the subsequent year. Dodder transmission tests continued for 3 months, until dodder bridge was broken. Symptom of green flower petals, as the most pronounced symptom, appeared on both periwinkles 4 months after. Grafted rootstock material did not show any symptoms for 3 years. Examination of tissue samples by TEM revealed some ovoid or spherical phytoplasma-like bodies in phloem tissue of currant shoots. Phloem cells filled with these bodies were observed in samples from symptomatic periwinkles. PCR products were obtained with mentioned primer pairs from the original currant plant, rooted planted cuttings, both symptomatic periwinkles and from dodder. No PCR products were obtained from symptomless grafted rootstock plants. The digestion of R16F2/R2 amplified fragments of the currant and periwinkle with restriction enzymes showed the same patterns of the strain 16SrI-C according to Lee *et al.* (1998). The nucleotide sequences of phytoplasma from currant and periwinkle were identical. The 16S partial sequence from currant of 1,613 bp was deposited in the GenBank under accession no. AY669063. Comparison with available sequences revealed a close relationship with sequences of the 16SrI-C subgroup.

Discussion

Similar symptoms of shoot deformation on red currant were observed in Germany on cv. Casa (Dr. E. Kruger, personal communication). Similar symptoms of twisting and branch deformation were reported in apple trees in the Czech Republic where phytoplasmas were detected by PCR assays and by electron microscopy. Using RFLP analyses these phytoplasmas were classified in the aster yellows group, subgroup 16SrI-C, 16SrI-B and in the apple proliferation group, subgroup 16SrX-A (Fránová, 2005). The presence of phytoplasma bodies in sieve elements of the affected currant plant supports the phytoplasma aetiology of the disease. In currant phloem tissue we found phytoplasma bodies in small numbers, but numerous bodies were observed in *C. roseus* after dodder transmission. *C. roseus* is a much better source of phytoplasmal nucleic acids than currants. In *C. roseus*, the phytoplasma numbers are usually higher and phytoplasma components are easier to obtain (Macone *et al.*, 1997). This is probably a reason why phytoplasma transmission by grafting into rootstock material was unsuccessful. So far we did not observe twisted branches and shoot malformations in black currants (Špak *et al.*, 2009).

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Phytoplasmas in apricot, peach and sour cherry orchards in East Bohemia, Czech Republic

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Abstract

Symptoms of prematurely yellowing and leaf roll in the summer were observed in apricot, peach and sour cherry orchards in the East Bohemia (Czech Republic) during 2009-2010. Samples from selected symptomatic trees were tested by PCR assays followed by RFLP analyses. Detected phytoplasmas were classified in the apple proliferation group, subgroup 16SrX-B, which representative strains are apricot chlorotic leaf roll and European stone fruit yellows. Although the majority of symptomatic trees was observed in apricot orchards, out of 54 apricot trees examined by PCR/RFLP assays, only 10 plants were positive (18.5%) to phytoplasma presence. In the peach orchards, phytoplasmas were detected in 4 trees out of 14 plants tested (28.6%). Total number of trees examined from sour cherry orchards was 25, out of these 9 plants revealed positive reaction (36%). DNA sequencing of phytoplasma strain from one sour cherry tree confirmed the RFLP identification as '*Candidatus* Phytoplasma prunorum'. Experiments for transmission of '*Ca. P. prunorum*' by double budding from positive trees to indicator plants were carried out in open field during 2009 and 2010. No symptoms typical for phytoplasma infection were observed to date.

Key words: '*Candidatus* Phytoplasma prunorum', PCR/RFLP, biological indexing, apricot, peach and sour cherry orchards.

Introduction

'*Candidatus* Phytoplasma prunorum' associated with of European stone fruit yellows (ESFY) disease is the quarantine phytoplasma infecting trees of the genus *Prunus* worldwide (Seemüller and Schneider, 2004). This pathogen can be transmitted by insect vector of psyllid species, *Cacopsylla pruni* (Scopoli) (Carraro *et al.*, 1998). Typical symptoms for ESFY are yellowing and leaf roll in summer. The disease is very dangerous, because infected plants die within one or two years. The first report of ESFY identification in Czech Republic was from declining apricot trees (Navrátil *et al.*, 1998). We report here the results of a survey on phytoplasmas infecting apricot, peach and cherry trees in East Bohemia during 2009-2010.

Materials and methods

The observation of phytoplasma disease symptoms was carried out in 2 apricot, 1 peach and 2 sour cherry orchards in East Bohemia during 2009-2010. Samples were collected from symptomatic branches during late summer and autumn. Altogether, 54 apricot trees, 14 peach trees and 25 sour cherry trees were examined.

DNA was extracted from phloem tissues of apricot and peach branches according to the modified method of Ahrens and Seemüller (1992), and from phloem tissues of leaves and branches of sour cherry using a phenol/chloroform method. The polymerase chain reaction (PCR) assay was carried out with two different primer pairs combinations. To amplify region that includes the 16S rRNA gene, the spacer region, and the start of 23S rRNA gene of the phytoplasmas, the primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) was used in direct PCR. PCR products were diluted with

sterile distilled water (1: 29) prior to amplification by nested PCR using R16F2n/R2 (Gundersen and Lee, 1996). Similarly, R16F2n/R2 was used in direct PCR as outer primer pair and fU5/rU3 (Lorenz *et al.*, 1995) was used as inner primer pair in further nested PCR. About 20 ng of each DNA preparation in water were added to the PCR mix (Schaff *et al.*, 1992) in a final reaction mixture volume of 25 µl. Approximately 200 ng of DNA of each positive PCR product were separately digested with 2.5 U of *Mse*I, *Ssp*I and *Rsa*I, *Bfm*I restriction enzymes (Fermentas, Lithuania) after R16F2n/R2 and fU5/rU3 amplification, respectively. Restriction patterns obtained were compared with those of positive controls and patterns described in the literature.

DNA isolated from one sour cherry tree cv. Morela pozdní was used for sequencing. A set of overlapping PCR products was generated by amplification with primers R16P1/U3 (position 2-1,230), R16F2n/R2 (position 152-1,379), R16Pc399/Pc1694 (position 399-1,694), R16F1/R0 (position 130-1,503) and 17RF758/P7 (position 758-1,818). PCR products were sequenced in both directions using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). Sequencing was performed in ABI PRISM 310 sequencer (PE Applied Biosystems, Foster City, CA, USA). The sequences were aligned with those of phytoplasmas available in the GenBank using World Wide Web service BLAST (<http://www.ncbi.nlm.nih.gov>).

The buds from phytoplasma positive trees were picked up in August 2009 and 2010. Indicator plants suitable for climatic condition of Czech Republic were inoculated on the virus-free rootstocks St. Julien for apricot and peach, Colt for sour cherry. The buds from infected trees were inoculated under indicator buds after 10-14 days. Indicators used were *Prunus persica* cv. GF-305 for apricot and peach trees, *P. avium* cv. Sam, *P. avium* cv. Bing, *P. avium* cv. F12/1 and *P. avium* cv. Canindex

for sour cherry trees. Indicators maintained in the field (3-5 plants for each) are visually inspected twice during the growing season for at least 2 years.

Results

Although 92 trees examined showed premature leaf yellowing and leaf roll symptoms mainly in the end of summer and in the beginning of autumn, as well as smaller size of leaves, sparse foliage and smaller under-ripened fruits, only 22 plants were positive for phytoplasma presence. Unexpectedly, one tree of sour cherry, from which specimens were collected as negative control, revealed also positive reaction in PCR. RFLP with *Mse*I, *Ssp*I and *Rsa*I, *Bfm*I endonucleases of the DNA sequences amplified by PCR with primer pair R16F2n/R2 and fU5/rU3, respectively, showed that phytoplasma from apricot, peach and sour cherry trees had a pattern identical to each other and to the phytoplasmas belonging to apple proliferation ribosomal group, subgroup 16SrX-B.

Phytoplasmas were identified in 10 apricot trees (18.5%), in 4 peach trees (28.6%) and in 9 sour cherry trees (36.0%). Moreover, to confirm the RFLP results, DNA amplified from 1 sour cherry tree cv. Morela pozdní was used for sequencing. Comparison of the 840 bp sequence obtained confirmed the RFLP classification and indicating 99% identity with '*Ca. P. prunorum*', strain ESFY-142 (accession No. AJ575108) from apricot from Spain. The results of biological indexing were not successful, the typical symptoms were not observed on the indicator plants till this time.

Discussion

Visual inspection of apricot, peach and sour cherry orchards in East Bohemia (Czech Republic) revealed frequent presence of plants with symptoms recalling phytoplasma infection. The PCR/RFLP results indicated the presence of phytoplasmas in 22 out of 92 examined symptomatic trees. Therefore, on the bases of up to now investigation, we can not consistently demonstrate the association of phytoplasmas with symptoms and the presence of other pathogens, like viruses, fungi or other bacteria can not be excluded. Moreover, one asymptomatic sour cherry plant was also positive in PCR/RFLP analyses for phytoplasma presence. Phytoplasmas attributed to subgroup 16SrX-B were identified in all PCR positive trees. Moreover, DNA sequence obtained from phytoplasma detected in sour cherry tree revealed the closest relationship with '*Ca. P. prunorum*', strain ESFY-142.

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Diversity among phytoplasmas infecting ornamental plants grown in India

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Abstract

Phytoplasmas were identified in five ornamental species grown in gardens of Uttar Pradesh and Uttarakhand, India and showing suspected phytoplasma symptoms through nested PCR assays. DNA from symptomatic ornamental plants was extracted and amplification of phytoplasma ribosomal DNA was done with the universal phytoplasma primer pairs. The 1.2 kbp amplicons were cloned, sequenced and deposited in GenBank database. Based on sequence identities and phylogenetic relationships, the new phytoplasma strains identified have been classified as related to '*Candidatus* Phytoplasma asteris' (16SrI) group.

Key words: phytoplasma, ornamental plants, nested PCR, phylogenetic analysis, India.

Introduction

Phytoplasmas are mollicutes associated with diseases of several plant species (Al-Saad and Khan 2006; Harrison *et al.*, 2008) and cause serious economic losses also in ornamental plants (Chaturvedi *et al.*, 2010a). Phytoplasma epidemics have compelled withdrawal of many ornamental varieties from cultivation. General yellowing and stunting of plants, proliferation of shoots, phyllody, virescence and reduced size of flowers and reddening of leaves are the common symptoms observed in ornamental plants (Chaturvedi *et al.*, 2010a). So far, 42 phytoplasmas belonging to 9 groups were identified in ornamental plants worldwide (Chaturvedi *et al.*, 2010a). Based on the 16Sr sequences identified phytoplasmas in ornamental plants in India mainly belong to 16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXII, 16SrXIII and 16SrXIV groups.

Little work has been done on occurrence and identification of phytoplasma in ornamental plants in India. Ajaykumar *et al.* (2007) first time recorded '*Candidatus* Phytoplasma asteris' associated with little leaf disease of *Portulaca grandiflora* and Samad *et al.* (2008) reported little leaf disease of *Portulaca grandiflora* at Lucknow. Raj *et al.* (2007a, 2007b; 2009) observed phytoplasma disease in *Chrysanthemum morifolium*, *Adenium obesum*, and *Gladiolus* at Lucknow. Chaturvedi *et al.* (2009a, 2009b; 2010b) reported little leaf disease in *Rosa alba*, *Catharanthus roseus*, and *Hibiscus rosa-sinensis* in Gorakhpur. Occurrence, identification, and characterization of phytoplasmas in five further ornamental species in India is reported.

Materials and methods

Samples from *Alstroemeria hybrids*, *Duranta erecta*, *Stebulus asper*, *Petunia hybrida* and *Zinnia elegans*

showing yellowing, phyllody, little leaf, proliferation of axillary buds and witches' broom were collected from ornamental plant from gardens at Mukteswara in Uttarakhand district and from Gorakhpur district of Uttar Pradesh, India. The total DNA was extracted from leaf/stem tissues following a CTAB. The initial PCR was performed using P1/P6 universal primers specific to the 16S rRNA gene (Deng and Hiruki, 1991). Cycle employed was with an initial denaturation at 94°C for 5 min, followed by 35 cycles (94°C for 1 min, 55°C for 60 s, and 72°C for 90 s) and a final extension at 72°C for 5 min. Further, nested PCR was carried out with primer R16F2n/R16R2 (Gundersen and Lee, 1996) as described above except that 2 µL of the 50-fold diluted amplified product from the initial reaction was used as template and the annealing temperature was 50°C. The amplicons obtained were resolved by electrophoresis through a 1.2% agarose gel. Cloning of the second round PCR products was performed. The 16S rRNA sequence generated from the phytoplasma strains identified in ornamental plants were assembled and edited using DNASTAR's Laser Gene software (DNASTAR) and a phylogenetic tree was constructed using Mega 4.2 tool.

Results

Primer P1/P6 amplified a fragment of approximately 1,500 bp, whereas the primer R16F2n/R16R2 amplifies a fragment of about 1.2 kb. Neither by direct nor by nested PCR assays was DNA amplified from template DNA extracted from healthy or non-symptomatic samples. The 1.2 kb obtained amplicons were cloned, sequenced and the nucleotide sequences of the phytoplasma detected in the five ornamental species were deposited in GenBank. Phylogenetic relationships among these phytoplasma sequences are shown in figure 1.

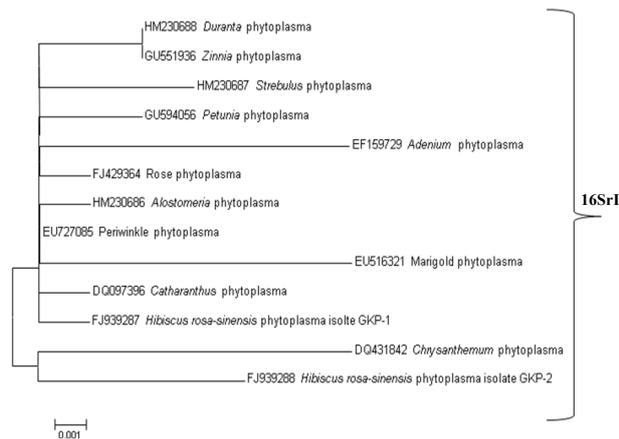


Figure 1. Phylogenetic tree constructed by using Clustal W algorithm and MEGA 4.2 version with 16Sr DNA sequences retrieved from GenBank from different phytoplasmas identified on ornamental plants in India.

The phylogenetic tree showed that *Alstroemeria* phytoplasma (HM230686), *Durlanta* phytoplasma (HM230688), *Strebulus* phytoplasma (HM230687), *Petunia* phytoplasma (GU594056) and *Zinnia* phytoplasma (GU551936) shared highest 99% similarity with the 16Sr RNA gene of other phytoplasmas of ornamental plants from India such as *Catharanthus* phytoplasma (DQ097396), periwinkle phytoplasma (EU727085), *Chrysanthemum* phytoplasma (DQ431842), *Adenium* phytoplasma (EF159729), rose phytoplasma (FJ429364), marigold phytoplasma (EU516321) and *Hibiscus* phytoplasma strains 1 and 2 (FJ939287, FJ939288), belonging to 16SrI group. Based on sequence identities and phylogenetic relationships, all the five phytoplasmas have been classified into ‘*Candidatus* Phytoplasma asteris’ (16SrI) group.

Discussion

Phytoplasma detection in symptomatic *Durlanta* and *Strebulus* is the first report in the world, however all the five ornamental plant species found infected with 16SrI phytoplasmas in Uttarakhand and Uttar Pradesh, are important. Seven other ornamental species were reported to be infected with phytoplasmas mainly belong to 16SrI and 16SrVI groups in India. The result of present study confirms that aster yellows group of phytoplasma predominates in ornamental species cultivated or naturally grown in India. Further identification of phytoplasmas in symptomatic ornamental species will facilitates the devising of control strategy towards management of such diseases in commercial grown ornamental crops in India. The infected ornamental plants may also acts as potential natural reservoir for phytoplasmas to other important agricultural commercial crops and vice-versa. Hence, an immediate attention is required for analyzing more samples for phytoplasma infection and to also look for the factors responsible for secondary spread of these phytoplasmas in nature.

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First report of stolbur phytoplasmas in *Prunus avium* in Bulgaria

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Abstract

Since 2005 the National Plant Protection Service in Bulgaria has included a monitoring program for detection, among other quarantine pests and pathogens, of quarantine phytoplasmas. During surveys conducted under this program, symptoms similar to those reported for phytoplasma diseases were observed in cherry trees. Sampling and molecular analyses allow to verify presence of stolbur phytoplasmas in symptomatic plants of cherry and bindweed in the same orchards suggesting the association of the disease in cherry with phytoplasmas.

Key words: phytoplasmas, nested PCR, stolbur, molecular identification.

Introduction

Stolbur, grapevine yellows, apple proliferation, pear decline and European stone fruit yellows are reported to be associated with serious phytoplasma diseases of vegetables, grapevine and fruit trees in Bulgaria (Topchiiska and Sakaliev, 2001; 2002; EPPO, 2006; Sakaliev *et al.*, 2007).

At the beginning of 2005, the National Plant Protection Service in Bulgaria (part of the Food safety agency) developed the monitoring program for quarantine pests on fruit trees and grapevine. The object of surveys was to verify the presence of quarantine phytoplasmas. The samples were tested at the Central Laboratory for Plant Quarantine and during these monitoring programs 'bois noir' infection in grapevine was associated with specific stolbur phytoplasma presence in cultivar Merlot (Avramov *et al.*, 2008). The stolbur disease is very common in Bulgaria and it was detected and identified in tomatoes (Bertaccini *et al.*, 1995), potatoes and other horticultural crops. During the monitoring a cherry disease possibly related to phytoplasma presence was observed in scattered plants and molecular analyses to verify this were carried out.

Materials and methods

Wilting, dying, and phloem necrosis were first observed in 2009 cherry (*Prunus avium* L.) plants located in South Eastern Bulgaria. Samples from symptomatic and asymptomatic cherry were collected in Stara Zagora region of Bulgaria together with bindweed (*Convolvulus arvensis* L.) growing under the symptomatic trees and analyzed at the Central Laboratory for Plant Quarantine.

Total DNA was extracted from leaf midribs, secondary roots and scrape of phloem from small branches or trunk (cherry) and from roots and leaves (bindweed) using a CTAB method (Doyle and Doyle, 1990) and the

Plant DNeasy mini kit (Qiagen GmbH, Hilden). Preliminary identification was carried out performing PCR assays with universal phytoplasma rDNA primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995), followed by nested PCR using the universal primers U3/U5 (Lorenz *et al.*, 1995). Reference controls included DNA extracts from asymptomatic cherry, naturally infected tomato samples showing typical stolbur symptoms, naturally infected apple showing apple proliferation symptoms, and PEY (*Pichis echioides* yellows, subgroup 16SrIX-C) and CX (X disease from peach, subgroup 16SrIII-A) infected *Catharanthus roseus*. Tubes without DNA were used as negative controls.

RFLP analysis of nested PCR products was performed using restriction enzymes *RsaI* (AfaI) and *AluI* (Amersham Biosciences, USA). Further amplification were carried out using P1A/P7A (Lee *et al.*, 2004; Martini, 2006) and R16F2/R2 (Lee *et al.*, 1995) primers in nested PCR on P1/P7 amplicons under published conditions; the final amplicons were subjected to RFLP analyses with *TruI*, *Tsp509I*, and *MboII* restriction enzymes (Fermentas Vilnius, Lithuania). Amplification and RFLP analyses of *tuf* gene (Langer and Maixner, 2004) were also performed on samples resulted infected by stolbur phytoplasmas using as reference strain stolbur from pepper from Serbia (STOL). Polyacrylamide 5% gels stained with ethidium bromide were employed to compare RFLP profiles to reference phytoplasmas for all obtained amplicons.

Results

Nested PCR results with general phytoplasma primers U5/U3, P1/P7A and R16F2/R2 provides positive results from both cherry and bindweed samples. In particular samples from roots and small branches of symptomatic cherry trees were positive as well as samples from

bindweed roots and leaves, negative results were obtained from negative controls employed (data not shown). RFLP analyses on both P1A/P7A and R16F2/R2 amplicons allow to identify phytoplasmas in cherry and bindweed as stolbur or 16SrXII-A phytoplasmas (Figure 1). RFLP analyses on *tuf* gene indicated that both cherry phytoplasmas could be assigned to *tuf* type-b as well as STOL reference strain and infected tomato from Bulgaria.

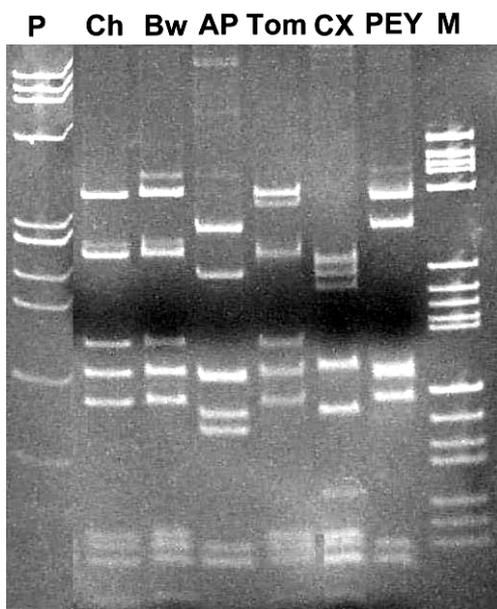


Figure 1. RFLP profiles of R16F2/R2 amplicons after *TruI* digest. Acronyms: Ch, cherry; Bw, bindweed; AP, apple proliferation; Tom, tomato infected by stolbur; CX, X disease phytoplasmas from peach in periwinkle (Canada; 16SrIII-A); PEY, *Pichris echioides* yellows in periwinkle (Italy, 16SrIX-C).

Discussion

Stolbur phytoplasmas were identified in the past in fruit trees showing symptoms referred to as Molière diseases, in these cases at least stolbur phytoplasmas were transmitted to periwinkle by dodder. Recently in Italy stolbur phytoplasmas were identified in cherry with lethal decline syndrome together with other phytoplasmas referable to ribosomal subgroup 16SrV-B (*Candidatus* *Phytoplasma ziziphae*), and 16SrIII-B (Paltrinieri *et al.*, 2008). To our knowledge, this is the first report of stolbur infecting cherry in Bulgaria; more research should be carried out in order to verify the epidemiology of the disease as well as the insect vector role in its spreading.

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Molecular detection of phytoplasmas infecting apple trees in Poland

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Abstract

In 2010 more than 120 apple trees from different regions of Poland were tested for phytoplasma presence. Shoots from symptomatic and asymptomatic plants were collected and total DNA was extracted from phloem tissue. DNA was subjected to a nested-polymerase chain reaction (PCR) for amplification of the 23S ribosomal RNA (rRNA) sequences using universal primers and primers specific for '*Candidatus Phytoplasma mali*' and '*Ca. P. asteris*'. Restriction fragment length polymorphism (RFLP) nucleotide sequence analysis of phytoplasmas' 16S-23S rDNA fragment analyses of PCR products made possible to identify '*Ca. P. mali*' in seven apple trees and '*Ca. P. asteris*' in two other trees.

Key words: apple proliferation, aster yellows, PCR/RFLP, sequencing, phylogenetic analysis.

Introduction

'*Candidatus Phytoplasma mali*', the agent associated with apple proliferation is one of the most economically important phytoplasmas in Europe. The disease was reported in all countries of Central and Southern Europe associated with serious economic losses in apple production. Symptoms include witches' brooms of affected shoots and enlarged stipules. Phytoplasmas of the aster yellows (AY) group (16SrI) occurred incidentally in apple orchards. The trees infected by '*Ca. P. asteris*' expressed different symptoms including leaf yellowing, shoot proliferation, branch twisting and rubbery wood (Bertaccini *et al.*, 1998; Fránová, 2005; Jomantiene and Davis, 2005).

The aim of this study was to determine the possible association of the phytoplasmas with symptoms of shoot proliferation and leaf deformation in apple trees in Poland.

Materials and methods

A hundred twenty seven symptomatic and asymptomatic apple trees of different cultivars grown in commercial orchards and home gardens were tested for phytoplasmas. Some of the trees showed shoot proliferation, witches' broom, leaf deformation, and enlarged stipules.

Total DNA was extracted from shoot phloem tissue using DNeasy Plant Mini Kit (Qiagen). DNA was subjected to PCR with P1/P7 followed by nested PCR with phytoplasma-universal primers R16F2n/R16R2 (Gundersen and Lee, 1996), as well as primers R16(I)F1/R1 and fAT/rAS specific to 16SrI, 16SrII, 16SrXII and 16SrXV groups, and apple proliferation phytoplasma (16SrX) groups, respectively. The fragments of the 16S rRNA gene amplified with R16F2n/R16R2 primers were digested by *MseI*, *HhaI*, *SspI*, and *RsaI* enzymes (Fermentas, Vilnius, Lithuania). The generated restriction patterns were analyzed by electrophoresis in 8%

polyacrylamide gels and compared with the reference strains (Lee *et al.*, 1998).

The partial nucleotide sequences of the 16S rDNA amplified with primers R16F2n/R16R2 were compared with sequences available in GenBank using the BLAST algorithm (<http://ncbi.nlm.nih.gov/BLAST/>). Multiple alignment was made using CLUSTALW of the DNASTAR's Lasergene software. Phylogenetic and molecular evolutionary analyses were carried out by the neighbor-joining method implemented in CLUSTALW of the genetic analysis software MEGA, 4.0 (Tamura *et al.*, 2007).

Results

PCR products (~1.2 kb) amplified with R16F2n/R16R2 primers were obtained from nine apple tree samples ('Shampion', 'Elstar', 'Golden Delicious', 'Yellow Transparent', 'Cox's Orange Pippin', 'Pinova', 'Evelina', and two trees of unknown cultivars). Products of the nested PCR with fAT/rAS specific for '*Ca. P. mali*' (apple proliferation, 16SrX group) were obtained for 16S rDNA fragments of phytoplasma strains from seven apple trees. Phytoplasma rDNA fragments from 'Pinova' and 'Evelina' trees were amplified by nested PCR with primer pair R16(I)F1/R1, but not with apple proliferation-specific primers fAT/rAS.

The resulting RFLP patterns of rDNA digested singly with *MseI*, *HhaI*, *SspI*, and *RsaI* enzymes revealed differences among phytoplasma strains and indicated that the apple trees were infected by phytoplasmas belonging to apple proliferation group, subgroup A ('*Ca. P. mali*'-related strains) and aster yellows group 16SrI, subgroup B ('*Ca. P. asteris*'-related strains).

Sequence analysis of the 16S rRNA gene fragment confirmed the presence of two different phytoplasmas in infected apple trees. Multiple alignments revealed that the phytoplasma detected in seven apple trees ('Shampion', 'Elstar', 'Golden Delicious', 'Yellow Transparent', 'Cox's Orange Pippin', and two trees of unknown

cultivars) shared a 100% nucleotide sequence identity with that of the 16S rDNA of 'Ca. P. mali' strain AT from Germany (GenBank ID: X68375). The phylogenetic analysis placed detected phytoplasma close to the other members of the apple proliferation group, subgroup A. In turn, 'Ca. P. asteris' strains from 'Pinova' and 'Evelina' were closely related with OAY isolate (GenBank ID: M30790), a reference strain of 16SrI-B group, 'Ca. P. asteris'.

Discussion

Results of PCR/RFLP and sequences analyses showed the presence of 'Ca. P. mali' and 'Ca. P. asteris' in apple trees in Poland. Witches' broom and leaf deformation were observed on shoots of the trees positively tested for 'Ca. P. asteris', while some trees infected by 'Ca. P. mali' showed shoot proliferation and incidentally enlarge stipules ('Golden Delicious'). Although the symptoms induced by 'Ca. P. mali' and 'Ca. P. asteris' differed, their association with these phytoplasmas was ambiguous.

Apple proliferation phytoplasma was reported in most of the European countries and its negative impact on apple production was evidenced. There are only few reports concerning an occurrence of phytoplasmas classified to aster yellows group in apple trees. Phytoplasma classified to 16SrI-B subgroup ('Ca. P. asteris') was detected in apple trees with leaf yellowing, shoot proliferation, and symptom 'sessile leaf' in Lithuania (Jomantiene and Davis, 2005). PCR/RFLP results confirmed the presence of phytoplasmas from the aster yellows (AY) group, subgroup B, (16SrI-B); in apple tree with rubbery wood symptom grown in the Czech Republic (Bertaccini *et al.*, 1998). The trees found in Czech Republic and United Kingdom showed diverse symptoms, and yielded positive results for phytoplasmas of subgroups 16SrI-B and 16SrI-C of AY group (Bertaccini *et al.*, 2001).

Occurrence of the phytoplasmas belonging to the same subgroups were also reported in apple trees with

branch twisting symptoms in Czech Republic (Fránová, 2005). The relevance of phytoplasmas from aster yellows group in fruit production is unclear. This is the first report of the natural occurrence of 'Ca. P. asteris' in *Malus* sp. in Poland.

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Generation of a specific monoclonal recombinant antibody against '*Candidatus Phytoplasma aurantifolia*' using phage display technology

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Abstract

Witches' broom disease of lime (WBDL) is a destructive disease caused by '*Candidatus Phytoplasma aurantifolia*' and is a limiting factor for lime production in Southern Iran. Conventional strategies for disease management have shown little success and new approaches based on genetic engineering need to be considered. Lack of natural resistance against phytoplasma diseases has highlighted the importance of alternative approaches and recombinant antibody mediated resistance is among them. The immunodominant membrane protein (IMP) is a major protein present on the surface of phytoplasma cells and is important for both diagnostics and interactions with plant hosts and insect vectors. Generation of specific recombinant antibodies with high binding ability against IMP proteins is beneficial for both diagnostic purposes and development of recombinant antibody-mediated resistance against disease.

Phage display is a powerful technology for generation of specific recombinant antibodies such as single chain variable fragment antibodies (scFv). This study describes generation of a specific scFv fragment through panning of naïve phage display libraries. For this aim, the gene encoding the IMP protein was isolated and cloned into a bacterial expression vector and recombinant IMP protein was expressed in bacterial cells and purified through affinity chromatography. Purified recombinant IMP protein was used for panning of naïve Tomlinson I and J scFv phage display libraries. Following three rounds of panning for selection and amplification of specific binders, capability of individual clones for production of specific scFvs was evaluated by an ELISA assay. The preliminary results showed generation of specific scFv recombinant antibodies with strong binding ability against the IMP protein. Complementary studies revealed that the scFvs are able to bind native IMP and could detect the presence of phytoplasma in infected plants. Further immunoassay analysis confirmed that generated scFvs are able to detect epitopes along the IMP amino acid residues. As far as we know, this is the first successful application of phage display libraries for generation of scFv recombinant antibodies against phytoplasma cells.

Key words: phage display, WBDL, IMP, '*Candidatus phytoplasma aurantifolia*', recombinant antibody, scFv.

Introduction

The witches' broom disease of lime (WBDL) caused by '*Candidatus Phytoplasma aurantifolia*', is the most destructive disease in lime tree throughout southern Iran. Phytoplasmas are phloem-limited bacterial pathogens that persistently colonize their plant hosts. They are wall-less prokaryotes belong to Mollicutes. They are known to have specific characteristics such as small genome size from 530 to 1350 Kb, low G+C percent, unculturable in cell free media, and transmission and spread by insect vectors mainly leafhoppers and planthoppers (Lee *et al.*, 2000).

Phytoplasmas are surrounded by a single cell membrane. The Immunodominant membrane proteins (IMPs) of phytoplasmas are major proteins located on the external surface of the cell membrane and appear to have important roles in pathogenicity in the host plant and insect cells (Hogenhout *et al.*, 2008). Due to the unique behaviour of IMP in pathogenicity of phytoplasma, they are good candidates for suppression of disease through a recombinant antibody-mediated resistance approach. Le Gall *et al.* (1998) generated an scFv fragment binding to the IMP protein of stolbur phytoplasma. The plants expressing this scFv showed the potential for recombinant antibody fragments for suppression

of phytoplasma diseases in transgenic plants (Le Gall *et al.*, 1998; Malembic-Maher *et al.*, 2005).

Phage display is a powerful technology for development of specific binders against almost any antigen. The main advantage of phage display is direct and physical linkage between phenotype and genotype. Several scFv phage display libraries have been developed by amplifying V_L and V_H regions of animal donors and fusing them to the pIII minor coat protein of a filamentous bacteriophage (Hust and Dubel, 2004). This article introduces application of phage display technology for generation of a specific scFv recombinant antibody that could be used for both specific detection of WBDL infected plants and immunomodulation of disease in transgenic plants.

Materials and methods

The gene encoding the IMP protein was isolated and cloned into a bacterial expression vector and the purified recombinant protein was obtained through affinity chromatography as previously described (Shahriyari *et al.*, 2010). Phage display processes were carried out by performing three rounds of panning on Tomlinson I and J scFv phage display libraries as previously described (Safarnejad *et al.*, 2008).

Panning was performed by immobilizing IMP recombinant protein (~50-100 µg/ml) overnight onto immunotubes (Nunc-Maxisorb), which had been washed with PBS, blocked with skimmed milk and incubated with phage suspension (~10¹³ CFU). Phage particles with affinity for the antigen were eluted using triethylamine and used for infection and amplification of exponentially growing *E. coli* (Migula) TG1 cells. The total eluted phage titre was determined after each round of panning. After the third round, individual colonies were randomly selected and analyzed for production of soluble scFv and binding activities against IMP by using blotting and ELISA analysis. Mini-induction of scFv recombinant proteins was performed in *E. coli* strain HB2151 harbouring scFv phagemid and by adding IPTG to a final concentration of 1 mM and further incubation at 30°C overnight. Soluble scFv was obtained by centrifugation and supernatant was applied for further serological analysis. In ELISA, 100 µg/ml antigens in PBS were directly coated on high-binding microtitre plates followed by a blocking step using 2% (W/V) skimmed milk. Around 100 µl of scFv solutions were then applied to the plates and incubated at 37°C for 2 hr. Bound scFs were detected using anti-c-myc monoclonal antibody 9E10 followed by AP conjugated goat-anti-mouse polyclonal antibodies. Colonies with high specificities were selected and subjected to further analysis.

Binding ability of the resultant scFv recombinant antibody against native IMP and intact phytoplasma cells was proved by applying ELISA, western blotting and dot blot immunoassays on WBDL infected plants.

Results and Discussion

The IMP protein was produced as a His-tagged fusion protein and purified by affinity chromatography in nickel-agarose columns. The SDS-PAGE results proved integrity and purity of recombinant protein and it was measured at 35 kDa. The total yield of purified IMP in the culture medium was calculated at around 6 mg/l.

The Tomlinson I and J naïve scFv libraries were screened for IMP binders using purified His-tagged IMP recombinant protein. Three rounds of panning were performed with 10¹³ of recombinant phage in each round. The results obtained after each round confirmed enrichment of IMP specific scFv throughout the panning processes. After the third round, individual colonies were randomly selected and their ability for production of soluble scFv was determined by western blot and dot blot assays. Presence of scFv in bacterial supernatant was shown by AP conjugated anti-c-myc monoclonal antibodies. These results revealed that around 50% of clones selected from the Tomlinson I library produced detectable scFv which was measured at 30 kDa. The binding activity of soluble scFv against IMP was measured by indirect ELISA. These results indicated that at least 6 individual clones produce specific scFv with strong binding ability to IMP. There was no detectable binding to negative control samples including GST and BSA proteins. Complementary ELISA and blotting analyses were carried out to determine the binding

specificity of scFvs against infected plant samples. These results proved that scFvs could react with phytoplasma cells presented in infected plants as well. Furthermore, presence of positive responses in western blot and ELISA analyses indicated that the corresponding epitope is a continuous type and presented on both recombinant and native IMP proteins. Based on the literature, the sole recombinant antibody generated against phytoplasma cells was produced by using pre-established hybridoma clone (Le Gall *et al.*, 1998). To our knowledge, this is the first report on obtaining a specific recombinant antibody against phytoplasmas by using scFv phage display libraries. Due to the presence of selective pressure in phage display for obtaining more soluble recombinant antibodies during the panning process, it seems that the resultant scFv fragments could be expressed well in plant cells and are suitable candidates for suppression of WBDL disease *in planta*.

Acknowledgements

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Phytoplasma's diversity in India

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Abstract

Phytoplasma have been associated with 45 plant species in India. Several fruits, vegetables, ornamentals, trees and other agriculturally important crop species are affected by phytoplasma diseases. Ten groups of phytoplasma have been identified in India and most of them have been reported from north – eastern parts of the country. Only few phytoplasmas have been recorded in Eastern, Western and Central India. These phytoplasmas affects sugarcane, sesame, ornamentals, oil crops, tree species, vegetables and many weed species. Aster yellows is the most prevalent group of phytoplasmas and has been associated with more than 31 diseases in India.

Key words: phytoplasma groups, diversity, India, epidemiology.

Introduction

Sandal spike was the first phytoplasma disease reported in India (Varma *et al.*, 1969). Thereafter a large number of phytoplasma diseases were described, which included brinjal little leaf disease (Varma *et al.*, 1969), grassy shoot disease of sugarcane (Chona *et al.*, 1960), rice yellow dwarf disease (Reddy and Jeyarajan, 1990), *Sesamum* phyllody (Vasudeva and Sahambi, 1955), white leaf disease of *Cynodon dactylon* (Singh *et al.*, 1978), little leaf disease of *Acanthospermum hispidum* (Raju and Muniyappa, 1981) and yellowing disease of *Urcthloa panicoides* (Muniyappa *et al.*, 1982). These reports are mainly based on bright-field and fluorescence microscopy, electron microscopic observations, tetracycline treatment and to a lesser extent on serological assays. Following the development of molecular tools, phytoplasmas have been characterized from as many as 45 plant species in India (Chaturvedi *et al.*, 2010; Rao *et al.*, 2011). Only a few of these phytoplasmas associated with more than 25 plant taxonomic families have been characterized for their taxonomic grouping. Taxonomic studies of phytoplasmas will help in understanding their diversity and spread in India. Study on phytoplasma vectors and alternate plant hosts and other modes of secondary spread in nature are also important and being attempted.

Analysis of 16S rDNA sequences of phytoplasmas indicated that ten groups exists in India affecting sugarcane, ornamentals, oil crops, tree species, vegetables and many weed species. Aster yellows phytoplasmas are the most common group of phytoplasmas in India associated with more than 31 diseases.

Materials and methods

GenBank sequences submitted for phytoplasma associated with different plant species in India were used for their comparison and phylogenetic analysis. The 16S rDNA sequences of different phytoplasmas identified were compared with each other using BLASTn pro-

gram. Sequences were aligned using BioEdit Sequence Alignment Editor. Phylogenetic tree was constructed (MEGA 4.0 software) using 100 bootstrap datasets. A comparative analysis has been discussed in the present paper and also the distribution of different identified phytoplasma groups were depicted in figure 1.

Results and discussion

Phytoplasma groups in India showed a wide geographical distribution especially in North Eastern and Southern parts of the country (figure 1). North – eastern parts has the most diverse phytoplasma groups followed by the Southern parts of India.



Figure 1. Distribution of 16S rDNA phytoplasma groups in India.

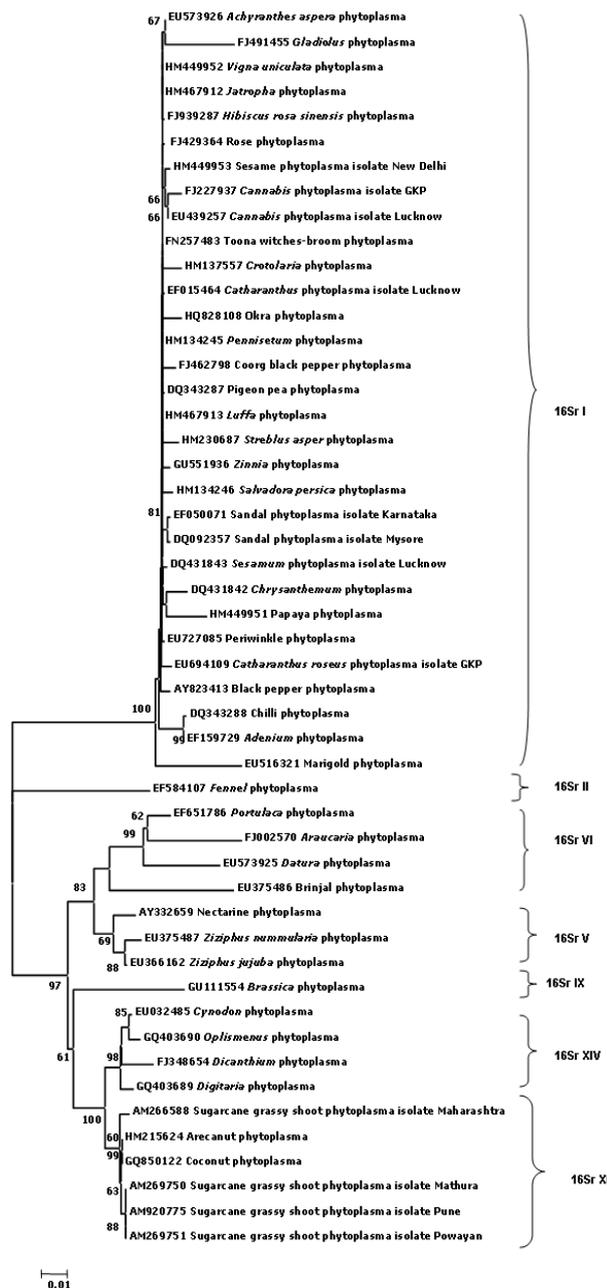


Figure 2. Phylogenetic tree constructed by using Clustal W algorithm and MEGA 4.0 version with 16Sr DNA sequences retrieved from GenBank from different phytoplasmas identified in ornamental and other types of plants in India.

Sugarcane grassy shoot associated with 16SrXI phytoplasmas, sesame phyllody associated 16SrIV and coconut wilt disease associated 16SrIV and 16SrXI are the most important diseases causing serious economic losses in India (Rao *et al.*, 2005; Khan *et al.*, 2007; Manimekhalai *et al.*, 2010) and need further investigation for their management.

Nucleotide sequence studies of 16S rDNA have shown that the '*Candidatus Phytoplasma asteris*', '*Ca. P. aurantifolia*', '*Ca. P. ulmi*', '*Ca. P. trifolii*', '*Ca. P.*

phoenicium', '*Ca. P. oryzae*', '*Ca. P. cynodontis*', respectively representing 16SrI, 16SrII, 16SrV, 16SrVI, 16SrIX, 16SrXI and 16SrXIV groups are the major groups associated with different plant species in India (figure 2). However, the aster yellows phytoplasma group (16SrI) is the most important which affects ornamentals, tree species, vegetables, sugarcane, fruit crops and pulses in India (figure 2).

Even though phytoplasma diseases are of common occurrence in India, only few of them have been fully characterized, particularly in North-Eastern and Southern parts of the country. Further studies of these phytoplasma diseases are prompted including their characterization, incidence, transmission and vector identification.

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Diversity among phytoplasma infecting various economically important plant species grown in India

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Abstract

Phytoplasma have been identified by electron microscopy observations of infected tissues, by polymerase chain reaction amplification with specific primers, restriction fragment length polymorphism, and sequence analysis of 16S ribosomal DNA gene. Various phytoplasma diseases have been detected and studied in a variety of crops, ornamental, vegetables and weed plants. They are classified into three major phytoplasma groups.

Key words: diseases, electron microscopy, PCR, sequencing, phytoplasma.

Introduction

Phytoplasmas are intracellular obligate prokaryotes which lack cell wall. Phytoplasma genome is very small (680-1,600 kb). They are transmitted in plant by leafhoppers, and cause typical yellowing, stunting of whole plant, virescence, phyllody, proliferation of axillary buds, witches' broom and die back symptoms inducing severe yield losses in a variety of plants of horticultural, agricultural and ornamental importance.

Several plant species are being grown in India as cereal crops, oil crops, fruit crops, vegetables, medicinal and ornamental plants. The cultivation of these plants has been drastically affected by several diseases caused by various types of pathogens such as fungi, bacteria, viruses, and phytoplasmas. Among them the latter play an important role since they are associated with diseases which affect the biomass yield and the production of these plants. Efforts have been made for detection, identification and possible management of phytoplasma diseases naturally occurring in various plant species in India so that their growth and yield may be improved.

Materials and methods

During a survey carried out in the last decade, the typical phytoplasma symptoms: yellows, virescence, phyllody, little leaf, proliferation of axillary buds and witches' broom were observed in various plant species growing at the National Botanical Research Institute (NBRI) gardens, kitchen gardens and agricultural fields in and around Lucknow, India.

Little leaf of *Withania somnifera*, chili, brinjal, *Datura* spp., *Cajanus cajan*, *Catharanthus roseus*, desert rose (*Adenium* spp.), rose (*Rosa alba*), and *Hibiscus* spp.; spike disease of sandal (*Santalum album*); yellows of chrysanthemum and *Achyranthes aspera*; witches' broom of *Ziziphus* spp., *Cannabis sativa*, *Parthenium hysterophorus*, *Sesamum indicum*, marigold and malformation and twisting of floral spikes in gladiolus were studied.

For phytoplasmas detection and identification the samples were collected from the symptomatic plant species listed below, and total DNA was extracted from approximately 100 mg leaf tissues employing a phytoplasma enrichment procedure (Ahrens and Seemüller, 1992). Direct polymerase chain reaction (PCR) using the total DNA and P1/P6 (Deng and Hiruki, 1991) universal primers, specific to 16S rDNA gene of phytoplasma was performed. Further nested PCR was carried out using 1: 10 diluted amplicons from the first stage (P1/P6) and R16F2n/R16R2 primers (Gundersen and Lee, 1996). The amplicons of ~1.3 kb obtained from nested PCR were cloned and sequenced.

Phylogenetic analysis using MEGA version 4.0 software was then carried out to determine the phylogenetic relationships among detected phytoplasmas.

Results

Direct PCR using P1/P6 primers resulted in the expected size bands of ~1.5 kb in some leaf samples. The nested PCR with R16F2n/R16R2 primers resulted in ~1.3 kb bands from many samples collected from various plant species indicating the association of a phytoplasmas with the symptomatic plants.

The partial sequence of 16S ribosomal DNA gene of phytoplasma associated with several plant species was obtained, deposited in Genbank and compared with other phytoplasmas strains using the BLASTn tool.

Phytoplasmas related to '*Candidatus* Phytoplasma asteris' were identified in little leaf of *Withania somnifera* (DQ151998), spike disease of sandal (*Santalum album*) (DQ092357, EF198362), *Catharanthus roseus* phyllody (DQ097396, EF015464), *Achyranthes aspera* (EU573926), *Cannabis sativa* (EU439257), Chili (DQ343288), *Parthenium hysterophorus* (EU375485, EU375488), *Cajanus cajan* (DQ343287), *Sesamum indicum* (DQ431843), chrysanthemum witches' broom (DQ431842), little leaf of desert rose (*Adenium* spp.), (EF159729), marigold witches' broom (EU516321), periwinkle little leaf (EU727085), little leaf disease of rose (*Rosa alba*)

(FJ429364), gladiolus with twisting of floral spikes (GQ338824), malformation of gladiolus (FJ491455), yellows and little leaf disease of *Hibiscus rosa-sinensis* (FJ939287, FJ939288).

Phytoplasmas related to '*Candidatus* Phytoplasma ziziphi' were identified in symptomatic samples of *Ziziphus nummularia* (EU375487); *Ziziphus jujube* (EU366162); *Datura innoxia* (EU573925) and phytoplasmas related to '*Candidatus* Phytoplasma trifolii' were identified in brinjal little leaf (EU375486).

Discussion

Based on phylogenetic analysis phytoplasma strains have been classified into three phytoplasma 16Sr groups ie. '*Ca. P. asteris*' (16SrI group); '*Ca. P. ziziphi*' (16SrV group); '*Ca. P. trifolii*' (16SrVI group). These results indicated that a clear-cut diversity exist among phytoplasma strains infecting various crops in India.

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Stolbur phytoplasma infection of kale crops (*Brassica oleracea* var. *gemmifera* L.) in Serbia

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Abstract

Kale plants (*Brassica oleracea* var. *gemmifera* L.) showing symptoms of reddening on leaf and petioles and overall stunting were found on locality Pančevo (south Banat) during inspection on vegetable crops carried out in 2010 in Serbia. The phytoplasma associated with the disease was detected through nested PCR with primer pairs P1/P7 and R16F2n/R2 followed by RFLP analysis with restriction enzyme *Mse*I. A total of 10 symptomatic and 4 asymptomatic plants were collected and analyzed for the presence of phytoplasma. Nested PCR amplified 16S ribosomal RNA fragments of phytoplasma in all samples of kale with symptoms, while all asymptomatic plants tested negative. Digestion with *Mse*I identified in infected kale the same pattern as a reference strain of stolbur phytoplasma belonging to 16SrXII-A group. Amplification of the elongation factor *tuf* gene and digestion with *Hpa*II restriction enzyme was performed for genotype differentiation of stolbur phytoplasmas detected in kale. Digestion of *tuf* gene indicated presence of *tuf*-type b of stolbur phytoplasma in all symptomatic kale plants. This is the first report of stolbur phytoplasma infecting kale crops in Serbia.

Key words: stolbur phytoplasma, Serbia, *tuf* gene, *Brassica oleracea* var. *gemmifera*.

Introduction

Phytoplasmas of the stolbur group (16SrXII-A) are widely distributed in Europe, infecting a wide range of cultivated and wild plants. In past 10 years, increasing incidence of stolbur phytoplasma was registered in different crops (grapevine, maize, sugar beet, potato, vegetable crops), suggesting progressive spread of diseases associated with this phytoplasma.

In the vegetable crops, severe yield losses caused by stolbur phytoplasma have been recorded in solanaceous crops (tomato, potato, pepper) and celery (Vicizian, 2002; Carraro *et al.*, 2008; Navratil *et al.*, 2009; Fialova *et al.*, 2009). Among phytoplasma associated diseases affecting kale crops, previous studies identified a phyllody of kale caused by phytoplasma belonging to aster yellows group (16SrI-B) (Marcone *et al.*, 2000).

Primary goal of this study was to identify and characterize phytoplasma in association with kale plants showing symptoms typical of phytoplasmas-associated diseases such as reddening and stunting.

Materials and methods

A total of 10 stunted plants of kale with red leaves and petioles (figure 1) were collected in 2010 in locality Pančevo (south Banat) and submitted to nested PCR analysis to identify presence of phytoplasma. Additional 4 asymptomatic plants were collected from the same locality and used as negative controls.

Nucleic acids were extracted from leaves and petioles using CTAB protocol previously described by Angelini *et al.* (2001). Phytoplasma presence was detected by amplifying 16S ribosomal RNA gene through nested

PCR analysis with universal primer pairs P1/P7 and R16F2n/R2 (Lee *et al.*, 1998). Restriction fragment length polymorphism (RFLP) analysis of the amplified DNA fragments from positive samples of kale was performed with *Mse*I restriction enzyme. Further stolbur characterization was performed by amplifying the elongation factor *tuf* gene with fTuf1/rTuf1 and fTufAY/rTufAY primers followed by digestion with *Hpa*II restriction enzyme (Langer and Maixner, 2004).

Results and discussion

Presence of phytoplasmas was detected in all kale plants exhibiting symptoms of reddening and stunting, while symptomless plants tested negative. Digestion of nested PCR products of 16S rRNA gene with endonuclease *Mse*I determined in all samples the same RFLP profile as reference strain of the stolbur phytoplasma belonging to 16SrXII-A subgroup (Lee *et al.*, 1998).

The *tuf* gene was amplified in all samples positive for stolbur presence. Digestion of TufAY PCR products with *Hpa*II restriction enzyme detected the presence of *tuf*-type b of stolbur phytoplasma in infected kale (figure 2). Identification of stolbur phytoplasma represents the first record on presence of this group of phytoplasma in association with kale crops in Serbia and South East Europe.

Diseases caused by the stolbur phytoplasma are considered typically epidemic because, under favourable conditions, they can spread quickly with a high incidence. The ability of phytoplasma to infect numerous wild and cultivated plants and its transmission by polyphagous planthoppers plays a key role in the spread of stolbur disease. Each of the three

stolbur genotypes differentiated based on *tuf* gene (*tuf*-types a, b, c) had been associated with different host plants of the main stolbur phytoplasma vector *Hyalesthes obsoletus* Signoret (Langer and Maixner, 2004). All strains of the *tuf*-type b genotype are known to be associated with bindweed *Convolvulus arvensis* L in German vineyards, however, this type of stolbur has been later detected in vegetable crops such as celery, pepper, potato, tomato and in diverse weed plants sampled from the vegetable plots (Fialova *et al.*, 2009).

In Serbia, naturally growing weeds can often be found along the borders and inside vegetable plots, presenting a real threat by continuously harboring stolbur phytoplasma or hosting planthoppers as potential vectors of phytoplasma from wild to cultivated plants.

Further study of stolbur disease in association with kale crops in Serbia are required, such as inspection of common weeds in and around vegetable plots, and identification of potential insect vectors.



Figure 1. A) healthy kale, and B) kale infected with stolbur phytoplasma expressing symptoms of petioles reddening and overall stunting. (In colour at www.bulletinofinsectology.org)

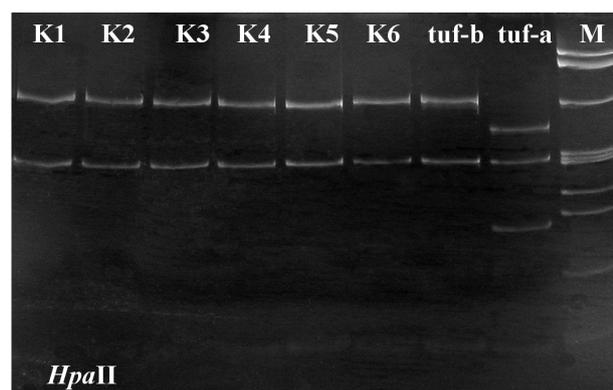


Figure 2. Polyacrilamide gel showing the *Hpa*II RFLP patterns of *tuf* gene of stolbur phytoplasmas from kale obtained with fTuf1/rTuf1 and fTufAY/rTufAY primers; K1-K6 – stolbur infected kale; tuf-b (stolbur *tuf*-type b from the Mosel region of Germany); tuf-a (stolbur *tuf*-type a, from Middle-Rhine region of Germany, provided by M. Maixner, Bernkastel-Kues); M: molecular weight marker ϕ X174/*Hae*III digested (Fermentas, Vilnius, Lithuania).

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Molecular detection of potato stolbur phytoplasma in Serbia

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Abstract

In August 2010 potato plants expressing symptoms of phytoplasma infection were collected from three localities in Vojvodina province of Serbia and analyzed for phytoplasma presence. Phytoplasma detection was performed by PCR/RFLP analyzes of 16S rRNA gene and by stolbur-specific PCR amplification with Stol11 primers. Stolbur phytoplasma was detected in all symptomatic potato plants analyzed. This is the first report of molecular detection of stolbur phytoplasma infecting potato in Serbia.

Key words: detection, stolbur phytoplasma, potato, aerial tubers, leaf discoloration, PCR, RFLP.

Introduction

Phytoplasmas are wall-less, non-culturable, phloem-limited, insect-transmitted plant pathogens from the class *Mollicutes*. They are associated with diseases in several hundred species of plants, many of which are economically important, including potato (*Solanum tuberosum* L.). Phytoplasma diseases of potato have become increasingly important in recent years (reviewed in Ember *et al.*, 2011), due to the epidemic appearance and geographic spread of the diseases, as well as significant yield losses in potato production and low quality of produced tubers. Different phytoplasma 16Sr groups are infecting potato worldwide, however many are causing similar symptoms. In Europe, potato stolbur phytoplasma is the most common, and by impact the most significant phytoplasma infecting potato and has a quarantine status in the European Union (EPPO/CABI, 1996).

In southeast Europe, including Serbia, presence of potato phytoplasma disease was for a long time, diagnosed solely on the basis of visual symptoms and/or presence of tentative insects-vectors (Ember *et al.*, 2011). First record of potato stolbur in Serbia is dating from the 1950's (Panjan, 1950), in time when the knowledge on stolbur "virus" was limited to descriptive records of symptoms expression on infected potatoes and general assumption that *Hyalesthes obsoletus* Signoret is the main cixiid vector of the disease. Since then, no attempt has been made on molecular characterization of phytoplasmas associated with symptomatic potato plants in Serbia.

Presence of symptoms typical for potato stolbur phytoplasma including reddening and upward rolling of the top leaves, shorten internodes and aerial tubers were observed in recent years in several localities in potato growing regions of Serbia. The main aim of this study was to identify phytoplasmas infecting potato in Serbia by employing PCR/RFLP methods of detection.

Materials and methods

During August 2010, potato growing areas of Bačka and Srem regions in Vojvodina province of Serbia were surveyed for the presence of potato phytoplasma. Samples of potato plants expressing symptoms of leaf yellowing, reddish or purplish discoloration, rolling of the top leaves and presence of aerial tubers (figure 1) were collected from three localities: Bački Petrovac, Titel and Zemun. Six symptomatic potato plants were sampled per site. From every plant leaves with petioles, stems and aerial tubers were collected and stored at -20°C before being processed. Leaves of two asymptomatic potatoes from each site were used as negative controls.

Total nucleic acids were extracted from potato plants using the CTAB method (Angelini *et al.*, 2001). Initial phytoplasmas identification was conducted on 16S rRNA gene using nested PCR procedure with the P1/P7 and R16F2n/R2 primers according to Lee *et al.* (1998). Obtained amplicons of the expected size (approximately 1,200 bp) were subjected to restriction analyses with *MseI* endonuclease, in order to identify 16S rRNA subgroup of detected phytoplasmas.



Figure 1. Symptoms of potato stolbur phytoplasma. Aerial tubers, yellowing and upward rolling of the top leaves in stolbur-infected potato plant.

To confirm the presence of stolbur phytoplasma, potato samples that were tested positive with the universal phytoplasma primers were also submitted to nested PCR with the stolbur-specific Stoll1 primers (Clair *et al.*, 2003) according to previously described protocol (Radonjić *et al.*, 2009).

Results and discussion

Molecular analyses of phytoplasma 16S rRNA PCR products from symptomatic potato plants revealed the presence of stolbur phytoplasma (16SrXII-A group) in all 18 samples analyzed, from all the three localities. Restriction digestion patterns of 16S gene fragment PCR products with *MseI* for all samples were identical to each other and to the maize redness (MR) sample from Serbia (figure 2). None of the symptomless plants were positive for the presence of phytoplasmas. Analyses with stolbur specific Stoll1 primers confirmed that all tested symptomatic potatoes were infected with stolbur phytoplasma (data not shown).

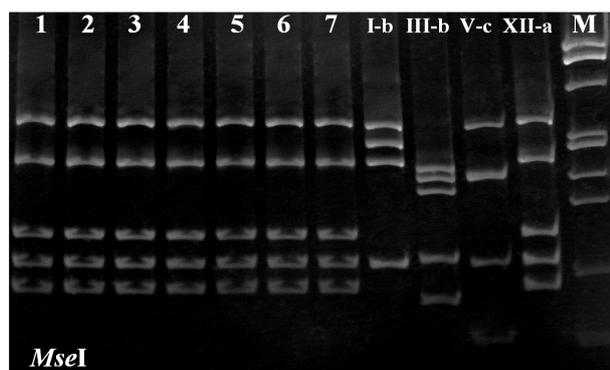


Figure 2. RFLP analyses of the 1,200 bp 16S rRNA gene fragment of stolbur phytoplasma infecting potato amplified by nested PCR with P1/P7 and F2nR2 primers, digested with *MseI* and separated by electrophoresis through 13% polyacrylamide gels. 1-7: symptomatic potato plants; I-b (16SrI-B, provided by E. Angelini, Conegliano); III-b (16SrIII-B, naturally infected *Cirsium arvense* with symptoms of multiple inflorescence disease, Serbia); V-C (FD-C, field growing infected grapevine from Central Serbia); XII-a (MR, field growing infected maize from South Banat region, Serbia); M: molecular weight marker *φX174/HaeIII* digested (Fermentas, Vilnius, Lithuania).

Although presence of potato phytoplasma is generally considered to be common in Serbia, our results represent the first detection of stolbur phytoplasma associated with previously observed and reported symptoms on potato. Frequency of infected plants and distribution of the disease in the territory of Serbia is yet not known, and will be determined in future studies. Attention should be given to the economic importance of potato production, especially considering recent report of stolbur induced yield losses of potato in Russia and

Romania (Ember *et al.*, 2011), as well as stolbur phytoplasma epidemics on maize in Serbia (Jović *et al.*, 2009). Due to widespread appearance of stolbur phytoplasma in Europe it is of great importance to determine epidemiological cycle and subsequently define proper management practice to control potato stolbur disease. Accurate identification of phytoplasmas by applying molecular tools, as well as identification of insect vector(s) and natural plant reservoirs of potato stolbur phytoplasma will be crucial to meet these demands.

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Molecular identification of 16SrIII and 16SrXII phytoplasma groups in *Chenopodium album* in Czech Republic

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Abstract

Chenopodium album L. plants showing symptoms of leaf yellowing, small leaves development, proliferation, plant and inflorescence stunting were examined for phytoplasma infection using PCR with universal phytoplasma primers R16F1/R0 and fU5/rU3. Only in two samples out of the 38 tested, the phytoplasma infection was confirmed. RFLP and sequence analyses based on partial 16S rDNA fragment confirmed that *C. album* is harbouring phytoplasmas belonging to 16SrIII (X-disease) group. This species has been also found infected with phytoplasmas belonging to 16SrXII group in Czech Republic.

Key words: weed, lambsquarters, phylogeny, X-disease, stolbur, phytoplasma.

Introduction

There are only a few data concerning the phytoplasma occurrence in *Chenopodium* species. Firstly the phytoplasma infection of *Chenopodium album* L. was reported by Seruga *et al.* (2003) during survey for phytoplasma detection in Croatian vineyards; phytoplasmas were identified by the use of nested PCR and RFLP analyses as belonging to the 16SrIII group. Tolu *et al.* (2006) obtained one positive out of seven *Chenopodium* spp. samples collected during the vineyards surveys in Italy and classified this phytoplasma into the subgroup 16SrII-E by RFLP analyses. *Chenopodium murale* L. was also identified as an alternative phytoplasma reservoir for the 16SrII phytoplasma associated with a lime decline disease in Saudi Arabia (Alhudaib *et al.*, 2009).

The aim of this work was to characterise phytoplasmas detected in *C. album* collected within tomato and pepper fields in the Czech Republic.

Materials and methods

Since the beginning of vegetation period from 2008 to 2010, 38 samples from *C. album* plants were collected in the horticultural region of south Moravia (Lednice) within tomato and pepper fields. The plants exhibited leaf yellowing, small leaves development, proliferation, plant and inflorescence stunting (figure 1).

The total DNA extraction was performed according to an enrichment procedure (Ahrens and Seemüller, 1992). DNA samples were subjected to PCR analysis with phytoplasma primer pairs in 16S rRNA gene. Nested-PCR assays were performed with primers pairs R16F1/R0 followed by fU5/rU3 (Lee *et al.*, 1995; Lorenz *et al.*, 1995). Identification of phytoplasmas was carried out by RFLP analyses using *AluI*, *MseI*, and *RsaI* according to Lee *et al.* (1998).

PCR products were directly cloned using pGEM-T (Promega) cloning kit, following the instructions of the manufacturer. Nucleotide sequences were obtained on automated ABIPRISM 3130 Genetic analyzer (Perkin

Elmer Applied Biosystems, Lincoln). Sequence data were analyzed by DNASTAR programme (Lasergene). The sequences were compared with those available in the GenBank using BLAST algorithm version 2.2.25.

Results

In two out of the 38 examined samples the presence and identity of phytoplasmas was confirmed by PCR/RFLP and sequence analysis. Products of expected length were obtained in fU5/rU3 PCR (879 bp). No amplification was observed in samples of healthy plant and water controls. The samples showed two distinct profiles in RFLP analyses, one corresponding to phytoplasmas belonging to 16SrIII and the second to 16SrXII ribosomal groups.

The identity of phytoplasma strains was confirmed by sequence analysis of fU5/rU3 PCR fragments. Strain number 2692 showed the 99% identity with stolbur phytoplasmas from grapevine from Italy, CH-1 (Acc. No. HQ589193), from potatoes from Russia, RuS93 (GU004375) and from tomato from Italy, PTV (GU004374); strain number 2909 showed 99% identity with X-disease phytoplasmas - *Cirsium* strain (FN298626) and *Taraxacum* strain (FN298621) from Finland and clover phyllody phytoplasma (HQ589196) from Italy.

Discussion

These findings agree with published results where the *Chenopodium* spp. is described as a sporadic host of different phytoplasmas. Up to date *Chenopodium* plants infected by 16SrIII group phytoplasmas were only in vineyards in Croatia (Seruga *et al.*, 2003). Other *Chenopodium* spp. showing yellowing and stunting associated with phytoplasmas belonging to 16SrII group have been reported from vineyard in Italy (Tolu *et al.*, 2006) and in case *C. murale* from citrus farm in Saudi Arabia (Alhudaib *et al.*, 2009). Although Özdemir *et al.* (2009) reported *C. album* L. as an alternative host of

tomato phytoplasmas, our results represents the first molecular identification of phytoplasma belonging to 16SrXII group in lambsquarters. The sporadic detection of phytoplasma infected lambsquarters plants in the locality with mass occurrence of phytoplasma diseases (Navrátil *et al.*, 2009) is probably connected with the low attractivity of weed plants for insect vectors compared to pepper and tomato plants.

Occurrence of phytoplasmas belonging to 16SrIII group was previously described in Czech Republic in *Trifolium* spp. (Fránová *et al.*, 2004), where it is not very common. Phytoplasmas belonging to the 16SrXII group are more common, causing the local epidemics in solanaceous crops (Fránová *et al.*, 2009; Navrátil *et al.*, 2009).



Figure 1. *C. album* plants. A. Infected plants showing leaf yellowing, small leaves development, proliferation, plant and inflorescence stunting. B. healthy plant.

Acknowledgements

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Development of specific detection primers for '*Candidatus phytoplasma pyri*'

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Abstract

Pear decline (PD) is an economically highly important disease in many pear growing regions. It is associated with a phytoplasma of the 16Sr group X: '*Candidatus Phytoplasma pyri*'. Despite its importance, reliable detection primers for direct identification of the pathogen by PCR are missing. Available PD-specific primers in the 16S-23S rDNA sequence detected only 80% of PD-infected field-collected samples from Southwest Germany. In order to select new primers sequence data of the 16S-23S rDNA region were obtained for two strains of '*Ca. P. pyri*'. PD-specific primers were selected *in silico* and their specificity was tested with reference strains of the 16Sr group X. They proved to be highly specific for '*Ca. P. pyri*'. The reliability of the primers for PD detection was evaluated with field samples of pear. The detection rate could be improved up to 90% with respect to phytoplasma detection in the same samples with highly sensitive universal ribosomal primers.

Key words: Pear decline, *Pyrus*, PCR detection, 16S rDNA, sequence analysis.

Introduction

Pear decline (PD) is one of the most important diseases of pear and induces a more or less quick decline of the tree. The disease presumably originates from Europe and has been introduced into North America as well as its vector (Seemüller, 1989). Today, PD probably occurs wherever pear is grown in Europe and North America. Recently, PD has also been reported from the Asian part of Turkey (Sertkaya *et al.*, 2008) and a similar decline of pear has been found in Taiwan (Liu *et al.*, 2007). New outbreaks of PD are currently observed across Europe.

Restriction fragment length polymorphisms (RFLP) and sequence analysis of ribosomal DNA (rDNA) revealed that the disease is associated with a distinct phytoplasma, '*Candidatus Phytoplasma pyri*', that is closely related to phytoplasmas associated with other important diseases of fruit crops: '*Candidatus Phytoplasma mali*' causing apple proliferation (AP) and '*Candidatus Phytoplasma prunorum*', the agent of European stone fruit yellows (ESFY) (Seemüller and Schneider, 2004). They all belong to the 16Sr group X, the Apple proliferation cluster.

Three psyllid species of the genus *Cacopsylla* are recognised or presumed vectors of PD: *Cacopsylla pyri* (Linnaeus), *C. pyricola* (Foerster) and *C. pyrisuga* (Foerster) (reviewed by Jarausch and Jarausch, 2010). As PD is of high economic relevance reliable detection of the pathogen in plants and insects is of paramount importance for sanitary measures as well as for insect vector identification. Currently, PCR technology is the method of choice for sensitive detection of phytoplasmas in woody plants and insects. Up to now, universal phytoplasma primers and specific primers both derived from the 16S rDNA sequence and the intergenic 16S-23S rDNA region are most widely used. However, due to the close relationship of '*Ca. P. pyri*' with '*Ca. P. mali*' and

'*Ca. P. prunorum*' most of the specific primers show cross reactivity with the DNA of other AP-group fruit tree phytoplasmas, especially with '*Ca. P. mali*'. Differentiation of the pathogens can only be achieved by RFLP analysis (Lorenz *et al.*, 1995) and sequence analyses. So far, only one of the ribosomal primer pairs (fPD/rPDS) shows a higher specificity and does not cross-amplify the target from '*Ca. P. mali*'. However, not all strains of '*Ca. P. pyri*' can be detected with these primers (Lorenz *et al.*, 1995). Thus, the objective of this study was to improve the reliability of the PD detection primers to allow a one-step identification of the pathogen in plants and insects.

Materials and methods

Plant material was derived from symptomatic and non-symptomatic pear trees in the Southwest of Germany and was sampled in late autumn when the concentration of the phytoplasma in the tree is highest. Phloem was prepared from branches and was extracted with a CTAB-based protocol as described by Maixner *et al.* (1995). Phytoplasma reference strains were maintained in micropropagated pear ('*Ca. P. pyri*', strain PD9267) as described in Jarausch *et al.* (2000), in micropropagated apple ('*Ca. P. mali*') as published by Jarausch *et al.* (1996) or in micropropagated *Prunus* ('*Ca. P. prunorum*') (Jarausch *et al.*, 1994). Shoot material from these plants was extracted with the same protocol.

PCR amplification of phytoplasma DNA was achieved with universal ribosomal primers fU5/rU4 (Ahrens and Seemüller, 1992) or with 16Sr group X-specific primers fO1/rO1 (Lorenz *et al.*, 1995). In addition, PD-specific ribosomal primers fPD/rPDS were applied (Lorenz *et al.*, 1995).

For sequence analysis, PCR products were cloned and sequenced with standard procedures.

Results and discussion

In total, 122 samples of pear trees were proven to be infected by phytoplasmas using PCR detection with universal ribosomal primers fU5/rU4. Almost all samples yielded specific PCR products with primers fO1/rO1. However, 'Ca. P. pyri' was detected in only 80% of the samples. This is in agreement with the data reported by Lorenz *et al.* (1995). This failure of detecting all 'Ca. P. pyri' strains can be due to an insufficient sensitivity of the primers or to genetic differences of the phytoplasma strains. Therefore, sequence data were produced for two strains for the 16S rDNA and the 16S-23S rDNA intergenic region. One sequence was obtained from the reference strain PD9267, the other from a field-collected isolate from Southwest Germany. Both sequences were almost identical to all other complete 16S-23S rDNA sequences of 'Ca. P. pyri' available in Genbank. One exception was the PD phytoplasma sequence accession Y16392 which has two important deletions compared to the other 'Ca. P. pyri' sequences. The primer fPD as well as the primer rPDS were both selected in these regions and their *in silico* specificity with regard to other 16Sr group X phytoplasmas is mainly based on these deletions. Corrected primers proved to be no longer specific when tested with other European fruit tree phytoplasmas. Therefore, new primers were selected in the 16S-23S rDNA sequence region and their specificity for 'Ca. P. pyri' was first checked *in silico*. The specificity of the primers was then evaluated with micropropagated plants highly infected with 'Ca. P. mali' and 'Ca. P. prunorum', respectively. No cross hybridization with these most closely related phytoplasmas was observed. The sensitivity and reliability of PD detection was then tested with the 122 field-collected pear samples tested positive with universal primers. Ninety percent of the samples yielded a specific PCR product with the new PD-specific primers. The majority of the non-reacting samples did not give rise to a PCR product with primers fO1/rO1 either, indicating a very low concentration of phytoplasma DNA. For these samples, the application of nested PCR approaches will be needed.

In conclusion, we developed specific primers for the direct detection of 'Ca. P. pyri'. Further studies with PD samples from different geographic regions are needed to confirm that the primers detect all strains of 'Ca. P. pyri'. The primers will be very valuable especially for the work with the different insect vectors of 'Ca. P. pyri' which might be infected with different types of phytoplasmas in nature.

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Rapid and automated analysis of single nucleotide polymorphisms (SNPs) in *secY* gene sequences for finer differentiation and characterization of phytoplasmas

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Abstract

The number of newly discovered phytoplasma strains has increased dramatically in the last decade. For epidemiological studies and for international quarantine, appropriate molecular tools are essential to identify and distinguish phytoplasmas at taxonomic ranks at and below ribosomal group level. Currently, use of RFLP analysis of the *secY* gene is one of the finest tools available to achieve the resolving power needed for fine differentiation of closely related phytoplasmas. In the present study, we developed a system for computer-simulated SNP-based analysis of *secY* sequences that incorporates SNPs located both within and outside of restriction sites, increasing the number of characters beyond those used in RFLP analyses. With the inclusion of the additional informative characters, the SNP-based system further enhances strain separation and characterization. This new system will provide additional molecular markers and should aid identification and characterization of strains that are ecologically distinct and/or originate from different geographical regions.

Key words: RFLP, SNP, differentiation, phytoplasmas, *secY* gene.

Introduction

Phytoplasmas comprise a group of genetically diverse plant pathogens that are transmitted by insect vectors, and are associated to a variety of economically important diseases (Hogenhout *et al.*, 2008). Several systems have been developed for differentiation of phytoplasma strains at different taxonomic levels (Lee *et al.*, 2010). RFLP analysis of 16S rRNA gene sequences has yielded a comprehensive classification scheme comprising at present 31 phytoplasma groups and more than 100 subgroups (Lee *et al.*, 1998; Zhao *et al.*, 2009), each group representing at least one distinct ‘*Candidatus* species’ level taxon.

Because the 16S rRNA gene is highly conserved, phytoplasmal 16S rRNA genes share similarities above 90% (Lee *et al.*, 2010). Thus, the relative genetic distances among phytoplasma strains, assessed on the basis of 16S rRNA gene sequence similarities, may not fully reveal the genetic heterogeneity of phytoplasmas, and genetically close, but biologically distinct, strains may remain unresolved. To facilitate the separation of such closely related strains, several less-conserved molecular markers have been employed for phytoplasma classification. For example, RFLP analysis of the *secY* gene for differentiation of strains within a given 16Sr group provides high resolving power, enabling differentiation of closely related strains (Lee *et al.*, 2010). SNPs have also been exploited as molecular markers separating phytoplasma lineages (Jomantiene *et al.*, 2011). In the present study, we explored analysis of SNPs in *secY* gene sequences for improving strain differentiation. In this approach, we focused on SNPs, within and outside of restriction sites, for comparative analyses among strains within a given group, with the expectation that this approach will further enhance the resolving power of *secY* gene analyses.

Materials and methods

Phytoplasma *secY* gene sequences from 13 strains representative of five subgroups within the 16SrV group (table 1) were aligned using the ClustalW algorithm implemented in the Megalign program of the Lasergene software package.

Table 1. Thirteen 16SrV strains used in this study.

Strain	Geographic origin	16S rDNA RFLP classification	<i>secY</i> gene GenBank acc. numbers
EY1	USA	16SrV-A	GU004330
EYEu	Italy	16SrV-A	AY197690
EY626	USA	16SrV-A	AY197691
CLY5	China	16SrV-B	AY197693
PY-In	India	16SrV-B	AY197694
JWB	China	16SrV-B	AY197695
AldY882	Germany	16SrV-C	AY197692
FD70	France	16SrV-C	AY197686
FD-C	Italy	16SrV-C	AY197688
AldY	Italy	16SrV-C	AY197684
SpaWB229	Italy	16SrV-C	AY197689
FD-D	Italy	16SrV-D	AY197685
RuS	Italy	16SrV-E	AY197696

The resulting dendrogram separated these strains into seven distinct branches. Each of these branches contains a single strain or a strain cluster. Nucleotide sequence variations in each alignment position were examined, and subgroup- or strain cluster-specific SNPs were identified computationally (Zhao *et al.*, unpublished).

Results

A total of 256 SNPs were identified in the *secY* sequences (ca. 1,233 bp) of strains classified in the 16SrV group. SNP similarities among the 13 16SrV group strains were calculated; the values ranged from 26.6% to 94.1% (figure 1). Among the seven specific strain or strain clusters, the average similarity ranged from 32.0% to 77.2%. Restriction sites and SNPs in *secY* genes of strains FD-D and FD-C are shown in figure 2.

	ALY	FD70	FD-D	FD-C	SpaWB	ALY882	RuS	EY1	EYEu	EY626	CLY5	PY-In	JWB
ALY	100	84.0	84.8	77.7	62.5	67.6	64.1	52.0	52.0	52.0	36.3	35.2	35.2
FD70		100	83.2	77.7	63.3	68.0	64.5	52.0	52.0	52.0	36.3	35.2	35.9
FD-D			100	76.2	60.9	66.0	61.7	50.4	50.4	50.4	35.2	34.0	34.0
FD-C				100	64.8	68.0	65.2	51.2	51.2	51.2	34.0	32.8	33.6
SpaWB					100	60.5	57.8	51.6	51.6	51.6	27.7	26.6	27.0
ALY882						100	69.9	52.7	52.7	52.7	33.6	32.4	32.0
RuS							100	51.2	51.2	51.2	32.0	30.9	31.2
EY1								100	93.8	93.8	31.2	30.1	30.5
EYEu									100	94.1	31.2	30.1	30.5
EY626										100	31.2	30.1	30.5
CLY5											100	87.5	87.1
PY-In												100	85.2
JWB													100

Figure 1. Base similarities (%) in 256 SNP positions in the *secY* genes of 13 phytoplasma strains classified in 16S rDNA RFLP group 16SrV. (In colour at www.bulletinofinsectology.org)

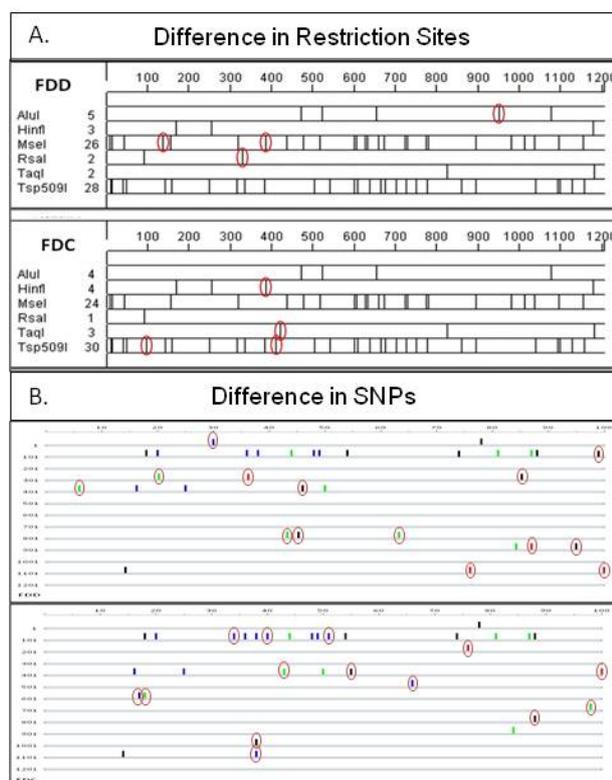


Figure 2. (A) Restriction sites in FD-D and FD-C. (B) SNPs in FD-D and FD-C (Color code for bases: A-green, T-red, C-blue, G-black). Ovals indicate differences between FD-D and FD-C. (In colour at www.bulletinofinsectology.org)

Discussion

Two decades of research have revealed extensive diversity of phytoplasma ecology and indicated a remarkable complexity of phytoplasma population structure. For epidemiological studies and for international quarantines, appropriate molecular tools are essential to identify and distinguish phytoplasmas at taxonomic ranks at and below 'Candidatus species' level. Identification and classification of strains may be achieved by RFLP analysis of *secY* sequences, but bases used for analysis in RFLP-based systems are limited to those within restriction sites. By maximizing inclusion of additional informative characters, the SNP-based analysis further enhances resolving power for strain differentiation and characterization. This new approach will provide additional molecular markers and should aid identification and characterization of strains that are ecologically distinct and/or originate from different geographical regions. A pattern recognition and pattern similarity coefficient calculation program is being developed to analyze query *secY* sequences for identification of SNP patterns.

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Epidemiological aspects of phytoplasmas in Chilean grapevines

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Abstract

Some Auchenorrhyncha specimens were captured, identified and tested to verify phytoplasma presence in Chilean vineyards; many of them belong to the subfamily Delthocephalinae and Agalliinae (family Cicadellidae) and to the families Cixiidae and Delphacidae, all known as potential phytoplasma vectors. Several individuals were positives to phytoplasma presence, in particular *Amplicephalus curtulus* Linnavuori & De Long, in which were detected phytoplasmas belonging to subgroup 16SrI-B and 16SrXII-A, and *Paratanus exitiosus* (Beamer) positive to phytoplasmas of the subgroups 16SrI-B, 16SrVII-A and 16SrXII-A. Phytoplasmas belonging to subgroup 16SrI-B and 16SrVII-A were identified in *Convolvulus arvensis* L. and *Polygonum aviculare* L.; to subgroup 16SrXII-A in *C. arvensis*; and to subgroup 16SrVII-A in *Galega officinalis* L. In three cases grapevine samples, weeds and insects collected in the same vineyard were positives to phytoplasmas of the same subgroup.

Key words: Auchenorrhyncha, grapevine yellows, nested-PCR, phytoplasmas, RFLP, sequencing, weed.

Introduction

Phytoplasmas found in Chilean grapevines showing yellows symptoms were identified as belonging to the ribosomal subgroups 16SrI-B and 16SrI-C ('*Candidatus* Phytoplasma asteris'), 16SrVII-A ('*Ca. P. fraxini*') and 16SrXII-A (stolbur or "bois noir") (Gajardo *et al.*, 2009). The presence of these pathogens in the plants depends on both propagation of infected plants and spreading by different insect species which feed on grapevine and also on the weeds growing near and/or in vineyards. There is no evidence of epidemic spread of yellows symptoms in the inspected vineyards so far; however a survey to verify the presence and identity of weeds and potential insect vectors was carried out.

Materials and methods

During 2009 and 2010 surveys were carried out in thirteen vineyards in four regions of Chile where phytoplasmas were detected: 4 vineyards in Valparaíso (V); four vineyards in Metropolitana de Santiago (RM); 1 vineyard in Libertador General Bernardo O'Higgins (VI); 4 vineyards in Maule (VII). From each vineyard weeds and insects (leafhopper and planthopper) were collected.

The insects were captured from December to March by sweeping with an entomological net. They were separated by the morphological characteristics and sex. From each individual photos were taken. For determination at genus and species level the male genitalia were

examined under a stereo-microscope. Weeds infected by phytoplasmas were identified at the species level.

Insects and weeds were tested in order to identify the phytoplasma presence. Total nucleic acids (TNA) was extracted with CTAB or chloroform/phenol methods, dissolved in Tris-EDTA pH 8.0 buffer, and maintained at 4°C; 20 ng/μl of nucleic acid were used for amplification. After direct PCR with primer pair P1/P7, nested PCR with R16F2n/R2 primers (Gundersen and Lee, 1996) was performed. PCR and nested PCR reactions were carried out following published protocol (Schaff *et al.*, 1992). Identification of detected phytoplasmas was done using RFLP analyses on amplified ribosomal DNA fragments with *TruI*, *RsaI*, *HhaI*, *Tsp509I*, *TaqI*, *AluI* (Fermentas, MBI, Vilnius, Lithuania) restriction enzymes. Selected R16F2/R2 amplicons identified after RFLP analyses were purified using Concert Rapid PCR Purification System (Life Technologies, Gaithersburg, MD, USA). DNA fragments were ligated into T/A cloning vector pTZ57R/T following the instructions of the InsT/Aclone PCR Product Cloning Kit (Fermentas, MBI, Vilnius, Lithuania). Putative recombinant clones were analysed by colony-PCR, sometimes in combination with the insert-specific primers. Amplicons obtained from three colonies per cloned fragment were subjected to RFLP analyses, as described above. Selected fragments from cloned DNAs were sequenced in both directions using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The sequences were then aligned with BLAST engine for local alignment (version Blast N 2.2.12).

Results and Discussion

From all the vineyards a total of 50 different weed samples and 200 specimens of leafhoppers and planthoppers were collected. Positive results were obtained only after nested amplification on P1/P7 amplicons. The phytoplasmas detected in weeds and insects were assigned by RFLP analyses to three different ribosomal subgroups and sequence analyses were performed for corroborate pathogen identification.

The most common weed species found positive to phytoplasmas were *Convolvulus arvensis* L. and *Polygonum aviculare* L.. Phytoplasma assigned to 16SrI-B and 16SrVII-A subgroups were identified in both species; 16SrXII-A subgroup phytoplasmas were identified in *C. arvensis*, and 16SrVII-A phytoplasmas were detected in one sample of *Galega officinalis* L.

Several insects belonging to the subfamily Deltocephalinae and Agalliinae (family Cicadellidae) and to the families Cixiidae and Delphacidae were found positives to phytoplasmas. The most common were *Amplipcephalus curtulus* Linnavuori & De Long in which phytoplasmas of 16SrI-B and 16SrXII-A subgroups were detected, and *Paratanus exitiosus* (Beamer) found positive to the subgroups 16SrI-B, 16SrVII-A and 16SrXII-A. In three cases the grapevine samples, weeds and insects collected in the same vineyard were positives to the same phytoplasma: Petit Syrah grapevine, *P. aviculare* and *P. exitiosus* infected by phytoplasmas belonging to the subgroup 16SrI-B; Carménère grapevine, *C. arvensis* and *P. exitiosus* by 16SrVII-A; Pinot noir grapevine, *C. arvensis* and *A. curtulus* by 16SrXII-A (table 1).

Table 1. Phytoplasmas detected in grapevines, weeds and insects from each of the three different vineyards.

Grapevine cultivar (Region)	Detected phytoplasmas		
	grapevine	weed	leafhopper
Petit Syrah (RM)	16SrI-C	16SrI-B (<i>P. aviculare</i>)	16SrI-B (<i>P. exitiosus</i>)
	16SrI-B	16SrVII-A (<i>P. aviculare</i> , <i>C. arvensis</i>)	16SrVII-A (<i>P. exitiosus</i>)
Carménère (RM)	16SrVII-A	16SrVII-A (<i>C. arvensis</i>)	16SrVII-A (<i>P. exitiosus</i>)
	16SrXII-A		16SrXII-A (<i>P. exitiosus</i>)
Pinot noir (V)	16SrVII-A	16SrXII-A (<i>C. arvensis</i>)	16SrXII-A (<i>A. curtulus</i>)
	16SrXII-A		16SrVII-A (<i>P. exitiosus</i>)

Since many individuals of *P. exitiosus* and *A. curtulus* were captured on the weeds it is very likely that they only occasionally feed on the grapevine (perhaps after weeding), transmitting phytoplasmas. In Chile *P. exitiosus* was found between the regions of Los Lagos (X) to Valparaíso (V), especially in the Bío Bío region (VIII) in sugarbeet crops (Casals *et al.*, 1999; Klein Koch and Waterhouse, 2000). *A. curtulus* was found only in the region of Los Lagos (X) (Zanol, 2007). In this study, the presence of *A. curtulus* in VII, VI, V and RM regions was observed for the first time.

P. aviculare and *C. arvensis* have been repeatedly found positive to different phytoplasmas, so we can conclude that these weeds represent a reservoir of phytoplasmas for grapevine in Chilean vineyards.

Assays to verify the phytoplasma transmission ability of the leafhoppers *A. curtulus* and *P. exitiosus* are in progress.

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An update on phytoplasma diseases in New Zealand

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Abstract

'*Candidatus Phytoplasma australiense*' occurs in New Zealand and Australia where it is associated with plant diseases in native, weed and crop plants. Between January 2009 and July 2010, four new diverse hosts of '*Ca. P. australiense*' have been identified in New Zealand: potato, Jerusalem cherry, swan plant and celery, as well as a new disease association in boysenberry. A 1.2 kb region of the 16S rRNA gene of the phytoplasma amplified from the new hosts were identical to each other. Partial *tuf* gene sequence analysis of 29 strains from the new plant hosts revealed that they belong to two separate subgroups, *tuf* variant VII and *tuf* variant IX. Two of the strains, one from potato and the other from celery, contained a mixed infection of both phytoplasma subgroups.

Key words: Mollicute, PCR, detection, epidemiology.

Introduction

'*Candidatus Phytoplasma australiense*' (16SrXII-B) is found in New Zealand and Australia where it is associated with a range of host plants. In New Zealand, the phytoplasma is historically associated with the diseases, *Phormium* yellow leaf, strawberry lethal yellows, *Cordylone* sudden decline, and *Coprosma* lethal decline (Liefting *et al.*, 2007). In Australia, this phytoplasma species has been associated with Australian grapevine yellows, Papaya dieback, as well as diseases in a range of other hosts including strawberry, pumpkin and bean (Streten and Gibb, 2006).

Sequence analysis of the *tuf* gene of different strains of '*Ca. P. australiense*' in New Zealand determined that there are nine different *tuf* variant groups (I-IX) that cluster into two distinct clades (Andersen *et al.*, 2006). Andersen *et al.* (2006) also analysed the available *tuf* gene sequences from Australian strains and determined that some strains formed a third distinct *tuf* gene clade while other strains clustered into one of the clades formed by the New Zealand isolates.

Here we provide an update on four new diverse hosts of '*Ca. P. australiense*' in New Zealand: potato (*Solanum tuberosum*), Jerusalem cherry (*Solanum pseudocapsicum*), swan plant (*Gomphocarpus fruticosus*) and celery (*Apium graveolens*), as well as a new association of '*Ca. P. australiense*' in boysenberry (*Rubus* hybrid). This phytoplasma was previously detected in boysenberry exhibiting different symptoms that were attributed to the fungus *Cercospora rubi* (Wood *et al.*, 1999). The *tuf* gene sequences of '*Ca. P. australiense*' from these new hosts were analysed extending the work of Andersen *et al.* (2006).

Materials and methods

Total plant DNA was extracted from leaf midribs and petioles, stems or tubers using an InviMag Plant DNA Mini Kit (Invitex, Berlin, Germany) and a KingFisher

mL workstation (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Initial screening of samples for phytoplasma infection was performed using the TaqMan real-time PCR assay of Christensen *et al.* (2004). For sequence analysis of the 16S rRNA gene, conventional nested-PCR was performed with the primer pairs, P1 (Deng and Hiruki, 1991)/P7 (Schneider *et al.*, 1995) followed by R16F2/R16R2 (Lee *et al.*, 1995). The *tuf* gene was amplified using the fTufAY/rTufAY primer pair (Schneider *et al.*, 1997).

Conventional PCR products were either sequenced directly or cloned into the pCR 4-TOPO vector (Invitrogen) according to the manufacturer's instructions.

The sequences were assembled and edited using Geneious Pro (Drummond *et al.*, 2010). Searches of the GenBank database for homologous sequences were performed using the BLASTn network service available at the National Centre for Biotechnology Information (Bethesda, MD, USA).

Results

Potato (*Solanum tuberosum*) plants exhibited upward rolling and purpling of the leaves. Symptoms in Jerusalem cherry (*Solanum pseudocapsicum*) included witches' broom, foliar yellowing and reduced leaf size. Swan plant, also known as milkweed (*Gomphocarpus fruticosus*), showed abnormal foliar yellowing and slight upward rolling of the leaves that resulted eventually in plant death. Celery (*Apium graveolens*) plants were observed to be showing unusual symptoms of pink and yellow foliage and leaf deformation. Symptoms in boysenberry plants become obvious close to flowering when the lateral branches become stunted and young leaves are chlorotic and smaller than normal. As the disease progresses, the older leaves become purple-bronze in colour, particularly towards the margin.

All symptomatic plants described above produced positive real-time PCR results. In order to identify the

phytoplasma present in these samples, a 1.2 kb region of the 16S rRNA gene generated from the R16F2/R16R2 primers was sequenced directly. BLAST analysis of the 1.2 kb region of the 16S rRNA gene amplified from symptomatic Jerusalem cherry, swan plant, celery and boysenberry showed 100% identity to 'Ca. P. australiense' 16S rRNA gene sequences in the GenBank database.

Partial sequences (~800-840 bp) of the *tuf* gene of 'Ca. P. australiense' amplified from 29 strains from the symptomatic plants described above were determined. Twenty-two of these sequences were identical to *tuf* variant VII and five sequences were identical to *tuf* variant IX of Andersen *et al.* (2006). Interestingly, these two *tuf* variants belong to distinct subgroups: *tuf* clade 1 (includes variants VIII and IX) isolates originate from both New Zealand and Australia whereas *tuf* clade 2 (includes variants I to VII) consists exclusively of isolates from New Zealand (Andersen *et al.*, 2006). None of the isolates in this study belonged to *tuf* variants I to VI and VIII and no new variants were discovered.

The sequences of the PCR products from two strains, one from celery and the other from potato indicated that these hosts contained a mixed phytoplasma population due to the presence of ambiguous bases. The *tuf* gene PCR amplicon from these two samples were cloned and the sequence was determined from 10 resulting clones. Sequence analysis of the clones from celery revealed that 7 were identical to variant VII and 3 were identical to variant IX, and for potato, 2 clones were of variant VII and 8 were of variant IX, thereby confirming the presence of two *tuf* gene types in these samples.

Discussion

Between January 2009 and July 2010, four new hosts (potato, Jerusalem cherry, swan plant and celery) of 'Ca. P. australiense' have been identified in New Zealand, as well as a new disease association in boysenberry. These are the first new hosts of 'Ca. P. australiense' to be recognised in New Zealand since *Coprosma* in 1998 (Beever *et al.*, 2004), although they may have gone unnoticed for several years.

The economic impact that 'Ca. P. australiense' will have on these newly identified hosts is potentially significant. Already, phytoplasma-infected potato tubers have failed quality control checks at the processing factory. In boysenberry, fruit from infected plants are unmarketable and the plants die within a year from when the symptoms are first noticeable. Although there have been no reports of the phytoplasma in commercially grown celery, the discolouration of the foliage that the phytoplasma induces in this crop would also render it unmarketable.

The polyphagous feeding behaviour of *Zeolarius oppositus*, the insect responsible for transmitting the phytoplasma into *Cordyline* and *Coprosma* (Beever *et al.*, 2008), suggests that it may also be moving the phytoplasma into the new hosts described here. *Z. oppositus* is especially abundant in grasses and sedges that commonly grow around crops in New Zealand. These hosts

may act as symptomless reservoirs of the phytoplasma and along with weed hosts such as Jerusalem cherry, play an important role in the spread of the phytoplasma. The diversity of the new hosts described in this paper emphasises the potential that 'Ca. P. australiense' has to spread into additional native plants and horticultural crops.

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Molecular identification of 'Candidatus Phytoplasma fraxini' in murta and peony in Chile

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Abstract

Plants of murta (*Ugni molinae* Turcz.) and peony (*Paeonia lactiflora* Pall.), exhibiting disease symptoms suggestive of possible phytoplasma infection were observed in Chile. Leaves were collected from six plants per species (three with and three without symptoms) and main leaf midribs were used for phytoplasma testing. Nested polymerase chain reaction (PCR) amplification allowed the detection of phytoplasmas in samples from plants that showed symptoms, but not in those from the asymptomatic ones. Cloning, sequencing and *in silico* restriction fragment length polymorphism (RFLP) of the 16S rRNA gene allowed identification of the phytoplasmas into ribosomal subgroup 16SrVII-A for both species.

Key words: *Ugni molinae*, peony, nested-PCR, phytoplasmas, *in silico* RFLP, sequencing.

Introduction

Murta (*Ugni molinae* Turcz., family Myrtaceae) is a native bushy plant present mainly in the South of Chile, between the region of Maule (VII) to Aysén del General Carlos Ibáñez del Campo (XI). The berries and leaves are high in antioxidant and analgesic compounds that are used in pharmacology. In murta witches' broom symptoms were observed during spring and summer time. During this period, leaves are smaller and yellowing. At the end of summer and during the autumn season, leaves turn reddish and twigs become necrotic and die. The berries, if present, are smaller and poor in sugar and flavorings. The first report of this disease, based on symptoms, has been made in the early '80s (Novoa, 1982). However, the first laboratory evidence for the presence of phytoplasmas in murta was obtained recently (Andrade *et al.*, 2009).

Peony (*Paeonia lactiflora* Pall., family Paeoniaceae) is cultivated mainly in the regions of Los Ríos (XIV), Araucanía (IX) and Libertador Bernardo O'Higgins (VI) and has been the main species of flowers exported by Chile in 2010, with shipments totaling 1.3 million US dollars. In one orchard of the VI region, plants of the peony variety Henry Bockstoe (*Paeonia lactiflora* x *officinalis*), with deep red flowers, showed: malformation, necrosis and downward rolling of leaves; green stripes on the petals; and drying up of flower bud. No fungal or bacterial isolation was obtained, and no viruses were found in both species. Molecular analyses were carried out during 2010 both in asymptomatic and symptomatic plants to verify the presence of phytoplasmas in murta and peony samples.

Materials and methods

Samples were collected in summer 2010 from three symptomatic and three asymptomatic plants of both species. Total nucleic acids were extracted from 1 g of main leaf

midribs (Prince *et al.*, 1993), dissolved in Tris-EDTA pH 8.0 buffer, and maintained at 4°C; 20 ng/μl of nucleic acid were used for amplification. After direct polymerase chain reaction (PCR) with primer pair P1/P7, nested PCR with R16F2n/R2 primers (Gundersen and Lee, 1996) was performed. PCR and nested PCR reactions were carried out following the published protocol of Schaff *et al.* (1992). Selected R16F2n/R2 amplicons were purified using Concert Rapid PCR Purification System (Life Technologies, Gaithersburg, MD, USA). DNA fragments were ligated into T/A cloning vector pTZ57R/T following the instructions of the InsT/Aclone PCR Product Cloning Kit (Fermentas, MBI, Vilnius, Lithuania). Putative recombinant clones were analysed by colony-PCR, sometimes in combination with the insert-specific primers. Amplicons obtained from three colonies per cloned fragment were sequenced in both directions using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The sequences were then aligned with BLAST engine for local alignment (version Blast N 2.2.12). Identification was done using *in silico* restriction fragment length polymorphism (RFLP) analyses on sequences belonging to ribosomal DNA amplified with primer pair R16F2n/R2 with *AluI* and *TaqI* restriction enzymes (Wei *et al.*, 2007).

Results and discussion

Positive results were obtained only after nested amplification on P1/P7. Cloned fragments from R16F2n/R2 amplicons was sequenced and subjected to *in silico* RFLP analysis that placed the phytoplasma in ribosomal subgroups 16SrVII-A ('Candidatus Phytoplasma fraxini') (figure 1). This phytoplasma was detected in all plants with symptoms from both species, but not in the asymptomatic ones. In all cases there was no sequence difference between the three cloned R16F2n/R2 fragments from the same sample (1,240 bp).

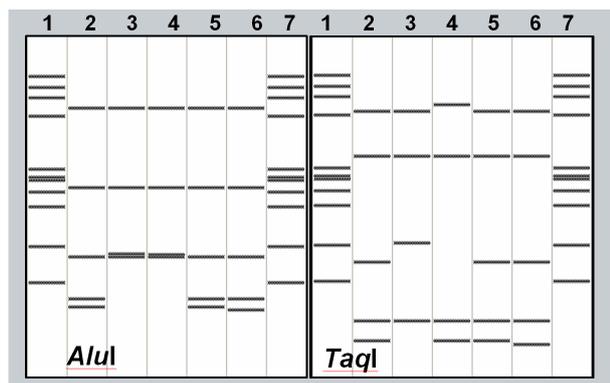


Figure 1. *In silico* RFLP analysis. 1 and 7: PhiX174: marker Φ X174 *Hae*III digested. Phytoplasmas: 2: 16SrVII-A, AF092209; 3: 16SrVII-B, AY034608; 4: 16SrVII-C, AY147038; 5: Chilean murta; 6: Chilean peony.

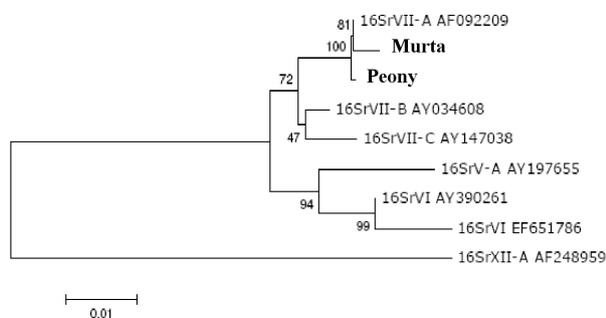


Figure 2. Phenetic tree constructed using neighbor-joining method with 16S rDNA region sequences of two phytoplasm strains from Chilean murta and peony, and related phytoplasmas.

The 16S rDNA (figure 2) sequences from murta and peony were 99.6 and 99.7% identical to AshY1 and AshY5 (Ash yellows phytoplasm - '*Ca. P. fraxini*' - 16SrVII-A) from the United States (Accession numbers AF092209 and AF105316), respectively (Griffiths *et al.*, 1999).

This is the first report of a 16SrVII-A phytoplasm in murta and peony, even though phytoplasm belonging to the same ribosomal subgroup were reported to infect grapevines in Chile (Gajardo *et al.*, 2009), ash and lilac in the United States and Canada (Griffiths *et al.*, 1999), ash in Colombia (Franco-Lara *et al.*, 2006), and peach in Italy and Canada (Paltrinieri *et al.*, 2003; Zunnoon-Khan *et al.*, 2010).

The evidence gathered to date suggests that the phytoplasm belonging to ribosomal subgroup 16SrVII-A is the most widespread in the different plant species of Chile. The presence of phytoplasm in Chilean murta and peony is probably the consequence of both vegetative propagation of infected plants and feeding activity of insect vectors. Moreover, the phytoplasm may overwinter in infected vectors and/or in other perennial plants that serve as reservoirs that spread again the phytoplasm in the following spring.

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Molecular detection and identification of group 16SrV and 16SrXII phytoplasmas associated with potatoes in Colombia

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Abstract

A severe disease was observed in the potato variety ‘Criolla Colombiana’. Main symptomatology consisted of discoloration or yellowing of the whole plant, apical leafroll, dwarfing, axillary buds and thicker internodes. Phytoplasmas related to 16SrV and 16SrXII groups were identified by nested PCR assays followed by real and virtual RFLP and sequence analyses. This is the first report of phytoplasma presence in potato in Colombia and the first identification of group 16SrV phytoplasmas in this crop.

Key words: Potato, Colombia, phytoplasma, PCR/RFLP analyses, sequencing.

Introduction

Potato (*Solanum tuberosum* L.) is a staple food and one of the most important agricultural crops in Colombia, reaching about 162,000 ha with a production of more than 2,721.396 tons/year. Phytoplasma diseases of potato have been reported in several countries around the world where at least nine phytoplasmas were identified such as 16SrI-A, 16SrI-B, 16SrII, 16SrIII-M, 16SrVI-A, 16SrXII-A, 16SrXIII, 16SrXVIII-A and 16SrXVIII-B (Paltrinieri and Bertaccini, 2007; Santos-Cervantes *et al.*, 2010). In 2011 in Colombia phytoplasma-related symptoms were found in potato for seed lots, multiplication in three areas in the municipalities of Guasca and Zipaquirá, (2,900 – 3,000 meters above sea level), in the variety ‘Criolla Colombiana’. Symptoms of the disease are discoloration or yellowing of leaflets, apical leafroll, dwarfing, axillary buds and thicker internodes (personal communication FEDEPAPA, 2011). Molecular analyses were carried out to verify phytoplasma association with this disease.

Materials and methods

Symptomatic leaves and stem of potatoes were collected from eight naturally diseased plants exhibiting symptoms, in the location of Guasca and Zipaquirá in the state of Boyacá. Total nucleic acids were extracted from 1 g of mixture of leaf midribs and stem phloem tissues of eight samples, dissolved in Tris-EDTA pH 8 buffer, and maintained at 4°C; 20 ng/μl of nucleic acid were used for amplification. Universal and specific primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) and R16F2/R2 (Lee *et al.*, 1995), and R16(I)F1/R1, R16(V)F1/R1 (Lee *et al.*, 1994) were used to prime amplification of phytoplasma 16S rDNA sequences in nested and second nested PCR assays.

Table 1. Results of PCR/RFLP analyses on potato.

Plant	Tissue	PCR/RFLP		
		16R _{758f} /16R _{1232r}	R16(I) F1/R1	R16(V) F1/R1
1	Leaf	16SrV+ -XII	16Sr	16SrV
	Stem	-	-	16SrV
2	Leaf	16SrXII	-	16SrV
	Stem	16SrXII	-	16SrV
3	Leaf	16SrV	16SrXII	16SrV
	Stem	16SrXII	16SrXII	16SrV
4	Leaf	16SrXII	16SrXII	16SrV
	Stem	-	-	16SrV
5	Leaf	-	-	16SrV
	Stem	-	-	16SrV
6	Leaf	-	-	16SrV
	Stem	16SrV	-	16SrV
7	Leaf	16SrXII	-	16SrV
	Stem	16SrV	-	16SrV
8	Leaf	16SrV	-	16SrV

-, no phytoplasma detected

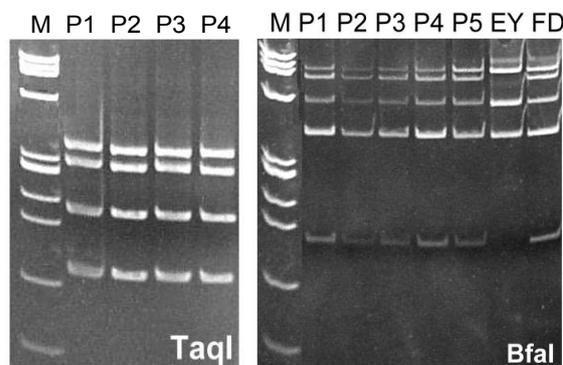


Figure 1. RFLP analyses of R16(V)F1/R1 and 16R_{758f}/V1731 amplicons. P, potatoes samples, EY, elm yellows (16SrV-A); FD, ‘flavescente dorée’ (16SrV-C). M, marker ΦX174 *Hae*III digested.

Further nested PCR were performed with 16R_{758F}/16R_{1232r} (Gibb *et al.*, 1995) and 16R_{758F}/V1731 (Martini *et al.*, 1999) primer pairs. All PCR reactions were carried out following the protocol of Schaff *et al.* (1992). Phytoplasma identification was done with RFLP analyses with *TruI*, *BfaI*, and *TaqI*, (Fermentas, Vilnius, Lithuania) restriction enzymes according with amplicons employed. Selected R16(V)F1/R1 and 16R_{758F}/V1731 amplicons were purified using Nucleospin extract (Macherey-Nagel, Germany) and directly sequenced in both directions using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The sequences were then aligned with BLAST engine for local alignment (<http://www.ncbi.nlm.nih.gov/BLAST/>). 16S rDNA sequences were retrieved from NCBI and used to construct phylogenetic trees. Minimum evolution analysis was carried out using the neighbor joining method and bootstrap replicated 500 times with the software MEGA 4.1 (<http://www.megasoftware.net/index.html>) (Kumar *et al.*, 2004). *Acholeplasma palmae* was used as the outgroup. To rule out other pathogens possibly associated with the disease ELISA tests for PLRV, PVX, PVY and PVS viruses were carried out.

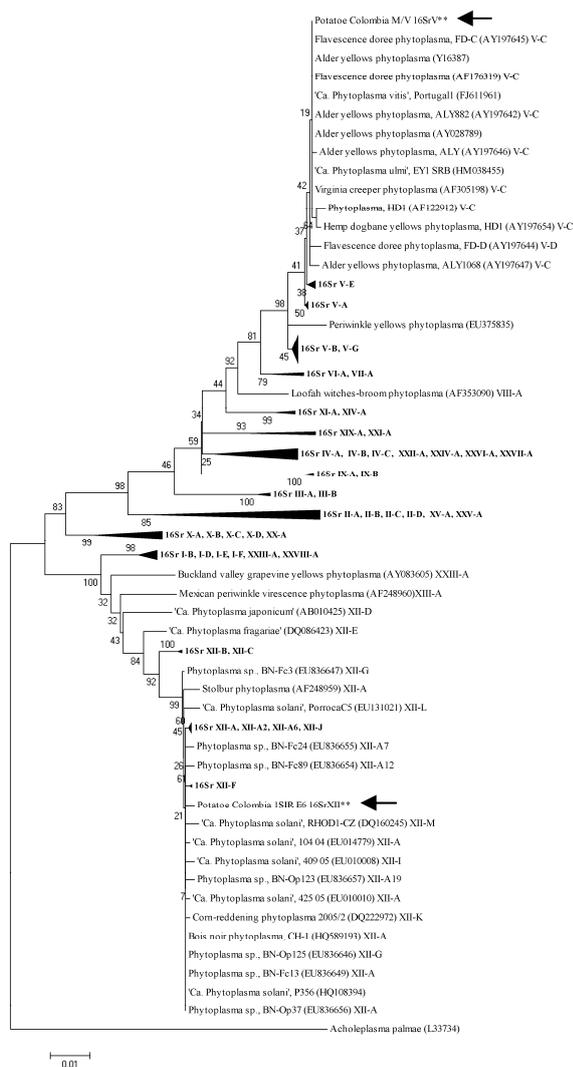


Figure 2. Phylogenetic tree from phytoplasma partial-length 16S rDNA. Sequences determined in this study are indicated by asterisks and arrows.

Results and discussion

All potato samples were negative in the tests for virus detection and positive for phytoplasma presence; RFLP analyses confirm the presence of 16SrXII phytoplasmas, while in samples amplified with 16SrV-group specific primers phytoplasmas related to 16SrV-C subgroup was determined by both RFLP analyses and sequencing (table 1; figure 1 and 2). In particular 16SrXII phytoplasmas were identified in 7 samples always in mixed infection with 16SrV phytoplasmas. This new potato disease reported in Colombia could cause severe economic losses since potato seed lots are grown in areas where high incidence of the disease has been already reported. This is the first identification of 16SrV phytoplasmas in potato and of its mixed infection with stolbur in Colombia and worldwide.

Acknowledgements

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***Turnera ulmifolia*, a new phytoplasma host species**

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Abstract

Turnera ulmifolia L., the yellow alder, is a widely distributed species in Brazil where, besides being an ornamental, it is used as a tea for the treatment of gastric diseases. In plants showing yellowing and witches' broom symptoms, a phytoplasma was detected by molecular analyses; its characterization by RFLP analyses of 16S rDNA gene allowed preliminary classification of this phytoplasma into the 16SrXIII ribosomal group. This is the first time that a phytoplasma from the 16SrXIII group has been reported in Brazil.

Key words: Brazil, chanana, witches' broom, yellow alder, phytoplasma.

Introduction

Turnera ulmifolia L. (Turneraceae), the yellow alder, is a perennial, dense, compact shrub native to tropical America. The species is widely distributed in Brazil, where it is popularly known as turnera, chanana and flor-do-guarujá. With showy yellow flowers that blossom year-round, turnera is adopted as an ornamental plant, being used as foundation, border, mass planting and ground cover (Lorenzi, 2008). In Brazilian folk medicine, turnera is also used as a tea for the treatment of diseases related mainly to gastric dysfunction. Research has produced data indicating that the plant extract has a significant antiulcerogenic effect (Gracioso *et al.*, 2002). Plants of *T. ulmifolia* exhibiting witches' broom growths (figure 1) and yellowing that are symptoms typically induced by phytoplasmas, have been observed in the state of Rio de Janeiro. The aim of the present work was to verify phytoplasma association with the *Turnera ulmifolia* witches' broom disease in Brazil and to molecularly identify detected phytoplasmas.

Materials and methods

Samples from *T. ulmifolia* exhibiting shoot proliferation and yellowing (figure 1) were collected in the location of Penedo, state of Rio de Janeiro, in 2003. DNA extraction procedure followed that of Montano *et al.* (2000). Reference phytoplasma strains in periwinkle were employed as control (figure 2) and a strain of erigeron witches' broom phytoplasmas [16SrVII-B, (Barros *et al.*, 2002)] from naturally infected plant from Brazil was also employed as a positive control. Universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) and R16F2/R2 (Lee *et al.*, 1995) were used to prime amplification of phytoplasma 16S rDNA sequences in nested PCR assays. Further nested PCR assays were carried out with primers F1/B6 (Davis and Lee, 1993; Padovan *et al.*, 1995). RFLP analyses were carried out with *TruI* on F1/B6 and R16F2/R2 amplicons and with *TaqI*, and *AluI* restriction enzymes

(Fermentas, Vilnius, Lithuania) on R16F2/R2 amplicons. Obtained patterns were compared with those of phytoplasma reference strains (Bertaccini *et al.*, 2000) on the same size amplicons. Further amplification for molecular characterization of detected phytoplasmas were carried out using rpF(I)1/rp(I)R1A primers to amplify the rplV (rpl22) and rpsC (rps3) genes; obtained amplicons were subjected to RFLP analyses with *TruI* under described conditions (Martini *et al.*, 2007).



Figure 1. *Turnera ulmifolia* asymptomatic (left and right) and with yellowing and witches' broom (arrow). (In colour at www.bulletinofinsectology.org)

Results

Phytoplasmas were detected in turnera plants exhibiting symptoms of witches' broom disease in direct, as well as in nested PCR tests. RFLP analyses with *AluI*, *TaqI* and *TruI* restriction enzymes on P1/P7, F1/B6 and on R16F2/R2 amplicons (figure 2 and Lee *et al.*, 1998) allowed the tentative phytoplasma affiliation to ribosomal subgroup 16SrXIII. The amplification of the rpl22-rps3 gene resulted in the expected 1.2 kb amplicons and the RFLP profile obtained after *TruI* digestion was clearly different from any of those available in the literature for the same gene.

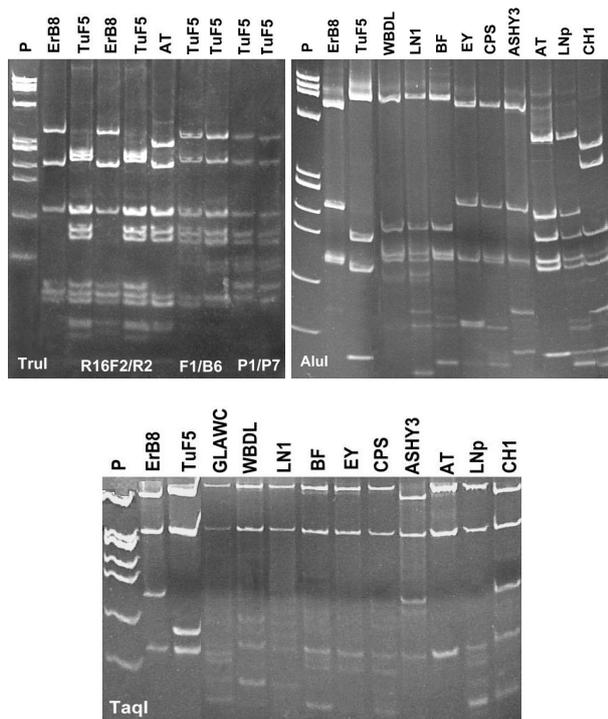


Figure 2. RFLP profiles with *TruI* of the three types of amplicons produced (top left) and with *AluI* (top right) and *TaqI* (bottom) of R16F2/R2 amplicons. Acronyms: ErB8, erigeron witches' broom; TuF5, turnera; GLAWC, 16SrI-B; WBDL 16SrII-B; LN1, 16SrIII-B; BF, 16SrIII-A; EY, 16SrV-A; CPS, 16SrVI-C; ASHY3, 16SrVII-A; AT, 16SrX-A; LNp, 16SrX-B; CH1, 16SrXII; P, marker Φ X174 *Hae*III digested.

Discussion

The PCR/RFLP results demonstrate that a phytoplasma is associated with *Turnera ulmifolia* and this is the first report of phytoplasma infection in the family Turneraceae family. The RFLP profiles obtained are referable to subgroup 16SrXIII (Lee *et al.*, 1998) for which *rp* gene profiles are not available in literature, further characterization of the phytoplasma is in progress.

In Brazil, the diseases associated with phytoplasmas have been reported in a wide range of families (Montano *et al.*, 2007), however 16SrXIII group phytoplasmas were not reported, although phytoplasmas genetically related with this group (98% homology on 16S rDNA) are listed in GenBank as associated with a papaya apical curl necrosis disease (EU719111).

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Larch is a new host for a group 16SrI, subgroup B, phytoplasma in Ukraine

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Abstract

A phytoplasma strain belonging to group 16SrI, subgroup B ('*Candidatus* Phytoplasma asteris'), the most widely spread phytoplasma worldwide, was detected in diseased larch (*Larix* sp.) in Ukraine. Detection, identification, and classification of the larch infecting phytoplasma were accomplished through RFLP and sequence analysis of 16S rRNA and ribosomal protein (*rp*) gene sequences amplified in polymerase chain reactions. The larch infecting phytoplasma was named larch dwarfed needle proliferation (LDNP) phytoplasma. The findings revealed *Larix* as a previously unknown host of phytoplasma and indicated that '*Ca. P. asteris*' is capable of infecting a gymnosperm, while raising questions concerning the identity of possible insect vector(s) involved in transmission of '*Ca. P. asteris*' to larch and perhaps to other gymnosperms.

Key words: Gymnosperm, Pinaceae, proliferation, polymerase chain reaction, mollicute.

Introduction

Class *Gymnospermae* (gymnosperms) contains several families including *Pinaceae*, within which are classified diverse woody plants, some of which are highly valuable for their timber (Lin *et al.*, 2010). One genus in the family is *Larix* (larches), trees 15 to 50 m height that constitute a major component of various forests in cool temperate regions of the northern hemisphere. Indeed, *Larix* has been cited as one of the most widely distributed tree genera in Eurasia (Lin *et al.*, 2010). Larches are conifers, but unlike members of most genera in the *Pinaceae*, the genus *Larix* is deciduous, the leaves, or needles, turning yellow and falling in autumn. In the present study we investigated the possible presence of phytoplasma in diseased larch growing in a public park in Ukraine.

Symptoms similar to those sometimes associated with plant infection by phytoplasma were observed in diseased larch in Oleski park (L'viv region, Ukraine). The trees exhibited general yellowing of needles, dwarfed needles, and proliferation and necrosis of needles. These symptoms, and recent reports of phytoplasma infections in other Gymnosperms (Davis *et al.*, 2010; Paltrinieri *et al.* 1998; Schneider *et al.* 2005; Sliwa *et al.*, 2007; Valiunas *et al.*, 2010), drew our attention to the possibility of phytoplasma-associated disease in larch. Samples of dwarfed needles were collected and analysed for the presence of phytoplasma by widely adopted molecular detection and identification methods (Lee *et al.*, 1998). The results revealed that larch was infected by a phytoplasma of group 16SrI, subgroup B ('*Candidatus* Phytoplasma asteris'), the most widely occurring phytoplasma worldwide, infecting numerous herbaceous and woody plants.

Materials and methods

Samples of symptomatic leaves (needles) from diseased larch were collected and assayed for possible phytoplasma infection. DNA was extracted from the yellowing, dwarfed and proliferated needles using Genomic DNA Purification Kit (Fermentas, Vilnius, Lithuania). Detection of phytoplasma 16S rDNA sequences was accomplished by the use of nested polymerase chain reactions (PCRs) primed by phytoplasma-universal primer pair P1/P7 followed by nested reactions primed by R16F2n/R16R2 (F2n/R2) (Lee *et al.*, 1998). Phytoplasma ribosomal protein (*rp*) gene sequences were amplified from larch DNA templates by using PCR primed by *rp* gene primer pair rpF1/rpR1. PCR was performed as described previously (Lee *et al.*, 1998). Annealing temperature for amplification of *rp* gene sequences was 50°C. Products (1.2 kbp, R16F2n/R2 segment) of the nested PCR were subjected to enzymatic restriction fragment length polymorphism (RFLP) analysis using restriction endonucleases *AluI*, *HaeIII*, *MseI*, *RsaI*, *HhaI*, *HinfI*, *HpaII*. (Fermentas, Vilnius, Lithuania). Digested products of 16S rDNA were analyzed using electrophoresis through 5% polyacrylamide gel for 16S rDNA. PCR products of *rp* gene sequences were digested with *AluI*, *MseI*, and *Tsp9065* and analysed using electrophoresis through 10% polyacrylamide gels. DNA bands were stained with ethidium bromide and visualized using a UV transilluminator. Classification of phytoplasma was accomplished through comparisons of RFLP patterns with patterns previously published, in accordance with the classification scheme of Lee *et al.* (1998). LDNP phytoplasma 16S rDNA sequence was subjected to computer-simulated restriction digestion and virtual gel plotting using *iPhyClassifier* (Zhao *et al.*, 2009).

Amplified 16S rRNA and *rp* gene products were cloned into *Escherichia coli* by using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and sequenced by automated sequencing of both strands to achieve a minimum of 3-fold coverage per base position.

Results

To determine the possible presence of phytoplasmas in diseased larch (*Larix* sp.), we examined total DNA samples prepared from the yellowing, dwarfed and proliferated needles. Two genetic markers (16S rRNA and *rp* gene sequences) were employed for the detection, identification and classification of phytoplasmas. The product amplified in nested PCR primed by R16F2n/R16R2 from the diseased larch samples was 1.2 kbp in size (data not shown). RFLP analysis of amplified 16S rDNA revealed patterns identical to those of group 16SrI, subgroup B phytoplasmas ('*Ca. P. asteris*') (figure 1). Amplification, RFLP and nucleotide sequence analysis of *rp* gene products confirmed identification of '*Ca. P. asteris*' presence in the larch samples (data not shown). The phytoplasma detected in larch was named larch dwarfed needle proliferation (LDNP) phytoplasma. Amplicons of 16S rDNA and ribosomal protein (*rp*) genes from strain LDNP were cloned, sequenced and deposited in the GenBank database under accession numbers JF747037 and JF767009, respectively.

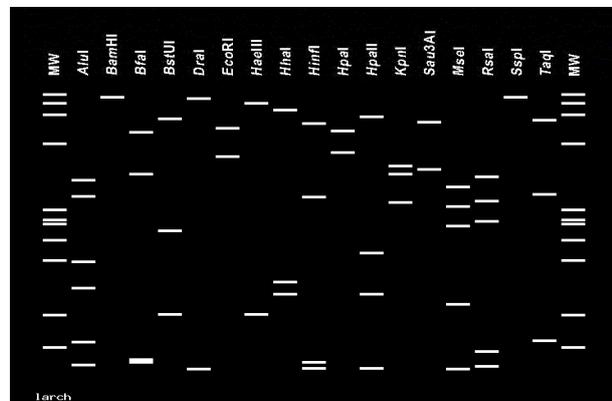


Figure 1. Virtual gel RFLP patterns from analyses of LDNP phytoplasma 16S rDNA R16F2n/R2 fragment.

Discussion

Phytoplasma infections in pine (family *Pinaceae*) have been detected in Spain, Germany, Poland, and Lithuania (Schneider *et al.*, 2005; Sliwa *et al.*, 2007; Valiunas *et al.*, 2010). Symptoms exhibited by the diseased pine trees were shoot-proliferation, dwarfed and proliferated needles, and stunting. The phytoplasma strains detected in pine belonged to group 16SrXXI, subgroup A ('*Candidatus Phytoplasma pini*'). Group 16SrIII phytoplasma was reported in cypress (Paltrinieri *et al.*, 1998). A juniper (*Juniperus occidentalis*) infecting phytoplasma in

Oregon was classified as a '*Candidatus Phytoplasma phoenicium*'-related strain and a member of group 16SrIX, subgroup E (Davis *et al.*, 2010). Our study revealed infection by a group 16SrI subgroup B phytoplasma in larch. In agreement with concepts expressed previously (Davis *et al.*, 2010), the present and other findings indicate that phytoplasma infections in gymnosperms may be more frequent than previously thought. It will be interesting to learn the identities of insect vectors transmitting the diverse phytoplasmas to gymnosperms and to learn the extent of phytoplasma infections in gymnosperms worldwide.

Acknowledgements

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Detection of phytoplasma infection in okra in Mauritius

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Abstract

Using PCR and RFLP techniques, 16SrXII and 16SrV phytoplasma groups were identified from okra (*Abelmoschus esculentus* Moench) leaf samples collected in Mauritius. The most frequent symptoms observed on okra plants were leaf yellowing, leaf distortion, curling and overall stunting. The phytoplasma infection was widely distributed across the island and a high incidence of 75 to 100% was recorded at inspected sites. This is the first report of the presence of phytoplasmas in okra in Mauritius.

Key words: Phytoplasmas, PCR/RFLP, okra, plant disease.

Introduction

Okra (*Abelmoschus esculentus* Moench) known also as lady's fingers or gumbo is a flowering plant in the Malvaceae family. It is valued for its edible green seed pods. Originating from Africa, the plant is cultivated in tropical, subtropical and warm temperate regions around the world. In Mauritius, okra is commonly known as lalo and its green pod is a popular vegetable. Major diseases commonly seen on okra are: powdery mildew and fruit rot caused by *Choanephora*. Annual production amounts to around 1,275 T over an area of 170 ha (Anonymous, 2010).



Figure 1. Top: okra plant showing stunting, leaf yellowing and distortions; bottom: close up of symptomatic leaves.

(In colour at www.bulletinofinsectology.org)

To date, there is no record of the occurrence of phytoplasma disease on okra in Mauritius. Phytoplasmas have only been recently identified in Mauritius on tomato (Dookun *et al.*, 1999; Gungoosingh-Bunwaree *et al.*, 2007), watercress and onion (Gungoosingh-Bunwaree *et al.*, 2010).

Since December 2010, okra growers across Mauritius reported a new disorder with unusual symptoms on the two local okra varieties. The most frequent symptoms comprised leaf yellowing, leaf distortions, upward leaf curling and overall stunting (figure 1).

A very high incidence of disorder ranging from 75 to 100% was encountered at the 36 okra planting sites visited across the country. A yield loss of around 40% was reported by okra growers. A high infestation of *Amrasca biguttula* (Hemiptera: Cicadellidae) was also reported at plantations inspected.

Materials and methods

Fresh symptomatic and asymptomatic okra leaf samples from the north and south of the island were employed for nucleic acid extraction to verify phytoplasma presence.

Total nucleic acids were extracted from 1 g of leaf midribs from symptomatic and asymptomatic okra leaves (Prince *et al.*, 1993), dissolved in Tris-EDTA pH 8 buffer, and maintained at 4°C. Forty ng/μl of nucleic acid were used for amplification. Samples employed as controls included DNA extracts from asymptomatic okra, STOL (stolbur from pepper from Serbia, 16SrXII-A), ULW (elm yellows from EU, 16SrV-A), GLAWC (gladiolus witches' broom from France, 16SrI-B) infected *Catharanthus roseus*. Tubes without DNA were used as no template controls. Direct PCR was carried out with primer pair P1/P7, followed by nested PCR with R16F2n/R2 (Gundersen and Lee, 1996) and with 16R_{758F}/16R_{1232R} (Gibb *et al.*, 1995).

After preliminary RFLP identification with *TruI* on amplicons obtained from the latter primers further nested PCR assays were carried out with primers

R16(V)F1/R1 and R16(I)F1/R1 (Lee *et al.*, 1995). PCR and nested PCR reactions were carried out following the protocol of Schaff *et al.* (1992).

Identification of detected phytoplasmas was done using RFLP analyses on 16R_{758F}/16R_{1232R} amplified ribosomal DNA fragments with *TruI*, *RsaI*, *HhaI*, *Tsp509I*, *TaqI*, *AluI* (Fermentas, MBI, Vilnius, Lithuania) restriction enzymes. Polyacrylamide 5% gels stained with ethidium bromide were employed to compare profiles to reference phytoplasmas (Bertaccini *et al.*, 2000).

Results

The 90% of symptomatic okra leaf sample tested positive to phytoplasma presence by using 16R_{758F}/16R_{1232R} primers in second nested PCR assays on R16F2/R2 amplicon size of about 500 bp. Asymptomatic negative controls and no template controls did not produce amplification. Preliminary RFLP analyses carried out with *TruI* restriction enzyme on these amplicons indicate that the profiles could be referable to phytoplasmas belonging to groups 16SrXII and 16SrV.

To further verify the phytoplasma identity, group specific primers were employed in nested PCR. Amplification with R16(I)F1/R1 primers allowed detection of phytoplasmas in all symptomatic samples tested and RFLP analyses with *TruI* confirmed that 16SrXII phytoplasmas are associated with the described symptoms. In some of the samples R16(V)F1/R1 primers provided amplification confirming the presence of 16SrV group phytoplasmas in mixed infection with 16SrXII phytoplasmas (stolbur).

Discussion

The above findings indicate that phytoplasmas are widening their host range in Mauritius. A phytoplasma of the 16SrV group was previously identified in tomato (Gungoosingh *et al.*, 2007), whilst a 16SrXII group phytoplasma was recently identified on onion and watercress in Mauritius (Gungoosingh *et al.*, 2010).

However it is the first time that these two phytoplasma groups are being identified in okra in Mauritius. Further studies need to be carried out in order to determine the correlation among yield loss, disease incidence, and role played by *Amrasca biguttula* in phytoplasma transmission across okra plantations and other crops in Mauritius.

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Association of a 16SrII group phytoplasma with dieback disease of papaya in India

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Abstract

During surveys, papaya plants showing dieback symptoms with yellowing and necrosis of leaves were collected from the Sugarcane Research Station campus, Gorakhpur, Eastern Uttar Pradesh, India in September 2010. DNA was extracted from infected and healthy plants and indexed in nested PCR with phytoplasma generic primers P1/P7 and R16F2n/R16R2. Nested PCR amplicons of 1.2 kbp were obtained from dieback symptomatic papaya samples. Following RFLP analysis, infected papaya samples exhibited identical *Hae*III and *Rsa*I profiles, which were typical of a 16SrII phytoplasma. None of the healthy papaya samples evaluated was found positive for the phytoplasmas. The amplification with phytoplasma primers and their RFLP profiling suggests that the dieback phytoplasma associated with papaya in India is a phytoplasma member of the 16SrII group ('*Candidatus* phytoplasma aurantifolia'). This is the first record of this phytoplasma associated with dieback disease of papaya in India.

Key words: Phytoplasma, papaya, 16SrII group, dieback disease, PCR and RFLP analysis.

Introduction

Carica papaya is one of the most common fruits grown in India and the area devoted to its cultivation has increased considerably in the last decade. *Carica papaya* (papaya) has numerous medicinal properties. A papaya paste was used traditionally for the relief of burns, cuts, rashes and stings. A compound known as papain is derived from the papaya fruit and has long been used as a natural antacid, for ulcer relief and to relieve constipation. The whole papaya fruit is an excellent source of dietary fiber, which is also necessary for digestive health.

Identification and molecular characterization of phytoplasmas in *C. papaya* has been reported in many countries. So far, phytoplasmas belonging to 6 ribosomal groups have been identified in *C. papaya* plants from all over the world (White *et al.*, 1998; Arocha *et al.*, 2007, 2009; Chaturvedi *et al.*, 2010; Alhudaib and Arocha, unpublished; Navarrete-Yabur *et al.*, unpublished).

Based on the sequences retrieved from GenBank, identified phytoplasmas of papaya mainly belong to the 16SrI, 16SrII, 16SrIII, 16SrX, 16SrXII and 16SrXVII groups. The peanut witches' broom group of phytoplasmas is the prevalent group of phytoplasmas identified in *C. papaya*. White *et al.* (1998) assigned the taxon '*Candidatus* Phytoplasma australasia' for papaya yellow crinkle and papaya mosaic disease on *C. papaya* plants in Australia. So far only, Kumar *et al.* (GenBank Ac. No. HM449951) have reported association of the '*Ca. P. asteris*' (16SrI group) with papaya dieback disease on *C. papaya* in India.

The molecular characterization of phytoplasmas causing yellowing and tip necrosis of leaves followed by dieback symptoms on *C. papaya* in India was carried out.

Materials and methods

During surveys on phytoplasma diseases symptomatic *C. papaya* plants showing dieback disease along with yellowing and necrosis of leaves were collected from the Sugarcane Research Station campus, Gorakhpur, Eastern Uttar Pradesh, India in 2009-2010. DNA from symptomatic *C. papaya* plants was extracted and amplification of phytoplasma ribosomal DNA (rDNA) was done with the universal phytoplasma primer pairs P1/P7 (Schneider *et al.*, 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996). Reactions were performed in a Minicycler with initial denaturation at 94°C for 2 min, followed by 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 52°C for 30 s and extension at 72°C for 30 s, with extension in the final cycle for 2 min. Total PCR volumes were 100 µl and contained 200 µM of each dNTP, 0.4 µM of each primer, 1 X DNA polymerase reaction buffer, 1 U *Taq* DNA polymerase (Boehringer) and 5 µl template DNA solution. Each reaction mixture was covered with 50 µl sterile mineral oil (Sigma). Five microlitres of each PCR product was subjected to electrophoresis in a 1.0% (w/v) agarose gel, stained with ethidium bromide and observed under UV illumination. Amplicons obtained with R16F2n/R16R2 primers were subjected to RFLP analyses with restriction enzymes *Hae*III and *Rsa*I to verify phytoplasma identity.

Results and discussion

The most peculiar symptoms observed on *C. papaya* plant includes yellowing, crinkling and leaf tip necrosis symptoms, drying of the upper leaves, which progresses to death of the entire plant (figure 1).



Figure 1. *Carica papaya* plant showing dieback symptom.
(In colour at www.bulletinofinsectology.org)

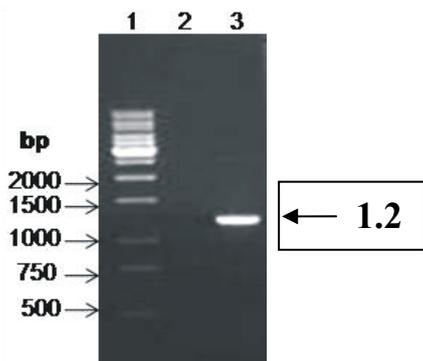


Figure 2. Lanes 1: 1kb ladder (MBI, Fermentas); lane 2: Symptomless papaya; lane 3: nested PCR amplicons from papaya infected leaf sample.

The 1.2 kb amplicon was obtained from nested PCR (figure 2). Neither by direct ('one-round') nor by nested PCR assays was DNA amplified from template DNA isolated from any of the healthy non-symptomatic samples. RFLP analysis with *Hae*III and *Rsa*I showed restriction profiles of phytoplasmas related to the 16SrII group. The amplification with phytoplasma nested primers and their RFLP profiling suggests that the die-back phytoplasma associated with papaya die-back disease in India is a member of the 16SrII group ('*Ca. P. aurantifolia*'). However, the only report available on association of phytoplasmas with *C. papaya* plants in

India concluded that it belonged to the 16SrI group (Ac. No. HM449951). Therefore, this is the first record of a 6SrII group phytoplasma association with dieback disease of papaya in India. Dieback disease of papaya associated with phytoplasmas has already been reported from Australia (Ac. No. Y18215), Cuba (Arocha *et al.*, 2005) and Ethiopia (Arocha *et al.*, 2006). However, association of 16SrII group phytoplasmas on papaya has only been recorded from Ethiopia (Arocha *et al.*, 2006) and Cuba (Ac. No. EU350564). This study confirms that the dieback disease of papaya is associated with two groups (16SrI and 16SrII) of phytoplasmas in India.

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Occurrence of a new stolbur strain in tobacco in Serbia

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Abstract

Stolbur phytoplasmas are associated with several important diseases on different crops worldwide. Although stolbur phytoplasmas are known to have low variability in 16S rDNA, some RFLP and single nucleotide polymorphisms among them were reported. To verify the presence and determine the identity of phytoplasmas present in tobacco in Serbia, PCR-RFLP and sequence analyses were performed on DNA extracts from 17 symptomatic plants. RFLP profiles of all positive samples except one were identical to those of 16SrXII-A subgroup, while strain 284/09 showed slightly different profile than others. The obtained results confirm presence and also show that there is variability in 16S rDNA among stolbur phytoplasmas in tobacco in Serbia. The revealed SNP was never reported in stolbur phytoplasmas before and is not present in any sequence deposited in the Genbank, which results in a unique RFLP profile. It is confirmed that the SNP is inside the 16S rDNA and is confirmed with PCR-RFLP analyses on three separate extractions, of which one was of the seedling after grafting with infected plant tissue. Relation of the SNP in the 16S rDNA with possible variations in other marker genes or some ecological properties of the strain are still to be defined.

Key words: stolbur, *Nicotiana tabacum*, PCR/RFLP.

Introduction

Stolbur phytoplasmas (16SrXII-A) are associated with several important diseases of both annual and perennial crops worldwide. In Serbia stolbur phytoplasmas were also reported in various plants and are associated with several economically important diseases, such as bois noir, corn reddening, stolbur on pepper (Martinović and Bjegović, 1950; Duduk *et al.*, 2004; Duduk and Bertaccini, 2006). Although stolbur phytoplasmas are known to have low variability in 16S rDNA, some RFLP and single nucleotide polymorphisms (SNPs) among them were reported (Quaglino *et al.*, 2009). However, non ribosomal DNA was also often tested for variability among stolbur phytoplasmas (Langer and Maixner, 2004; Pacifiko *et al.*, 2006).

Survey for phytoplasma presence, identification and possible variability in tobacco in Serbia was performed.

Materials and methods

To verify the presence and determine the identity of phytoplasmas present in tobacco, molecular assays were performed on DNA extracts from 17 symptomatic plants collected during 2009 in Ečka, Serbia. Total DNA extraction was performed using CTAB protocol described by Angelini *et al.* (2001). Polymerase chain reaction (PCR) was performed, for amplification of phytoplasma 16S rRNA gene, spacer region and part of 23S rRNA gene, using phytoplasma-universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995). The reaction conditions were as reported in literature (Lee *et al.*, 1995). Products amplified by PCR assays were visualised and the positive ones were subjected to the restriction fragment length polymorphism (RFLP) analysis. *TruI* (Fermentas, Vilnius, Lithuania) restriction endonuclease was used, according to the manufacturer's instructions. For a selected strain (284/09) nu-

cleic acids extraction and PCR-RFLP analyses was repeated on the same plant and on a seedling plant after stem tissue grafting and symptoms appearance. The P1/P7-amplified products of two selected samples, were purified using Metabion mi-PCR purification kit (Metabion International AG, Martinsried, Germany) and sequenced in both directions with two forward primers P1 and R16F2 (Lee *et al.*, 1995) and one reverse primer P7, using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The sequences were assembled using Pregap4 from the Staden program package (Staden *et al.*, 2000), aligned using Clustal X (Thompson *et al.*, 1997) and searched for SNPs in Bioedit program (Hall, 1999). The obtained sequences were compared with 16Sr sequences of phytoplasmas in the GenBank database using blast (v. Blast N 2.2.18) at the National Center for Biotechnology Information.

Results

RFLP profiles of all positive samples except one, were identical to those of 16SrXII-A subgroup (Lee *et al.*, 1998), while strain 284/09 showed slightly different profile than others (figure 1a), suggesting the presence of a new restriction site. To verify this finding new extraction was carried out and the results were confirmed. In order to maintain the strain and to verify persistence of this polymorphism, grafting of the plant material was performed on a tobacco seedling. One month after grafting and symptoms expression, nucleic acids were extracted and the presence of the same strain in grafted plant was confirmed (figure 1b).

Alignment of the sequences obtained from two samples 142/09 and 284/09 (1,704 and 1,703 bp respectively) showed SNP only on one position [184 (A/G)] which is a recognition site for the *TruI* restriction enzyme and is inside of the 16SrRNA gene.

Blast search of 142/09 and 284/09 phytoplasma 16S

rDNA sequences showed 100% and 99% respectively, homology with a stolbur phytoplasma strain from potato from Russia (EU344887). It was also observed that on the position 184, nt A was present only in 284/09 strain, while 142/09 and all other sequences of stolbur deposited in the GenBank had nt G in that position.

Tobacco seedling grafted with 284/09, together with the sequenced field infected sample (142/09) was transferred to *in vitro* in MS medium and deposited to Phytoplasma collection at the Plant Pathology, DiSTA - *Alma Mater Studiorum* - University of Bologna, Italy (Bertaccini, 2010).

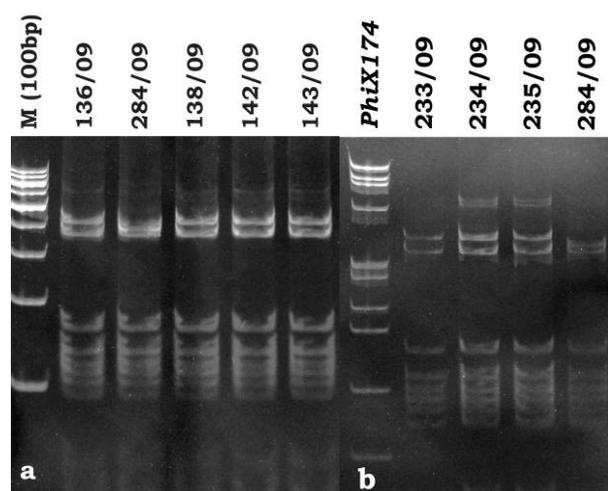


Figure 1. Differential *TruI* RFLP profiles obtained from P1/P7 amplicons of phytoplasmas from tobacco.

Discussion

The obtained results confirm presence of stolbur phytoplasma in tobacco plants in Serbia. It is also shown that there is variability in 16S rDNA among stolbur phytoplasmas in tobacco in Serbia. While strain 142/09 has regular stolbur RFLP profile with 100% homology of 16S rDNA sequence with a stolbur strain from the Genbank, the strain 284/09 represents a variant of stolbur phytoplasma with a SNP on *TruI* restriction site. The SNP on the position 184 was never reported in stolbur phytoplasmas before and is not present in any sequence deposited in the Genbank, which results in a unique RFLP profile. It is confirmed that the SNP is inside the 16S rDNA (also inside the 16RF2/R2 amplified region) and is confirmed with PCR-RFLP analyses on three separate extractions, of which one was of the seedling after grafting with infected plant tissue.

Relation of the SNP in the 16S rDNA with possible variations in other marker genes or some ecological properties of the strain are still to be defined.

Acknowledgements

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The first report of a phytoplasma associated with pot marigold phyllody in Iran

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Abstract

Pot marigold phyllody was observed in a Botanical Garden in Yazd province of Iran. The disease agent was transmitted from pot marigold to pot marigold and periwinkle via dodder inoculation. Polymerase chain reaction using phytoplasma-specific primer pair P1/P7 or nested PCR using primer pair P1/P7 followed by R16F2n/R16R2, amplified products of expected size (1.8 and 1.2 kbp, respectively) from symptomatic pot marigold in the field and from symptomatic dodder inoculated pot marigold and periwinkle plants. Restriction fragment length polymorphism analysis of R16F2n/R16R2 primed nested PCR products indicated association of a peanut witches' broom related phytoplasma (16SrII) in naturally and experimentally infected pot marigold plants. This is the first report of pot marigold phyllody in Iran.

Key words: Pot marigold phyllody, 16SrII group, Yazd, Iran.

Introduction

Pot marigold (*Calendula officinalis* L.) is a plant in the genus *Calendula* in the family Asteraceae native to southern Europe and cultivated in temperate regions around the world. It is a herbaceous ornamental plant with many medicinal, culinary and cosmetic uses.

Phytoplasmas belonging to aster yellows (16SrI) group were identified in diseased pot marigold from Italy (Marcone *et al.*, 1997) and Canada (Wang and Hiruki, 2001). During a survey in 2006 in Yazd Botanical Garden, 30 km North west of Yazd (Yazd province, Iran), pot marigold phyllody (PMP) was observed (Esmailzadeh-Hosseini *et al.*, 2008). In the present study the identification of phytoplasmas associated with PMP was carried out.

Materials and methods

A pot marigold plant with typical phyllody symptoms was selected in Yazd Botanical Garden, transferred to greenhouse and used as the source of the disease agent for dodder (*Cuscuta campestris* Yunck.) transmission and molecular studies. Infection of dodder-inoculated plants was verified by PCR. Total DNA was extracted from midrib tissue of 10 naturally phyllody affected pot marigold, dodder inoculated and healthy plants using Zhang *et al.* (1998) procedure. DNA samples were tested for presence of phytoplasma by direct PCR using P1/P7 (Schneider *et al.*, 1995) and nested PCR using P1/P7 and R16F2n/R16R2 (Gundersen and Lee, 1996) primer pairs. For identification of phytoplasma associated with pot marigold phyllody, R16F2n/R16R2 primed nested PCR products from naturally infected marigold and experimentally infected marigold and periwinkle plants were digested with *AluI*, *HinfI*, *MseI*, and *RsaI* restriction enzymes and digestion profiles were compared with those

of known phytoplasmas. Furthermore, ELISA test using polyclonal antibody against Yazd alfalfa witches' broom phytoplasmas (Esmailzadeh-Hosseini *et al.*, 2003) was also used for identification of PMP agent.

Results and Discussion

Characteristic symptoms of the PMP disease were leaf size reduction, yellowing, phyllody, virescence, proliferation and sterility in the flower, proliferation of axillary buds along the stem, witches' broom and stunting (figure 1).

Up to 12% of the pot marigold plants were found infected in the field. Under greenhouse conditions, the agent of PMP was transmitted from naturally infected pot marigold to pot marigold and periwinkle via dodder inoculation.



Figure 1. Pot marigold phyllody in Yazd Botanical Garden of Yazd province.
(In colour at www.bulletinofinsectology.org)



Figure 2. Virescence, phyllody and witches' broom in a pot marigold plant dodder inoculated with PMP agent (left) compared with a healthy plant (right). (In colour at www.bulletinofinsectology.org)

Four of 5 pot marigold and all 5 periwinkle plants parasitized by dodder from infected pot marigold developed disease symptoms. The duration of the latent period in dodder-inoculated plants ranged from 6 to 11 weeks. In dodder inoculated marigold (figure 2), disease symptoms were similar to those of naturally infected pot marigold plants. The major symptoms shown by experimentally infected periwinkle plants were small leaves, virescence, phyllody, yellowing and stunting. DNA fragments of approximately 1,800 and 1,200 bp were amplified by direct and universal primer pairs P1/P7 and R16F2n/R16R2, respectively by direct and nested PCR from total nucleic acid samples extracted from 10 naturally phyllody-affected pot marigold and all symptomatic experimentally inoculated plants. No amplification was observed in DNA samples from symptomless plants and water control. R16F2n/R16R2 primed nested PCR products (1.2 kbp) were analyzed by digestion with *AluI*, *HinfI*, *MseI* and *RsaI* enzymes. Collectively, RFLP patterns analyzed with these enzymes were similar to those of peanut witches' broom, 16SrII group phytoplasmas (Lee *et al.*, 1998). ELISA test using polyclonal antibody prepared against Yazd alfalfa witches' broom (YAWB) phytoplasma showed that PMP phytoplasma is serologically related to YAWB agent, a 16SrII group related phytoplasma.

On the basis of disease symptoms, dodder transmission and positive reaction in PCR and ELISA tests, PMP in Yazd has phytoplasmal etiology. This is the first report of pot marigold phyllody disease in Iran. On the basis of RFLP analysis, this phytoplasma is related to

'*Candidatus* Phytoplasma aurantifolia', 16SrII group phytoplasma. In other countries, an aster yellows-related phytoplasma (16SrI) was associated with the same disease (Marcone *et al.*, 1997; Wang and Hiruki, 2001) but no reports were found of pot marigold as a host for the 16SrII phytoplasma group. Alfalfa witches' broom is a peanut witches' broom related phytoplasma (16SrII) that is prevalent in Yazd province (Salehi *et al.*, 1995). It is yet to be determined whether the agents of YAWB and PMP are the same phytoplasma.

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Detection of stolbur phytoplasma in willow in Spain

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Abstract

Preliminary results of nested-PCR indicated that phytoplasmas were detected in willow (*Salix babylonica* Linn) showing yellows, ball-like structures and small leaves symptoms collected in Valencia Province (Eastern Spain). RFLP analyses showed that the phytoplasmas belonged to the stolbur group (16SrXII).

Key words: willow, witches' broom, phytoplasma, nested-PCR, RFLP, stolbur.

Introduction

Willow (*Salix babylonica* Linn) is a traditional tree normally grown in urban areas in Valencia Province (Eastern Spain). For several years, symptoms characteristic of diseases potentially associated with phytoplasmas presence have been observed in willows in Valencia. Affected trees showed yellows, thin leaves and ball-like structures (figure 1).

The main objective of the present work was to verify phytoplasma presence in some affected willow tree showing abnormal symptoms and to identify the phytoplasma group present in those samples. The results presented here are preliminary and the work is in progress.

Materials and methods

Samples from different willow trees with phytoplasma-like symptoms were collected in 2003, 2010 and 2011.

Healthy samples of willow, positive samples for stolbur and for '*Ca. P. asteris*' were also included in the assay as negative and positive controls, respectively. Total DNA was extracted as described Green *et al.* (1999).

A nested-PCR was performed using the universal phytoplasma primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) in the first amplification followed by R16F2n/R16R2 (Gundersen and Lee, 1996) in the second amplification to detect phytoplasmas in the affected trees.

The PCR products were analysed in 1.2% agarose in TAE buffer gels, stained with ethidium bromide and visualized with a UV transilluminator.

Restriction fragment length polymorphism (RFLP) analyses of the nested-PCR products (1.2 kb 16S rDNA fragments) were used for identification of the putative phytoplasma detected (Lee *et al.*, 1998) with *Hha*I,

*Mse*I, *Rsa*I and *Taq*I endonucleases (Fermentas, Vilnius, Lithuania) in 5% polyacrylamide gels.

Results and discussion

Fragments of the expected size (1.2 kb) were only amplified from symptomatic samples and positive controls. No amplification was produced from healthy samples or water controls. The RFLP profiles when compared with control phytoplasma profiles (figure 2) and with profiles of other phytoplasma 16S rRNA groups described by Lee *et al.* (1998) indicated that the phytoplasmas present in willow trees belong to the stolbur group, 16SrXII.

During 2008, aster yellows group 16SrI (subgroup 16SrI-C) phytoplasma was associated with a yellows-type disease of willows in China (Wei *et al.*, 2009); to our knowledge, this work represents the first report of phytoplasmas in willow trees in Spain.



Figure 1. Willow branch showing symptoms of ball-like structures.

(In colour at www.bulletinofinsectology.org)

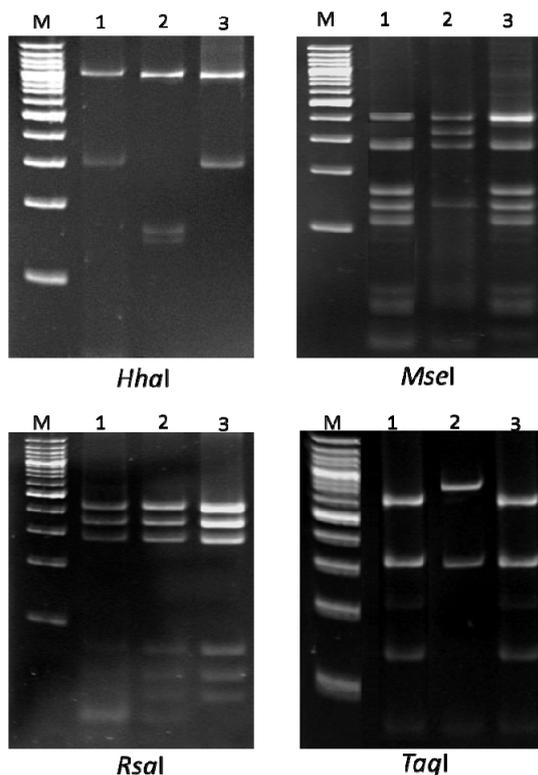


Figure 2. Polyacrylamide 5% gel of the RFLP analyses of 16S rDNAs (nested-PCR products amplified with primers R16F2n/R16R2) using endonucleases *HhaI*, *MseI*, *RsaI* and *TaqI* of willow sample (lane 1), positive control aster yellows phytoplasma (lane 2) and positive control stolbur phytoplasma. Lane M, 100 bp DNA marker (Fermentas, Vilnius, Lithuania).

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Association of 'Candidatus Phytoplasma phoenicium' with GF-677 witches' broom in Iran

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Abstract

Symptoms of GF-677 witches' broom (GFWB) disease were observed in Beedzard and Estahban areas in Fars province of Iran. Polymerase chain reaction (PCR) using phytoplasma-specific primer pair P1/P7 or nested PCR using P1/P7 followed by R16F2n/R16R2 amplified products of expected size (1.8 and 1.2 kbp, respectively) from symptomatic GF-677 trees. Restriction fragment length polymorphism (RFLP) analysis and sequence homology of 16S rDNA indicated that GFWB related to almond witches' broom phytoplasma. This is the first report of the natural occurrence of GFWB and the molecular characterization of the GFWB phytoplasma.

Key words: GF-677 witches' broom, 'Candidatus Phytoplasma phoenicium', Iran.

Introduction

GF-677 (*Prunus amygdalus* x *Prunus persica*) with strong roots and a good potential for pests and diseases (Fasolo *et al.*, 1987) is one of the most suitable rootstocks for almond and peach used in calcareous soils to overcome lime-induced chlorosis (Kester, 1970; Fasolo *et al.*, 1987; Hartmann *et al.*, 1990). GF-677 cuttings were imported from France to Iran and planted in Beedzard, Estahban and Neyreez areas (Fars province) for further propagation and distribution to other Iranian stone fruit growing areas. During a survey conducted in 2009, witches' broom disease of GF-677 trees was observed in the Beedzard and Estahban regions. Because witches' broom is a typical symptom for phytoplasma infection, polymerase chain reaction (PCR) using phytoplasma specific primers was performed to detect phytoplasmas in symptomatic GF-677 trees. This paper describes the occurrence of witches' broom disease in GF-677 trees and the molecular detection and identification of the associated phytoplasma.

Materials and a methods

Leaf samples from 20 witches' broom affected and 3 symptomless GF-677 trees from Beedzard and Estahban areas were collected. Total DNA was extracted from fresh midrib tissue using the small-scale procedure of Zhang *et al.* (1998) as modified by Abou-Jawdah *et al.* (2002). DNA samples were tested for phytoplasma infection by direct PCR using the universal phytoplasma primer pair P1/P7 (Schneider *et al.*, 1995) which amplify a 1800 bp fragment of the 16S rRNA ribosomal operon the 16S-23S intergenic spacer region (SR) and a portion of the 5' region of the 23S rRNA gene. To increase the sensitivity of assays, a nested PCR was performed using a 1: 40 dilution of P1/P7-primed PCR products as templates and primer pair R16F2n/R16R2 (Gundersen and

Lee, 1996), which yielded a 1,200-bp fragment of the 16S rRNA gene. Eight microlitres (approximately 200 ng) of nested PCR products (1.2 kbp) from Beedzard and Estahban strains were individually digested with the restriction enzymes *AluI*, *HhaI*, *HinfI*, *HpaII*, *MseI*, *RsaI*, *Sau3AI*, *TaqI* and *ThaI* (Roche), following the manufacturer's instructions. RFLP profiles were analyzed by electrophoresis of digested DNA in a 2% agarose gel, staining with ethidium bromide and visualization with a UV transilluminator. The profiles were compared with previously published data (Lee *et al.*, 1998; Abou-Jawdah *et al.*, 2002). Ribosomal DNA products amplified by R16F2n/R16R2 primer pair were ligated onto the pTZ57R/T vector and cloned into *Escherichia coli* DH5 α cells using InsT/Aclone PCR Product Cloning Kit (Fermentas, Vilnius, Lithuania) according to manufacturer instructions and sequencing was performed by SEQ LAB (Germany). Using sequenced fragments BLAST search was performed to determine the closest phytoplasma relatives of Beedzard and Estahban strains.

Results and Xiscussion

Characteristic symptoms of the disease were internode shortening, chlorosis, reduced size of leaves especially in the broom, proliferation of slender upright shoots, witches' broom, stunting and dieback (figures 1 and 2). Up to 10% of the trees were found infected in Estahban area. With universal primer pair P1/P7 the target DNA fragments of approximately 1,800 bp were amplified by direct PCR from total nucleic acid samples extracted from 14 out of 20 witches' broom-affected GF-677 trees but not from the 3 symptomless samples, collected as negative controls. Nested PCR with primer pair P1/P7 followed by primer pair R16F2n/R16R2 yielded fragments of approximately 1.2 kbp from all witches' broom affected GF-677 trees tested but not from healthy control plants.



Figure 1. Internode shortening, chlorosis, reduced leaf size and stunting in a GF-677 tree from Neyreez, infected with a ‘*Ca. P. phoenicium*’ related phytoplasma (middle), compared with healthy trees (right and left). (In colour at www.bulletinofinsectology.org)



Figure 2. Proliferation of slender upright shoots and witches’ broom in a GF-677 tree from Neyreez, infected with a ‘*Ca. P. phoenicium*’ related phytoplasma. (In colour at www.bulletinofinsectology.org)

After enzymatic digestion, Beedzard and Estahban strains showed identical profiles, undistinguishable from those of the almond witches’ broom phytoplasma (AWBP) (Abou-Jawdah *et al.*, 2002) ‘*Ca. P. phoenicium*’. Blast search showed that the 16S rRNA gene sequences of Beedzard (GenBank accession No.

JF781493) and Estahban (GenBank accession No. F781494) isolates shared a 99% of identity with the 16S rRNA gene sequence of AWBP.

Based on disease symptoms and PCR amplification of the 16S rRNA gene with universal phytoplasma primers, GF- 677 witches’ broom (GFWB) is associated with phytoplasma presence. RFLP analysis and sequence homology of 16S rDNA indicated that GFWB phytoplasma is molecularly indistinguishable from AWBP. Almond witches’ broom is an important disease in Fars province of Iran (Salehi *et al.*, 2006). Infected almond trees may be the source of GFWB phytoplasma. However, infection of GF-677 trees with AWBP suggests involvement of a vector. This is the first report of ‘*Ca. P. phoenicium*’ associated with witches’ broom disease of GF-677 trees in Iran.

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Screening for phytoplasma presence in leafhoppers and planthoppers collected in Bulgarian vineyards

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Abstract

In the beginning of 2003, the Bulgarian Food Safety Agency started the monitoring program for quarantine pests on grapevine. The object of surveys was to verify the possible presence of 'flavescence dorée' phytoplasmas. At the same period entomological surveys were carried out with yellow sticky traps in the vineyards surveyed. Identification of *Scaphoideus titanus* Ball. was done in the summer of 2006 in Varna and Veliko Turnovo regions. For the control of natural spreading of phytoplasmas in Bulgarian vineyards individual testing by PCR of the collected planthoppers and leafhoppers in order to define phytoplasma presence and increase knowledge on the grapevine phytoplasma vectors in Bulgaria was carried out.

Key words: *Scaphoideus titanus* Ball., nested PCR, grapevine phytoplasmas, insect vectors.

Introduction

'Flavescence dorée' (FD) and 'bois noir' (BN), are serious diseases of grapevine (*Vitis vinifera* L.) in temperate European areas. FD, quarantine organisms in Bulgaria and in EU, is associated with phytoplasmas that belongs to the elm yellows (EY) group (16SrV group) (Bertaccini *et al.*, 1995). This phytoplasma is naturally transmitted by the leafhopper *Scaphoideus titanus* Ball (Hemiptera, Cicadomorpha, Cicadellidae). BN diseases is associated with stolbur (STOL, 16SrXII) phytoplasmas, it is the second economically important yellows of grapevine in Europe and is transmitted by the planthopper *Hyalestes obsoletus* Signoret. The disease was reported in Bulgaria since 2006 (EPPO, 2006; Sakalieva *et al.*, 2007; Avramov *et al.*, 2008).

In the beginning of 2003, the National Plant Protection Service in Bulgaria (now Bulgarian Food Safety Agency) started the monitoring program for quarantine pests on grapevine. The object of surveys was to verify the presence of grapevine yellows diseases, especially FD. The samples were checked for phytoplasmas in the Central Laboratory for Plant Quarantine. At the same period entomological observations were done with yellow sticky traps. For the control of natural phytoplasma spreading in vineyards individual testing of the collected planthoppers and leafhoppers in order to define phytoplasma infection and increase knowledge on the grapevine phytoplasma vectors.

Materials and methods

Insect collection. Since 2008 yellow sticky traps sampling have been conducted from July until September in vineyards and 230 specimens in total between adult insects and nymphs were captured and examined. The leafhoppers and planthoppers were collected in all regions of Bulgaria, where vineyards are present except *Scaphoideus titanus* Ball witch is spread only in a North part of Bulgaria (table 1).

DNA extraction. Total nucleic acids were extracted from single leafhoppers by the method of Doyle and Doyle (1990) as adapted by Marzachi *et al.* (1998). Each individual was ground in a 1.5 ml Eppendorf tube along with 500 µl of pre-heated (60°C) CTAB buffer [2% w/v cetyl-trimethyl-ammonium-bromide, 1.4 M NaCl, 20mM EDTA pH 8.0, 100 mM Tris-Cl, pH 8.0, 02.% v/v β-mercaptoethanol]. After incubation for 30 min at 60°C, nucleic acid was extracted with 1 vol of 24:1 chloroform:isoamyl alcohol, precipitated with 1 vol of cold isopropyl alcohol and freed from salts by 70% (v/v) ethanol washings. Final products are suspended in 200 µl of sterile double distilled water or TA buffer [10 mM Tris and 1 mM EDTA, pH 8].

PCR assays. DNA extracted from insect specimens was tested by nested PCR (Lee *et al.*, 1993), using two pairs of generic primers (P1/P7, R16F2/R16R2) that amplify phytoplasma 16S rDNA and followed by RFLP analyses for phytoplasma identification using restriction enzymes *RsaI* (*AfaI*) and *AluI* (Amersham Biosciences, USA). PCR and RFLP products were analysed by electrophoresis on 1.5% agarose gel followed by staining with ethidium bromide and visualization of DNA bands with a UV transilluminator.

Results and Discussion

After entomological identification the specimens were distributed as follows: *S. titanus* - 48 units, *H. obsoletus* - 57 units, *Reptalus* spp. - 52 units, *Empoasca vitis* Goethe - 23 units, *Neotalitrus fenestratus* Her. Schaffer - 16 units, *Zigina rhamni* F. - 12 units, *Eupteryx* spp. - 11 units and *Philaenus* spp. - 11 units.

H. obsoletus known as vector of stolbur phytoplasma in Bulgaria and BN disease of grapevine was identified in the regions of Bourgas, Varna, Vratsa, Rousse, Veliko Tarnovo, Stara Zagora and Pleven. For the first two years of the monitoring programme *S. titanus* was not detected; it was first identified in the summer of 2006 in Veliko Turnovo region.

Table 1. Results of molecular tests on collected leafhopper and planthopper with yellow sticky traps in vineyards in Bulgaria*.

Species	Regions (number of captured specimens)	PCR tests carried out	Positive results
<i>S. titanus</i>	Vratsa (14), Montana (9), Russe (6), Veliko Turnovo (6), Vidin (6), Pleven (4), Lovech (3)	48	none
<i>H. obsoletus</i>	Veliko Turnovo (10), Varna (12), St Zagora (6), Russe (6), Lovech (4), Vratsa (3), Vidin (4), Plovdiv (5), Pleven (4), Bourgas (3)	57	12 (In all 10 regions)
<i>Reptalus</i> spp.	St. Zagora (10), Plovdiv (10), Pleven (8), Veliko Turnovo (7), Lovech (3), Vratsa (3), Russe (2), Varna (2), Bourgas (2), Vidin (2), Blagoevgrad (2), Haskovo (1)	52	10 (In 9 out of 12 regions)
<i>Empoasca</i> spp.	Plovdiv (8), Pleven (5), Vratsa (4), Bourgas (3), St. Zagora (2), Russe (1)	23	none
<i>N. fenestratus</i>	Lovech (3), Vratsa (3), St Zagora (2), Vidin (2), Varna (2), Plovdiv (1), Veliko Turnovo (1), Bourgas (1), Blagoevgrad (1)	16	none
<i>Zigina</i> spp.	Plovdiv (4), Bourgas (3), Vratsa (2), St Zagora (2), Varna (1)	12	none
<i>Eupteryx</i> spp.	Pleven (3), Veliko Turnovo (3), Bourgas (2), Plovdiv (1), Vidin (1), Haskovo (1)	11	none
<i>Philaenus</i> spp.	Plovdiv (4), Pleven (3), Varna (3), Bourgas (1)	11	none

* Not all identified insects were tested: approximately equal numbers from different regions were selected.

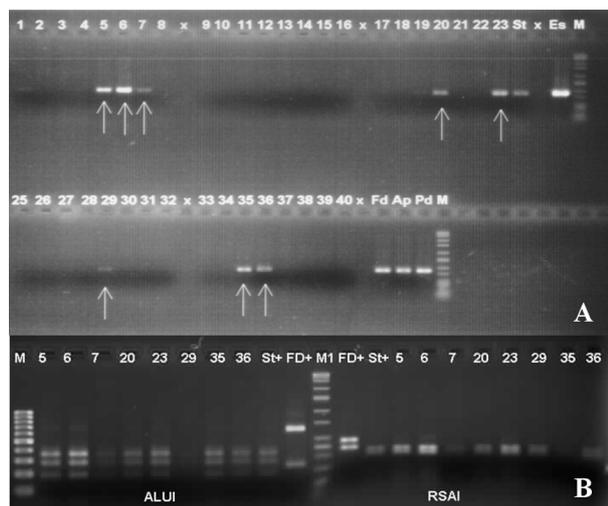


Figure 1. Results of nested PCR. A) P1/P7 followed by R16F2n/R16R2. Line 1 - 40 insect samples, St, Fd, Es, Ap, Pd – positive controls for stolbur, FD, ESFY, PD. M – wide range marker, Sigma, 10 kbp, lines 5, 6, 7, 20, 23, 29, 35, 36 are samples with positive results for phytoplasma presence. B) RFLP results in agarose gel.

After the performing nested PCR analysis the phytoplasma presence was detected in 22 insects. *S. titanus* was never found to carry phytoplasmas. All positive samples produced showed 16SrXII profiles (figure 1) suggesting the presence of stolbur phytoplasmas associated with BN presence in vineyards. Infection of BN phytoplasma presence was confirmed only in the cixiids *H. obsoletus* - 12 and *Reptalus* species – 10 positive results. Phytoplasma infection was not detected into the others species (table 1). Based on the results of RFLP analyses, tested phytoplasma isolates were classified as ‘bois noir’.

No FD infections were found in vineyards in Bulgaria

inspite of the presence of the FD vector *S. titanus*. There is good evidence that *H. obsoletus* and *Reptalus* spp. are the insect vector of grapevine phytoplasma infections detected in Bulgaria so far.

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Evaluation of colour traps to monitor insect vectors of sugarcane white leaf phytoplasma

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Abstract

The present study was conducted to evaluate the attractiveness of various colour sticky traps and light traps at sugarcane field in Phandon village, Kumpawapi district, Udon Thani Province, Northeast region of Thailand for the insect vectors of sugarcane white leaf phytoplasma, the leafhoppers *Matsumuratettix hiroglyphicus* (Matsumura) and *Yamatotettix flavovittatus* Matsumura. In sticky traps experiment, yellow, blue, white, green, orange and colorless (control) were used. A higher number of the putative vectors, *Matsumuratettix hiroglyphicus* and *Yamatotettix flavovittatus* were trapped on blue and yellow as compared to white, orange, green and colorless (control) sticky traps. In light traps experiment, yellow, green, black light-blue and black color light sources were used. With regard to treatment colour light traps with black light-blue trapped significantly higher numbers of both leafhoppers followed by black, green and yellow traps. The light traps were found significantly more attractive to both species of insect vectors compared to sticky traps, as lower number of insect vectors were trapped on sticky traps. In conclusion, a trap with black light-blue colour was the best attractive equipment for monitoring the of insect vectors of sugarcane white leaf phytoplasma in sugarcane fields.

Key words: *Matsumuratettix hiroglyphicus*, *Yamatotettix flavovittatus*, color sticky traps, light traps.

Introduction

Sugarcane is an important economic crop grown in Thailand. Sugarcane white leaf (SCWL) is the most destructive disease of sugarcane in this country and it is caused by plant pathogenic phytoplasma, spread through sugarcane stocks and transmitted plant to plant by leafhoppers, *Matsumuratettix hiroglyphicus* (Matsumura) and *Yamatotettix flavovittatus* Matsumura. Their transmission rates were found 55 and 45 percent respectively (Hanboonsong *et al.*, 2006). Thus, to monitor the population level of phytoplasma insect vectors *Matsumuratettix hiroglyphicus* and *Yamatotettix flavovittatus* in sugarcane fields is important for management of the disease.

Trapping methods are principal tools in insect pest management programs. The ability to attract specific arthropod species is depending on the different trapping equipments. The different colour sticky traps are used to monitor leafhopper species on many crops (Kersting *et al.*, 1997; Chu *et al.*, 2000; Lessio and Alma, 2004; Raja and Arivudainambi, 2004).

The yellow colour traps were significantly more attractive for vector of sesame phyllody phytoplasma, *Orosius orientalis* (Kersting *et al.*, 1997) and potato leafhopper, *Empoasca fabae* (de Gooyer *et al.*, 1998). Ramamurthy *et al.* (2010) studied the different numbers of insects species caught by light traps with different light sources.

The objective of this study was to evaluate the most suitable trapping method for monitoring populations of *Matsumuratettix hiroglyphicus* and *Yamatotettix flavovittatus* in sugarcane fields.

Materials and methods

This experiment was conducted at farmers' field in Phandon village, Kumpawapi district, Udon Thani Province, Northeast region of Thailand during May to October 2010. It was conducted to evaluate the attractiveness of *Matsumuratettix hiroglyphicus* and *Yamatotettix flavovittatus* to (30 cm x 20 cm) sticky card of yellow, blue, white, green, orange and colourless (control) traps. The experiment was carried out using Randomized Complete Block Design with six replications. The colour sticky cards were placed in the field above the plant canopy and adjusted to canopy height later in the season. The traps were collected and replaced every two weeks.

Light traps with different colour light sources; yellow, green, black light-blue (BLB) and black light with glue on transparent plastic sheets (50 cm x 50 cm) and two replications were used in this experiment. It was carried out at the experimental field of sticky color traps. The traps were placed inside the field of 1 m distance from the border row. All light traps were adjusted to a height of 1 m and checked every two weeks at dark hours of 18:00 – 21:00 p.m..

All different colour sticky traps and light traps that caught insects were kept in plastic boxes and transferred to the laboratory for identification of leafhoppers species and counting of individuals at each evaluation date. Data were subjected to analysis of variance (ANOVA) and the treatment means were separated by least significant difference (LSD) at 5% probability level.

Table 1. Mean numbers of *M. hiroglyphicus* and *Y. flavovittatus* caught on sticky traps of different colours in sugarcane field from June to October 2010.

Sticky trap colours	Mean numbers of	
	<i>M. hiroglyphicus</i> (\pm SE)/trap/day	<i>Y. flavovittatus</i> (\pm SE)/trap/day
Blue	3.08 \pm 0.31a	10.73 \pm 0.92a
Yellow	2.50 \pm 0.16b	7.88 \pm 1.02b
Orange	0.83 \pm 0.16c	5.83 \pm 1.08c
White	0.75 \pm 0.13c	4.42 \pm 0.48cd
Green	0.61 \pm 0.08cd	4.08 \pm 0.45d
Colorless	0.19 \pm 0.07d	2.19 \pm 0.45e

*Means within a column not followed by the same letter are significantly different ($p < 0.05$) by LSD.

Table 2. Mean numbers of *M. hiroglyphicus* and *Y. flavovittatus* caught on light traps with different colours light sources in sugarcane field from June to October 2010.

Light trap with colour light source	Mean numbers of	
	<i>M. hiroglyphicus</i> (\pm SE)/trap/day	<i>Y. flavovittatus</i> (\pm SE)/trap/day
Black light-blue	203.38 \pm 37.88a	449.65 \pm 30.07a
Black	127.75 \pm 30.50b	174.75 \pm 30.03b
Green	54.75 \pm 12.75bc	119.79 \pm 10.36b
Yellow	10.88 \pm 1.13c	22.00 \pm 5.72c

*Means within a column not followed by the same letter are significantly different ($p < 0.05$) by LSD.

Results

The results of the study indicated that blue colour sticky traps were the most attractive followed by yellow for the leafhopper vectors of sugarcane white leaf phytoplasma, *M. hiroglyphicus* and *Y. flavovittatus*. The orange, white, green and colourless (control) sticky traps were less attractive than yellow (table 1).

On the other hand, mean number of insect vectors captured on light traps with different colour light sources exhibited a significant difference at all treatments. The results showed that the highest population of *M. hiroglyphicus* and *Y. flavovittatus* was trapped on light traps with black light-blue followed by black, green and yellow light, respectively (table 2). This result clearly indicated that black light-blue light trap was the most attractive for the main insect vectors of SCWL phytoplasma.

Discussion

In the previous study, the yellow and orange color sticky traps were significantly attractive for leafhopper, *Empoasca decipiens* in cotton (Demirel and Yildirim, 2008). The red colour sticky traps caught more *Scaphoideus titanus*, grapevine ‘flavescence dorée’ phyto-

plasma vector than white, yellow or blue (Lessio and Alma, 2004). Our results showed that significantly higher numbers of *M. hiroglyphicus* and *Y. flavovittatus* were generally trapped on colour light traps. Our results indicate that the active movement behaviour of SCWL insect vectors could be evening or when it is dark and it showed insect vectors are attracted by light source. We suggest that light trap with black light-blue light source is the best for monitoring the populations of the insect vector species, *M. hiroglyphicus* and *Y. flavovittatus* in sugarcane fields.

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Presence of phytoplasmas in hemipterans in Czech vineyards

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Abstract

A survey to monitor the presence of stolbur phytoplasma in its putative vectors was conducted in infected vineyards of South Moravia. Collected hemipterans were analysed using polymerase chain reaction and detected phytoplasmas were identified after restriction fragment length polymorphism of 16S rRNA gene. In the majority of the samples tested, phytoplasmas of the aster yellows group were detected.

Key words: phytoplasma, vectors, hemipterans, nested PCR, RFLP.

Introduction

The preliminary survey of hemipterans as putative vectors of phytoplasmas was conducted in 2008-2009 in the South Moravian region of the Czech Republic, in vineyards infected with Potato stolbur phytoplasma.

The most important vector of stolbur phytoplasma is the planthopper *Hyalesthes obsoletus* Signoret (Sforza *et al.*, 1998). Other reported stolbur phytoplasma vectors occurring in the Czech Republic are *Reptalus panzeri* (P. Low), *Euscelis incisus* (Kirschbaum), *Macrostelus laevis* (Ribaut), *Macrostelus quadripunctulatus* (Kirschbaum), *Macrostelus cristatus* (Ribaut), *Macrostelus viridigriseus* (Edwards), *Speudotettix subfuscus* (Fallen), *Anoscopus albifrons* L., *Aphrodes bicinctus* (Schrank) and true bugs of the genus *Lygus* Hahn (Neklyudova and Dikit, 1973; Palermo *et al.*, 2004). Vectors of aster yellows disease are leafhoppers (family Cicadellidae).

The hemipterans tested for phytoplasma presence by PCR were members of Cixiidae, Delphacidae, Cicadellidae, Aphrophoridae and Miridae family.

Materials and methods

In infected vineyards, the two groups of hemipterans, polyphagous and insects preferring Poaceae family plants, were collected from grapevine plants, weeds, and cover crop between vine rows, see table 1.

Collected insect material was determined and sorted by sex, development stadium and locality of finding and divided to 157 laboratory samples. Each sample consisted of one to ten individuals and was freeze-stored in absolute ethanol.

The total DNA was extracted using High Pure PCR template Preparation Kit (Roche) according to the manufacturer's protocol for the isolation of nucleic acids from mammalian tissue. Purified DNA was eluted twice with 50 µl of elution buffer.

Presence of phytoplasma was analysed using PCR with R16R0/R16F1 primer pair followed by nested amplification of 16S rRNA phytoplasma gene with R16F2n/R16R2 primers (Gundersen and Lee, 1996). Positive samples were further analysed using restriction fragment length polymorphism of PCR product to iden-

tify the detected phytoplasma. Moreover, the stolbur phytoplasma positive samples were characterised by analysis of non-ribosomal *tuf* gene sequence amplified using PCR with primer pair fTufAY/rTufAY (Schneider *et al.*, 1997) followed by nested PCR with fTufAY2/rTufAY2 primers in combination with restriction analysis (Pasquini *et al.*, 2007).

Results

Out of 157 samples tested, 39 resulted phytoplasma positive using 16S rRNA nested-PCR. Although all insect samples were collected in stolbur phytoplasma infected vineyards, the phytoplasma was detected only in one sample after restriction fragment length polymorphism analysis with *AluI*, *FspBI*, *HaeIII*, *HhaI*, *HinfI*, *MseI*, *MspI*, *RsaI* and *Sau3AI* enzymes (Lee *et al.*, 1998). *Tuf* gene sequence analysis revealed the detected stolbur phytoplasma to belong to the *tuf*-type b (data not shown).

Digestion of PCR products of the remaining 38 phytoplasma positive samples with the same nine enzymes revealed that 81% of samples contained aster yellows phytoplasmas, subgroups B, C and F according to Marcone *et al.* (2000). Eight percent was identified as X-disease phytoplasma, subgroup B and 3% of samples belonged to the Bermuda grass white leaf phytoplasma ('*Candidatus* Phytoplasma cynodontis') according to Lee *et al.* (1998). Three percent revealed mixed infection of aster yellows and X-disease phytoplasma and five percent of samples resulted in unknown restriction profiles. Overview of the results is shown in table 1.

Discussion and conclusions

The obtained results indicate that some of the collected hemipterans species might be putative vectors of phytoplasmas in Czech Republic. Although the vineyards were stolbur infected, the stolbur phytoplasma was detected only in *H. obsoletus*. In most of the positive samples were found phytoplasmas of the aster yellows group, primarily in *Jassargus obtusivalvis*, *Euscelis incisus* and *Javesella pellucida*.

Table 1. Summary of hemipteran species and phytoplasmas detected in them according with sex or stadium of development. Polyphagous insects are marked with *.

Phytoplasma identified	Species tested	No. of samples		
		male	female	larvae
Aster yellows 16SrI-B	<i>Macrosteles quadripunctulatus</i> (Kirschbaum)*	1	0	0
	<i>Hardya tenuis</i> (Germar)*	1	0	0
Aster yellows 16SrI-C	<i>Jassargus obtusivalvis</i> (Kirschbaum)	5	2	0
	<i>Javesella pellucida</i> (F.)	1	0	0
	<i>Laodelphax striatella</i> (Fallen)	0	1	0
	<i>Aphrodes bicinctus</i> (Schrank)*	1	0	0
Aster yellows 16SrI-F	<i>Errastunus ocellaris</i> (Fallen)*	1	1	0
	<i>Euscelidius variegatus</i> (Kirschbaum)*	0	1	0
	<i>Euscelis incisus</i> (Kirschbaum)*	2	3	1
	<i>Hardya tenuis</i> (Germar)*	0	1	0
	<i>Jassargus obtusivalvis</i> (Kirschbaum)	3	0	1
	<i>Javesella pellucida</i> (F.)	2	1	0
	<i>Laodelphax striatella</i> (Fallen)	1	0	0
	<i>Philaenus spumarius</i> (L.)*	0	1	0
	<i>Euscelis incisus</i> (Kirschbaum)*	1	0	0
	<i>Jassargus obtusivalvis</i> (Kirschbaum)	0	1	0
X-disease 16SrIII-B	<i>Lygus rugulipennis</i> (Poppius)*	0	0	1
	<i>Euscelis incisus</i> (Kirschbaum)*	0	1	0
X-disease + aster yellows 16SrIII-B + 16SrI-F	<i>Jassargus obtusivalvis</i> (Kirschbaum)	1	0	0
Bermudagrass white leaf 16SrXIV	<i>Hyalesthes obsoletus</i> Signoret*	1	0	0
Unidentified	<i>Laodelphax striatella</i> (Fallen)	1	1	0

The tuf-type b of stolbur phytoplasma correspond to the type earlier reported in Czech vineyards (Fialová *et al.*, 2009).

Up to now, the presence of bermudagrass white leaf phytoplasma as well as the aster yellows phytoplasma, subgroup 16SrI-F was not reported in Czech Republic, and their presence in plant hosts needs to be confirmed.

Acknowledgements

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Monitoring of psyllid species (Hemiptera, Psylloidea) in apple and pear orchards in East Bohemia

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Abstract

During the years 2009–2010, a monitoring of the psyllid species occurring in apple and pear orchards with different types of plantation management was carried out in orchards in East Bohemia. Species known as vectors of quarantine phytoplasmas ‘*Candidatus Phytoplasma mali*’ and ‘*Candidatus Phytoplasma pyri*’, were studied in priority. The infection with phytoplasmas was determined by amplification of DNA using polymerase chain reaction with subsequent restriction fragment length polymorphism analysis in selected individuals. Specimens of *Cacopsylla picta*, *C. pyricola*, *C. pyri*, *C. pyrisuga* were found to be phytoplasma infected with in the studied territories.

Key words: psyllid species, vectors of phytoplasmas, ‘*Candidatus Phytoplasma mali*’, ‘*Candidatus Phytoplasma pyri*’, PCR/RFLP.

Introduction

‘*Candidatus Phytoplasma mali*’ and ‘*Candidatus Phytoplasma pyri*’ are associated with serious diseases in apple and pear growing areas. ‘*Ca. P. mali*’ is the agent associated with apple proliferation (AP) and ‘*Ca. P. pyri*’ is the agent associated with pear decline (PD) (Seemüller and Schneider, 2004). Psyllids of *Cacopsylla* genus (Hemiptera, Psylloidea) are vectors of these phytoplasmas. In Central and Southern Europe *Cacopsylla picta* (Foerster 1848) has been determined as a vector of AP, (Frisinghelli *et al.*, 2000; Jarausch *et al.*, 2003) whereas *C. melanoneura* (Foerster 1848) was only confirmed as vector of AP in northwestern Italy (Tedeschi *et al.*, 2002). PD is transmitted in Europe by *C. pyri* (García-Chapa *et al.*, 2005), *C. pyrisuga* (Križanac *et al.*, 2008) and *C. pyricola* (Jensen *et al.*, 1964).

The aim of this study was to monitor the occurrence of these known phytoplasma vector in apple and pear orchards with different types of plantation management: conventional, organic, integrated production and old deserted orchards under the climatic conditions in Czech Republic.

Materials and methods

The observation of occurrence of known AP and PD vector species was carried out in 4 apple plantations and 4 pear plantations with conventional, organic, integrated production and old deserted orchards in East Bohemia. The insects were collected with sweep-netting from March to September at every 2 weeks or more often. Insects were determined, numbered and then stored at -20°C in absolute ethanol for later identification. The infection of psyllid species with phytoplasmas was determined by PCR. Total DNA was extracted from two in-

dividuals of the same *Cacopsylla* species, using a commercial kit (Wizard Genomic DNA Purification Kit, Promega, USA). DNA products, diluted with sterile water in proportion 1: 10, was amplified by 35 cycles in a thermocycler (Techne). Nested PCR was carried out with the primers R16F2n/R2 (Gundersen and Lee, 1996) and fU5/rU3 (Lorenz *et al.*, 1995). Final products were submitted to RFLP analyses using *RsaI* and *BfmI* (Fermentas, Vilnius, Lithuania). The PCR and RFLP products were analyzed on 1.5% agarose gels in TBE buffer and stained with SYBR Green.

Results

In table 1 there are mentioned species of *Cacopsylla*, tested for the presence of phytoplasmas by PCR. Individuals of *C. mali*, *C. melanoneura* and *C. pyri* were tested in the year 2009. The presence of phytoplasma was found in the samples of *C. pyri*. Abundance of *C. pyri* (mainly in commercial and integrated orchards), *C. pyricola*, *C. pyrisuga*, *C. picta* and *C. mali* (mainly in organic and old deserted orchards) was observed, but only several individuals of *C. melanoneura* were collected during the growing season 2010. Owing to the low quantity of collected individuals, *C. melanoneura* has not been tested by PCR and testing of *C. mali* samples is in progress.

The comparison of abundance of psyllid species in apple and pear orchards with different types of plantation management during two growing seasons showed disparities within a single years as well as with regard to plantation management. In 2010, a high incidence of *C. pyri* in pear orchards, especially in integrated and commercial orchards, and of *C. mali* in apple orchards, mainly in organic and old deserted orchards was observed (figure 1).

Table 1. Results of phytoplasmas detection in *Cacopsylla* species by PCR in years 2009 and 2010.

Species/year of observation	Number of tested samples and results of PCR					
	2 0 0 9			2 0 1 0		
	total	positive	%	total	positive	%
<i>Cacopsylla mali</i>	45	0	0	0	0	0
<i>Cacopsylla melanoneura</i>	7	0	0	0	0	0
<i>Cacopsylla picta</i>	0	0	0	90	4	4.4
<i>Cacopsylla pyri</i>	454	1	0.2	512	10	2
<i>Cacopsylla pyrisuga</i>	0	0	0	47	1	2.1
<i>Cacopsylla pyricola</i>	0	0	0	17	1	5.9

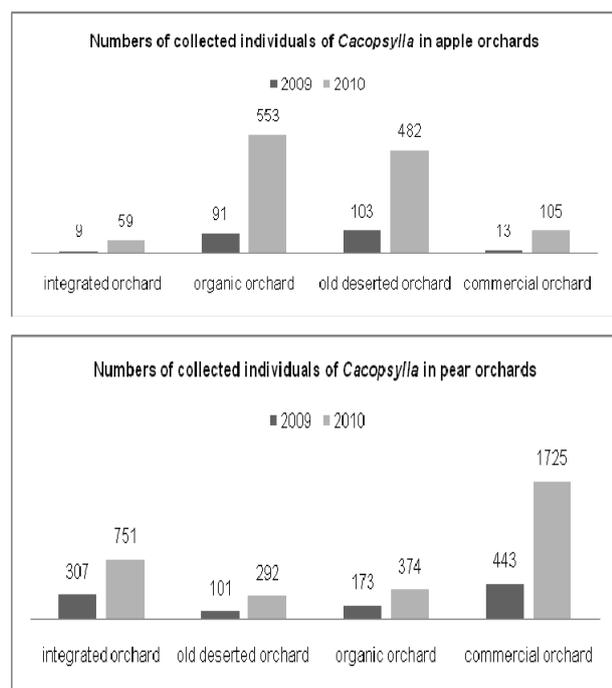


Figure 1. Numbers of collected individuals of *Cacopsylla* spp. in apple and pear orchards with different plantation management in years 2009 and 2010.

Discussion

C. pyri represents the big problem for the quality production in pear plantations with commercial and integrated plantation management. A huge amount of psyllid species, especially *C. mali*, occur in the organic and old deserted orchards. According to the PCR/RFLP examination of phytoplasma presence, *C. pyri*, *C. pyricola* and *C. pyrisuga* can be vectors of 'Ca. P. pyri' in Czech Republic. Samples of *C. mali* collected in the year 2010 have not been tested yet, however, the presence of the AP in this vector was not described in other countries to this date (Jarausch *et al.*, 2003). Further work to verify the situation toward better disease management in orchards is in progress.

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Preliminary survey of potential vectors of '*Candidatus Phytoplasma phoenicium*' in Lebanon and probability of occurrence of apricot chlorotic leaf roll (ACLR) phytoplasma

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Abstract

Malaise traps and sticky yellow traps were placed in two almond orchards infected with almond witches' broom (AlmWB) phytoplasma in 2004 and in 2010. Collected insects were tested by nested PCR using universal primers for detection of phytoplasma. In 2004 *Asymmetrasca decedens*, *Euscelidius* sp. and *Fieberiella* sp. gave positive results with the universal primers and also with the pigeon pea group specific primers. The mere presence of phytoplasma in an insect is not a proof that it is a vector, but it may help narrowing the choice of insects for conducting actual transmission tests. In 2010, early in the season, in over 20 species surveyed we were not able to detect phytoplasma except in *Psammotettix provincialis*. The PCR amplicon was sequenced (1180 bp) and found to be most closely related to the Aster yellows (AY) group 16SrI, subgroup F which includes apricot chlorotic leaf roll (ACLR) phytoplasma. Late in the season, most PCR tests were negative, the major reason was correlated with DNA degradation which resulted from the hot summer temperatures combined with the method of insect collection. The implications of these findings are discussed.

Key words: almond witches' broom, nested PCR, *Asymmetrasca decedens*, *Euscelidius* sp., *Fieberiella* sp., *Psammotettix provincialis*.

Introduction

Almond witches' broom (AlmWB) phytoplasma was reported as a devastating almond disease that killed over 100,000 almond trees in Lebanon (Abou-Jawdah *et al.*, 2002). AlmWB has been tentatively called '*Candidatus Phytoplasma phoenicium*' and belongs to the pigeon pea phytoplasma group 16Sr IX (Abou-Jawdah *et al.*, 2002; Verdin *et al.*, 2003). Grafting experiments showed that it is transmitted to peach and nectarine but not to apricot (Abou-Jawdah *et al.*, 2003). These results were confirmed recently as AlmWB was reported to cause severe epidemics on peach and nectarine in South Lebanon (Abou-Jawdah *et al.*, 2009). A similar disease was reported in Iran. The rapid and extensive spread of the disease suggests the presence of efficient vector(s) and calls for an integrated disease management approach which would require information about the disease epidemiology. The present work tries to identify potential vectors in order to start transmission tests for identification of the real vector(s).

Materials and methods

Leafhoppers were collected at bi-weekly intervals from Malaise traps and from yellow sticky traps installed in two AlmWB infected orchards. Insects were removed and identified directly or stored in 70% ethanol until identification. In 2004 the insects in the malaise trap

were killed by pyrethroid pesticide releasing pellets; while in 2010, the insects were trapped in 70% ethanol. DNA was extracted from individual insects for large-sized species, or from groups of 5 insects in the case of *Asymmetrasca decedens* (Paoli) and similar small-sized species. Phytoplasma was detected either by nested PCRs using universal primer pairs P1/P7 and R16F2n/R16R2 (Gundersen and Lee, 1996), or by PCR using the primer pair (AlwF2/R2) for detection of pigeon pea taxonomic group (Abou-Jawdah *et al.*, 2003).

Results and discussion

Among the 10 genera of leafhopper tested in 2004, seven genera gave positive results with the group specific primers and only three with the universal primers (table 1). *A. decedens*, *Euscelidius* sp. and *Fieberiella* sp. gave positive results using both the phytoplasma universal primer pairs and the pigeon pea group specific primers. From our experience using both techniques the group specific primers give more sensitive detection than the universal primers; thus probably the phytoplasma concentration is higher in these three genera and therefore may be given priority in transmission assays. *Euscelidius* and *Fieberiella* genera include known phytoplasma vectors, while relatively a limited number of reports are available on *Empoasca*, a relative to *Asymmetrasca*, as a potential phytoplasma vector (Pastore *et al.*, 2004).

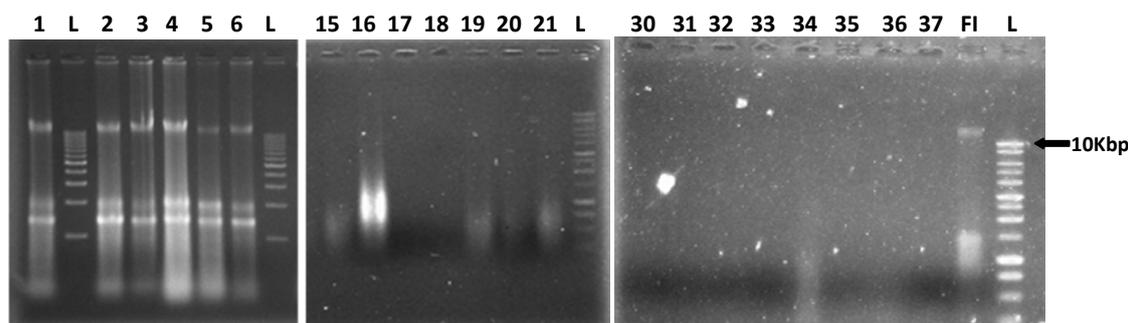


Figure 1. Gel electrophoresis of DNA extracts from different insects collected at three different dates. 1-6: Spring, 15-21: mid Summer, 30-37: late Summer. FI is a freshly collected insect, L= 1kbp ladder.

Table 1. Detection of phytoplasma by PCR and nested PCR in DNA extracts of 10 leafhopper genera collected in 2004 from North Lebanon.

Insect	No. of PCR positive samples/No. of total samples	
	Nested with R16F2/R2n	primers AlwF2/R2
<i>Allygus</i> sp.	0/2	1/2
<i>Asymmetrasca decedens</i>	2/5	5/5
<i>Cicadulina</i> sp.	0/2	0/2
<i>Empoasca decipiens</i>	0/8	3/8
<i>Euscelidius</i> sp.	1/14	10/14
<i>Euscelis</i> sp.	0/1	0/1
<i>Fieberiella</i> sp.	1/1	1/1
<i>Laylatina</i> sp.	0/8	1/8
<i>Thamnotettix</i> sp.	0/3	1/3
<i>Zygina</i> sp.	0/3	0/3

In 2010, Lebanon experienced a very hot summer and the leafhoppers were trapped in glass jars containing 70% ethanol and collected at biweekly intervals. This may have led to DNA degradation in samples collected starting from June (figure 1). Therefore, in hot climates it would be necessary to adapt the trapping method to preserve the insect DNA.

DNA extracts from one leafhopper *Psamnotettix provincialis* (Ribaut) collected early in the 2010 season gave positive results in nested PCR tests. The amplicon sequence was most closely related to aster yellows (AY) 16SrI subgroup-F (16SrI-F) which includes apricot chlorotic leaf roll (ACLR) and leafhopper borne, CVB.

In conclusion, field observation suggests the presence of more than one AlmWB vector (primary and secondary), one responsible for long distance migration and feeds only occasionally on stone fruits, and another like *A. decedens* which may be a much less efficient vector but is present in large numbers in stone fruit orchards. These observations coupled with PCR results must be confirmed by transmission assays. Other insects in the Psyllidae or Cixiidae should be also monitored.

In Lebanon, in AlmWB infested areas, apricot is considered as one of the possible replacement crops for almond since it was proven to be resistant to AlmWB. However, the probability of occurrence of ACLR phytoplasma which lead to the decline of apricot trees in some European regions, must be thoroughly investigated. Due

to the diversity of AY group, surveys must be conducted using PCR tests based not only on 16Sr DNA sequences but also on secY and rp gene sequences (Lee *et al.*, 2005). The epidemic potential of AlmWB calls for a regional and international cooperation to eradicate it or prevent its further spread.

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'*Candidatus Phytoplasma mali*': identification of potential insect vectors in Spanish apple orchards

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Abstract

Samplings realized in apple plots of different geographical areas of Spain indicated the presence of the two species of *Cacopsylla* reported as vectors of the apple proliferation disease. The population evolution of *Cacopsylla picta* and *C. melanoneura* was followed during two years in the two regions of Spain where these species were identified. The population showed two peaks, one for adults re-immigrants, which in the two years occurred in early April and other for new generations between June and July.

Key words: Apple proliferation, '*Candidatus Phytoplasma mali*', *Cacopsylla picta*, *Cacopsylla melanoneura*.

Introduction

Apple proliferation (AP) disease is associated with '*Candidatus Phytoplasma mali*' and it is one of the most important phytoplasma diseases in Europe causing considerable economic losses in apple orchards. This phytoplasma is present in many countries of Europe such as, Germany, France, Switzerland and Italy. Some of the symptoms of AP are witches' broom, elongated stipules, chlorosis and early leaf reddening. The fruits have lower size and worse quality. In Spain it has been found in commercial plots of different varieties of apple in Asturias and the Basque Country and also in plant material from several nurseries.

In the last years there has been a significant increase of the disease in Europe. This new outbreak (Danet *et al.*, 2011) may be due to the emergence of new isolates or to the presence of more effective vectors. For this reason, the identification and control of vectors and a good understanding of the population dynamics, insect infectivity and the host plants for both, phytoplasma and vectors in a specific region are of great importance.

In Europe two psyllid species have been described as vectors of the phytoplasma: *Cacopsylla picta* (syn. *C. costalis*) and *Cacopsylla melanoneura*. However, their distribution, infectivity and transmission capacity vary between different geographical areas (Jarausch *et al.*, 2007; Tedeschi *et al.*, 2003; Mayer *et al.*, 2009). During two years, the search of psyllids, vectors of '*Ca. P. mali*', and of other potential vectors was extended to different geographical areas of Spain.

Materials and methods

The insects were captured on yellow sticky traps (10 x 16 cm) placed in apple orchards of different geographical areas of Spain (Catalonia, Asturias and Basque

Country), during 2010 and 2011. The traps were replaced every 15 days. The insect capture was done from February until the beginning of August. All specimens captured were separated and classified for DNA extraction and PCR analysis.

DNA from insects was extracted by grinding 1-5 insects, depending on the species, following the methods used in previous studies (Garcia-Chapa *et al.*, 2005).

Results

In the sampled plots were caught several species of psyllids and leafhoppers were caught. The two species of *Cacopsylla* reported as vector of the disease have been identified in the Basque Country and Asturias. In a plot located in Oberlaún (Basque Country), with a higher incidence of the disease *Cacopsylla picta* and some individuals of *C. melanoneura* have been identified. In Aduna (Basque Country) and Siero (Asturias) *C. melanoneura* has been identified. The population evolution of both these species showed two population peaks, one for adults re-immigrants, which for both years occurred in early April and another one for new generations between June and July (figure 1). The higher population of these species, known as vectors of the '*Ca. P. mali*', has been found in the most affected plot (Oberlaún, Guipúzcoa) (figure 1, table 1).

Other species of cicadellidae known as potential vectors of phytoplasmas were captured in the sampled plots (table 1). In the plots of Catalonia located very close to plum, peach and pear plots, other *Cacopsylla* species such as *C. pruni* and *C. pyri* were captured (table 1).

The PCR analyses of the two species cited as vectors of the phytoplasma (*C. picta* and *C. melanoneura*) are being carried out with the aim to determine the percentage of individuals carrying the phytoplasma.

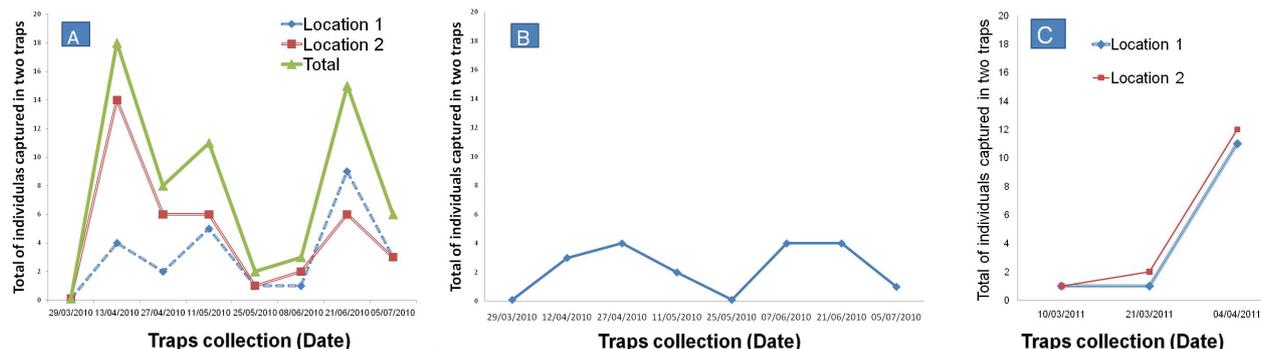


Figure 1. Population evolution of *C. picta* in (A) Oberlaún (Basque Country) and of *C. melanoneura* in (B) Siero (Asturias) in 2010. (C) Population evolution in 2011 in Oberlaún (Basque Country). (In colour at www.bulletinofinsectology.org)

Table 1. Species of cicadelidae and psyllidae captured in different apple orchards of Basque Country, Asturias and Catalonia.

Species	Basque Country	Asturias	Catalonia
<i>Agallia</i> sp.	0	0	2
<i>Cacopsylla picta</i>	72	1	0
<i>Cacopsylla melanoneura</i>	9	12	0
<i>Cacopsylla</i> sp.	0	0	24
<i>Cacopsylla crataegui</i>	0	0	1
<i>Cacopsylla pruni</i>	0	0	72
<i>Cacopsylla pulchella</i>	0	0	41
<i>Cacopsylla pyri</i>	0	0	44
<i>Cercopidae</i> sp.	0	6	0
<i>Cixidae</i> sp.	8	0	1
<i>Empoasca</i> sp.	44	3	0
<i>Erythroneura</i> sp.	10	1	0
<i>Euscelidius variegatus</i>	0	2	0
<i>Jassidae</i>	2	10	0
<i>Macrostes</i> sp.	1	4	0
<i>Psillidae</i>	1	2	15
<i>Triozidae</i>	0	0	6
<i>Zygina</i>	28	8	0

Discussion

In Spain *C. picta* is also the insect species that can be related with a higher apple proliferation incidence in the sampled plots such as happens in other European areas (Jaraush *et al.*, 2007). In other plots where the disease is present but has a lower incidence, *C. melanoneura* was identified. In the other sampled areas of Spain where the disease is not present, these species have not been identified. The absence of vectors can explain why the disease is not present, despite that the phytoplasma was sporadically identified in some cases also in certified plant material (Batlle *et al.*, 2004).

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***Hyalesthes obsoletus*, a vector of stolbur phytoplasma: current situation in South Moravia, Czech Republic**

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Abstract

A survey for the presence of *Hyalesthes obsoletus* Signoret in 2010 confirmed a dramatic increase in number of captured individuals at observed localities in agriculturally important areas in southern Moravia, Czech Republic. Population density from the end of June to the middle of July, varies with locality. A preferred host plant *Urtica dioica* L. was present. Average percentage of stolbur phytoplasma positive *H. obsoletus* specimens varied from 31 to 59%.

Key words: weeds, *Urtica dioica* L., insect vectors, Hemiptera.

Introduction

During the recent years, the insect vector of stolbur phytoplasmas *Hyalesthes obsoletus* Signoret caught the attention of many researchers in connection with the study of phytoplasma infections of grapevines, i.e. with the local outbreak of bois noir (BN) disease (Sforza *et al.*, 1998), or the spread of grapevine yellows (VK) (Weber and Maixner, 1998).

H. obsoletus presence could be connected with local epidemics of stolbur disease in solanaceous plants as they play an important role in spreading stolbur phytoplasma in main natural reservoirs, *Urtica dioica* L. and *Convolvus arvensis* L. (Alma and Tedeschi, 2010).

The preliminary results of our study focused on the occurrence and infectivity of *H. obsoletus* Signoret in three agriculturally important localities.

Materials and methods

Three localities from an agriculturally important area in the Czech Republic were chosen for the monitoring the stolbur phytoplasma vector. They were two bois noir infected vineyards, Březí and Perná localities, and one horticulture locality Lednice, where there are tomato and pepper plantations with high incidences stolbur phytoplasma.

H. obsoletus populations were surveyed from June 2008 to August 2010 using sweep nets ca every 10 days (depending on the weather) on bindweeds and stinging nettles. In each locality the weeds were swept 150 times. The spectrum of potential phytoplasma vectors was identified.

Infectivity of *H. obsoletus* individuals was analysed by the phytoplasma-specific PCR. The extracts of total DNA from single psyllid individuals were obtained using a commercial kit (Wizard Genomic DNA Purification Kit, Promega, USA). The detection of phytoplasmas in single individuals of *H. obsoletus* Signoret was performed using nested-PCR with universal P1/P7 primer pair followed by R16F2/R2 pair (Deng and Hi-

ruki, 1991; Gundersen and Lee, 1996; Lee *et al.*, 1993; Schneider *et al.*, 1995) derived from 16S rDNA. Identification of phytoplasmas was done in subsequent RFLP analyses using *AluI*, *MseI*, and *RsaI* according to Lee *et al.* (1998).

Results

In the vineyard in Březí, where nettles are rarely found, only 18 specimens were captured. The percentage of stolbur phytoplasma-positive specimens varied from 0% to 67%, with average 31%. The maximum number of *H. obsoletus* individuals was noted at the end of June (figure 1A).

In the vineyard in Perná, 90 *H. obsoletus* were captured, all on the nettle. The occurrence culminated at the end of the first half of August. The percentage of stolbur phytoplasma-positive specimens varied from 29% to 50%, with average 39% (figure 1B).

Sporadic occurrence of a few individuals of *H. obsoletus* was noted only at Lednice during the period 2008-2009. The situation changed completely in 2010 when the number of captured individuals dramatically increased. In this year a total of 556 individuals was captured, mostly on nettles. The occurrence culminated during the second half of July. The percentage of stolbur phytoplasma-positive specimens varied from 21% to 86%, with average 59% (figure 1C).

In all the phytoplasma positive individuals only stolbur phytoplasma presence was identified by PCR/RFLP analyses.

During the year 2010 a total of 6,450 individuals of bugs, leafhoppers, planthoppers and psyllids were collected. Other potential stolbur vectors, such as *Aphrodes bicinctus* (Schrank), *Euscelidius variegates* Zachvatkin, *Euscelis incisus* (Kirschbaum), *Lygus rugulipennis* Poppius, *Psammotettix alienus* (Dahlbom), *Psammotettix confinis* (Dahlbom), and *Reptalus panzeri* (Löw) were caught, too. A molecular analyses of the individuals and the evaluation of their infectivity are in preparation.

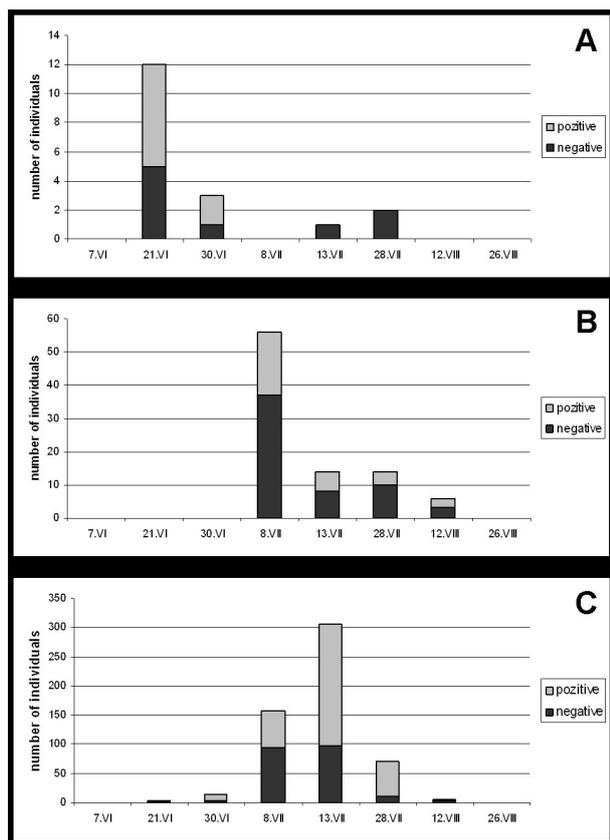


Figure 1. Population dynamics and infectivity of *H. obsoletus* in (A) Břeží, (B) Perná, (C) Lednice localities.

Discussion

The planthopper *H. obsoletus* is the main vector of ‘bois noir’ phytoplasma (stolbur) (Weber and Maixner, 1998; Forte *et al.*, 2010). It prefers herbaceous plant hosts *C. arvensis* and *U. dioica*; other reported hosts such as *Calystegium sepium* (L.) R. BR., *Lamium orvala* L., *Lavandula* sp., and *Vitex agnus-castus* L. were described (Alma and Tedeschi, 2010; Kessler *et al.*, 2011) are of minor importance in Czech Republic.

Population dynamics recorded on the localities studied in Czech Republic are similar to those of *H. obsoletus* in European countries and areas with the similar climate conditions. A comparable vector dynamics described Forte *et al.* (2010), a culmination delayed a few days was noted in North-Eastern Italy (Lessio *et al.*, 2007).

Infestation level of *H. obsoletus* varied from 31 to 59% which is comparable to vector activity detected in the Mosel Valley in Germany, where ca 30% of planthoppers were stolbur positive (Weber and Maixner, 1998).

Results presented in this study represent the first mass occurrence of *H. obsoletus* Signoret in the Czech Republic from the 1950’s.

Acknowledgements

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Potential new hemipteran vectors of stolbur phytoplasma in Serbian vineyards

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Abstract

The diversity of Auchenorrhyncha species was studied in vineyards affected by 'bois noir' (BN) and their surroundings in Serbia. Auchenorrhyncha specimens were collected for identification, statistic analysis and for stolbur phytoplasma presence detection. A total number of collected species was 49, belonging to 8 families. The most numerous was family Cicadellidae with 30 species, followed by Cixiidae (7), Delphacidae (4), Aphrophoridae (3). Families Dictyopharidae, Issidae, Cercopidae and Membracidae were present with only one species. The most abundant species in all inspected sites was *Psammotettix alienus* (Dahlbom). *Dictyophara europaea* (L.), *Hyalesthes obsoletus* Signoret, *Euscelis incisus* (Kirschbaum) and *Reptalus quinquecostatus* (Dufour) were numerous, as well. The presence of BN was detected in 4 Auchenorrhyncha species: *H. obsoletus*, *R. quinquecostatus*, *Reptalus panzeri* (Low) and *D. europaea*. This is the first record of BN phytoplasmas in *D. europaea*.

Key words: 'bois noir', stolbur, *Dictyophara europaea*, vector, grapevine.

Introduction

'Bois noir' (BN) is the most widespread grapevine yellows in Europe and Mediterranean area. BN is a disease associated with stolbur phytoplasma presence (16SrXII) and for the past two decades was registered as one of the economically most important grapevine diseases in Europe.

Stolbur phytoplasma infects a wide range of wild and cultivated herbaceous plants and can be transmitted by the cixiid planthoppers *Hyalesthes obsoletus* Signoret, *Pentastiridius leporinus* (L.) and *Reptalus panzeri* (Low) (Maixner *et al.*, 1995; Gatineau *et al.*, 2001; Jović *et al.*, 2007) and the leafhoppers *Macrostelus quadripunctulatus* (Kirschbaum) and *Anaceratagallia ribauti* (Ossiannilsson) (Battle *et al.*, 2008, Riedle-Bauer *et al.*, 2008). Stolbur phytoplasma has also been detected in several other cixiid and leafhopper species (Trivellone *et al.*, 2005), but their vector role has not yet been confirmed. The aims of this study were to determine the diversity of Auchenorrhyncha species present in vineyards and to identify new potential insect vectors of stolbur phytoplasma.

Materials and methods

The diversity of Auchenorrhyncha species was studied in three vineyards: Topola (central Serbia), Vršac (northern Serbia) and Rajac (eastern Serbia), where a high percentage of BN infected plants were present.

Insect sampling was carried out in 2006 and 2007, from mid-May to the end of September. Potential hemipteran vectors including leafhoppers and planthoppers were collected using sweep nets and mouth-aspirators from grapevines and weeds along the borders of vineyards, and stored in 96% ethanol for later species and phytoplasma identification.

Total nucleic acids were extracted from individual insects using a modified CTAB method (Gatineau *et al.*, 2001). Phytoplasma was identified in insects using a modification of the stolbur phytoplasma-specific nested PCR protocol, with primers Stol11f/r, R16F2/R1, and R16F3/R2, according to Radonjić *et al.* (2009).

Results and discussion

In total, 4,971 Auchenorrhyncha specimens were collected in and around vineyards, belonging to eight families and 49 species (table 1). The most abundant was the Cicadellidae with 30 species, followed by Cixiidae 7 species, Delphacidae 4 species, Aphrophoridae 3 species, and Dictyopharidae, Issidae, Cercopidae and Membracidae with only one species recorded. The predominant species were *Psammotettix alienus* (Dahlbom) (29.4%). *Dictyophara europaea* (L.) (10%), *H. obsoletus* (9.2%), *Euscelis incisus* (Kirschbaum) (6.4%), *Reptalus quinquecostatus* (Dufour) (5.8%), *Neoliturus fenestratus* (Herrich-Schaffer) and *Errastunus ocellaris* (Fallen) (about 4.2%), were numerous as well, while presence of *Laodelphax striatella* (Fallen), *Philaenus spumarius* (L.), *Doratura impudica* Horvath and *Zyginidia pullula* (Boheman) was between 2.5 and 3.3%.

PCR analyses using DNA from collected adult insects indicated that 4 out of 49 collected species harbored the stolbur phytoplasma: 38% of *H. obsoletus* (91/240), 15% of *R. quinquecostatus* (44/289), 8% of *R. panzeri* (4/49) and 12% of *D. europaea* (41/341) specimens.

In this survey, relatively dense populations of the *D. europaea* were recorded in vineyards and their surroundings, with 12% of captured specimens positive for BN phytoplasma presence.

Table 1. Auchenorrhyncha species collected in and around vineyards affected by BN.

Species
Cixiidae/ Cixinae
<i>Cixius wagneri</i> (China 1942)
<i>Cixius similis</i> (Kirschbaum 1868)
<i>Hyalesthes obsoletus</i> (Signoret 1865)
<i>Reptalus cuspidatus</i> (Fieber 1876)
<i>Reptalus panzeri</i> (Low 1883)
<i>Reptalus quinquecostatus</i> (Dufour 1833)
<i>Tachycixius desertorum</i> (Fieber 1876)
Delphacidae/ Delphacinae
<i>Asiraca clavicornis</i> (F. 1794)
<i>Dicranotropis hamata</i> (Boheman 1847)
<i>Laodelphax striatella</i> (Fallen 1826)
<i>Stenocranus major</i> (Kirschbaum 1868)
Dictyopharidae /Dictyopharinae
<i>Dictyophara europaea</i> (L. 1767)
Membracidae /Smiliinae
<i>Stictocephala bisonia</i> (Kopp et Yonke 1977)
Issidae /Issinae
<i>Issus coleoptratus</i> (F. 1781)
Cercopidae /Haematoloma
<i>Lepyronia coleoptrata</i> (L. 1758)
Aphrophoridae /Aphrophorinae
<i>Philaenus spumarius</i> (L. 1758)
<i>Neophilaenus campestris</i> (Fallen 1805)
<i>Aphrophora alni</i> (Fallen 1805)
Cicadellidae/ Typhlocybinae
<i>Empoasca vitis</i> (Gothé 1875)
<i>Eupterix</i> sp.
<i>Zyginidia pullula</i> (Boheman 1845)
Cicadellidae/ Deltocephalinae
<i>Scaphoideus titanus</i> (Ball 1932)
<i>Fieberiella septentrionalis</i> Wagner 1963
<i>Jassargus obtusivalis</i> (Kirschbaum 1868)
<i>Errastunus ocellaris</i> (Fallen 1806)
<i>Neotalitrus fenestratus</i> (Herrich-Schaffer 1834)
<i>Mocydia crocea</i> (Herrich-Schaffer 1837)
<i>Psammodictya alienus</i> (Dahlbom 1850)
<i>Psammodictya confinis</i> (Dahlbom 1850)
<i>Euscelis incisus</i> (Kirschbaum 1858)
<i>Goniagnathus brevis</i> (Herrich-Schaffer 1835)
<i>Macrostelus</i> sp.
<i>Platymetopius major</i> (Kirschbaum 1868)
<i>Recilia schmidgeni</i> (Wagner 1939)
<i>Doratura impudica</i> Horvath 1897
<i>Allygidius commutatus</i> (Fieber 1872)
<i>Graphocraerus ventralis</i> (Fallen 1806)
<i>Ophiola decumana</i> (Kontkanen 1949)
<i>Streptanus confinis</i> (Reuter 1880)
<i>Mocuellus collinus</i> (Boheman 1850)
<i>Allygus</i> sp.
Cicadellidae /Cicadellinae
<i>Cicadella viridis</i> (L. 1758)
<i>Macropsis fuscata</i> (Zetterstedt 1828)
<i>Macropsis</i> sp.
Cicadellidae/ Aphrodinae
<i>Aphrodes</i> sp.
Cicadellidae/ Idiocerinae
<i>Idiocerus</i> sp.
Cicadellidae /Dorycephalinae
<i>Eupelix cuspidata</i> (Fabricius 1775)
Cicadellidae /Agalliinae
<i>Anaceratagallia ribauti</i> (Ossiannilsson 1938)
<i>Dryodurgades reticulatus</i> (Herrich-Schaffer 1834)

This is the first record of stolbur phytoplasma presence in *D. europaea*. Until now, it was reported that *D. europaea* is infected with ‘flavescence dorée’ phytoplasma and preliminary transmission experiments showed that this species is able to transmit this phytoplasma from clematis to grapevine (Filippin *et al.*, 2009). *D. europaea* is polyphagous insect present in vineyards and herbaceous plants along the borders of vineyards from the middle of May to the end of September. Its ability to carry stolbur phytoplasma gives a new light in the epidemiology of BN disease in vineyards, but further studies are required to assess its vector role.

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First report of potential phytoplasma vectors *Euscelis incisus* and *Macrosteles sexnotatus* in Lithuania

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Abstract

Phytoplasma strains of four 16Sr RFLP groups have been reported from various plant hosts in Lithuania, but there are no published data about insect species that serve as vectors of the phytoplasmas in the country. In the present study, phytoplasma strains belonging to subgroups 16SrIII-P and 16SrI-C were identified in leafhoppers collected from a meadow and orchard. Phytoplasma strains belonging to subgroup 16SrI-C were found in *Euscelis incisus* and *Macrosteles sexnotatus*. Phytoplasma strains belonging to subgroup 16SrIII-P were detected in *E. incisus*. These results indicate that *E. incisus* and *M. sexnotatus* feed on plant hosts of these phytoplasmas and that they possibly act as vectors of strains classified in subgroups 16SrI-C and 16SrIII-P, the latter subgroup containing phytoplasma strains that have to date been found only in Lithuania.

Key words: *Euscelis incisus*, *Macrosteles sexnotatus*, phytoplasma vectors, 16S rDNA, 16SrI-C, 16SrIII-P.

Introduction

Phytoplasmas are phytopathogenic wall-less bacteria that cause hundreds of plant diseases worldwide. Phytoplasmas are transmitted by phloem-feeding insects, mainly leafhoppers, but also could be transmitted by grafting, vegetative propagation, and by parasitic plants such as dodder (*Cuscuta* spp.) (Weintraub and Beanland, 2006).

The main group of phytoplasma-transmitting insects belongs to order Hemiptera, most vectors being affiliated with subgroups Auchenorrhyncha (leafhoppers and planthoppers) and Sternorrhyncha (psyllids) (Hogenhout *et al.*, 2008). These insects are highly effective vectors of phytoplasmas because the feeding manner and habitats are the same for nymphs and adults; they are exclusively phloem-feeding insects; they do not extensively damage tissues while feeding; and they transmit phytoplasmas in a persistent, propagative manner (Weintraub and Beanland, 2006).

Thus far, phytoplasmas classified in four 16Sr groups (16SrI, 16SrIII, 16SrV, and 16SrXII) and twelve subgroups have been reported from various plant hosts in Lithuania (reviewed in Valiunas *et al.*, 2009), but there are no data about insect species that serve as phytoplasma vectors in the country. The objective of this study was to gain knowledge about potential vectors of phytoplasmas in Lithuania by detecting and identifying/classifying phytoplasma strains that are carried by insects.

Materials and methods

Samples of putative insect vectors (leafhoppers) of phytoplasmas were collected by sweeping net from a meadow and orchard in the Kaunas district of Lithuania,

where phytoplasma-infected plants had previously been found. Leafhoppers were preserved in 90% ethanol. Adult male specimens were identified to the species level by morphological analysis under a stereomicroscope. DNA was extracted separately from each of 10 individuals of *Euscelis incisus* (Kirschbaum) and from each of 10 individuals of *Macrosteles sexnotatus* (Fallen) using Doyle and Doyle protocol, modified by Marzachi *et al.* (1998). Extracted DNA was used as template in nested PCRs primed by universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996), and PCRs were carried out and products were analysed as previously described (Lee *et al.*, 1998). Amplified DNA products of nested PCRs primed by primer pair R16F2n/R16R2 were analysed by single enzyme digestion, according to manufacturer's instructions, with *AluI*, *BfaI*, *HaeIII*, *HhaI*, *HinfI*, *HpaII*, *KpnI*, *MseI*, and *RsaI* (Fermentas, Vilnius, Lithuania). Restriction fragment length polymorphism (RFLP) patterns were analysed by electrophoresis through 5% polyacrylamide gel. The DNA size marker was phiX174 DNA/*BsuRI* (*HaeIII*) (Fermentas, Vilnius, Lithuania). RFLP patterns were compared with patterns previously published (Jomantiene *et al.*, 2002; Lee *et al.*, 1998).

Results

Two species of leafhoppers, *E. incisus* and *M. sexnotatus*, were identified among the insects collected from the meadow and orchard. Phytoplasma characteristic 1.2 kbp DNA amplicons were obtained in PCRs containing template DNAs from all ten *E. incisus* individuals and from three of ten *M. sexnotatus* individuals, indicating the presence of phytoplasmas in these insects (data not shown). Comparisons of RFLP patterns of the PCR

products with patterns published for 16S rDNAs from other phytoplasmas revealed that the phytoplasmas found in *E. incisus* belonged to subgroup 16SrIII-P (dandelion virescence phytoplasma subgroup) and to subgroup 16SrI-C (clover phyllody phytoplasma subgroup); the phytoplasma strains found in *M. sexnotatus* were all affiliated with subgroup 16SrI-C (data not shown).

Table 1. Phytoplasma strains detected in insects.

Insect species	Detected phytoplasma	
	Strain	16Sr subgroup
<i>Euscelis incisus</i>	CPh (clover phyllody)	16SrI-C
	DanVir (dandelion virescence)	16SrIII-P
<i>Macrosteles sexnotatus</i>	CPh (clover phyllody)	16SrI-C

Discussion

The data obtained so far in the present study have highlighted two leafhopper species as possible vectors of two different phytoplasmas in Lithuania. *E. incisus* is a known vector of phytoplasma strains that are classified in 16S rDNA RFLP groups including 16SrXII-A, 16SrVI, and 16SrI-B (Weintraub and Beanland, 2006). A close relative, *E. lineolatus*, transmits subgroup 16SrI-C phytoplasma strains (Weintraub and Beanland, 2006). Our results indicate that *E. incisus* may also transmit strains belonging to subgroup 16SrI-C in Lithuania, and additionally it could be a potential vector for phytoplasma strains classified in subgroup 16SrIII-P, for which no insect vector has been previously identified.

M. sexnotatus is a confirmed vector of phytoplasma strains classified in subgroup 16SrI-B, and close relatives (*M. cristata* and *M. laevis*) transmit subgroup 16SrI-C strains (Weintraub and Beanland, 2006). Based on our findings, it is possible that *M. sexnotatus* is also a vector of phytoplasma strains classified in subgroup 16SrI-C.

In the meadow where insects were collected in the present study, phytoplasmas belonging to the same subgroups were previously found in dandelion (*Taraxacum officinale*) (Jomantiene *et al.*, 2002) and clover (*Trifolium repens*) (Staniulis *et al.*, 2000), inferring that *E. incisus* and *M. sexnotatus* may be involved in phytoplasma transmission to these plant species.

Further studies are needed to determine whether the two leafhopper species found in this study to carry phytoplasmas in Lithuania are also capable of transmitting

the phytoplasmas to plants. Continued study should reveal additional insect species as candidate vectors of phytoplasmas in this region.

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Preliminary investigations on *Graminella nigrifrons* as a potential vector for phytoplasmas identified at the Canadian Clonal Genebank

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Abstract

Prunus and *Pyrus* species from the Canadian Clonal Genebank infected with putative phytoplasma diseases, as well as leafhopper species collected from *Prunus* and *Pyrus* fields were molecularly tested for phytoplasma presence. Preliminary results showed that *Graminella nigrifrons* may be a potential vector for phytoplasma group 16SrI ('*Candidatus* Phytoplasma asteris'), 16SrVII ('*Candidatus* Phytoplasma fraxini') and 16SrX-C ('*Candidatus* Phytoplasma pyri'). Results also showed that *G. nigrifrons* may be able to transmit multiple phytoplasmas within the same location. *G. nigrifrons* appears to have a complex ecology therefore further transmission trials are required to verify its phytoplasma vector role in fruit trees.

Key words: phytoplasma, *Graminella nigrifrons*, phytoplasma vector, ecology.

Introduction

Graminella nigrifrons (Forbes), the black-faced leafhopper is widely distributed in North America. This member of Membracoidea, Cicadellidae, Deltocephalinae is the known vector of the maize bushy stunt phytoplasma, ('*Candidatus* Phytoplasma asteris' group, subgroup 16SrI-B), and of several maize viruses (Beirne, 1956). Recently, phytoplasmas of groups 16SrI, 16SrVII ('*Candidatus* Phytoplasma fraxini') and 16SrX-C ('*Candidatus* Phytoplasma pyri') have been reported affecting *Prunus* and *Pyrus* species in Ontario, Canada, and are included in the Canadian Clonal Genebank (CCG) (Hunter *et al.*, 2010, Zunnoon-Khan *et al.*, 2010a, 2010b). Vector management is an effective control strategy for phytoplasma diseases (Weintraub and Beanland, 2006). Surveys to identify potential vector species for *Prunus* and *Pyrus* phytoplasma diseases at CCG were conducted to assess the importance of insect management in phytoplasma spreading.

Materials and methods

During June-August/2010, over 500 leafhopper specimens were collected by sweep net in *Prunus* and *Pyrus* in four CCG fields: T4 Centre-apricot, T4 East-peach, T3 West plum and cherry, and T4 West-pear (Table 1), and subjected to identification and PCR testing. Random leaf samples from peach and apricot trees from each field showing symptoms of decline, leaf reddening, witches' broom and peach rosette-like (Zunnoon-Khan *et al.*,

2010a, 2010b) were also collected and tested (table 1). Total DNA was extracted from leafhopper specimens and plant samples (FastDNA Spin kit, MP Biomedicals, USA). Nested PCR was performed with primers specific for phytoplasma 16S rRNA, R16mF2/R1 (Gundersen and Lee, 1996) and fU5/rU3 (Lorenz *et al.*, 1995). PCR products were subjected to RFLP with *Mse*I and *Alu*I restriction endonucleases. Representative PCR products of the expected size (880 bp) from samples from each field were purified (EZNA Cycle Pure kit, Omega Bio-Tek, USA), cloned (pGEM-T Easy Vector, Promega), and sequenced (Princess Margaret Hospital, Toronto, Canada).

Results

The four fields surveyed yielded specimens of *Balclutha impicta* (Van Duzee), *Delphacodes campestris* (renamed *Muirodelphax arvensis* in 2010), and *Graminella nigrifrons*. Only *G. nigrifrons* produced PCR amplicons for phytoplasmas; 263 out of 322 (81.7%) samples collected (table 1). PCR/RFLP confirmed phytoplasmas in plant samples as members of groups 16SrI-B (peach-almond acc. PRU0382, cultivar Kando; peach acc. PRU0445, cultivar HW271; apricot acc. PRU0134, cultivar Harglow; apricot acc. PRU0142, cultivar Sundrop); 16SrVII-A (peach acc. PRU0164, cultivar Harcrest; peach acc. PRU0168, cultivar Harrow Diamond; peach acc. PRU0176, cultivar Siberian C; peach acc. PRU0180, cultivar Vanity; plum acc. PRU0406, cultivar Pembina); 16SrX-C (peach acc. PRU0336, cultivar Redhaven; apricot acc. PRU0147, cultivar Wescot).

Table 1. Phytoplasma groups identified in *G. nigrifrons* specimens and in plant samples randomly collected from *Prunus* and *Pyrus* fields at the CCG.

Field	Accession No.	<i>G. nigrifrons</i> PCR positive/tested	Phytoplasma 16S ribosomal groups
T4 East-peach	PRU0168, PRU0176, PRU0180, PRU0445, PRU0382, PRU0336	79/89	16SrI, 16SrVII, 16SrX
T4 Centre-apricot	PRU0134, PRU0142, PRU0147	39/85	16SrI, 16SrVII, 16SrX
T3 West-plum and cherry	PRU0406	81/81	16SrI, 16SrX
T4 West-pear	PYR0190	63/66	16SrI, 16SrX



Figure 1. *G. nigrifrons*. Picture taken by Tom Murray. (In colour at www.bulletinofinsectology.org)

BLAST analysis of partial 16S rDNA sequences of representative phytoplasmas detected in *G. nigrifrons* samples showed 99% of sequence identity with those from groups 16SrI, 16SrX and 16SrVII at NCBI and those from plant samples, including those of phytoplasmas previously reported at CCG in peach (16SrI-B, HQ450211 and 16SrVII-A, GU223903), as well as, a 98.8% of sequence identity with that of the phytoplasma reported in pear associated with pear decline in Ontario (16SrX-C, GU565960).

Discussion

More than 75% of all confirmed phytoplasma vector species are monophagous to polyphagous members of Deltocephalinae, and transmit one or more phytoplasma taxa (Weintraub and Beanland, 2006).

Three different phytoplasma groups have been identified in *G. nigrifrons* that we collected from four fields planted with peach, apricot, plum and pear, suggesting that this leafhopper is polyphagous and may contribute to disease spread by moving from field to field. Phytoplasma of groups 16SrI and 16SrVII were simultaneously detected in *G. nigrifrons* from the field T4-Centre apricot; while those from groups 16SrI and 16SrX were found in fields T3-West plum and cherry; T4-East-peach, and T4-West pear, which suggest that *G. nigrifrons* populations possess a complex ecology.

The 16SrX and 16SrI were the predominant phytoplasma groups identified in *G. nigrifrons*. This supports previous findings of *G. nigrifrons* as a vector for group 16SrI; however this is the first report of group 16SrX being carried by *G. nigrifrons* and affecting *Prunus* species in Ontario, Canada.

A highest 16S rDNA sequence identity was found between phytoplasmas identified in insect and plant samples. This suggests that *G. nigrifrons* may be a potential vector for 16SrI, 16SrVII and 16SrX phytoplasmas. Transmission trials are required to determine its vector role and ecological factors influencing transmission of phytoplasma diseases in *Prunus* and *Pyrus* at the CCG.

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***Cacopsylla melanoneura* (Foerster): aestivation and overwintering habitats in Northwest Italy**

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Abstract

Cacopsylla melanoneura (Foerster) is considered one of the most important vectors of 'Candidatus Phytoplasma mali', the phytoplasma associated with apple proliferation disease. In the last years several studies concerning the biology, the infectivity and the transmission activity of this psyllid highlighted the crucial role of the overwintered adults in the epidemiology of the disease. However very few information on the overwintering sites are available. The aestivation and overwintering habitats of *C. melanoneura* in Northwest Italy were studied. Samplings were carried out in conifer forests, chosen by following the direction of warm ascending currents. The altitudinal distribution of the psyllid as well as the shelter plants were investigated. Adults of *C. melanoneura* were collected on *Abies alba* (Miller), *Picea abies* (L.), *Pinus sylvestris* (L.) and *Larix decidua* (Miller) predominantly at an altitude range between 1,350 and 1,650 m a.s.l. It was possible in this way for the first time to follow the whole life cycle of *C. melanoneura* throughout the year.

Key words: *Cacopsylla melanoneura*, shelter plants, conifers, aestivation, overwintering, apple proliferation.

Introduction

The psyllid *Cacopsylla melanoneura* (Foerster) (Hemiptera: Psylloidea) is considered one of the most important vectors of 'Candidatus Phytoplasma mali', the prokaryote associated with the apple proliferation (AP) disease, (Tedeschi *et al.*, 2002). Studies on the epidemiology of AP and on the infectivity of the vector showed evidence of the crucial role of overwintered adults of *C. melanoneura* in transmitting 'Ca. P. mali' (Tedeschi *et al.*, 2002, 2003, Tedeschi and Alma, 2004; Pedrazzoli *et al.*, 2007) when they reach, often already infected, apple and hawthorn plants at the end of the winter. On the contrary fairly little is known about newly emerged *C. melanoneura* behaviour after the migration to shelter plants. For this reason, new surveys were carried out to identify some aestivation and overwintering sites of *C. melanoneura* in Northwest Italy. In the present work we studied the altitudinal distribution of *C. melanoneura* and the suitability of different conifer species as shelter plants during aestivation and overwintering periods.

Materials and methods

The area of study (Aosta Valley and Piedmont, Northwest Italy) was chosen according to the incidence of AP disease and to the abundance of *C. melanoneura* specimens recorded in the last years.

Coniferous forests, potentially reachable by the psyllids, were spotted, as suggested by Čermák and Lauterer (2008), following the direction of warm ascending currents. These flows rise from apple orchards located in lowlands up to the mountains and promote psyllid movements. Samplings were carried out once a week from June to December at different altitudes by means

of a sweep net with a 5 m telescopic handle enabling to reach the highest parts of the plants. Samples were collected at different altitudes between 800 and 2,077 m a.s.l. on different species of conifers: *Abies alba* (Miller), *Picea abies* (L.), *Pinus sylvestris* (L.), *Larix decidua* (Miller) and *Pinus cembra* (L.). Once the most suitable shelter plants were localized, newly emerged adults of *C. melanoneura* were collected from apple and hawthorn plants and caged on branches of different species of conifers between 1,442 and 1,636 m a.s.l. to follow the entire cycle of the insects until the re-immigration period into apple orchards or on hawthorn plants. A total of 1,405 specimens were isolated in 74 net cages until the end of January when normally the first remigrants appear on apple and hawthorn plants. Then the cages were brought to the laboratory and alive psyllids counted.

Results

Forty-nine samplings were carried out at different altitudes between 800 m. and 2,077 m. a.s.l. A total number of 266 *C. melanoneura* specimens were collected during aestivation and overwintering periods on conifers. *C. melanoneura* was found on Norway spruce, European silver-fir, Scots pine, larch, but not on Swiss stone-pine. The highest psyllid density was recorded on Norway spruce followed by European silver-fir, larch and Scot's pine. Norway spruce was the dominant conifer species in the forests under investigation, followed by larch while the other species were sporadic.

For this reason the altitude distribution was in depth analysed only in relation to *P. abies* and the results are shown in figure 1. *C. melanoneura* was more common at the altitude ranging between 1,350 and 1,650 m a.s.l..

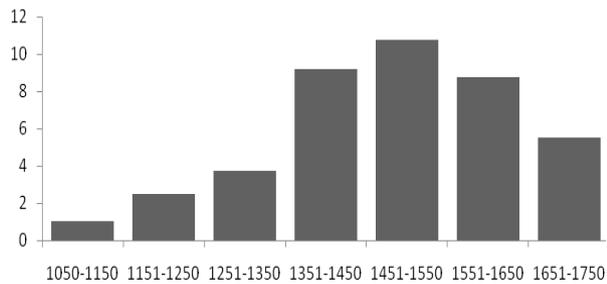


Figure 1. Altitudinal distribution of *Cacopsylla melanoneura* (Foerster) captured on *Picea abies* (L.).

In January living specimens were found in the net cages placed on European silver-fir, Norway spruce, Swiss stone-pine and Scots pine. All the psyllids isolated on juniper died. *C. melanoneura* alive specimens were observed on larch only until November, before the needle fall. On January all the psyllids isolated on larch were dead. Among the 1,405 individuals isolated on different species of conifers only 19.2% survived. *A. alba* revealed to be the best shelter plant with 36.5% of survivals, followed by *P. cembra* (20.4%), *P. abies* (19.9%) and *P. sylvestris* (3.1%).

Discussion

The present work allowed to localize for the first time the habitats of *C. melanoneura* in Northwest Italy during the aestivation and overwintering period. As proposed by Čermák and Lauterer (2008) the warm ascending currents should be fundamental for psyllid movements, allowing quite long migrations. In our case the distance between the shelter plants and the closest apple orchard was around 2 km, but we cannot exclude longer migration distances considering the location of the other orchards. The samplings by means of the telescopic sweep net enabled to identify the shelter plant species colonized by *C. melanoneura* and to determine the altitudinal distribution of the psyllid. Almost all the conifers analyzed seem suitable as shelter plants. No specimens were found on *P. cembra*, but very few plants of this species were analysed. Anyhow the experiments with the net cages revealed that *P. cembra* is a suitable shelter plants. In the case of larch, all the specimens isolated in the net cages died before January. We have to consider that larch is the only deciduous conifer in our regions and probably this is the reason why all the psyllids died. Considering also that we were able to collect *C. melanoneura* on larch until needle fall, the results obtained make us suppose that *C. melanoneura* has some feeding activity on conifers, as proposed also by Jackson *et al.* (1990), and maybe also some movement activity between different shelter plants.

In the present work it was not possible to define some preference towards the different conifers. Most of the psyllids were collected on Norway spruce, but this is the predominant species in the investigated area. So there is a higher probability for *C. melanoneura* to reach this species rather than the others.

The study on the altitudinal distribution revealed that *C. melanoneura* finds the best climate conditions for aestivation and overwintering between 1,350 and 1,650 m a.s.l.. It will be interesting to analyse if during the period June-January some vertical migration occur within the same forest according to changes of climatic conditions. So further studies are required to deepen this aspect. The finding of the sites colonised by *C. melanoneura* during a quite long period of the year, after the migration from apple and hawthorn plants opens new perspectives on the study of the phytoplasma-vector relationships during the season and thus of the epidemiology of AP. Moreover these information could provide new indications for prompt control strategies, for instance exactly forecasting the migration to apple orchards in relation to the climatic conditions in the overwintering sites.

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Aster yellows phytoplasma in grapevines: identification of vectors in South Africa

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Abstract

Since the discovery of aster yellows phytoplasma on grapevine in South Africa in 2006, a concerted effort by various research groups to identify the vector(s) was co-ordinated by Winetech. This included qualitative and quantitative surveys for two years of leaf- and planthoppers occurring in vineyards in affected areas, testing specimens of leaf- and planthopper species for the presence of aster yellows phytoplasma with PCR, transmission experiments, and relating presence of leaf- and planthoppers in the field to the time of disease transmission. Twenty-eight leaf- and planthopper species/species groups have been recorded from vineyards in aster yellows-infected regions. The four most abundant species/species groups were *Acia lineatifrons* (Naudé), *Austroagallia* spp., *Cicadulina* spp. and *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae). *Austroagallia* spp. and *M. fuscovaria* repeatedly tested positive for the presence of AY. Results of transmission experiments and a field trial to determine leaf-/planthopper abundance in vineyards along with time of transmission in the field suggest that *M. fuscovaria* is a vector of aster yellows phytoplasma in South Africa. The concerted effort between industry and researchers allowed for fast identification of a potential vector of aster yellows phytoplasma in South Africa.

Key words: Cicadellidae, Coelidiinae, leafhopper, *Mgenia fuscovaria*, phytoplasma.

Introduction

A phytoplasma was recorded for the first time from grapevine, *Vitis vinifera* L. (Vitaceae), in South Africa in 2006 (Engelbrecht *et al.*, 2010). Aster yellows phytoplasma (AY), 16SrI group (Engelbrecht *et al.*, 2010), until recently had been reported to occur in grapevine in two regions but lately was also found in a third region, also in the Western Cape Province. Aster yellows disease (AY) has been recorded in other countries from a broad host range (Hogenhout *et al.*, 2008). Economic losses caused by infection of cultivated plants can be severe. Phytoplasmas can be transmitted through phloem-feeding insect vectors (Auchenorrhyncha (Cicadellidae, Fulgomorpha, Psyllidae)) (Weintraub and Beanland, 2006). Identification of vectors is essential if disease spread is to be managed.

In general, identification of vectors of phytoplasmas of grapevine (grapevine yellows) has proved difficult, and in several instances these have not been identified (Constable, 2010). Due to the urgency of identifying vector(s) in order to manage the disease effectively, whilst recognizing the challenges this entails, Winetech (Wine Industry Network for Expertise and Technology) initiated a multidisciplinary programme in 2008 involving researchers from several organizations for rapid identification of the vector(s) of AY in South Africa.

Materials and methods

To identify potential vectors, the programme commenced in 2008 with qualitative and quantitative surveys to identify leaf-/planthopper and other potential vector species present in vineyards and to determine their abundance. Insects in two grapevine growing areas in four vineyards each where AY occurs were monitored with vacuum sampling (DVAC) for two years. Where possible, specimens of potential vectors were identified to species level. Subsamples of potential vector species collected were tested for the presence of phytoplasma with nested PCR (Engelbrecht *et al.*, 2010). To confirm that species that tested positive are vectors of AY, transmission experiments using field-collected insects from highly AY-infected vineyards were conducted. Field-collected insects, rather than laboratory-reared insects, were used because of the difficulties experienced in establishing cultures. Individual phytoplasma-free grapevine (cv. Chardonnay) or *Nicotiana benthamiana* L. (Solanaceae) plants served as recipient plants. To confirm that insects and plants from collection sites for transmission experiments were infected with AY, leaves from subsamples of plants and subsamples of leafhopper specimens were tested for AY with real-time PCR (Angelini *et al.*, 2007). Recipient plants were confirmed AY-free in the same manner prior to transmission experiments. Plants were tested starting five weeks post-AY transmission.

In addition, identification of vector(s) was done by monitoring leaf-/planthopper populations for 12 months and correlating this to the time of AY infection of grapevines in the field. To this end, 10 AY-free grapevine plants (cv. Cabernet franc, cv. Chardonnay, cv. Chenin blanc) together with 10 yellow sticky traps and, when available, periwinkle (*Catharanthus roseus* (L.) G. Don (Apocynaceae)) plants, were placed in two AY-infected vineyards, the rationale being that plants would only become infected when AY-infected vectors are present and feed on them. Bait plants were replaced weekly for a year, treated with a systemic insecticide and maintained in insect-free enclosures and were subsequently tested for AY with real-time PCR (Angelini *et al.*, 2007) starting seven weeks after exposure in the field. All leaf-/planthopper species collected on the yellow sticky traps were identified to species level where possible.

Results

Since 2008, 28 leaf-/planthopper species/species groups have been recorded from grapevine and vegetation within vineyards in regions where AY occurs. The most abundant species/species groups were *Acia lineatifrons* (Naudé), *Austroagallia* spp., *Cicadulina* spp. and *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae). Amongst these, *Austroagallia* spp. and *M. fuscovaria* tested positive for phytoplasma. Furthermore, *M. fuscovaria*, was collected in the largest number from grapevines, with very few collected from weeds. In subsequent transmission experiments with field-collected *M. fuscovaria*, and based on real-time PCR tests, AY was transmitted to three out of 20 grapevine plants. None of the control plants, not exposed to *M. fuscovaria* and kept under the same conditions as recipient plants, tested positive for AY. Thus far, AY-transmission by field-collected *Austroagallia* spp. to individual *N. benthamiana* plants has not been demonstrated.

A comparison of the time of AY infection in vineyards with leaf-/planthopper abundance over a period of 28 weeks also suggests that AY transmission was due to *M. fuscovaria*, which is the most prevalent species in the vineyard where AY-infected bait plants were recorded. The only other species whose presence coincided with the time of exposure of bait plants and the latter becoming infected with AY, were single individuals belonging to three species collected in the course of four different weeks, whereas *M. fuscovaria* occurred in large numbers on these occasions.

Discussion

Because *M. fuscovaria* was the dominant species and in some instances the only species found on the traps in the weeks when AY-infected bait plants were obtained it is likely that AY-transmission was due to *M. fuscovaria* feeding. This, together with the transmission of AY to

healthy grapevine plants maintained in insect-free facilities using field-collected *M. fuscovaria*, suggests that this species is a vector of AY in South Africa. Planned work includes transmission experiments with AY from grapevine to grapevine with specimens of the *Austroagallia* species group to determine if they, too, are vectors.

The qualitative and quantitative surveys together with direct tests of species for AY allowed us to focus on specific species for transmission experiments to assess whether they serve as vectors. Monitoring leaf-/planthopper populations together with determining the time of AY transmission not only facilitated the identification of potential vectors, but will also assist with the development and timing of control measures. The concerted effort by a multi-disciplinary team co-ordinated by Winetech has resulted in a rapid identification of a potential vector of AY in South Africa.

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Phytoplasmas identical to coconut lethal yellowing phytoplasmas from Zambesia (Mozambique) found in a pentatomide bug in Cabo Delgado province

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Abstract

Phytoplasmas are associated with several syndromes of lethal yellowing type of coconut. Only in Florida the insect vector of the local coconut lethal yellows was identified to be *Myndus crudus* (Cixiidae). In Mozambique, phytoplasmas are associated with a coconut lethal yellowing type syndrome. In the Cabo Delgado province, some pentatomids of the species *Platacantha lutea* were found carrying the same phytoplasmas as those identified in the diseased coconut on which they were found. As some pentatomids are vectors of other phloem-restricted pathogens associated with a coconut lethal yellowing type syndrome, hypothesis of a possible pentatomid as a vector is proposed.

Key words: Pentatomidae, *Platacantha lutea*, vector, Derbidae, coconut lethal yellowing.

Introduction

Several syndromes of lethal yellowing type (LYTS) occur worldwide on coconut and other palm species. For most of them, phytoplasmas are specifically associated with the syndrome. However, the phytoplasmas belong to different 16SrDNA groups and inside each group, it is possible to identify several subgroups. For instance, the phytoplasmas associated with the most studied LYTS - coconut lethal yellowing (LY) occurring in the Caribbean- belong to the group 16SrIV. But in this group, the number of subgroups has been continuously increasing these last years, now reaching six subgroups (Harrison *et al.*, 2008).

Other phytoplasmas are associated with LYTS in West Africa, as for instance those associated with Cape St Paul Wilt in Ghana (CSPW), “maladie de Kaïncopé” in Togo or Awka disease in Nigeria (Dollet *et al.*, 2008). Until the end of the 90s, they were listed in the same groups as the LY phytoplasmas, the 16SrIV (Harrison *et al.*, 1994; Lee *et al.*, 1998). Then, they were included in a new group, 16SrXXII (Wei *et al.*, 2007). In Mozambique - East Africa- phytoplasmas very close to those associated with CSPW occur in coconuts, causing a LYTS (Mpunami *et al.*, 1999).

Among all these LYTS only one insect vector has been identified: *Myndus crudus* (Homoptera: Cixiidae), vector of the coconut LY in Florida (Howard *et al.*, 1983). Search for the insect vector(s) of LYTS in other countries stays fruitless. In Ghana another cixiid – *Myndus adiopodoumensis*, now known as *Myndodus adiopodoumensis* (Ceotto and Bourgoin, 2008)- was suspected to be the vector, but it was impossible to prove it (Philippe *et al.*, 2009). In only one insect - Derbidae – the CSPW phytoplasma was detected by PCR but no transmission of the syndrome could be obtained (Philippe *et al.*, 2007). In Tanzania, some insects were found PCR positive for phytoplasmas but experimental tran-

smision never occurred (Mpunami *et al.*, 2000). Then, because of the diversity of the phytoplasmas involved in the coconut LYTS and because of the diversity of the fauna existing in the different regions where these LYTS occur, we can imagine there could be a diversity of insect vectors involved in the transmission of the different coconut LYTS. We describe in this article, for the first time, the possibility of a pentatomid bug as a phytoplasma vector of the coconut LYTS in Cabo Delgado province.

Materials and methods

A survey was made in Zambesia and Cabo Delgado provinces in September 2007. Samples were taken from palms with LYTS. Every time when it was possible diseased trees were cut and leaves and flowers examined. Samples from flowers or trunk and insects were kept dried until arrival in the laboratory. DNA extractions were performed with CTAB or with DNeasy Blood and tissue Kit QIAGEN, and direct PCR with primers P1/P7 or nested PCR (P1/P7 followed with G813/AKSR) were run according to already described protocols (Tymon *et al.*, 1997). PCR products were cloned and sequenced. Sequences were analysed with BLAST. Insect species were determined by J. M. Maldes.

Results

In Zambesia province all diseased coconut samples had a 16SrDNA sequence very close to the sequences published by Mpunami *et al.*, (1999) corresponding to the local LYTS (16SrXXII). No insect – apart from several derbids – were found on diseased coconuts.

In Cabo Delgado province, in Mecufi, (south of Pemba), in a hamlet south of the village four palms at



Figure 1. *P. lutea* found on a diseased coconut in Mecufi, Cabo Delgado province. (In colour at www.bulletinofinsectology.org)

different stages of LYTS were observed. One palm at the beginning of the yellowing was cut and dissected. The removal of the leaves one by one revealed more than 40 predominantly green pentatomid bugs (figure 1) identified as *Platycanthis lutea* Westwood, 1837 (Pentatominae – Eysarcorini). 7 *P. lutea* out of 12 individuals tested and a sample of the dissected palm tree were found positive by direct PCR. Using nested PCR 10 of 12 *P. lutea* were positive. The 16SrDNA sequences obtained from the bugs and from the palm were all identical or very close (99%) to the 16SrXXII sequences.

Discussion

These results show for the first time that pentatomids (Heteroptera) can carry phytoplasma associated with coconut LYTS. It could be only the consequence of insects feeding in the sap of an infected coconut tree. However when we performed similar tests with a wide range of insects feeding on coconut affected by the CSPW in Ghana we rarely found a positive derbid (Phillippe *et al.*, 2007). So far, apart from the only positive result from *Myndus crudus* (Auchenorrhyncha) in Florida (Howard *et al.*, 1983) the vector(s) of the different LYTS associated with phytoplasmas are not yet clearly identified. May be one must think of an insect outside the Auchenorrhyncha group. As a matter of fact, a pentatomid (Hemiptera) is the vector of a coconut LYTS in Latin America: several species of *Lincus* sp. (Pentatomidae) are vector of phloem restricted trypanosomatids (*Phytomonas* sp.) responsible for “Hartrot” of the coconut (Louise *et al.*, 1986; Dollet, 2001).

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Development of innovative methods for trapping phytoplasma vectors by attractive infochemicals

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Abstract

Some examples of attempts to develop biotechnical control methods for psyllids vectoring fruit tree phytoplasmas by sticky traps lured with newly detected infochemicals are reported. The apple psyllid *Cacopsylla picta* is the main vector of 'Candidatus Phytoplasma mali', the agent associated with apple proliferation disease in Germany and most neighbouring countries. Complex interactions between *Malus domestica*, the psyllid *C. picta*, and the phytoplasma were investigated in laboratory and field experiments. Results showed that emigrants of *C. picta* are able to distinguish the odours of healthy and infected apple trees and preferred the odours of infected trees. This means that the phytoplasma directly manipulates both the plant physiology by producing an attractive compound, and the psyllid behaviour, resulting in a better spread within its host plant population. The compound responsible for the attraction of the vector was collected from the headspace of infected apple plants and identified by gas chromatography coupled with mass spectrometry. It attracted both genders of *C. picta* and will be used for the development of traps for monitoring or mass trapping.

Key words: 'Candidatus Phytoplasma mali', apple proliferation, *Cacopsylla picta*, *Cacopsylla melanoneura*, infochemicals, monitoring, mass trapping.

Introduction

During their evolution, pathogens and their hosts must pursue different strategies to avoid reciprocal detrimental effects. Investigating multitrophic systems, up to now only limited information is available on the evolution of interactions between plants, phytoplasmas, and their insect vectors. The results of investigations on the chemically mediated interactions of the cell wall-lacking bacterium 'Candidatus Phytoplasma mali', its host plant apple tree (*Malus domestica*), its main vector *Cacopsylla picta* and the (partial) vector *C. melanoneura* (Hemiptera: Psyllidae) (Mayer *et al.*, 2009) are reported. The apple proliferation phytoplasma causes major economic yield losses by inducing tasteless dwarf fruits on its host plant. It can modify the emitted volatiles of its host plant, resulting in their increasing attractiveness for its vector, compared to a closely related non-vector (Mayer *et al.*, 2008a, 2008b, 2011). Furthermore, we present our attempts to use these findings for the development of innovative biotechnical control methods for psyllid species vectoring fruit tree phytoplasmas by sticky traps lured with newly detected infochemicals.

Materials and methods

Investigations were done by measuring the population dynamics of closely related species on their alternate host plants. Infections of vector insects and plants were analyzed by PCR using specific primers selective for fruit tree phytoplasmas. Further, the olfactory preferences of psyllids were investigated in Y-shaped olfac-

tometer bioassays. Fitness parameters of insects were measured on infected and non-infected plants. Host plant odours were collected using different headspace sampling methods, thermodesorption and afterwards analysed using gas chromatographs coupled with mass spectrometers.

Results and discussion

We could show that the phytoplasma directly manipulates the plant physiology and indirectly the vector behaviour by attracting *C. picta* to infected plants, leading to a better spread within its host plant population (Mayer *et al.*, 2008a, 2008b). In contrast, its vector evolved mechanisms to minimize harmful effects emanated by the phytoplasma: the infection is tolerated by adults and detrimental effects to the offspring are avoided by an adapted oviposition behaviour (Mayer *et al.*, 2011). The compound responsible for the attraction of the vector was collected from headspace of infected apple plants and identified by gas chromatography coupled with mass spectrometry. The sesquiterpene β -caryophyllene attracted both genders of *C. picta* and will be used for the development of traps for monitoring and/or mass trapping of *C. picta* (Gross and Mayer, 2010).

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Population dynamics of *Cacopsylla pruni* and 'Candidatus Phytoplasma prunorum' infection in North-Eastern Italy

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Abstract

A two year surveys was conducted in Friuli Venezia Giulia region (north-eastern Italy) with the aims to improve knowledge about biological characteristics of *Cacopsylla pruni* Scopoli, and to ascertain its role in the spread of different 'Candidatus Phytoplasma prunorum' strains. Insects were captured starting from March till the end of April in apricot orchards with high European stone fruit yellows incidence. For phytoplasma detection a nested PCR protocol based on *aceF* gene was adopted. Results confirmed that the percentage of phytoplasma positive insects increased from March to April, because they fed on infected trees. During the reimmigration season the percentage of females was higher than that of males, especially after coupling time. Besides a very high percentage of infected *C. pruni*, no significant differences related to the sex were observed. 'Ca. P. prunorum' strains of *aceF*-A, *aceF*-C and *aceF*-E subgroups, were mainly found in the captured *C. pruni* population.

Key words: *aceF* gene, nested PCR, European stone fruit yellows.

Introduction

Cacopsylla pruni (Scopoli) is the vector of 'Candidatus Phytoplasma prunorum', associated with European stone fruit yellows (ESFY) disease (Carraro *et al.*, 1998). *C. pruni* is a univoltine psyllid widespread in Europe. In spring eggs of *C. pruni* are laid by reimmigrant adults on *Prunus* spp.; individuals of new generation develop on *Prunus* spp. and new adults abandon the original host in summer to overwinter on conifers (Thébaud *et al.*, 2009). In Friuli Venezia Giulia (FVG) region, north-eastern Italy, several *Prunus* species were reported as natural host of ESFY (Carraro *et al.*, 2002) and different strains of 'Ca. P. prunorum' with different virulence were observed (Ermacora *et al.*, 2010). Recently, in order to investigate phytoplasma strains variability, molecular tools for 'Ca. P. prunorum' detection and characterization were developed (Danet *et al.*, 2008, Martini *et al.*, 2010). Although in our conditions *C. pruni* population density usually does not cause direct damages to the crops their ability to transmit 'Ca. P. prunorum' requires a correct orchard management in order to prevent the spread of ESFY. With the aim to monitor the presence of *C. pruni* and evaluate their infectivity for a correct integrated pest management, an extensive survey was conducted during 2010 and 2011 in an area with high ESFY disease pressure. Another aim of this research was to acquire more knowledge about the biology of *C. pruni*.

Materials and methods

During 2010 and 2011, from March till the end of April, a total of 383 individuals of reimmigrant *C. pruni* were collected in apricot orchards located in areas with high ESFY disease pressure. In the monitored orchards

ESFY incidence was very high (about 90% of PCR positive plants) and management program was insecticide free in order to avoid interferences with the natural dynamic of *C. pruni* population. Insects were captured by the beating method, and immediately stored in 80% ethanol. *C. pruni* were individually observed under stereomicroscope for proper identification and sex characterization. Nucleic acids were extracted from the insects according the protocol proposed by Doyle and Doyle (1990) with slight modifications.

Protocol adopted for 'Ca. P. prunorum' detection in the samples was based on *aceF* gene, with a direct PCR with primers AceFf1/AceFr1 followed by nested-PCR with primers pair AceFf2/AceFr2 (Danet *et al.*, 2008). RFLP of nested-PCR amplicons using endonucleases *Bpi*I, *Hae*III and *Tsp*509I was adopted for characterization of 'Ca. P. prunorum' strains (Martini *et al.*, 2010).

Results

During the two considered years, presence of reimmigrant *C. pruni* was detected from March 22 till the end of April, with a peak of population in the period 12-16 April. Mating period lasting from the beginning till the 20th of April. Male and female percentages varied in the considered period with a substantial reduction of male presence. Percentage of insects that hosted 'Ca. P. prunorum' increased during their presence on apricots without significant differences between males and females. Considering the characterization of 'Ca. P. prunorum' strains present in the insect analysed population, a clear prevalence of strains of *aceF*-A, *aceF*-C and *aceF*-E subgroups was reported, *aceF*-B strains were rare and *aceF*-D strains were not detected; in 19% of the cases we found mixed infections.

Table 1. Presence of males and females of *C. pruni* during the sampling period and their infectivity.

	Capture period					
	22-25 March	30 March-02 April	05-09 April	12-16 April	19-22 April	26-30 April
% males	34.6	10.5	38.9	36.1	25.0	11.5
% females	65.4	89.5	61.1	63.9	75.0	88.5
% males ' <i>Ca. P. prunorum</i> ' positive	57.9	75.0	42.3	71.4	100.0	33.3
% females ' <i>Ca. P. prunorum</i> ' positive	55.6	61.8	63.7	84.6	80.0	82.6

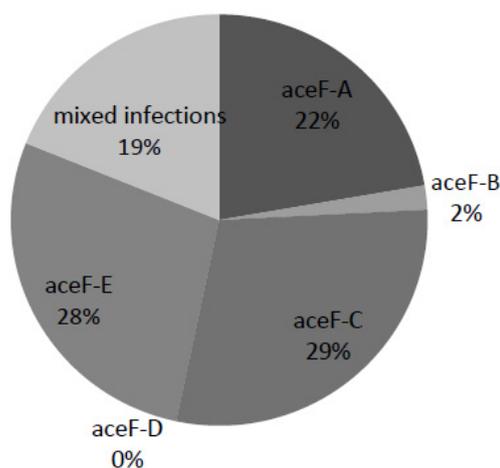


Figure 1. Characterization of '*Ca. P. prunorum*' strains in *C. pruni* based on *aceF* gene.

Discussion

This survey confirmed the presence of *C. pruni* and its high infectivity in the FVG region, where ESFY disease is endemic and affects wild and cultivated *Prunus*. As reported by Carraro *et al.* (2004) it was confirmed that overwintering adults infectivity increase after their reimmigration on infected *Prunus* spp.. In our case the infection rate of the first capture reimmigrants was 56.4% and reached a plateau slightly exceeding 80% in the last two captures. Results obtained on the male/female percentages when the reimmigrant individuals are present in the orchards, evidenced the longevity of females that at the end of the monitored period represented 88.5% of the population. The longevity of females could be explained from an evolutionary point of view considering the crucial role of the females for the species survival. Another hypothesis to explain the variability of the male presence during the monitoring period and the changes in their mean infectivity could be a mortality of the males after the copulation. Concerning '*Ca. P. prunorum*' molecular characterization, the obtained results indicated mainly the presence of strains of AceF-A,-C, and -E subgroups and a sporadic

presence of AceF-B strains. Mixed infections were also detected and explained with the presence of the same strains in plants of *Prunus* spp. in the same area.

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Survey on the presence of *Cacopsylla pruni* in Turkey: preliminary results

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Abstract

Although '*Candidatus Phytoplasma prunorum*' has been previously detected in different regions of Turkey, its vectors have not been identified yet. A survey was conducted in 2010 and 2011 in order to determine the presence of *Cacopsylla pruni* populations in six different fruit tree producing areas. The psyllid was found in Adana, Mersin, Bursa and Yalova on either *Prunus spinosa* or wild plums (*Prunus* spp.) and also on fir trees. The mean percentage of individuals of *C. pruni* infected with *Ca. P. prunorum* was around 23% in Mersin.

Key words: '*Candidatus Phytoplasma prunorum*', *Prunus* spp., psyllid vector.

Introduction

Stone fruits are important fruit crops in Turkey, where local land-races and new varieties are cultivated. In recent years, diseases caused by phytoplasmas have become increasingly important in stone fruit trees in this country. Severe decline of plum and apricot trees growing in nurseries and commercial orchards has recently reached alarming levels. For example, in foreign (e.g. 'Precoce de Tyrinthe', 'Fraccaso') and local (e.g. 'Sakit', 'Şekerpare', 'Alkayısı') apricot cultivars symptoms have been observed, i.e. deformation and rolling of the leaves, reduced yield, and decline; '*Candidatus Phytoplasma prunorum*' was detected on apricot or plums from different regions of Turkey (Jarausch *et al.*, 2000; Çağlayan *et al.*, 2004; Ulubaş Serçe *et al.*, 2006).

Despite these records, no information was presented on the presence of vectors of phytoplasma diseases and their prevalence in Turkish orchards. Therefore, the main objective of this research was to survey the presence of *Cacopsylla pruni* Scopoli the expected vector of '*Ca. P. prunorum*' in Turkey.

Materials and methods

Sixteen localities from six provinces where apricot, peach and plum growing is important in Turkey, were selected for surveys of *C. pruni* (figure 1). These surveys were carried out during the springtime of 2010 and 2011, respectively. Psyllids were essentially collected from wild *Prunus* species (e.g. *P. spinosa*, *P. cerasifera*), or *Prunus* orchards, and conifers (table 1).

DNA was extracted from *C. pruni* and then the presence of '*Ca. P. prunorum*' was determined from individual insects with the primers ESFYf/r as described by Thébaud *et al.* (2009).

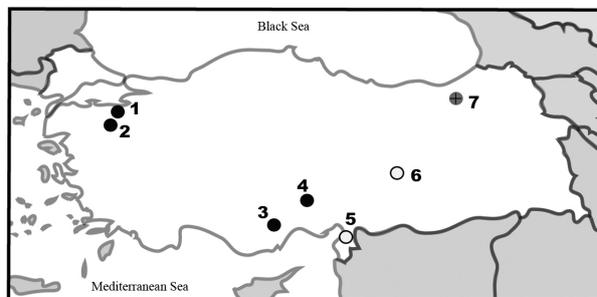


Figure 1. Sampled localities where the psyllid *C. pruni* was found (●) or not found (○) in Turkey in springs 2010 and 2011. Locality 7 was described by Güçlü and Burckhardt, 1996. See table 1 for more details on the host plants and the number of collected psyllids.

Results

Remigrant *C. pruni* individuals were found and collected at the end of March in all surveyed localities except those from Hatay and Malatya (table 1). Specimens on *P. spinosa* were found only in Bursa and Yalova provinces. In Adana and Mersin, the psyllids were abundant on wild *Prunus* and *Prunus* trees inside apple orchards. Psyllids have been found but very rarely on other host plants: *Malus domestica*, *Cydonia oblongua* and *Crataegus* ssp. Many overwintered individuals were collected from *Abies nordmanniana* subsp. *bornmulleriana* and *Pinus* ssp. from Uludağ, but never from other locations even there has been fir trees in the forests.

Nymphs of *C. pruni* were first detected on wild *Prunus* trees in the first half of May 2010 in Mersin. In June 2010 nymphs were also collected from apricot and peach trees.

The higher percentage of remigrant individuals of *C. pruni* infected with '*Ca. P. prunorum*' was around 23% and was found in Mersin (7 positives out of 30 specimen tested).

Table 1. Localities sampled during springs 2010 and 2011, and number of *C. pruni* collected on different host plants. “0” indicates that psyllids have been searched but not found, “-” not investigated. Pspi: *Prunus spinosa*, Psp: *Prunus* sp., Pdo: *P. domestica*, Cra: *Crataegus* sp., Cyd: *Cydonia oblongua*, App: apple, Apri: apricot, Con: conifers.

Province	District	Host-plant							
		Pspi	Psp	Pdo	Cra	Cyd	App	Apri	Con
1-Yalova	Center	27	-	-	-	-	-	-	-
	Keles	2	-	-	-	-	-	-	-
2-Bursa	Orhaneli	11	3	-	1	-	-	-	-
	Nilüfer	65	-	-	-	-	-	-	-
	Uludağ	-	-	-	-	-	-	-	143
3-Mersin	Silifke	-	> 100	-	2	-	1	0	-
	Mut	-	-	-	-	-	-	0	-
4-Adana	Pozanti	-	180	-	-	1	-	-	-
	Ceyhan	0	-	-	-	-	-	-	-
5-Hatay	Samandağ	0	0	-	-	-	-	-	-
	Center	0	0	-	-	-	-	-	-
	Yayladağ	0	0	-	-	-	-	-	-
	Iskenderun	0	0	-	-	-	-	-	-
6-Malatya	Doğanşehir	-	0	0	-	-	-	-	-
	Yeşilyurt	-	0	-	-	-	-	-	-
	Akçadağ	-	0	-	-	-	-	-	-

Discussion

These preliminary data presented here are the results of two consecutive spring surveys of *C. pruni* in Turkey. They exhibit limited distribution of the psyllid in some-surveyed areas, but abundant in the localities of the provinces of Bursa, Adana and Mersin. These are important provinces for growing apricot, peach and plum trees, where ‘*Ca. P. prunorum*’ infections have been recorded in previous research (Çağlayan *et al.*, 2004; Ulubaş Serçe *et al.*, 2006). Overwintered psyllids have been caught on conifers at Uludağ (National park in the Bursa province, 1510 m alt.), near (10-25 km) localities where we have found them on *P. spinosa*. Actually, in this region, the psyllids could complete their life cycles, and so they could play a major role in the phytoplasma dissemination at a local scale (Thébaud *et al.*, 2009).

Fortunately *C. pruni* has not been found near Malatya, the most important apricot growing province. In this region, the conifers are not common and the nearest conifer forests are 100-120 km as the crow flies. Thus, the insect would not be able to survive in that province.

In France, the highest range of phytoplasma infected remigrant *C. pruni* was recorded as 15% but is generally around 3% (Yvon *et al.*, 2004). In Turkey, this range was determined as 23% what seemed considerably high. This situation clearly revealed the potential spread risk of ‘*Ca. P. prunorum*’ by *C. pruni* in Turkey and the necessity of taking precautions.

Turkey is a centre of diversity of a wide range of fruit trees. To determine if only one or both genetic groups identified in *C. pruni* is found in Turkey will inform about the speciation history of the species, which can also be linked to phytoplasma transmission capacity. For this objective, all specimens collected in this survey will be typed for phytoplasma and for determining their genetic groups A or B.

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Stolbur phytoplasma interaction with vector longevity in alternative plants

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Abstract

The epidemiology of phytoplasma-induced plant diseases is significantly influenced by the interaction between phytoplasma and the vectoring insect. Infection with phytoplasma may increase or lower fitness, or change the behaviour of the vector. The cixiid *Hyalesthes obsoletus* vectors stolbur 16SrXII-A phytoplasma from bindweed and stinging nettle to grapevine, where it causes the yellows disease 'bois noir'. Here we present first experiments studying the effect of stolbur infection on longevity in *H. obsoletus*. Preliminary tests of female *H. obsoletus* sampled on bindweed and stinging nettle, respectively, showed that females survive significantly longer on the own plant, whereas no effect of stolbur infection was observed on other host plant.

Key words: *Hyalesthes obsoletus*, Cixiidae, 'bois noir', longevity, host plant attraction, host races.

Introduction

Despite the important role of vectors in the epidemiology of phytoplasma-induced plant diseases, few studies have addressed how phytoplasma affect the insect vector. Generally, negative effects (virulence) of symbiont infection may accrue if vectors acquire new "mal-adapted" strains, e.g. by feeding on new host plants, or by competition between different symbiont strains, whereas symbionts should become benign towards the vector when it is the more mobile species, free symbionts rarely disperse and strains do not compete (Elliot *et al.*, 2003). Symbionts may also influence the behaviour of vectors, for example by manipulating them to find host plants (Mayer *et al.*, 2008). Benefits of infection to the vector by phytoplasma include increased longevity (Beanland *et al.*, 2000; Ebbert and Nault, 2001) and fecundity (Beanland *et al.*, 2000), while studies showing negative effects include reduced life span (Garcia-Salazar *et al.*, 1991) and lower fecundity and longevity (Bressan *et al.*, 2005) of the vector.

Stolbur 16SrXII-A phytoplasma induces the grapevine yellows disease 'bois noir'. Stolbur is transmitted to grapevine from the natural host plants of both stolbur and its main vector, *Hyalesthes obsoletus* (Cixiidae). Grapevine itself is a dead end host and does not contribute to the epidemiology of bois noir.

In Germany, *H. obsoletus* has two host races associated with bindweed (*Convolvulus arvensis*) and stinging nettle (*Urtica dioica*), respectively. Bindweed harbours the stolbur tuf-type b strain, whereas the tuf-type a strain is associated with stinging nettle. Hence, there are two epidemiological cycles. The "nettle cycle" is younger than the "bindweed cycle" in Germany. In a newly arisen, non-adapted infectious pathway one might expect detrimental vector-symbiont interactions, whereas a co-adapted system should show neutral or positive interactions.

We are investigating how stolbur infection influences *H. obsoletus* life-history by studying longevity and host

plant attraction of *H. obsoletus* to own and alternative host plants in relation to infection with 16SrXII-A phytoplasma. In this paper, we present preliminary results regarding longevity of females.

Materials and methods

H. obsoletus used for analyses were caught in June and July 2010 in the Mosel Valley, Germany. Individuals were studied for longevity on own and alternative host plants relative to infection with stolbur. Longevity was defined as the number of days surviving on plants in the laboratory. All individuals of one host race were caught on the same day to ensure equal mean survival estimates between treatments within each host race. Individuals were kept in large plastic cups with small plant twigs with 2-3 leaves. The plant twigs were immersed in vials that contained water and were sealed with foil to avoid evaporation. Survival was checked on a daily basis, and analysed for each cohort separately. Different rates of survival were tested with Kaplan-Meier survival analysis (Log-Rank-tests). All specimens were checked for stolbur infection using the *tuf* gene via nested PCR.

Results and Discussion

Females of *H. obsoletus* host races lived significantly longer on the own host plant (figure 1A, D), as previously reported for *H. obsoletus* caught on stinging nettle in Switzerland (Kessler *et al.*, 2011). Different survival times may be influenced by phenotypic conditioning of the larvae to the host plant rather than by an evolutionary adaptation. However, because *H. obsoletus* in the Mosel Valley has genetically divergent populations (M. Imo, unpublished data) we contend that the different survival rates are further evidence for specialisation of host races.

There was no significant effect of stolbur infection on longevity in either host race on either plant (figures 1B, C, E, F). Lack of significant effects of infection might be influenced by low infection rates that led to low statistical power: 15% *H. obsoletus* caught on stinging nettle and 28% on bindweed were infected. A higher sample size will shed more light on this result. Lack of negative stolbur-effects on longevity suggests that the vector-phytoplasma interaction has not evolved recently but is part of a co-evolved system. However, future in-

vestigations will include analyses of longevity in males, which might differ from females. We found no mixed infections of tuf-type a and b, and the tuf type of all infected vectors corresponded to that of the field host plant. Homogeneous infection patterns are predicted to cause evolution of benevolence towards the vector. If one assumes that the stinging nettle host race in Germany is only recently evolved (M. Imo, unpublished results) our results imply that *H. obsoletus* is adapted to stolbur in general rather than to specific strains.

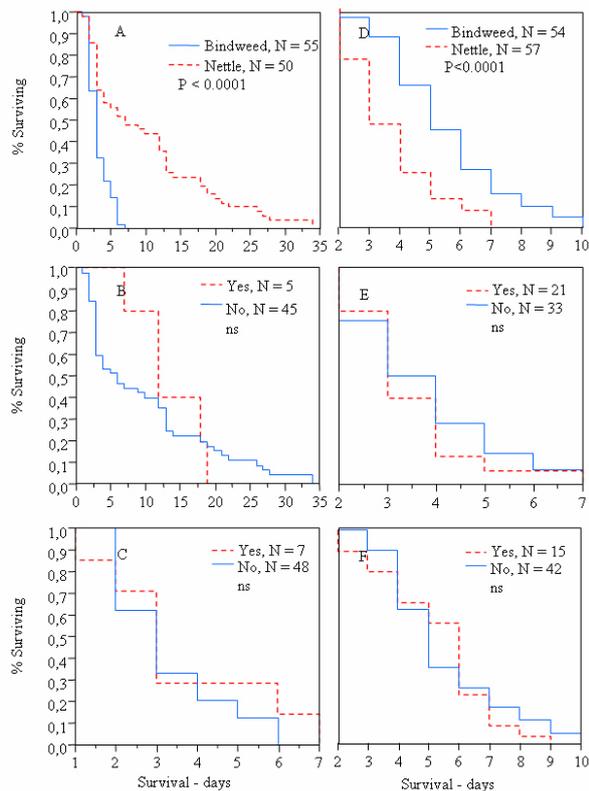


Figure 1. Survival of female *H. obsoletus*. 1) Survival of stinging nettle females; on stinging nettle or bindweed (A), infected and non-infected females on stinging nettle (B), infected and non-infected females on bindweed (C). 2) Survival of bindweed females; on stinging nettle or bindweed (D), infected and non-infected females on bindweed (E), infected and non-infected females on stinging nettle (F). Yes = infected; No = not infected; N = sample size; ns = not significant. (In colour at www.bulletinofinsectology.org)

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***In vitro* interactions between immunodominant membrane protein of lime witches' broom phytoplasma and leafhopper vector proteins**

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Abstract

Lime witches' broom phytoplasma (16SrII, LWB) causes major losses to lime cultivation in Iran. The role of its immunodominant membrane protein (IMP) in the specific interaction with vector proteins was addressed. The carboxy terminal moiety of LWB IMP, coding the extracellular part of the protein, was expressed (LWB fΔIMP) as a tool to study IMP interactions with insect proteins. In preliminary affinity chromatography experiments, leafhopper vector proteins with different sizes interacted with LWB fΔIMP.

Key words: *Hishimonus phycitis*, immunodominant membrane protein, protein interaction.

Introduction

Immunodominant membrane proteins of phytoplasmas are in direct contact with the surrounding host cell environment, and therefore they are good candidates for interaction with host proteins. Studies on two 16SrI related phytoplasmas showed that their antigenic membrane protein (AMP) interacts specifically with certain insect vector proteins and might have a role in determining vector specificity (Suzuki *et al.*, 2006; Galetto *et al.*, 2010). The role of phytoplasma IMP and IdPA type membrane proteins in interactions with insect vector is still unknown. Preliminary studies showed that lime witches' broom (LWB) phytoplasma (16SrII), the causal agent of a devastating disease of lime in Iran, has the major (immunodominant) membrane protein homologous to IMP type of phytoplasma membrane proteins. Moreover, preliminary studies have indicated that some amino acids in the extracytoplasmic region of the IMPs of 16srII phytoplasmas (including LWB) are under positive selection. A more rapid evolution of this part of the gene compared to other genomic regions may further support a role for IMP in host-pathogen interactions, as already suggested for AMP (Kakizawa *et al.*, 2006). The main objective of this study was to determine the interaction of LWB IMP with proteins of its leafhopper vector *Hishimonus phycitis* (Distant).

Materials and methods

The IMP ORF of LWB excluding its transmembrane domain was amplified by PCR and cloned in pQE30 vector to express the truncated IMP protein in fusion with 6X histidine residues (LWB fΔIMP). The recombinant protein was purified and coupled to a resin affinity column. Crude extracts of leafhoppers, *H. phycitis*, *Macrostelus quadripunctulatus* (Kirschbaum), *Euscelidus*

variegatus (Kirschbaum), *Empoasca decipiens* Paoli and *Scaphoideus titanus* Ball, were separately loaded on the IMP affinity column followed by elution of specifically bound proteins. Crude extract of the non vector aphid *Myzus persicae* (Sulzer) was separately loaded as control. All crude extracts were also loaded on affinity column coupled to a polyhistidine peptide, as control. Eluted proteins were subjected to SDS-PAGE followed by gel staining. Protein bands resolved on the gel were excised and identified by mass spectrometry. In Western blot experiment, proteins separated on SDS-PAGE were electro-blotted on a PVDF membrane. After blocking, the membranes were incubated with commercial primary antisera for 2 h at room temperature, then with HRP-conjugated secondary antibodies at room temperature for 1 h. Membranes were developed with Super Signal West Pico reagents (Pierce) and bands were visualized by VersaDoc model 4000 Imaging system (BioRad).

Results

The LWB IMP extracellular domain (LWB ΔIMP) was successfully expressed in *E. coli* after cloning in pQE30 expression vector.

A total of 16 protein bands, detected by SDS PAGE following elution from the LWB ΔIMP-coupled affinity column, were subjected to mass spectrometry analysis for identification (table 1). P40 bands (40 kDa protein band) from different leafhopper species were identified with high confidence as actin. P50, P45 and P200 bands showed spectra matching with those of ATP synthase beta, arginine kinase and myosin heavy chain, respectively. No matches were found for P90, P150, P55 and P80 bands. No bands were detected when extracts from the aphid *M. persicae* were eluted from the LWB fΔIMP-coupled affinity column.

Table 1. Leafhopper proteins retained in affinity column packed with LWB ΔIMP, their observed molecular weight, results of MS/MS identification, and summary of western blot experiments to confirm MS/MS identifications.

Insect species	Band Size (kDa)	MS/MS protein identification	Western blot (ATP synthase beta)	Western blot (actin)
<i>Hishimonus phycitis</i>	P90	Unknown		
	P50	Unknown	+	+
	P150	Unknown		
<i>Macrosteles quadripunctulatus</i>	P90	Unknown		
	P80	Unknown	+	+
	P40	Actin		
<i>Euscelidius variegatus</i>	P45	Arginin kinase		
	P90	Unknown	+	+
	P40	N/A		
<i>Empoasca decipiens</i>	P90	Unknown		
	P50	ATP synthase beta	+	+
	P40	N/A		
<i>Scaphoideus titanus</i>	P200	Myosin		
	P90	Unknown	+	+
	P55	ATP synthase beta		
	P40	Actin		

Anti actin and anti ATP synthase beta subunit polyclonal antibodies were employed in Western blot to confirm the presence of the corresponding proteins in insect eluates from LWB ΔIMP affinity column. Actin and ATP synthase beta protein bands were detected on eluates of all leafhopper species. No signal was detected in the western blots with corresponding eluates of the aphid *M. persicae*, and from leafhopper eluates of the poly-histidine control column.

Discussion

We showed the *in vitro* interaction between lime witches' broom IMP and some proteins of leafhoppers vectors of phytoplasmas, so far not known as LWB vectors. Two of these proteins, including actin and ATP synthase beta were previously shown to participate in specific interactions with AMP of two 16SrI related phytoplasmas (Suzuki *et al.*, 2006; Galetto *et al.*, 2010). Sequence analysis are in progress to evaluate the existence of positive selection acting on the exposed moiety of LWB IMP, as reported for other IDPs (Kakizawa *et al.*, 2009).

We showed that LWB IMP interacted *in vitro* under non denaturing conditions with several proteins from *H. phycitis*, candidate vector of the disease in Iran, as well as with other leafhoppers vectors of phytoplasmas in 16Sr groups I and V. IMP, however, did not interact with proteins of the aphid *M. persicae* which is not known as phytoplasma vector. One band (p90) was present among interacting proteins obtained from all leafhoppers, although peptides from its trypsin digestion did not match with any known sequence. Consequently, p90 could not be identified. IMP-interacting proteins p50 and p150 (*H. phycitis*), p80 (*M. quadripunctulatus*), and p40 (*E. variegatus* and *E. decipiens*) could not be identified for the same reason. These preliminary experiments showed that IMP of LWB phytoplasma

interacts with several leafhopper proteins involved in cell cytoskeleton, organelle mobility, and energy production.

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Effects of temperature and CO₂ on phytoplasma multiplication pattern in vector and plant

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Abstract

Multiplication patterns of two phytoplasmas, chrysanthemum yellows and ‘flavescence dorée’ were characterized over time in insect vectors and plant hosts under different climatic (temperature, T, and CO₂) conditions. Acquisition and transmission experiments were run in parallel in two phytotrons (P1 and P2). *Euscelidius variegatus* and daisy were used as chrysanthemum yellows hosts, *Scaphoideus titanus* and broad bean as ‘flavescence dorée’ hosts. Phytoplasma concentration was measured three times in insects and four in plants, at different days after acquisition and inoculation, respectively. Latent period in the vector was also evaluated for both phytoplasmas under the two conditions. On average, phytoplasma multiplication was faster under cooler conditions in insects (P1, 18-22°C; CO₂ 400 ppm) and under warmer conditions in plants (P2, 22-26°C; CO₂ 800 ppm). An influence of T and CO₂ concentrations was observed for chrysanthemum yellows latency in the vector only. Results suggest that T and CO₂ influence on phytoplasma multiplication is host-dependent.

Key words: leafhopper vectors, *Chrysanthemum carinatum*, *Vicia faba*, phytotron, global warming.

Introduction

Climate change is predicted to have a progressively negative effect on the yield of food crops. As with all species, plant pathogens will have varying responses to climate change. Whilst the life cycle of some pathogens will be limited by increasing temperatures, other climatic factors, such as increasing atmospheric CO₂, may provide more favourable conditions for pathogens. Moreover, climatic variability can affect not only the pathogen, but also plant host and insect vector, as well as the interactions between or among these organisms (Luck *et al.*, 2011).

To date, no studies have linked changes of phytoplasma disease impact to changes in climatic conditions (Foissac and Wilson, 2010). Aim of this work was to monitor multiplication rate of two different phytoplasmas in plant and insect vector in two phytotrons under different temperature (T) and CO₂ levels, in order to investigate effects of global warming on phytoplasma epidemiology.

Materials and methods

Healthy colonies of *Euscelidius variegatus* Kirschbaum were reared on oats, *Avena sativa* (L.), of *Scaphoideus titanus* Ball on grapevine, *Vitis vinifera* (L.), and broad bean, *Vicia faba* (L.). Chrysanthemum yellows phytoplasma (‘*Candidatus* Phytoplasma asteris’, CY, 16SrI-B), isolated in the Liguria Region was maintained by insect transmission on daisy, *Chrysanthemum carinatum* Schousboe. ‘Flavescence dorée’ phytoplasma, FD 16SrV-C/secY-C isolated in Piemonte, was routinely maintained on broad beans by transmission with *E. variegatus*.

E. variegatus and daisy were used as host species to characterize CY multiplication pattern under different experimental conditions; *S. titanus* and broad bean were used for FD. Two phytotrons were set as described in table 1.

All acquisition and transmission experiments were performed in parallel in the two phytotrons. To identify the CY latent period and to characterize CY multiplication pattern in insect, nymphs of *E. variegatus* were isolated on infected daisy for one week of acquisition access period (AAP) and then maintained on healthy oats. Groups of ten insects randomly sampled were transferred on two daisies for 2/3 days for transmission, starting from 18 days after acquisition (dpa) until 39 dpa, for 9 successive inoculation access periods (IAP). After IAPs, transmission was evaluated by symptom observation and confirmed by PCR. To characterize CY multiplication pattern in insect, *E. variegatus* adults were sampled for DNA extraction, phytoplasma detection and quantification by real time PCR at 10, 20 and 30 dpa (about 15 insects for each sampling date from each phytotron). To characterize CY multiplication pattern in host plant, 6 daisies were inoculated in each phytotron with infective *E. variegatus*. Leaf portions were sampled for DNA extraction, phytoplasma detection and quantification at 5, 8, 13 and 15 days after inoculation (dpi), according to Saracco *et al.* (2006).

To investigate FD multiplication pattern in insects and plants, and latency, experiments were carried out similarly as detailed for CY. Ten consecutive IAPs were performed to evaluate latent period every 2/3 days, starting from 20 to 44 dpa. *S. titanus* individuals were sampled at 10, 20 and 30 to measure FD titer in insect. Six broad beans were inoculated in each phytotron and leaf portions were sampled for phytoplasma detection and quantification at 17, 23, 30 and 37 dpi.

Table 1. Conditions of the two phytotrons employed.

Phytotron	Temperature °C		CO ₂ (ppm)	Relative humidity (%)	PAR* (μmol m ⁻² s ⁻¹) (16 hours photoperiod)
	Min.	Max			
P1	18	22	400	85-90	0 (night) - 600 (day)
P2	22	26	800	85-90	0 (night) - 600 (day)

* PAR = Photosynthetically active radiation.

Total DNA was extracted from single insects and from 100 mg of leaf tissues and analysed by nested-PCR for the presence of CY and FD with ribosomal primers R16F2/R2 followed by group specific primers R16(I)F1/R1 or R16(V)F1/R1. For quantitative real time PCR, the procedures described in Marzachi and Bosco (2005) were followed for CY and FD quantification, respectively.

Results

Considering all *E. variegatus* sampled at different dates in the two phytotrons, CY was detected in 65 samples out of 74 (88%). No significant differences were found among the frequencies of CY-infected *E. variegatus* sampled at each date in both phytotrons. Two way ANOVA was run on CY titers in *E. variegatus* for phytotron and sampling dates. No significant difference between P1 and P2 was found, regardless sampling dates. However at 10 dpa, CY titer in P1 was significantly higher than in P2. CY titer significantly increased over time in *E. variegatus* in both phytotrons. *E. variegatus* showed a shorter latency in P2 than in P1.

All daisies exposed to CY vectors in both phytotrons were infected, and tested CY-positive in PCR since the first sampling date (5 dpi). Two way ANOVA for phytotron and sampling date was run on CY titers measured in daisies. Average titers were always higher in plants kept in P2, but the difference was significant only at 5 dpi. CY titers significantly increased over time in daisies maintained in both P1 and P2.

Considering all *S. titanus* sampled at different dates in the two phytotrons, FD was detected in 65 samples out of 114 (57%). No significant differences were found among sampling dates and phytotrons. Two way ANOVA for phytotron and sampling date was run on FD titers measured in *S. titanus*. FD titers measured in P1 were significantly higher than in P2 at any sampling date. FD titer significantly increased over time in *S. titanus* in both phytotrons. The same FD latent period in *S. titanus* was recorded in P1 and P2.

All broad beans exposed to FD vectors in both phytotrons were infected. However, in PCR assays two, four and six out of six inoculated plants tested FD-positive at 17, 23 and 30 dpi, respectively, in both phytotrons. Two way ANOVA for phytotron and sampling date was run on FD titers measured in broad beans. Average titers were higher in plants kept in P2, but this difference was significant only at 30 dpi. FD titers significantly increased over time in broad bean, in both phytotrons.

Discussion

The tested experimental conditions did not affect acquisition and transmission capabilities of both CY and FD vectors. On the contrary, higher temperature and CO₂ concentration resulted in a shorter CY latent period in *E. variegatus*. While no clear influence of environmental conditions was observed on CY titer in both plant and vector, FD multiplication in *S. titanus* was faster at lower T and CO₂ concentration. Overall, phytoplasma multiplication was faster under cooler conditions in insects and under warmer conditions in plants. We can suggest that the effect of environmental conditions on phytoplasma multiplication is host-dependent. These are the first experimental results addressing aspects of phytoplasma epidemiology under global warming scenario.

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Transmission of maize bushy stunt phytoplasma by *Dalbulus maidis* leafhopper

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Abstract

The maize bushy stunt phytoplasma (MBS) transmission by *Dalbulus maidis* was studied using two colonies of leafhoppers caged for five days in two MBS symptomatic plants. After the latent period, one leafhopper was confined per seedling in two different maize genotypes (popcorn hybrid and L22 inbred line). Leafhoppers samples were submitted to PCR analyzes before and after inoculation. Colony 1 and 2 had 27.5% and 3.3% of infected leafhoppers, respectively. Maize bushy stunt phytoplasma transmission to popcorn was almost 100% and around 30% to L22.

Key words: MBS, mollicutes, *Dalbulus maidis*, *Zea mays*.

Introduction

The corn leafhopper *Dalbulus maidis* transmits mollicutes maize bushy stunt phytoplasma (MBS) and corn stunt spiropasma (CSS) in a persistent manner (Nault, 1980). Studies about MBS transmission by *D. maidis* show influence of the MBS strains from different regions, temperature, and spiropasma acquisition (Moya-Raygosa and Nault, 1998; Legrand and Power, 1994; Oliveira *et al.*, 2007). Understanding of the best condition for maximum transmission efficiency of MBS can contribute to improve the methodology for its inoculation. In this study the MBS transmission by infected leafhopper *D. maidis* was studied by PCR analyzes.

Materials and methods

Two experiments were carried out in screen house conditions. Two colonies of young adults of *D. maidis* (around 300 individuals/colony) were given a 5-day acquisition access period (AAP) on two MBS infected plants, that presented typical symptoms, followed by 39 and 44 days of incubation period on healthy plants (Nault, 1980), for colonies 1 and 2, respectively. Each experiment was carried out with 60 plants of a popcorn maize hybrid and 60 plants of susceptible maize L22 inbred line. It was used one leafhopper per plant with an inoculation access period (IAP) of six days in eight days old seedlings. Colony 1 was used in experiment 1 and colony 2 in experiment 2. The control consisted of six popcorn and six L22 plants with healthy leafhoppers. The MBS strain used in the experiments was obtained from popcorn plants. It has been cultivated for six years, in the same popcorn. A total of 132 pots with 5 kg of substrate and one plant per pot, cultivated for 60 days, were used in each experiment. Plant symptoms were evaluated weekly. The temperatures and relative humidity data were registered at the meteorological station of Sete Lagoas. Samples of 10 leafhoppers before testing, 10 leafhoppers after IAP from popcorn and 10 from L22 were tested by PCR (Lee *et al.*, 1993) using primers

R16F2 and R16R2 for phytoplasma detection. DNA from plants infected with MBS was used as positive control.

Results

PCR analyzes showed weak bands of 1.2 kb and non-specific bands in 21 leafhoppers samples from colony 1 and one specific 1.2 kb band in eight leafhoppers samples from colony 2 (table 1). The MBS transmission was confirmed by plant symptoms only for leafhoppers samples that presented strong bands. Based on strong band percentage, leafhoppers infection was estimated in 27.5 and 3.3% in colonies 1 and 2, respectively. The MBS transmission to popcorn from infected leafhoppers was almost 100%, and around 30% for L22 inbred line (table 2). The environmental climatic variables are presented in table 3.

Discussion

MBS acquisition and transmission was dependent on the plant source and on the environmental temperature condition for AAP. The low MBS acquisition under minimum temperature of 17°C might be responsible for low MBS concentration in leafhoppers that was not enough for transmission and resulted on PCR with weak bands. Results suggest that could be convenient to use at least three leafhoppers for inoculation, after AAP and latent period. Popcorn maize showed to be more susceptible to the MBS than L22. The variability of MBS geographic isolates (Moya-Raygosa and Nault, 1998) and temperature adaptation might help MBS to survive in areas with different maize cultivars and environmental conditions, supporting this emergent disease spreading. PCR analyzes of leafhoppers can help on the decision of how many individuals are needed for an efficient inoculation, since confirmed their presence only when observed a 1.2 kb strong band as a PCR product.

Table 1. Results for PCR analyzes and transmission by infected leafhoppers.

Leafhoppers samples	Experiment 1 (colony 1)		Symptomatic plants
	weak ¹	strong	
10 leafhoppers before IAP test	7	0	Not tested
10 leafhoppers after IAP in popcorn	7	0	0
10 leafhoppers after IAP in L22	5	0	0

Leafhoppers samples	Experiment 2 (colony 2)		Symptomatic plants
	weak ¹	strong	
10 leafhoppers before IAP test	2	3	Not tested
10 leafhoppers after IAP in popcorn	0	3	3
10 leafhoppers after IAP in L22	0	2	2

¹ Band of 1.2 kb and non-specific bands; IAP, Inoculation access period.

Table 2. Results of transmission by leafhopper colonies 1 and 2.

	Experiment 1 (colony 1)		Experiment 2 (colony 2)	
	Popcorn	L22	Popcorn	L22
Number of MBS symptomatic plants	2	0	15	5
Percentage of MBS symptomatic plants	3.3	0	25	8.3
Percentage of MBS transmission (from strong band percentage 27.5)	-	-	92.5	30.7

Table 3. Average and standard deviation of maximum and minimum temperatures (Tmax, Tmin) and relative air humidity (RH) at Embrapa Maize and Sorghum (meteorological station of Sete Lagoas - INMET), during MBS acquisition access period (AAP), latent period (LP) and inoculation access period (IAP) by leafhoppers colonies (experiments 1 and 2).

Period	Experiment 1			Experiment 2		
	Tmax (°C)	Tmin (°C)	RH (%)	Tmax (°C)	Tmin (°C)	RH (%)
AAP	32.1 ± 1.2	17.4 ± 0.9	57.7 ± 4.4	30.4 ± 3.7	20.4 ± 0.5	75.2 ± 17.4
PL	29.6 ± 2.1	19.5 ± 1	75.1 ± 14.5	30.1 ± 3.3	19.2 ± 1	72.1 ± 14.5
IAP	32.7 ± 0.6	18.5 ± 0.3	58.9 ± 2.4	32.8 ± 0.8	18.8 ± 0.7	60.2 ± 4.19

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Hosts of stolbur phytoplasmas in maize redness affected fields

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Abstract

The plant host range of a phytoplasma is strongly dependent on the host range of its insect vector. Maize redness in Serbia is caused by stolbur phytoplasma (subgroup 16SrXII-A) and is transmitted by the cixiid planthopper, *Reptalus panzeri* (Löw). *R. panzeri* was the only potential vector found to be infected with stolbur phytoplasma in and around maize redness affected fields, and the phytoplasma was only found in monocotyledonous plants including maize, Johnsongrass, and wheat. Other known stolbur phytoplasma vectors and weedy plant hosts tested were not infected. These results are discussed with respect to potential differentiation of the pathogen in different host-vector systems.

Key words: Class *Mollicutes*; South Banat region; stolbur phytoplasma; vector.

Introduction

All phytoplasmas have a dual host infection cycle, multiplying in both their plant host and insect vector (Gasparich, 2010). The plant host ranges of phytoplasmas are generally considered to be controlled by the host range of their specific insect vector. Stolbur phytoplasma (subgroup 16SrXII-A) has a broad plant host range that includes important solanaceous crops, grapevine, celery, sugarbeet, strawberry, lavender and maize (Garnier *et al.*, 2000; Jović *et al.*, 2007). The phytoplasma also infects a number of dicotyledonous weeds, the most important of which are bindweeds (*Convolvulus arvensis* L. and *Calystegia sepium* (L.) (R. Br.) and nettle (*Urtica dioica* L.). Known vectors of stolbur phytoplasma include the cixiid planthoppers *Hyaletthes obsoletus* Signoret, *Pentastiridius leporinus* (L.) and *Reptalus panzeri* (Löw). Bindweeds and nettle are the primary hosts of *H. obsoletus*. *P. leporinus* and *R. panzeri* are polyphagous, with early stages of development occurring on grasses.

Maize redness (MR) in the Banat region of Serbia is associated with stolbur phytoplasma transmitted by *R. panzeri* (Duduk and Bertaccini, 2006; Jović *et al.* 2007; 2009). The disease can cause catastrophic losses in maize (Bekavac *et al.*, 2008). The initial disease symptom, reddening of the leaf midrib, appears in late July. Reddening of leaves and stalks intensifies with time, and plants become dry by early September. Critically, ear development and seed set are adversely affected by the disease.

MR transmission in South Banat was associated only with *R. panzeri*. Very few *H. obsoletus* individuals were found in MR affected fields, and no *Pentastiridius* species were present (Jović *et al.*, 2009). In contrast, ‘bois noir’ of grapevine associated with stolbur phytoplasmas is transmitted only with *H. obsoletus*. The association of stolbur phytoplasma diseases with different vectors,

suggests key differences in disease etiology and epidemiology between monocot and dicot hosts.

Materials and methods

Experiments were carried out as described in Jović *et al.* (2009). Briefly, insects were collected in July from MR affected maize fields. Insects were stored in 80% ethanol at -20°C prior to analysis. The DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used to isolate DNA from individual insects. Stolbur phytoplasma was detected in DNA samples using nested PCR (Clair *et al.*, 2003). Weedy plants were selected randomly from the borders of MR affected maize fields. Genomic DNA was extracted from the roots of individual plants (Angelini *et al.*, 2001). Winter wheat was collected in the spring from a field that had been planted after maize in an MR affected field in Samoš. DNA was isolated from individual wheat roots using the DNeasy Plant Mini Kit (Qiagen). Samples were assayed for the presence of stolbur phytoplasma by nested PCR.

Results

Phytoplasmas were detected in three of 13 Auchenorrhyncha species collected from MR affected fields (table 1). *R. panzeri* individuals were infected with stolbur phytoplasma, while *Mocycdia crocea* (Herrich-Schaffer) and *Psammotettix alienus* (Dahlbom) were positive for phytoplasmas from the aster yellows group (16SrI-C and I-B). No phytoplasmas were detected in any of 139 *Zyginidia pullula* (Boheman) and 42 *Laodelphax striatella* (Fallen) individuals tested. Fewer than ten individuals were collected for eight other species, and phytoplasmas were not detected in these insects.

Table 1. Phytoplasmas in insect species collected from maize redness affected fields in Serbia (adapted from Jovic *et al.* 2007, 2009).

Species	# Positive/# Tested ^a	16Sr Group ^b
<i>Reptalus panzeri</i>	70/404	XII-A
<i>Mocytia crocea</i>	1/6	I-C
<i>Psammotettix alienus</i>	13/187	I-C (12) I-B (1)

^a Number of phytoplasma positive specimens/# specimens tested.

^b Classification of phytoplasmas into 16Sr groups by digestion with *TruI* (Lee *et al.*, 1998).

Roots of weeds were sampled from in and around MR affected fields. Of the seven species tested, 7 of 33 Johnsongrass (*Sorghum halepense* (L.) Pers.) samples were positive for stolbur phytoplasma. Phytoplasma was not detected in any of the broadleaf weeds, including *Medicago sativa* L., *Datura stramonium* L., bindweed (*Convolvulus arvensis*), *Solanum nigrum* L. and *Bilderdykia convolvulus* (L.) Dumort and *Sinapis arvensis* L.. *R. panzeri* transmitted stolbur phytoplasma to 5.5% of wheat seedlings under laboratory conditions, and the phytoplasma was detected in about 2.5% of roots sampled from wheat planted into an MR affected maize field.

Discussion

Of the hemipteran species identified in MR affected fields in South Banat Serbia, only *R. panzeri* was found to be infected with stolbur phytoplasma (table 1, Jović, *et al.*, 2009). In particular, very few *H. obsoletus* individuals and no *Pentastiridius* species were found. Populations of vectors of other phytoplasmas were similarly low. Insects previously shown to harbor stolbur phytoplasma such as *M. crocea* and *P. alienus*, which can harbor several phytoplasmas including stolbur (Tothova, 2004), were present, but these did not carry stolbur phytoplasma. While it is possible that some other species are capable to transmitting stolbur phytoplasma to maize, *R. panzeri* is likely to be the major vector of MR in South Banat.

Two monocots, Johnsongrass and wheat were identified as hosts of stolbur phytoplasma in MR affected areas. In contrast, the phytoplasma was not detected in dicotyledonous weeds previously shown to be hosts, including *C. arvensis*, *M. sativa*, *D. stramonium* and *S. nigrum* (Garnier, 2000). These results, with distinctly different plant and insect vector hosts for stolbur phytoplasma associated with MR raises the possibility of pathogen diversification. Strains of stolbur from different hosts can be distinguished on the basis of *tuf* and *vmp* genes, and genome size (Cimerman *et al.*, 2009; Marccone *et al.*, 1999; Riolo *et al.*, 2007). We are working to identify genomic characteristics that distinguish stolbur phytoplasma from MR affected plants from strains infecting other vectors and plant hosts.

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Molecular evidence of phytoplasmas in winter oilseed rape, tomato and corn seedlings

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Abstract

To evaluate the seed transmission of phytoplasmas in three herbaceous hosts material from different geographical origin but all collected from infected or symptomatic mother plants was used. Almost 1,000 seeds were germinated under controlled conditions and 652 seedlings were obtained. Samples were grouped in 214 samples and 74 of them resulted positive to phytoplasma presence after three to 90 days from germination by nested PCR assays on 16S ribosomal gene. All the tested species i.e. winter oilseed rape, tomato and corn resulted carrying phytoplasmas belonging to the ribosomal groups retrieved in the infected mother plants.

Key words: Phytoplasma, seed transmission, aster yellows, stolbur, elm yellows.

Introduction

Phytoplasma seed transmission is still a controversial issue considering the very poor connection of embryo with the mother plant. However it is well known that flower structures are the most colonized by phytoplasmas in many plant species. Most likely the seed production in infected plants is quite reduced, however recent studies have highlight the fact that late infections, especially in herbaceous crops, allow the normal seed production increasing chances of transmission of the pathogens through the seeds (Olivier e Galka, 2008). After the first report of 16SrII phytoplasma in alfalfa seedlings (Khan *et al.*, 2002), recently stolbur phytoplasmas were again identified in pea seedlings (Zwolinska *et al.*, 2010). To verify the seed transmissibility trials in sterile conditions on a relevant number of seedlings collected from field infected mother plants of tomato, oilseed rape and corn were carried out.

Materials and methods

Winter oil sees rape seeds were collected in Veneto (Northern Italy) from aster yellows and stolbur infected mother plants (Mori *et al.*, 2010; Contaldo *et al.*, unpublished). Two batches of seeds were analyzed Excalibur 2008 (same batch used to seed the field where infection was detected) and Excalibur 2009 (produced from the infected plants). A total of 50 and 325 seed respectively for each batch were employed. A total of 128 tomato seeds from Bulgaria (varieties Rila, Marti, Kristi, Trapezitza, Milyana, and UC 82-A) collected from symptomatic tomato plants (figure 1) were tested. For corn from Serbia 186 seeds tested were from 4 cobs collected from plants showing reddening (Duduk and Bertaccini, 2006) and resulted positive to stolbur phytoplasmas

(Mitrovic *et al.*, unpublished). Further 287 seeds from 11 Italian cobs were collected in Mantova province (Northern Italy) and derived from plants showing reddening (Calari *et al.*, 2010) resulted either positive or negative to phytoplasmas presence. Surface sterilized seed were grown in insect proof environments, *in vitro* or in sterile wet substrate (figure 2) for 10 days to 3 months before testing for phytoplasmas presence. Batches of 3 to 20 individual seedlings (about 100 mg at least each) clean from all maternal tissues were subjected to DNA extraction, for some of the corn seedlings a separation into roots and leaflets was also carried out for testing. The DNaesy Plant Minikit (Qiagen) and a CTAB method (Angelini *et al.*, 2001) were employed for nucleic acid extraction and nested PCR with 16S ribosomal primers was carried out under reported conditions (Calari *et al.*, 2010). Phytoplasma identification was performed by RFLP analyses and/or sequencing of the obtained amplicons.

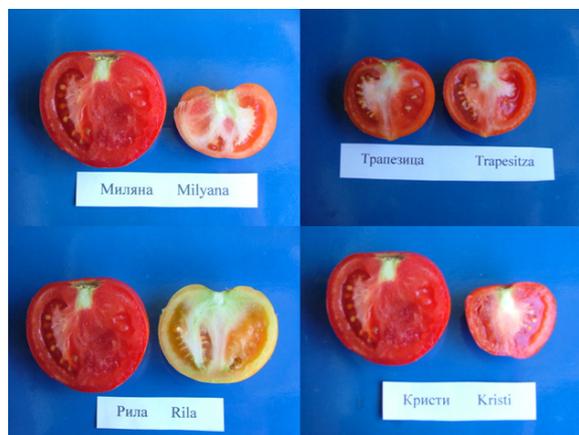


Figure 1. In each picture: healthy (left) and stolbur infected (right) tomato from which seeds were used.

Results

Germinating percentages for winter oilseed rape and tomato seeds ranged from 30 to 100%. For corn there was a difference in germination rate between the Serbian seeds (average 70%) and the Italian ones (average 54%).

In seedlings from Excalibur 2009 a total of 30 seed batches was tested and both 16SrI-B and stolbur (16SrXII) phytoplasmas were identified: 7 batches resulted infected with 16SrXII phytoplasmas and 3 with 16SrI-B phytoplasmas. Seedling from Excalibur 2008 five out of the seven batches tested resulted positive for 16SV and 16SrII phytoplasmas.

In tomato 23 batches were tested and only Rila and Milyana resulted negative to phytoplasmas presence, two batches were positive to 16SrXII (stolbur) and 5 to 16SrI-B (aster yellows) phytoplasmas.

The tested batches of Italian corn were 55 and 17 batches resulted positive to phytoplasmas presence, seedling from 5 of the tested cobs resulted all negative. Phytoplasmas identified in these samples were 16SrI-B, 16SrXII and 16SrV in some cases in mixed infections, phytoplasmas were detected in both leaves and roots. The 37 batches tested from seedling germinated from Serbian cobs show phytoplasma presence in all samples but 7, all cobs were positive.



Figure 2. Corn seeds under germination process before testing.

Discussion

The long distance spread of phytoplasmas is quite easily achieved for woody plants of agricultural interest by infected propagation material such as cutting, corms and micropropagated plant or insect vectors accidentally carried with plant material. On the contrary epidemic outbreak associated with phytoplasmas not present in a certain eco-system such as small island or similar in annual herbaceous crops allow to hypothesize a role of the phytoplasma seed transmission. Considering the ability of seed producing of plants that are infected by phytoplasmas in late stages or their life (De La Rue *et al.*, 2002; Cordova *et al.*, 2003), and the viability of malformed seeds (Nečas *et al.*, 2008; Nipah *et al.*, 2007; Olivier e Galka, 2008) it is possible to assume that a low percentage of seed transmission should be considered. In this work the presence of phytoplasmas in seedling was not correlated to the germination percentages, however evident differences in the number of batches positive to phytoplasmas infection was found for example between corn in Italy

and in Serbia. To better define the statistical relevance of the reported results work is in progress in oilseed rape to verify the ability of the phytoplasmas seed transmitted to induce symptoms in mature plants.

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Study of the expression of defense related protein genes in stolbur C and stolbur PO phytoplasma-infected tomato

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Abstract

Phytoplasma are phloem-restricted plant pathogenic bacteria that cause hundreds of diseases. They are not cultivated *in vitro* and can be transmitted through insect vectors or grafting. Researches have been investigating defense pathways to different pathogens (bacteria, fungi...) but little is known about defense pathways activated in phytoplasma infected plants. In this study, the expression of genes related to salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) pathways have been investigated in stolbur C phytoplasma and stolbur PO phytoplasma-infected tomato. We noted elevated level of expression for Pathogenesis Related (PR) genes. Transcription of SA and ET marker genes was up-regulated in stolbur PO and stolbur C-infected tomato, while transcription of PIN2, a gene regulated by JA, increased only in stolbur C-infected tomato.

Key words: phytoplasma, PR protein genes, salicylic acid, jasmonic acid, RT-PCR, gene expression.

Introduction

Phytoplasmas are plant pathogenic wall-less bacteria restricted to phloem sieve tubes. They induce plant disorders such as leaf yellowing, growth aberrations and flower malformations. The pathogenicity mechanism of these bacteria are yet not well understood. Recent data revealed that phytoplasmas effectors can induce specific symptoms (Zhang *et al.*, 2004, Hoshi *et al.*, 2009). However, it is not known if the plant defense pathways are activated in response to the infection. The three major plant defense pathways involve salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Pieterse *et al.*, 2001). Here, the expression of marker genes of these pathways was studied in tomato infected with two different isolates of stolbur phytoplasma. In spite of similar multiplication rates in plant, these two isolates, ie stolbur C and PO, induce distinct symptoms. Flowers from stolbur PO-infected plants show hypertrophied sepals, aborted stamens and petals. This is never observed in tomato infected with stolbur C isolate. The activation of the different defense pathways was examined in stolbur C and PO-infected plants. SA dependent pathway was found to be activated in infected tomato, while JA pathway seemed to be activated only in stolbur C-infected tomato.

Materials and methods

Tomato (*Solanum lycopersicum* cv Ailsa Craig) plants were infected with the stolbur phytoplasma by grafting. RNA were extracted from tomato leaves with Tri-reagent (SIGMA) and treated with DNase, following the supplier protocol.

Semi-quantitative RT-PCR was done with primers specific for each studied gene. For each tube of RT, 1 µg of DNA, 0.55 µM of an oligodT18, 0.01 M DTT, 2.2 mM dNTP, 280 U of Rnase OUT were mixed in a final volume of 19 µl. After 5 min at 65°C and 5 min at

45°C, 1 µl (200 U) of Superscript II reverse transcriptase (Invitrogen) was added and incubated for 1 hour. Five min at 75°C allowed the enzyme denaturation. PCR were done with 1 µl of cDNA, 0.35 mM MgCl₂, 0.9 mM dNTP, 2 µg/ml BSA, 4.4 µM each primer (3' and 5') and 2.2 U *Taq* DNA polymerase (Promega), in a final volume of 25 µl. Hybridization temperature depends on primers used.

RT-PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The intensity of the bands was measured with a FluorS and the associated software Quantity One (Biorad).

The cDNA obtained after reverse transcription were diluted according to 100 ng/µl. Quantitative real-time RT-PCR SYBRgreen assays were performed on a Roche Light Cycler480. Each reaction tube contained 1X SYBRgreen Fluorescein Mix (Applied Biosystem, Foster city, USA), 250 nM of each primer and 1 µl of RT-PCR product in a final volume of 25 µl. The thermal cycling programme was: 95°C for 15 min, 45 cycles with 95°C for 20 seconds, annealing temperature according to primer tested for 40 seconds, 72°C for 40 seconds. This was followed by a melt curve programme: 95°C for 30 seconds for 1 cycle, 60°C for 10 seconds for 1 cycle, melting step from 60°C to 100°C for 10 seconds for 80 cycles (step of 0.5°C) and final cycle of 72°C for 10 min. All samples were amplified in triplicate from four distinct RNA batches extracted from four distinct tomato samples.

Efficiency of the primer pairs was determined using dilution of the RT-PCR product (pure-1/10-1/100-1/1000-1/10000) and using the Light-Cycler480 software.

Method of calculation:

$$\text{Relative Gene Expression} = \frac{\text{Efficiency}^{(\text{Ct healthy} - \text{Ct infected})} (\text{tested gene})}{\text{Efficiency}^{(\text{Ct healthy} - \text{Ct infected})} (\text{control gene})}$$

Phytoplasma detection was carried out also by nested PCR. Primers used were R16mF2/R16mR1 and R16F2n/R16R, yielding a 1250 bp DNA fragment (Gundersen and Lee, 1996). First amplification was as follow: 94°C 4 minutes, 35 cycles of 94°C 1 minute, 60°C 1 minute, 72°C 1 minute and 30 seconds, with an extension at 72°C for 10 minutes. An aliquot (1/40) of the first PCR product was used for the nested amplification. Cycles are as follow: 94°C 4 minutes, 35 cycles of 94°C 1 minute, 55°C 1 minute, 72°C 1 minute and 30 seconds, with an extension at 72°C for 10 minutes. Eight µl of product were analyzed on agarose gel and quantified using Quantity One software (Biorad).

Results and discussion

Leaves were collected on healthy tomato plants or on tomato plants infected with stolbur C or stolbur PO phytoplasma. After RNA extraction, the infection was verified by nested RT-PCR with primers U3-U5. Stolbur C and stolbur PO phytoplasmas were detected in leaves of infected tomato at similar levels.

Q-RT-PCR specific to defense genes from SA, ET and JA pathways were carried out. Ten genes were tested: PR1a, PR1b, PR2a, PR2b, PR5, PR10, LOXD, PIN2, PAL, CHS2, that includes genes encoding enzymes for the biosynthesis of SA and JA, transcription factor and Pathogenesis Related protein genes (PR). Each expression was tested on stolbur C and stolbur PO-infected plants. The up- or down-regulation was estimated with the relative gene expression calculation. Examples of data are shown in figure 1. Increased transcripts levels for SA/ET pathways marker genes were observed in stolbur C and stolbur PO-infected tomato (table 1). Wound inducible gene PIN2 regulated by methyl jasmonate was up-regulated in stolbur C tomato leaves and down-regulated in stolbur PO-infected tomato leaves. Same results were observed for the phenylalanine ammonia-lyase gene PAL. Conversely, chalcone synthase gene CHS2 was activated in stolbur PO tomato and repressed in stolbur C tomato leaves.

These results were confirmed from semi-quantitative RT-PCR experiments done with the same RNA preparations.

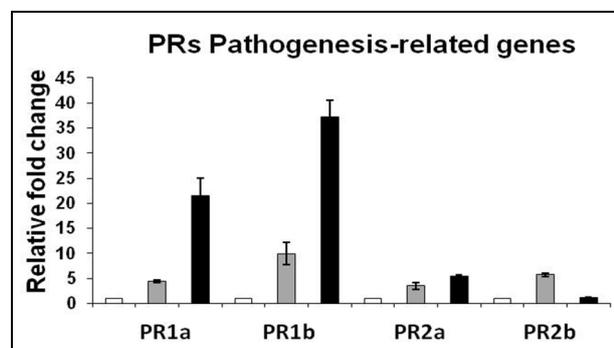


Figure 1. Histogram representing the relative gene expression of PR1a, PR1b, PR2a and PR2b genes, in healthy (white), stolbur C (grey) and stolbur PO (black) infected tomato leaves.

Table 1. Relative gene expression of 10 genes compared with those in healthy plants. RNA lots extracted from stolbur C (C) or stolbur PO-infected tomato leaves (PO).

Genes	RNA lot 1		RNA lot 2		RNA lot 3	
	C	PO	C	PO	C	PO
PR1ac	4,42	21,50	6,54	56,50	1,42	1,98
PR1bas	9,92	37,20	9,05	73,80	1,91	3,12
PR2ac	3,50	5,44	6,94	5,50	0,78	0,46
PR2bas	4,30	0,60	1,98	1,07	0,75	0,03
PR5	5,04	18,40	13,70	39,00	0,92	3,41
PR10	1,84	11,90	2,24	2,95	2,04	1,73
LOXD	1,68	0,07	0,31	0,07	0,85	0,58
PIN2	1,98	0,67	1,58	0,06	2,78	0,13
PAL	1,60	0,05	1,96	0,01	2,75	0,01
CHS2	0,28	6,54	0,19	1,54	0,50	2,25

Conclusions

Stolbur C and PO phytoplasma induced different symptoms on tomato leaves and flowers. They also induced distinct patterns of defense gene expression. SA and ET dependent defense pathways were activated in stolbur PO-infected tomato indicated by the high level of expression of SA/ET related genes. In turn, PIN2 (JA pathway) was repressed. In stolbur C-infected tomato, both SA/ET as well as JA pathways were activated indicated by increased transcription level of PIN2.

The role of these defense pathways on the development of the phytoplasma infection will be further investigated by studying the effect of pre-activation of these pathways on the phytoplasma infection.

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Gene expression analysis and cytochemical investigations in 'Candidatus Phytoplasma mali'-resistant and -susceptible *Malus* genotypes grown *in vitro*

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Abstract

A gene expression study was carried out in 'Candidatus Phytoplasma mali'-resistant and -susceptible apple genotypes infected with 'Ca. P. mali'. Genes involved in the general response against stress and pathogens were found to be differentially expressed in the 'Ca. P. mali' resistant wild genotype *Malus sieboldii*. Furthermore, cellular modifications and ultrastructural features were investigated through cytochemical analysis performed on healthy and 'Ca. P. mali'-infected *in vitro* plants of *Malus sieboldii* and the 'Ca. P. mali'-susceptible *Malus x domestica* cv. Golden Delicious. Preliminary results showed that while the cellular organisation in the susceptible genotype was deeply affected by the presence of 'Ca. P. mali' only few localised structural modifications were observed in the resistant genotype.

Key words: 'Candidatus Phytoplasma mali', cytochemical analysis, gene expression, TEM.

Introduction

Apple proliferation (AP) disease is the most important graft-transmissible and vector-borne disease of apple in Europe and is caused by 'Candidatus Phytoplasma mali'. Genetic resistance against 'Ca. P. mali' was observed in the wild genotype *Malus sieboldii* (MS) and in few hybrids derived from the crossings of MS with the 'Ca. P. mali'-susceptible *Malus x domestica* (Kartte and Seemüller, 1991). So far, the resistance mechanism against 'Ca. P. mali' is still poorly understood and only little information is available. A form of induced resistance, characterised by the stable remission of AP symptoms and disappearance of the phytoplasma from the canopy, has been observed in old susceptible apple trees known to be infected since several years (Musetti *et al.*, 2004). This phenomenon called "recovery" seems to occur stochastically while the resistance in MS was shown to be a reproducible and inheritable trait (Bisognin *et al.*, 2008a; Seemüller *et al.*, 2008; Jarausch *et al.*, 2010). Recently, cytochemical studies showed that in recovered plants there is a localised production of reactive oxygen species (ROS) with structural changes in the intracellular composition of the phloem tissue (Musetti *et al.*, 2010). However, in both resistance and "recovery" the mechanisms that act in the plant response against the pathogen are still largely unknown.

In this study, a gene expression analysis was carried out through real time quantitative RT-PCR (qRT-PCR) on differentially expressed genes previously individuated through cDNA-Amplified Fragment Polymorphism (cDNA-AFLP) (Moser *et al.*, 2007). Moreover, in order to investigate if resistance in MS could be characterised by features similar to that observed in the "recovery"

phenomenon a cytochemical analysis was performed in healthy and 'Ca. P. mali'-infected *in vitro* plants.

Materials and methods

Plant material

Healthy and infected plants were those described in Bisognin *et al.* (2008b). Micropropagation was done as described in Ciccotti *et al.* (2008). The genotypes used in this study were the 'Ca. P. mali'-susceptible *Malus x domestica* cv. Golden Delicious and the 'Ca. P. mali'-resistant *Malus sieboldii*.

RNA extraction

Total RNA of the *in vitro* plants was extracted according to the protocol described in Moser *et al.* (2004).

Gene expression analysis

The gene expression level was measured through reverse transcription quantitative PCR using the Invitrogen One-step qRT-PCR kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. The reactions were performed on a Chromo4 instrument (Biorad, Germany). The data analysis was performed as described in Peirson *et al.* (2003).

Cytochemical analysis

The material was collected from healthy and 'Ca. P. mali'-infected *in vitro* plants and treated as described by Musetti *et al.* (2004). Visualization of the samples was performed under a Transmission Electron Microscope (Philips CM10, Eindhoven, The Netherlands) operating at 80 kV.

Results

In a previous work (Moser *et al.*, 2007), several differentially expressed genes were individuated through cDNA-AFLP analysis on healthy and 'Ca. P. mali'-infected *in vitro* plants. Based on their putative function a subgroup of genes related to stress and pathogen response was chosen for the real time qRT-PCR analysis. Among these targets, the expression levels of three genes putatively involved in electron transport and H₂O₂ production and signaling were found to be differentially regulated in the 'Ca. P. mali'-resistant MS but not in the susceptible Golden Delicious (GD). In order to gain more information on the type of response a cytochemical analysis was carried out on the *in vitro* material to investigate the infected plants at a cellular level. The comparison between healthy and 'Ca. P. mali'-infected MS and GD showed that while the cellular organisation in the susceptible genotype was deeply affected by the presence of 'Ca. P. mali' in the resistant genotypes only few limited structural modifications were observed. Interestingly, by comparison of healthy tissues from both resistant and susceptible *in vitro* apple plants, it appeared that the lumen of the cell was reduced in different sieve tube members by nacreous layers. In addition, sieve plates tended to present callose accumulation, and vacuolar phenolics were present in the parenchymal cells.

Discussion

In this study we adopted an integrated approach. Our objective was to verify the indications obtained from the analysis at a molecular level with an analysis at a cellular level. The study of the gene expression in 'Ca. P. mali'-resistant and -susceptible genotypes after the infection with 'Ca. P. mali' showed that there is a broad range of metabolisms that are affected by the phytoplasma presence. In the resistant genotype it seems that a general response against stress and pathogen is activated. This resembles a type of reaction that was also observed in the "recovery" phenomenon. In order to gain more information on ultrastructural features and modifications at cellular level a cytochemical analysis was performed. Preliminary results on healthy and 'Ca. P. mali'-infected MS and GD *in vitro* plants showed that in GD there is a dramatic change in the cellular organisation while in MS this was localised to very few cells. Structural features of MS phloem cells, already evident in the healthy tissue, could be correlated to the ability of the genotype to contain the phytoplasma spread.

So far, the indications obtained from the gene expression analysis were not confirmed by the cytochemical analysis of the phloem tissues. Further investigation will be necessary to better integrate the two approaches. Nevertheless, the data obtained from the TEM analysis showed once more the power of this technique in plant-pathogen interaction studies.

Acknowledgements

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Molecular differentiation of severe and mild strains of ‘*Candidatus Phytoplasma mali*’ and evidence that their interaction in multiply infected trees determines disease severity

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Abstract

Previous work has shown that multiple infections of apple trees by distinctly different strains of ‘*Candidatus Phytoplasma mali*’ are widespread. In the current study, pathological data of infected trees with single or multiple phytoplasma were analyzed and compared with molecular data based on a *hflB* gene of the infecting phytoplasmas. Single-strand conformation polymorphism and sequence analysis of a variable *hflB* gene fragment revealed that mild and severe strains can be distinguished by their SSCP profiles and their phylogenetic clustering. Analysis of cloned sequences from mild and severe single-strain accessions resulted in two groups of reads that clustered, according to their virulence, distantly in the phylogram. Based on this data, the clustering patterns of multiple-strain accession sequences indicated that nearly all of them were composed of mild and severe strains. Our data indicate that the virulence of multiple-strain accessions is determined by the ratio of the occurring mild and severe strains in that mild accessions were characterized by the predominance of sequences representing mild strains and vice versa. There is evidence that shifts in the population may occur that drastically alter virulence of multiple-strain accessions.

Key words: Apple proliferation, ‘*Candidatus Phytoplasma mali*’, virulence, multiple infections, classification.

Introduction

Apple proliferation (AP), associated with ‘*Candidatus Phytoplasma mali*’, is one of the most important phytoplasma diseases in Europe. AP induces a range of symptoms that are either specific, such as witches brooms, rosettes, and enlarged stipules, or largely nonspecific, such as foliar reddening, yellowing, growth suppression and undersized fruits. However, symptom expression is often subject to fluctuation. Diseased trees may recover and may show no or only mild symptoms for shorter or longer periods after which severe symptoms may reappear. Symptom development also depends on the virulence of the infecting AP phytoplasma strains (Seemüller and Schneider, 2007).

‘*Ca. P. mali*’ is, at the level of ribosomal DNA sequences, a homogenous species throughout Europe. To elucidate the molecular diversity within this taxon, several other approaches have been employed. However, the genotypes delineated in these efforts were unrelated to phenotypical traits such as virulence and other pathological characteristics. High resolution of ‘*Ca. P. mali*’ genotypes was achieved by single-strand conformation polymorphism (SSCP) analysis of a fragment of the ATP00464-type *hflB* gene of strain AT (Schneider and Seemüller, 2009). In continuing this work, we identified DNA samples yielding more complex SSCP profiles that suggested the presence of multiple infections in a single tree. Cloning of PCR products of such samples resulted in clone populations showing distinct profile polymorphisms and diverse sequences that clustered distantly when subjected to phylogenetic analysis. The collective data indicated that

they were composed of two or three distinct ‘*Ca. P. mali*’ strains (Seemüller *et al.*, 2010).

Recent work also suggested that multiple infections are of pathological relevance, probably due to antagonistic strain interactions leading to shifts in the populations (Seemüller *et al.*, 2010). In the research presented here we characterized the AP phytoplasmas present in singly and multiply infected trees. By linking virulence of the infecting phytoplasmas with their SSCP and sequence data, it was possible to distinguish and classify mild and severe strains at the molecular level.

Materials and methods

Root or shoot scions from 27 donor trees naturally infected by ‘*Ca. P. mali*’ were collected in Germany, France and Italy to graft-inoculate healthy rootstocks. The recipient trees were grown in the open or in an unheated greenhouse. The occurrence of AP symptoms was annually recorded using a rating scheme from 0 (no symptom) to 3 (severe symptoms such as witches brooms, stunting or reduced fruit size). The figures obtained were used for evaluating both symptomatology and virulence.

Root and/or the current season’s shoot samples were collected from infected apple trees. DNA was extracted from phloem preparations of roots or shoots using a cetyltrimethylammonium bromide procedure (Seemüller and Schneider, 2007). PCR amplification was performed employing primer pair *hflB3_1/rHflB3* (5’-TTCTAGCTATTTCATCGTGAA-3’/5’-CGGCGCGAT TAGTAGCTCC-3’) that specifically amplifies a 528-bp

fragment of ATP00464 *hflB* gene homologues from all AP phytoplasma strains (Schneider and Seemüller, 2009).

hflB gene fragments obtained by PCR amplification were ligated into pGEM-T Easy vector system (Promega) and transformed to *Escherichia coli* XL1 Blue cells (Stratagene). Colony PCR was employed to amplify the inserts of recombinant plasmids using the primer pair described above. The PCR products were subjected to SSCP analysis. Selected cloned fragments were sequenced using M13 forward and reverse primers. Alignment of sequences was performed with ClustalX2 (Thompson *et al.*, 1997). For phylogenetic comparison a 454-bp fragment was selected and analyzed employing the MEGA4 program using Neighbor-Joining parameters and bootstrap analysis (Tamura *et al.*, 2007).

Results and discussion

In the current study the virulence of 27 '*Ca. P. mali*' accessions collected from three different groups of trees was observed over six to 17 years. These trees showed all major traits of AP disease, namely expression of severe symptoms at the onset of disease, inconsistent symptomatology, recovery, reappearance of disease and great differences in the virulence of the infecting phytoplasmas. From our work, there is evidence that a major reason for the unusual symptomatology of AP is the obviously widespread occurrence of multiple infections. Our study revealed that about half of the accessions examined were multiply infected and were composed of two to five distinct strains. The real percentage of multiple infections is probably even higher considering the fact that sometimes many cloned fragments had to be examined to identify this phenomenon.

In our attempt to molecularly characterize the virulence of '*Ca. P. mali*' strains we investigated the relationship between symptomatology and molecular traits of the infecting phytoplasmas. This effort was greatly facilitated by the availability of single-strain accessions. Cloning of *hflB* gene fragments of severe and mild strains of such accessions resulted in two types of sequences that clustered separately in phylogenetic analysis. This fact and the sequence diversity among the various single-strain accessions enabled us to prove that nearly all multiple-strain accessions were composed of mild and severe strains and to classify the cloned sequences accordingly. The separated clustering of clones representing severe and mild strains was supported by a range of molecular markers at the nucleotide and amino acid level.

The molecular classification of the components of multiple-strain accessions reflects their virulence. Cloned sequences of highly virulent accessions clustered predominantly with sequences of severe strains whereas sequences of mild accessions clustered mainly with sequences of mild strains. However, shifts in the phytoplasma composition may occur that drastically alter virulence. There is indication that growing condi-

tions trigger such shifts that seem to play an important role in the symptomatology of AP phytoplasma-infected trees. Our data also indicate that another mechanism may exist leading to low virulence. A few accessions proved to be highly virulent in the field but did not develop any symptom following graft transmission and greenhouse growing. As sequences representing mild and severe strains were present in equal numbers, it is conceivable that virulence factors were inhibited by action of the mild strain components.

In this study, we succeeded to classify phytoplasma virulence at the molecular level. In this way, it was possible to resolve the phytopathogenic composition of multiple-strain accessions and to estimate their importance for symptom expression and course of disease. As shown for one accession, the potential of multiple-strain accessions is not restricted to symptom expression in apple alone; it also affects the host range. Following dodder transmission, two mild strains of this accession grew specifically in either periwinkle or tobacco. The early observations with aster yellows phytoplasmas (Freitag, 1964; Kunkel, 1955) indicate that multiple phytoplasma infections and strain interactions are not restricted to the AP agent but are probably widespread phenomena in phytoplasma. They are not only important to better understand symptomatology and course of disease, but they also affect detection (primer and antibody specificity), identification and classification.

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Symptomatological detection and biochemical changes of the trees infected by apricot chlorotic leafroll phytoplasma

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Abstract

The ecological conditions in the South-Eastern part of Romania are very favorable for the apricot culture (*Prunus armeniaca*). Unfortunately, the same conditions are favorable also for some pathogens (viruses, phytoplasma, bacteria or fungi), the causal agents for many dangerous diseases. The final syndrome of this diseases complex is known as apoplexy or premature dye back of the apricots. Recognition of this diseases complex is very important for the study and cure of the phenomena. If for the majority of the apricot diseases the symptoms are easy to be evidenced, the ones produced by apricot chlorotic leafroll phytoplasma, a key pathogenic agent in premature dye back of the apricots, are more difficult to be distinguished. This paper refers to the researches carried out in order to realize the symptomatological detection of these phytoplasma presence and the quantification of the biochemical changes in the leaves of some apricot cultivars with different degrees of susceptibility to apricot chlorotic leafroll, compared with the leaf biochemical pattern of the same healthy cultivars. The biochemical changes studied are referred to the leaf cell plasma content in polyphenol-oxidase, total phenols and tannins, free amino-acids, soluble dry mater, water, carbohydrates and assimilation pigments.

Key words: biochemical changes, polyphenol-oxidase, phenols, tannins, free amino-acids, photoassimilate pigments.

Introduction

Every year, in the South-Eastern part of Romania, an important number of apricots die from unknown diseases. Researches carried out in many European countries mention that on this fruit species, 80% of the dead trees or of the suffering ones, are infected by the apricot chlorotic leafroll (ACLR) (Cornaggia *et al.*, 1994; Jaraus *et al.*, 1998; Labonne *et al.*, 2000). ACLR is a quarantine disease (OEPP/EPPO, 1986) belonging to the phytoplasma group (Morvan, 1977). The disease was also evidenced on many fruit species, although some of them (plum) are not showing symptoms (Németh, 1986).

The researches carried out during 2006-2010 were focused mainly on detection of the disease symptoms under the eco-pedo-climatic conditions from Research Station for Fruit Growing Constanța, for early and correct diagnosis of this disease. Moreover, during the period of study, were the biochemical changes that occurred in the cellular juice of the leaves sampled were assessed from the ACLR-diseased trees compared to the biochemical changes occurred in the cellular juice of the leaves sampled from healthy trees.

Materials and methods

In order to describe the symptoms produced by natural infections with ACLR, periodical observation were done during vegetation and tree dormancy period. Trees with symptoms were marked and details of external and internal macroscopic display of pathogen and their evolution in time were observed for an easier recognition of the disease in the commercial apricot orchards.

For the quantification of the biochemical changes in the leaves of the apricot cultivars the biological material used in this study was represented by some apricot cultivars among those more commonly grown in Romania (Goldrich, Harcot, Dacia, Earliril, Royale and Olimp) integrated in four different classes of resistance (function of the F% and I value faced to the presence of the ACLR) through natural infection. Biochemical analyses of the cellular juice from leaves were performed during two vegetation periods: in fully activity (June) at the end of the period (October). The determinations were made using the known methodologies and they refer to the content in:

- Polyphenol-oxidase - titrimetical method.
- Total phenols, spectrophotometrical method.
- Tannins, lowenthal, Neubaur method.
- Free amino-acids, spectrophotometrical method.
- Dry matter, gravimetrical method.
- Assimilate pigments, Hagars Bertensath method.
- Carbohydrates-titrimetrie by Bertland, Jijin method.
- Crude protein, spectrophotometrical method.

Results and discussion

In the spring, the ACLR symptoms are mainly the early leafing, preceding or occurring along with the blooming, the phenomena being spotted first in one or two branches.

When the summer starts, the leaves from the attacked branches are smaller in size with green-yellow color. The typical symptoms occurs at the end of the summer, when the apricot leaves roll along the main stalk and vein, develop a cone (or spoon) shape, and fall prematurely. The fruits, numberless, are smaller, slight

asymmetric, and rip later or remain green and prematurely fall. The apricot branches growth slows down, the internodes are shorter and after a variable tie period the internodes are drying. A section through these branches evidence the phloem oxidation and necrosis, in the structures where the pathogen develops (Morvan, 1977). In the orchard, the disease either occurs in isolated apricots, or affects the trees in groups. As regard the transmission, the researches did not identify *Cacopsylla pruni* species as vector (Carraro *et al.*, 1998), but revealed the disease transmission with the shoots used for grafting or budding from diseased apricot trees.

As regard the biochemical changes the content in proteins ferments-polyphenol oxidase was found in higher quantities in cultivars showing resistance to ACLR., compared to smaller quantities in the susceptible ones (table 1). This can be explained by the fact that the polyphenol-oxidase plays a role in the catalase synthesis of the phytoalexins and other components as indicators of the infection process.

Table 1. The content of polyphenols, total phenols and tannoid substances of some apricot cultivars.

Cultivar	Resistance	Polyphenol oxidase	Tannoid substances	Total phenols
Goldrich	V.S.A.	0.35	2.98	80.0
Harcot	S.A.	0.30	2.78	73.0
Dacia	M.A.	0.25	2.67	62.6
Royale	H.A.	0.15	2.50	50.0
Earliril	V.H.A.	0.10	2.48	42.0
Olimp	V.H.A.	0.05	2.40	40.0

The content in tannoid substances, that plays a role in the inhibition of the infection process is higher in the resistant cultivars. The phenols substances are the first ones opposing to reproduction of the pathogen in the plant. Through their oxidation the chinons obtained, are very toxic substances also for the pathogenic agents.

Regarding the free amino-acids content, our results have evinced that the most susceptible varieties have a higher content. This fact may be explained both by the reduction in the protein synthesis and the increase in the protein decomposition due to the disturbance induced by the pathogenic agent.

As for the dry substance content the higher resistant cultivars have presented an increased content in terms of mean annual value (table 2).

There is a direct correlation between the water content of the leaves and the different behavior of the apricot cultivars under the ACLR infection.

The quantity of a; b; c forms of assimilation pigments, especially of the (a+b)/c ratio are an obvious indicator regarding the appearance of some factors which disturb

the normal metabolic processes. Thus, the (a+b)/c ratio was much smaller in the susceptible cultivars than in the resistant cultivars.

Table 2. The content in dry substance and total water in some apricot cultivars.

Cultivar	Wet weight	Dry weight	Dry subst.	Total water
Goldrich	53.0	18.7	35.3	64.7
Harcot	68.0	23.2	33.6	66.4
Dacia	54.0	17.3	32.1	67.9
Royale	55.0	26.1	32.0	67.9
Earliril	41.0	12.9	31.9	68.1
Olimp	46.0	14.2	26.3	73.7

The percentage variation of the glucids in the resistant cultivars presented an elevated quantity of direct reducing and total forms. The mean value of crude protein (%) is decreasing with the increasing of the sensitivity to the ACLR infection. This could be explained by the reduction in the capacity of the proteins synthesis and the intensification of the proteins decomposition in the case of some cultivars with a higher susceptibility to the disease.

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Proteome responses of *Vitis vinifera* L. to ‘flavescence dorée’ phytoplasma infection

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Abstract

‘Flavescence dorée’ is a serious phytoplasma disease affecting grapevine in several European countries. We are currently studying the interaction of ‘flavescence dorée’ phytoplasma with its natural plant host, by monitoring the effects of infection on the protein expression profile. The red-berried *V. vinifera* cv. Nebbiolo and Barbera were the source of plant material, as they are widely grown in Piedmont and produce wines of high quality. Moreover, the two varieties show a different sensitivity to the disease: ‘Nebbiolo’ is generally considered more tolerant to ‘flavescence dorée’ infection and shows milder symptoms than ‘Barbera’. Total proteins from midrib tissues were separated by two dimensional gel electrophoresis, and differentially expressed spots ($p \leq 0.05$, $|\text{ratio}| > 2$) were identified by mass spectrometry (MALDI-TOF-TOF) analysis. The proteins were further analyzed by Blat2GO software, in order to study their function and involvement in biological processes. By this way, we could develop interaction maps, showing the possible involvement of the proteins in several biological pathways.

Key words: grapevine, 2-DE, recovery, biological pathways.

Introduction

‘Flavescence dorée’ (FD) is a serious disease of the cultivated grapevine (*Vitis vinifera* L.), causing significant reduction in yield and fruit quality. The disease is caused by an obligate biotrophic phytopathogenic bacterium in the class Mollicutes, ‘flavescence dorée’ phytoplasma (FDp). FD may cause epidemics and is subject to quarantine restrictions in Europe. Two typical cultivars of Piedmont, ‘Barbera’ and ‘Nebbiolo’, naturally infected by FDp, were considered. Barbera was chosen for its high sensitivity to FDp infection, which can frequently result in whole plants turning purple. Nebbiolo is generally more tolerant to infection and shows milder symptoms. Often, a spontaneous remission of symptoms (known as recovery) may occur and interestingly, beside the high incidence to FD, ‘Barbera’ shows a high recovery phenotype after the initial infection with FDp (Morone *et al.*, 2009).

In the last decade, 2-DE coupled to peptide analysis by mass spectrometry has become important in the study of plant biology and plant pathology. So far, proteomic studies in grapevine have been mostly applied to berry quality, and to uncover proteome changes during plant development, ripening or response to abiotic stress (Giribaldi and Giuffrida, 2010).

In this study, we are using 2-DE to explore the proteome changes in the leaves of *V. vinifera* infected by FDp or recovered. We have so far performed the analysis on not-infected and infected plants. The analysis on recovered plants is in progress. Our goals are: i) obtainment of 2-DE maps from total protein extracts of not-infected, infected and recovered plants; ii) comparison of not-infected, infected and recovered proteome profiles of the two cultivars; iv) identification of differentially expressed spots; iv) development of maps showing the participation of the proteins in biological pathways.

Materials and methods

A vineyard of ‘Nebbiolo’ grapevines located in Vezza d’Alba and a vineyard of ‘Barbera’ grapevines in Cocconato (Cuneo province, Piedmont, northern Italy), in August 2008 and 2009, respectively were monitored. The vineyards had already been monitored for 3 years for phytoplasma infection, and a map of infected, recovered (i.e. symptomless-plants found positive for FDp in previous years) and healthy plants was already available at the beginning of the study. The sanitary status of the collected material was checked by molecular assays: each sample was tested for phytoplasma infection (‘flavescence dorée’ and ‘bois noir’ phytoplasma) and for other grapevine viruses (i.e., GLRaV-1, GLRaV-2, GLRaV-3, GFLV, GFkV, GVA, GVB, ArMV). FDp strains were characterized and corresponded to subgroup FD-C. Not-infected and FDp-infected samples were selected. At least three biological replicates/thesis (not-infected versus infected) were considered. Total proteins were extracted from midribs isolated from symptomatic or “healthy” leaves following a TCA/acetone protocol (Margaria and Palmano, 2011) and quantified using BSA as standard.

2-DE, gel analysis and MS. Isoelectrofocusing (IEF) was carried out with 400 μg of protein extract using immobilized pH gradient (IPG) strips 17 cm-long, pH interval 4-7 (ReadyStrip IPG Strips, BioRad). Sample loading was performed by passive rehydration for 1 h followed by active rehydration for 12 h at 50 V in a Protein IEF cell apparatus (BioRad). IEF migration was performed at 20°C using a scalar gradient until reaching 10.000 V; the final value was 60.000 Vh. Second dimension SDS-PAGE was performed after strips equilibration, in 12% acrylamide gels using the PowerPac Universal apparatus (BioRad) in a buffer containing 25 mM Tris, 0.192 M glycine and 0.1% SDS. Running conditions were: 30 min at 16 mA per gel and 6 hours at

35mA per gel. Gels were stained using a colloidal Coomassie brilliant blue (CBB) G-250 procedure and scanned with Versadoc Imaging System (BioRad). Image elaboration and analysis were carried out with the PDQuest Software (Biorad). Spots showing significant variation between healthy and infected samples were selected according to the results of Student's *t*-tests ($p \leq 0.05$). Molecular weights and *pI* of spots were predicted according to migration of 2-D standards (BioRad). Gel fragments were excised and analyzed at the Genomics & Proteomics Laboratories Technology Facility, University of York.

The protein data were organized into functional categories according to the Blast2GO program version 2.4.6 (Conesa *et al.*, 2005) with default parameters. The same software was used for building up biological pathway maps.

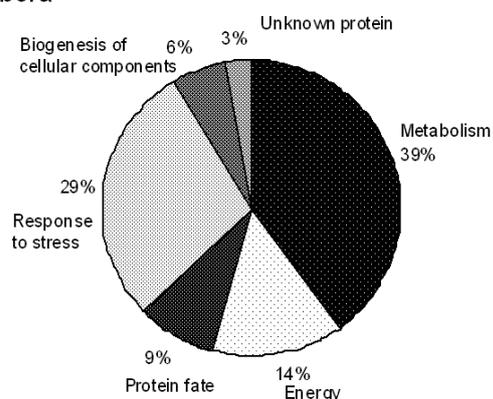
Results and discussion

The high potential of proteomics offers a high throughput tool to study the specific interaction grapevine-FDp from a wide point of view. We successfully performed a proteomic analysis of the differentially expressed spots in FDP-infected 'Nebbiolo' and 'Barbera' grapevines.

According to the set parameters and to Student's *t*-test, a mean of 5% of the detected proteins was differentially were up-regulated in infected tissues. Proteins were grouped in several functional categories, including metabolism, energy, photosynthesis, protein destination, protein synthesis, response to stress. Pie charts representing the distribution of the spots into functional categories are given in figure. 1. Beside common spots, some proteins were exclusively expressed in one cultivar: potentially they could be associated to the differential response to FD. However, further work will be necessary to assess whether the differences in the proteome profiles are effective or instead related to other factors, including the sampling date or variations in protein expression during the season.

Our comparative analysis between the three sanitary status considered (not-infected, infected, recovered), on cultivars showing different levels of sensitivity to FDP, could be critical for better understanding the grapevine responses to phytoplasmas infection and, in a wide view, the complex mechanisms of susceptibility in plants. Moreover, the work could give important information to address the impact of FDP-infection on the quality of the final product, given that some proteins involved in the race between host and pathogen are major allergens in grapes and wine (Pastorello *et al.*, 2003).

Barbera



Nebbiolo

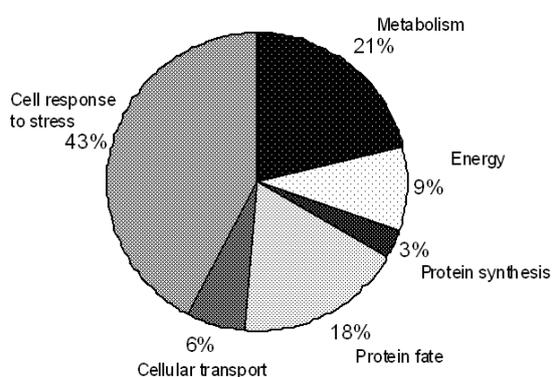


Figure 1. Pie charts representing the functional category distribution of the spots which were differentially expressed in 'flavescence dorée' phytoplasma-infected 'Barbera' (upper pie) and 'Nebbiolo' (lower pie) grapevines, in comparison to not-infected plants.

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Genetic control of the response to a '*Candidatus* *Phytoplasma trifolii*' strain by *Solanum peruvianum*

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Abstract

A report describing the strong resistance and tolerance of the accession PI128655 of *Solanum peruvianum* to two BLTVA strains (beet leafhopper transmitted virescence agent; '*Candidatus* *Phytoplasma trifolii*') was published almost twenty years ago. This work was revisited using another BLTVA strain and the PI128655 plants. No resistance or tolerance was observed. Instead the plants showed either one of two different sets of symptoms. A genetic control of the response to the phytoplasma isolated by the PI128655 plants was demonstrated using clones of host plants. However we obtained experimental evidence that the BLTVA isolate lacked homogeneity or stability, which could also explain the occurrence of two sets of symptoms. A possible hypothesis is that the PI128655 plants, by interfering with the competition between the 'components' of the BLTVA isolate, can alter the final composition of the phytoplasma population and therefore influence the induced symptoms.

Key words: BLTVA, '*Candidatus* *Phytoplasma trifolii*', *Solanum peruvianum*, mixed infection, plasmid, branched inflorescence.

Introduction

Although a promising strategy for controlling diseases caused by phytoplasma infection, host resistance is still rarely applied and remains poorly understood. So far, one of the best documented cases of host plant resistance is the interaction between the apple proliferation phytoplasma ('*Candidatus* *Phytoplasma mali*') and hosts derived from *Malus sieboldii* stocks (Seemüller *et al.*, 2009). In the resistant rootstocks, the phytoplasma concentration was lower than in the susceptible rootstocks M9 and M11 and the phloem did not show sieve tube necrosis and starch depletion. The molecular basis for this resistance is still unknown.

A report describing the complete resistance or tolerance of the PI128655 accession of *Solanum peruvianum* to two BLTVA (beet leafhopper transmitted virescence agent; 16SrVI group; '*Ca. P. trifolii*') strains was published almost twenty years ago (Thomas and Hassan, 1992) with no follow-up since then. Because these experiments demonstrated a case of resistance or tolerance of a herbaceous species relatively close to cultivated tomato, we decided to revisit the interaction between *S. peruvianum* accession PI128655 and BLTVA and assess whether this pathosystem could constitute a good starting point to investigate plant-phytoplasma interactions.

Materials and methods

The BLTVA strain used in this study was kindly provided by Jim Crosslin (USDA-ARS, USA). It originates from a potato tuber produced by a plant naturally infected in a field in Moxee, Washington (USA) and maintained by *in vitro* culture (J. Crosslin, personal communication). For convenience, we transferred the phytoplasma strain from potato to tomato (cv Ailsa Craig) by grafting. It was then further propagated in tomato by successive grafting. The BLTVA strain used in

this study was confirmed to belong to the 16SrVI group ('*Ca. P. trifolii*') (data not shown) but is presumably not the same as the ones used in the experiments of Thomas and Hassan (1992). The *S. peruvianum* accession PI128655 was retrieved from the USDA-ARS Plant Genetic Resource Unit (USA).

Plants were cultivated in an insect-proof greenhouse under natural light. Healthy plants approximately one-month old were inoculated by side-grafting two pieces of stem from infected plants. Except when explicitly mentioned, infected tomato cv Ailsa Craig was used as the source of inoculum.

Cuttings were obtained by taking a piece of stem composed of 2-3 nodes from healthy plants. The cuttings were dipped into a commercial growth hormones rooting powder (Bayer or Rhizopon) and rooted in soil.

Results

Inoculation of the PI128655 plants with one BLTVA strain induced symptoms in all plants tested. However, based on the symptoms produced, the infected plants ranged within two types. Type I plants displayed a growth vigor and a leaf pigmentation similar to the healthy plants, but produced branched inflorescences bearing a greatly increased number of flowers or buds. In these plants, flowers could occasionally show partly attached sepals or inflated buds reminiscent of big bud symptoms (Shaw *et al.*, 1993), or some signs of floral reversion. When the symptoms were more pronounced, buds were replaced by meristematic, cauliflower-like structures, corresponding to a continuous branching and a perpetually delayed flower development. Type II plants showed a reduction in growth vigor, chlorosis at the margin of the leaflets and/or paleness of the leaves, and an absence of flowers due to an early growth arrest of the buds (buds remain smaller than 1 mm). A few type II plants produced cauliflower-like structures after

some time. Clones of individual PI128655 plants were produced by taking cuttings (3 clones for 6 different individuals representing the two plant types). Inoculation of the cuttings with BLTVA resulted in plants displaying symptoms of the same type as the mother plant, thus showing that the symptoms induced upon infection were reproducible, and demonstrating that the factor controlling the formation of symptoms of type I or type II is the genotype of the plant, rather than random or culture conditions.

The formation of two symptoms sets might also originate from the BLTVA strain being a mixture of two strains with different virulence. The homogeneity of the BLTVA strain was assessed as follows. In a first step, PI128655 plants of type I and II were inoculated with BLTVA using infected stem pieces from the same Ailsa Craig plant. Next, healthy type I plants grown from cuttings were inoculated with stem pieces taken either from a type I or a type II infected plant. The type I stem pieces induced mild symptoms on the type I stock, consisting of branched inflorescences with many flowers. The infected type II stem pieces induced clearly stronger symptoms with some plants showing chlorosis, small leaves and cauliflower-like structures without flowers. This experiment therefore suggests that the BLTVA isolate may not be homogenous or may somehow vary in its virulence, since a stable, homogeneous phytoplasma strain should induce the same symptoms when infecting the same type I test plants, whatever the origin of the infected stem piece (type I or type II).

Discussion

This study was initiated with the idea to reproduce the results obtained by Thomas and Hassam (1992) and to investigate the *S. peruvianum*/BLTVA resistance/tolerance mechanism at work. However, we observed no resistance/tolerance as described by Thomas and Hassam (1992), most likely because the BLTVA strain that we used was different. All tested plants showed some symptoms: two types of plants were recognized based on the symptoms developed after inoculation with BLTVA. The occurrence of two symptom sets may be explained by some heterogeneity or lack of stability of the BLTVA strain after passaging in different host plants, as shown by the stronger symptoms displayed by type I plants when inoculated with infected pieces from type II donors compared to type I donors. Virus detection tests should be performed in order to eliminate the possibility that the BLTVA strain also contains a virus inducing differential symptoms between type I and type II plants. Apart from a virus contamination, the heterogeneity may come from the existence of two “components” i.e. from two phytoplasma strains with distinct virulence properties occurring in a mixed infection, or alternatively phytoplasma cells with variable contents of plasmids involved in virulence, as the BLTVA phytoplasma do possess plasmids (Liefting *et al.*, 2004).

Moreover, the inoculation of clones obtained by cuttings revealed that the plant response to the BLTVA infection is genetically controlled by the host PI128655 plants, resulting in two distinct sets of symptoms.

The variation of symptoms thus appears associated with both some phytoplasma heterogeneity and a genetic control by the host plant. Our preferred hypothesis is that the genotype of the host plant differentially influences the multiplication of the “components” of the BLTVA strain, rather than directly controlling the plant response. The accumulation of one “component” in the host would simultaneously (i) prevent the other “component” to accumulate further and (ii) define the general trend of symptoms by imposing its own pathogenic properties. Such effects of the competition between phytoplasma strains of different virulence were already suggested for instance in the case of mixed infection of apple trees by ‘*Ca. P. mali*’ (Seemüller *et al.*, 2010).

The hypotheses of the above scenario will have to be experimentally tested. The perspectives for this work will therefore focus on the search for the putative “components” of the BLTVA strain and their molecular characterization. Each “component” should then be quantified in type I and type II plants. Other perspectives include infection challenges of PI128655 plants with other ‘*Ca. P. trifolii*’ strains or phytoplasma other than ‘*Ca. P. trifolii*’. Finally, the herbaceous nature of the host may allow a genetic analysis of the plant genes involved in the response to the phytoplasma infection.

Acknowledgements

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Symptom expression and 'Candidatus Phytoplasma prunorum' concentration in different *Prunus* species

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Abstract

A SYBR[®] Green I real-time PCR assay has been used for specific detection and quantification of 'Candidatus Phytoplasma prunorum' in different *Prunus* species such as *P. armeniaca*, *P. salicina*, *P. persica* and *P. tomentosa* grown in a screenhouse and infected by means of the vector. Infection level of 'Ca. P. prunorum' in plant samples, expressed as 'Ca. P. prunorum' GU per ng of plant DNA, was achieved by the standard curve quantification method choosing *rplV* (*rpl22*) gene as target for phytoplasma quantification and plant 18S rDNA to normalize the data. Visual inspections of the plants maintained in the screenhouse and qualitative molecular data demonstrated that the species *P. armeniaca* and *P. salicina* are the most susceptible and sensitive, that *P. persica* is less susceptible but quite sensitive and finally that *P. tomentosa* is less susceptible and quite tolerant. Quantitative molecular data for the first time demonstrated, for the first time, that 'Ca. P. prunorum' titre increases during the vegetative season, and that symptom expression is correlated with its infection level.

Key words: ribosomal protein gene, SYBR[®] Green I real-time PCR, European stone fruit yellows, symptom expression.

Introduction

'Candidatus Phytoplasma prunorum' is associated with European stone fruit yellows, a quarantine phytoplasma disease present mainly in Europe and also in Turkey (Sertkaya *et al.*, 2005). Together with 'Ca. P. mali' and 'Ca. P. pyri', it belongs to a major ribosomal group, the apple proliferation (AP) phytoplasma group (16SrX) (Marcone *et al.*, 2010).

European stone fruit yellows (ESFY) is a destructive phytoplasma disease that has a wide range of host plants among cultivated and spontaneous stone fruits, which have large differences in terms of symptom expression and susceptibility (Marcone *et al.*, 2010). Detection of 'Ca. P. prunorum' is carried out by molecular methods, and real-time PCR represents the most recent innovation for detection and quantification of phytoplasmas.

The present study is part of a wider project to improve knowledge about the dynamics of diffusion and colonization of ESFY on 13 different *Prunus* species grown in semi-controlled conditions (screenhouse) and infected by means of the vector. The species that resulted to be the most susceptible to ESFY in the experiment conditions were *P. salicina*, *P. armeniaca*, *P. persica* and *P. tomentosa*. The aims of the present work were to evaluate 'Ca. P. prunorum' concentration in the four different *Prunus* sp. during the vegetative season and to verify a possible correlation between symptom expression and 'Ca. P. prunorum' titre.

Materials and methods

Symptom expression had been monitored monthly during the vegetative season. In June 2009, for qualitative analyses, leaves were collected randomly from all plants of four species *Prunus salicina*, *P. armeniaca*, *P. persica* and *P. tomentosa* grown in a screenhouse. For quantitative analyses, plant material was collected in

July and September from plants of the four species which gave positive results in the qualitative analysis. Total DNAs were extracted from phytoplasma infected plants using a CTAB extraction method modified (Martini *et al.*, 2009) from Doyle and Doyle (1990). The presence of 'Ca. P. prunorum' in plants was assayed by qualitative 'Ca. P. prunorum' specific real-time PCR assay (Martini *et al.*, 2007).

'Ca. P. prunorum' was quantified by SYBR[®] Green I real-time PCR as the number of 'Ca. P. prunorum' genome units (GU)/ng of plant DNA according to Martini *et al.*, 2007. Ribosomal protein (rp) gene *rplV* (*rpl22*) and 18S rDNA were chosen respectively as targets for amplification of 'Ca. P. prunorum' and plant DNA. A standard curve was established by 1:10 serial dilutions of a plasmid containing rp genes of LNp phytoplasma, starting from 1 ng/μl to 1 fg/μl in 20 ng/μl of total DNA from healthy periwinkle. To quantify plant DNA a standard curve was prepared with 1:10 serial dilutions of total DNA from a healthy plant of each of the four species starting at 50 ng/μl to 5 pg/μl. Statistical analyses were performed with the INSTAT GRAPHPAD software package using an ANOVA two-tailed.

Results

Results of symptom expression monitoring and qualitative analysis (June) are reported in table 1.

Table 1. Number of symptomatic and 'Ca. P. prunorum' infected plants (*: mild symptoms).

<i>Prunus</i> sp.	Symptomatic plants	Phytoplasma positive plants
<i>P. armeniaca</i>	7/10	8/10
<i>P. salicina</i>	9/9	9/9
<i>P. persica</i>	2/9	3/9
<i>P. tomentosa</i>	3/10*	5/10

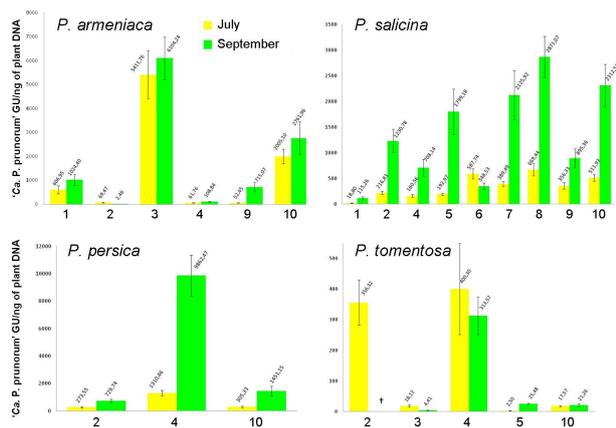


Figure 1. ‘*Ca. P. prunorum*’ concentration (phytoplasma GU/ng of plant DNA) in each *Prunus* sp. plant in July and September.

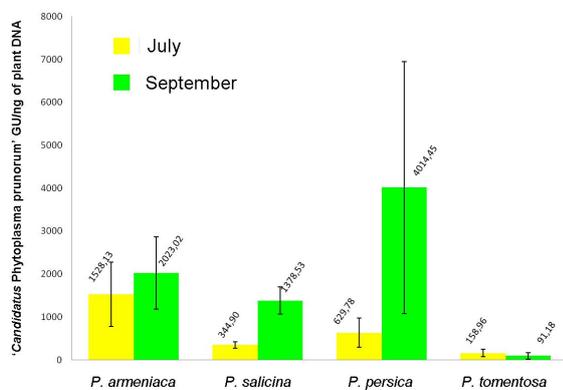


Figure 2. Comparison of the average of ‘*Ca. P. prunorum*’ concentration (phytoplasma GU/ng of plant DNA) among the four *Prunus* sp. in July and September.

The four *Prunus* sp. showed the typical symptoms of the disease, even with slight differences in severity between species and between plants within the same species. Qualitative real-time PCR analysis demonstrated that the number of ‘*Ca. P. prunorum*’ infected plants was higher than the number of symptomatic plants.

All data on ‘*Ca. P. prunorum*’ infection level in July and September, obtained by quantitative real-time PCR analyses for each plant of the four *Prunus* species, are reported in figure 1. For the great majority of plants the phytoplasma concentration was higher in September than in July. Moreover, the phytoplasma concentration values in *P. salicina* appeared to be the most uniform.

Comparing the average of infection level of ‘*Ca. P. prunorum*’ obtained from the four species (figure 2), the average differed significantly in September comparing to July respectively in *P. armeniaca* (1.3 times higher, $P=0.0337$) and *P. salicina* (2.05 times higher, $P=0.0064$).

Discussion

In *P. armeniaca*, *P. salicina* and *P. persica*, but not in *P. tomentosa*, the infection level of ‘*Ca. P. prunorum*’ increased during the vegetative season from July to September, when the plant metabolism is higher and the symptoms become more evident.

Regarding susceptibility, *P. armeniaca* and *P. salicina* appeared to be the most susceptible, whereas *P. persica* and *P. tomentosa* were the least. Concerning symptom expression the species *P. armeniaca*, *P. salicina* and *P. persica* demonstrated a higher sensitivity compared to *P. tomentosa*, which confirmed its tolerant behaviour. A higher *Prunus* sp. sensitivity correlated with a higher average of ‘*Ca. P. prunorum*’ infection level; moreover plants of the four species, showing heavier symptoms in September correlated with the highest values of ‘*Ca. P. prunorum*’ concentration in the same period. All these data demonstrated that, in our case, a correlation exists between symptom expression and phytoplasma concentration.

To our knowledge, it is the first time that quantitative molecular data have demonstrated how and how much the phytoplasma concentration changes during the vegetative season, and that symptom expression is correlated with phytoplasma infection level.

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***Arabidopsis thaliana* as a model plant for understanding phytoplasma interactions with plant and insect hosts**

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Abstract

With increasingly strict restrictions being imposed upon the utilization of environmentally damaging pesticides, research must focus upon the development of phytoplasma-resistant crops as a strategy to control disease outbreak. An enhanced understanding of the mechanisms by which phytoplasmas infect their plant hosts will facilitate achieving this objective. In this study, we report that Aster Yellows phytoplasma strain Witches Broom (AY-WB; ‘*Candidatus* Phytoplasma asteris’) readily infects the model plant *Arabidopsis thaliana* as transmitted by the aster leafhopper *Macrostelus quadrilineatus*. Inoculated plants exhibit symptoms that are characteristic of infection with AY-WB, including witches broom, virescence, phyllody and increased leafhopper fecundity indicating that the AY-WB-*Arabidopsis thaliana* interaction represents an experimental pathosystem to enable research into phytoplasma virulence. Previously, 56 candidate effectors were identified by mining the fully sequenced AY-WB genome for genes encoding secreted proteins. Studying these effectors resulted in the identification of three AY-WB effectors that induce various phenotypes in *A. thaliana*, including phyllody and increased leafhopper fecundity. Thus, the model plant *A. thaliana* has allowed rapid progress with understanding how phytoplasma effectors alter plant development and plant-insect interactions.

Key words: effector, phyllody, virescence, phytoplasma, plant-microbe interactions.

Introduction

Phytoplasmas comprise a group of insect-transmitted plant pathogens that cause a variety of devastating diseases in agricultural crops as diverse as apples, coconuts, carrots, and canola, lettuce and grapevine (Hogenhout *et al.*, 2008). Infected plants cannot be readily cured of phytoplasmas, current strategies to limit the occurrence of phytoplasma outbreaks focus primarily upon removing infected plants and reducing insect vector populations. As countries within the European Union (and throughout the developed world) move to prohibit the widespread application of environmentally damaging pesticides, new mechanisms of controlling phytoplasma dissemination must be advanced. An improved understanding of the molecular mechanisms by which phytoplasmas infect plants is necessary to facilitate the development of resistant crops.

Aster yellows witches’ broom phytoplasma strain (AY-WB; ‘*Candidatus* Phytoplasma asteris’) is transmitted by the polyphagous leafhopper *Macrostelus quadrilineatus* and is capable of infecting a broad range of plants, including lettuce and China aster (Zhang *et al.*, 2004). Symptoms exhibited by plants infected with AY-WB include stunting and yellowing, witches broom (an increased production of axillary stems), virescence (greening of non-green tissues), and phyllody (the generation of leaf-like flowers). Indeed, many phytoplasma strains elicit one or more of these symptoms in infected plants (Lee *et al.*, 2004). We have hypothesized that phytoplasmas such as AY-WB secrete effector (virulence) proteins that modulate developmental processes in plants, resulting in the symptoms that are characteristic of infection by these bacterial pathogens (Hogenhout *et al.*, 2008). The genome of AY-WB has been sequenced (Bai *et al.*, 2006) and mined to enable the iden-

tification of candidate effectors (Bai *et al.*, 2009). We wish to establish a model system in which the molecular mechanisms underlying phytoplasma virulence might be dissected, and thus we exposed the model plant *Arabidopsis thaliana* to AY-WB carrier leafhoppers and observed disease progression in this host.

Materials and methods

Arabidopsis thaliana ecotype Col0 plants were exposed to uninfected (healthy control) and AY-WB carrier *M. quadrilineatus* (Forbes) to enable phytoplasma inoculation. To avoid egg-laying, males only were caged with 3 to 4 week old *A. thaliana* plants (2 leafhoppers per plant) for one week. Prior to insect exposure, plants were germinated in growth chambers under a short-day photoperiod (10 hour light/14 hour dark) at 22°C. After insect exposure, and for the duration of the experiment, plants were transferred to a growth chamber set to a long-day photoperiod (16 hour light/8 hour dark) at 23°C light/20°C dark.

Results

The three and four-week old *A. thaliana* plants that were inoculated with AY-WB exhibited symptoms that are characteristic of infection with this phytoplasma strain. Particularly, infected plants appeared to be bushier than control plants exposed to non-carrier insects, consistent with a witches’ broom phenotype. Furthermore, petals produced by infected plants were green whereas healthy plants exclusively produced flowers with white petals (figure 1). Examination of flowers on infected plants revealed evidence of virescence (white petals were con-

verted to green petals) and phyllody (flowers had a leaf-like appearance). In addition, *M. quadrilineatus* produced ca. 60% more progeny on AY-WB-infected versus healthy *A. thaliana* and the AY-WB-infected plants became feeding and reproductive hosts for the maize specialist leafhopper *Dalbulus maidis*, which does not normally use *A. thaliana* (Hogenhout *et al.*, submitted).

Previously, we proposed that phytoplasma produce effectors that actively induce the various phenotypes observed in phytoplasma-infected plants (Hogenhout *et al.*, 2008). We mined the fully sequenced genome of AY-WB (Bai *et al.*, 2006) for genes encoding secreted AY-WB proteins (SAPs) and resulted in the identification of 56 candidate effectors (Bai *et al.*, 2009). Generation of transgenic *A. thaliana* lines expressing these effector genes revealed three AY-WB effectors that alter *A. thaliana* phenotypes, including one effector that induces the production of leafy flowers similarly to those observed in AY-WB-infected plants (figure 1), and another that stimulates egg laying of *M. quadrilineatus*, a phenotype that is also observed in AY-WB-infected *A. thaliana* (Hogenhout *et al.*, submitted). Thus, the model plant *A. thaliana* has enabled us to make quick progress in elucidating the mechanism by which phytoplasmas interfere with plant development and plant-insect interactions.



Figure 1. (A) *A. thaliana* plants infected with phytoplasma AY-WB produce green leaf-like flowers. (B). Flowers from healthy *A. thaliana*. Scale bar, 1mm.

Discussion

Phytoplasmas are the agents associated with numerous devastating diseases that affect agricultural crops, causing reductions in crop yield and quality. Nonetheless, the mechanisms by which phytoplasmas such as AY-WB successfully colonize plants and insects remain largely unknown. The inability to cultivate these bacteria in artificial media has hindered the study of phytoplasmas, and we wished to establish a model system in which the molecular events that underpin the role of phytoplasma infection on symptoms induction may be revealed. The leafhopper *M. quadrilineatus* can utilize a

variety of species as feeding and reproductive hosts, and is capable of vectoring AY-WB, a phytoplasma with an equally broad plant host range. In this study, we have determined that *M. quadrilineatus* can transmit AY-WB to *A. thaliana*. Furthermore, infected plants exhibit classical symptoms of disease, suggesting that the model plant *A. thaliana* may offer an appropriate experimental system from which to identify the phytoplasma effectors that modulate plant development. Indeed, we identified three AY-WB effectors that induce various phenotypes in *A. thaliana* that resemble those observed in AY-WB-infected *A. thaliana* enabling us to make rapid progress in understanding the mechanisms by which these effectors alter plant development and plant-insect interactions.

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Symptom remission and recovery in 'bois noir' infected grapevines

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Abstract

The disease progress of 'bois noir' has been studied from 1996 to 2010 in two vineyards of the middle Rhine valley in Germany. This period was characterized by two severe outbreaks in 1997 and 2007, followed by endemic periods with decreasing incidence. Although the highest yearly incidence was 50% in cv. Riesling and 14% in cv. Pinot noir, the cumulative incidence reached 91% and 40%, respectively. The mean rates of symptom remission were 38% (Riesling) and 59% (Pinot noir), and the average duration of the symptomless period was 3.3 years and 6.6 years, respectively. Based on the comparison of the probabilities to develop disease symptoms it is proposed to deem vines in the first and second asymptomatic year to be temporary free of symptoms (symptom remission), while they should be considered recovered thereafter.

Key words: 'Bois noir', disease progress, remission, recovery.

Introduction

The disease progress of 'bois noir' (BN) is subject to the antagonistic influence of new infection and to the disappearance of symptoms from infected vines. This interaction leads to a considerable temporal variation of disease incidence. Occasional outbreaks of the disease are followed by rather endemic periods with constant or decreasing levels of disease. The spontaneous disappearance of symptoms from infected vines is typical for grapevine yellows (Caudwell, 1961) and related to physiological alterations in the recovering vines (Musetti *et al.*, 2007). Various attempts aim to promote the spontaneous recovery of infected vines by pruning, other cultivation practices, or the stimulation of grapevine defense mechanisms (Romanazzi *et al.*, 2009). It is necessary for the sound interpretation of field and laboratory results to distinguish between the temporal remission of symptoms (latent infection) and the complete recovery of previously diseased vines. A long term study is carried out in different vineyards of the middle Rhine valley, one of the most affected German wine growing areas, with the aim of a better understanding of the temporal aspects of BN disease progress. The data presented here are derived from a subset of the monitoring plots that had been analyzed before for a shorter period (Maixner, 2006).

Materials and methods

The two vineyards were established by the same grower in 1994 with cv. Pinot noir (PN, 819 vines) and cv. Riesling (RI, 611 vines). After the only sporadic occurrence of symptoms in 1995 the systematic monitoring by visual inspection of all vines started in 1996 and was carried out since then every year in September. The yearly disease incidence was calculated as the ratio of symptomatic to all vines, while the rate of new infection was estimated as the proportion of symptomatic vines in the group of previously healthy plants. The rate of re-

mission is the ratio of asymptomatic vines in the group of previously symptomatic ones. Based on previous observations (Maixner, 2006) and the results of the current study, vines were considered to be recovered from BN when they stayed symptomless for more than two years. Vines were grouped according to their diseases history to study the influence of previous symptomatic or asymptomatic periods on the disease. Group data of different years were combined. The data were analyzed by ANOVA with the arcsin-transformed proportions as dependent variables. Tukey's HSD test was applied for multiple comparisons of means. Some parameters were compared between 'endemic' (decreasing or constant incidence) and 'epidemic' years (increasing incidence).

Results and discussion

Two severe outbreaks of BN were observed in the period between 1996 and 2010 (figure 1), and an additional peak was observed in 2001. Peak incidence levels in Riesling were 50%, 42%, and 43% in the respective years. Rates of new infection reached 47%, 25%, and 20%. The average disease incidence was significantly lower in the less susceptible cv. Pinot noir (5.9%) compared to Riesling (34%), but the fluctuations were synchronous. The maximum incidence in the Pinot noir vineyard was observed in 1997 (13.7%). The cumulative incidence from 1995 to 2010 was much higher with 40% (Pinot noir) and 91% (Riesling). The differences between the levels of current and cumulative incidence underline the importance of the spontaneous recovery of infected vines.

Endemic and epidemic years did not differ significantly with respect to BN incidence in Riesling but in Pinot noir, probably because the range between minimum and maximum disease levels was lower in Riesling (factor 4.3) compared to Pinot noir (7.7). In both cultivars the rates of new infection were significantly higher in epidemic years (factor 2.2 to 3.0) while the values for remission were significantly lower. Recovery

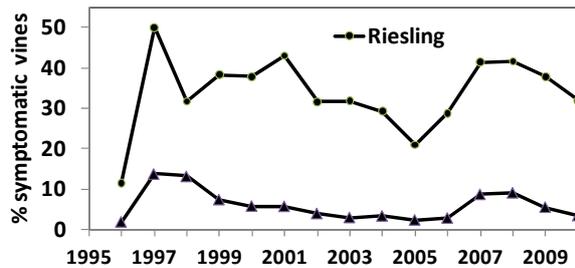


Figure 1. Temporal variation of ‘bois noir’ incidence between 1996 and 2010 in two vineyards of the middle Rhine valley.

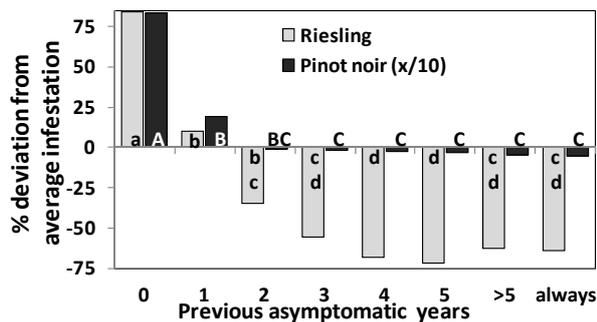


Figure 2. Influence of preceding asymptomatic periods on symptom expression. The within-group incidences are compared to the average incidence of all vines.

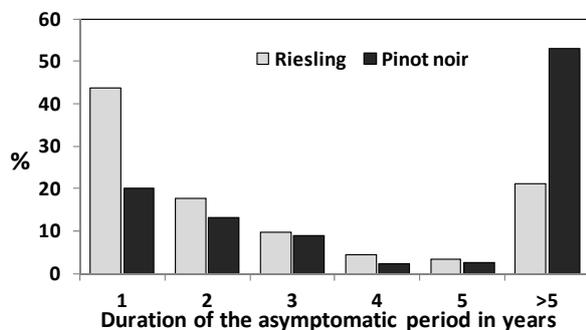


Figure 3. Frequency distributions of Riesling and Pinot noir vines showing symptom remission with respect to the duration of the asymptomatic period.

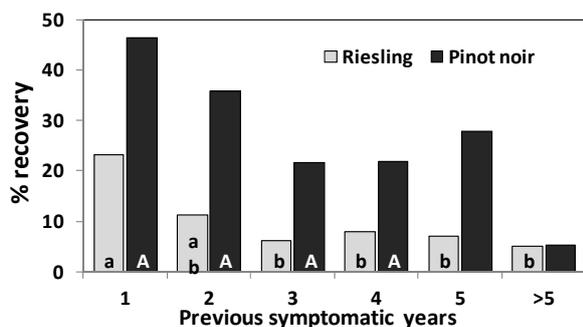


Figure 4. Influence of the length of the preceding symptomatic period on the recovery of vines.

was slightly reduced in epidemic years by 22 and 27%, but the differences were not significant. The strongly varying infection pressure (rate of new infection) seems to be the primary factor for the variability of BN incidence.

The rates of symptom expression were calculated separately for different cohorts according to the previous asymptomatic periods (figure 2). A single asymptomatic year reduced the probability of symptom expression significantly in both cultivars, although it still exceeded the average value of the whole plot. In all other groups the incidence was less than the mean, but it was not significantly different between the groups of one and two asymptomatic years. From the third asymptomatic year the groups were not significantly different nor could be distinguished from the vines that were always healthy before. These results support the previous suggestion to consider a minimum asymptomatic period of three years as a mark to distinguish between temporal remission and permanent recovery of BN infected vines (Maixner, 2006).

The risk of symptom expression of plants with four and five asymptomatic years was less than in the always healthy vines, though not significantly different. More data need to be analyzed to confirm this observation, because it could indicate a reduced susceptibility to new infection after recovery due to physiological changes in recovering and recovered grapes (Musetti *et al.*, 2007).

The rates of remission and recovery averaged 38% (15%) in Riesling and 59% (39%) in Pinot noir, while the mean duration of the asymptomatic period was 3.3 years and 6.6 years, respectively. Most of the recovered vines (asymptomatic period ≥ 3 years) stayed free of symptoms for more than five years (figure 3).

As shown in figure 4 the rate of recovery decreased with the increasing duration of the previous symptomatic period. The chance of Riesling vines to recover was significantly higher after one symptomatic year compared to longer periods. This is probably related to the initially limited distribution of the phytoplasmas in the infected plants that might favor their elimination by pruning.

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Phytochemical effects of phytoplasma infections on essential oil of *Monarda fistulosa* L.

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Abstract

Two essential oils hydrodistilled from the aerial parts of phytoplasma-infected plants of *Monarda fistulosa* L. (wild bergamot) growing at the Herb Garden of Casola Valsenio (Emilia Romagna Region; Italy), have been analyzed and compared by GC and GC/MS. The first oil was extracted from wild bergamot plants showing phytoplasma symptoms (virescence, yellows and stunting) and infected by aster yellows and stolbur phytoplasmas; the second oil was distilled from asymptomatic plants in which stolbur phytoplasmas were identified. These two oils were similar in composition to a third oil, of Italian origin, from *M. fistulosa* cultivated in Trentino Region. The oil from plants infected with aster yellows plus stolbur infected plants showed an increase in the quantity of some monoterpenes, as well as in the content of α -caryophyllene and a marked decrease in the content of thymol; the oil from symptomless material (in which stolbur phytoplasmas were detected) yielded a significant increase in thymol concentration and a marked decrease in monoterpene compounds.

Key words: wild bergamot, PCR, RFLP, phytoplasmas, essential oils, GC/MS.

Introduction

Monarda fistulosa L. (“wild bergamot”) is an annual or perennial medicinal plant known for its strong therapeutic effects: its essential oil is characterized by high antibacterial, antimycotic, and anti-inflammatory activities and, for this reason, has been recently proposed for the treatment of seborrhoea (Zhilyakova *et al.*, 2009).

16SrXII-A “stolbur” (Bellardi *et al.*, 2011). In 2010, a study to verify the correlations among the presence of phytoplasma, symptom expression, and the effects of these prokaryotes on essential oil composition was carried out.

Materials and methods

During Summer 2010, molecular tests to confirm the presence of phytoplasma were carried out in *M. fistulosa* plants belonging to the same crop surveyed in 2009, (Herb Garden, Casola Valsenio, Italy). Starting in July, increasing percentages of plants showing phytoplasma symptoms (more than 80%) were observed. Symptomless (=SLP) and symptomatic plants (=SP) (figure 1) were labelled by visual inspection of their aerial parts and tested at blooming stage to verify phytoplasma presence and to determine their identity. After a chloroform/phenol extraction (Prince *et al.*, 1993), plants were tested by direct PCR with primers P1/P7 followed by nested PCR with primers R16F2/R2 (Lee *et al.*, 1995). RFLP analyses were performed with *TruI* and *Tsp509I* for 16 hours at 65°C and with *HhaI* for 16 hours at 37°C and analysed on 5% polyacrilamide gels after ethidium bromide staining.

About 900 gr fresh aerial part material of SP and about 120 gr of SLP were collected in August at the end of flowering, and hydrodistilled; the two oils were separated from water and kept in tightly closed amber vials before analyses. Identification of the compounds was made by combined gas chromatography mass spectrometry (GC/MS) and by comparison of retention times of *M. fistulosa* components with those of a control oil from plants grown in Trentino region (Northern Italy) (table 1).



Figure 1. Flowers of *Monarda fistulosa*: symptomless (a) and symptomatic (b).

During a survey carried out in 2009 in Italy, wild bergamot showing yellows, stunting, virescence and flower bud proliferation, was found for the first time, to be infected by a phytoplasma belonging to 16Sr subgroup

Table 1. Composition of *M. fistulosa* oils.

Compound	Symptomless (SLP)	% Content Symptomatic (SP)	Control	R _i
α-Thujene	0.40 ± 0.03	4.05 ± 0.11	2.60 ± 0.08	923
α-Pinene	0.38 ± 0.03	3.04 ± 0.08	2.36 ± 0.07	942
β-Pinene	1.60 ± 0.04	10.92 ± 0.21	3.10 ± 0.10	978
Myrcene	4.91 ± 0.21	3.92 ± 0.11	8.05 ± 0.13	986
α-Phellandrene	2.78 ± 0.16	8.60 ± 0.31	13.70 ± 0.21	1003
β-Phellandrene	9.96 ± 0.33	14.42 ± 0.13	17.02 ± 0.27	1005
p-Cymene	12.38 ± 0.25	14.86 ± 0.24	13.49 ± 0.19	1024
Δ ³ -Carene	2.92 ± 0.12	0.84 ± 0.03	3.95 ± 0.09	1027
β-Terpineol	0.89 ± 0.053	0.51 ± 0.03	0.44 ± 0.02	1137
α-Terpineol	2.00 ± 0.11	0.80 ± 0.04	0.61 ± 0.04	1178
Carvacrol methyl ether	9.83 ± 0.31	9.08 ± 0.22	3.51 ± 0.09	1205
Thymol	43.57 ± 0.55	20.79 ± 0.43	26.48 ± 0.39	1282
α-Caryophyllene	0.27 ± 0.01	2.65 ± 0.09	0.40 ± 0.02	1425
β-Caryophyllene	2.90 ± 0.09	1.07 ± 0.05	0.69 ± 0.03	1428
Epibicyclosiquiphellandrene	1.25 ± 0.09	0.49 ± 0.03	0.22 ± 0.01	1431
Germacrene D	3.16 ± 0.18	3.22 ± 0.10	2.44 ± 0.09	1486
γ-Cadinene	0.35 ± 0.04	0.47 ± 0.02	0.19 ± 0.01	1504
δ-Cadinene	0.45 ± 0.03	0.27 ± 0.02	0.75 ± 0.03	1508

Results and discussion

In 2009, only 50% of the plants showed phytoplasma symptoms and were found to be infected only by stolbur, while in 2010, phytoplasmas were detected from both symptomless and symptomatic plants (there were no phytoplasma-free plants). Both direct and nested PCR, as well as RFLP analyses on 16Sr DNA sequences confirmed that in all SPL wild bergamot plants were infected by stolbur phytoplasma and SP plants were infected by mixed phytoplasmas: stolbur and another phytoplasma belonging to ribosomal subgroup 16SrI-B (Aster yellows, ‘*Candidatus* Phytoplasma asteris’: AY). Considering that every single plant, with or without symptoms, was phytoplasma-infected, no phytoplasma-free material has been collected to obtain control oil from healthy plants. The increasing of phytoplasma infection (100%) inside the Herb Garden occurred as a consequence of the presence of leafhoppers, weeds and other medicinal phytoplasma-infected plants, such as *Echinacea purpurea* Moench, *Digitalis lutea* L., *D. lanata* Ehrh., *Grindelia robusta* L., recently reported as “new natural host” of AY and/or stolbur phytoplasmas (Bellardi *et al.*, 2007).

Differences in compositions between the oils from SP and SLP were observed. As shown in table 1, in which the percentage presence of 18 components is listed according to their elution order, the oil from SP showed an increase in the quantity of some monoterpenes like α-thujene, α- and β-pinene, and β-phellandrene as well as in the content of α-caryophyllene and a marked decrease in the content of thymol. On the other hand, oil from SLP yielded only a significant increase in thymol concentration as the only relevant modification and a marked decrease in monoterpene compounds.

Composition of the oils obtained by distillation varies significantly among different *M. fistulosa* hybrids

(Mazza and Marshall, 1992); thymol accounted for c.a. 20% of the oil from SP, but over 40% of that from SLP. The thymol reduction in mixed phytoplasma infected plants has particular relevance since thymol is one of the main constituents of the plants having antibacterial and antimycotic activity. Further studies are in progress to compare therapeutic effects of the studied essential oils.

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Phytoplasma titer in diseased lavender is not correlated to lavender tolerance to stolbur phytoplasma

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Abstract

Yellow decline of lavender is associated with stolbur phytoplasma. It is an economically important disease in south-eastern France, and there is no curative control method. In the past few years, susceptible and tolerant lavenders (*Lavandula spp.*) or lavandins (*Lavandula latifolia* x *lavandula angustifolia*) were proposed as disease management strategy to growers. However, the bases of tolerance were unknown. In order to establish these possible bases, we compared the titers of stolbur phytoplasma in the leaves and shoots of different lavenders and lavandins, either sensitive or tolerant. Results showed that symptom severity was not correlated to the tolerance status of all cultivars tested, and that symptom severity was not correlated to the phytoplasma titer.

Key words: yellow decline of lavender, Q-PCR, stolbur phytoplasma, lavandin.

Introduction

In south-eastern France, lavender essential oil production is endangered by lavender decline. Lavander (*Lavandula angustifolia*.) and lavandin (*L. latifolia* x *L. angustifolia*) cultures are affected by yellow decline ("deperissement jaune" in French). The disease is associated with the presence of stolbur phytoplasma, which is transmitted by *Hyalesthes obsoletus* (Cousin *et al.*, 1970, 1971, Moreau *et al.*, 1974, E. Boudon-Padieu, personal communication). Stolbur phytoplasma is also spread by vegetative propagation through lavender and lavandin nurseries.

Up to now, no curative methods exist against stolbur phytoplasma. The only way is to eliminate diseased plants, apply insecticide treatments against insect vectors and plant phytosanitary certified material. Control of phytoplasma infection with resistant plant has always been very limited. However, as alternatives to susceptible plants, tolerant lavenders(ins) were proposed to growers to reduce the damage caused by lavender decline. Up to now, little is known about the mechanism of this tolerance, which could involve interactions between the plant and the phytoplasma or interactions between the plant and the insect vector. In order to identify the bases of tolerance to the decline, we intended to quantify the stolbur phytoplasma in lavenders(ins).

Materials and methods

Lavenders and lavandins were sampled in fields in South-East France. Lavenders were classified as susceptible (Lavande Bleue, lavande fine, Carla, B7, Maillettes, Matherone) or tolerant (Rapido, Diva). Lavandins were classified as susceptible (Abrial) or tolerant (Grosso, Sumian).

DNA extraction was done from 0.3-0.5 g of symptomatic leafy shoots with CTAB method and the final

DNA pellet was resuspended in 50µl TE 1X (Murray and Thomson, 1980).

For setting an internal reference used in real-time PCR quantification, amplification of a 976 bp fragment of MAP gene was achieved using primers adkF2 (5'-GTTGGTTCG CAGAATTTGTCC-3') and if1R2 (5'-CCAGAAACATAAGCGGTAATCGT-3') for 35 cycles (94°C 40 sec, 55°C 40 sec, 72°C 40 sec).

MAP PCR product was cloned in pGEM-T easy plasmid (Promega) following the manufacturer instruction. Q-PCR was carried out using primers MapStol-F and MapStol-R with a method modified from Pelletier and colleagues (Pelletier *et al.*, 2009). The primers and probe for 'flavescence dorée' phytoplasma were removed, as well as the endogenous control targeting a grapevine tRNA which was replaced by a polyvalent endogenous control amplifying the COX gene (Weller *et al.*, 2000). Results were analyzed using MxPro QPCR software (Agilent). The number of phytoplasmas was calculated for all plants, and the difference between mean values were evaluated by Chi-square tests.

Results

Obtainment of the quantification standard. A fragment of the stolbur phytoplasma MAP gene used for the detection (Pelletier *et al.*, 2009) was amplified from DNA extracted from infected lavenders(ins) using primers adkF2 and if1R2. The obtained product was cloned in pGEM-T Easy and sequenced. The plasmid was then quantified and diluted in order to obtain a range standard from 10¹ to 10⁸ plasmids/µl. This standard was tested in duplicate in each Q-PCR assay.

Absence of correlation between phytoplasma titer and symptom severity (severity index). A total of 2,500 lavenders(ins), susceptible or tolerant, were collected in 2008, 2009 and 2010. Each plant was assigned a severity index (from 0: no symptoms, to 7: dead plant) and

tested in Q-PCR assay 692 were positives for the presence of stolbur phytoplasma. Results showed that phytoplasma titer was not significantly correlated to the severity index.

The phytoplasma titer was measured in susceptible and tolerant lavenders(ins). Figure 1 shows the mean titer of phytoplasma in susceptible and tolerant lavandins in spring and autumn. There was a 2.4 fold and 1.2 fold differences in phytoplasma mean titer between susceptible and tolerant lavandins in spring and autumn respectively. However, these differences were not statistically significant (student impaired t-test). The same differences were observed between susceptible and tolerant lavenders in spring and autumn. The only significant difference in phytoplasma titer was observed for susceptible lavender between spring and autumn.

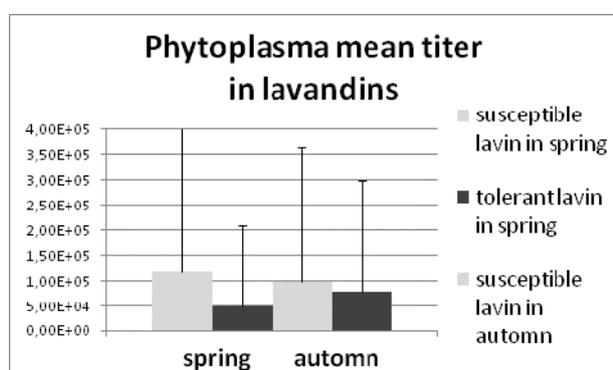


Figure 1. Histogram representing mean titer of phytoplasma (phytoplasma/ μ l) in susceptible (grey) or tolerant (black) lavandins (lavin), in spring and in autumn. Sampling was done in 2008-2009 and 2010.

Time course evolution of phytoplasma titer in lavenders(ins) of different susceptibility was studied. In one year, samples were collected every month on the same lavenders(ins) (from May to November 2010 except in August). Six different cultivars were analyzed: lavandins Abrial (susceptible, clonal), Grosso (tolerant, clonal), Blue-lavander (susceptible, populations), Rapido lavender (tolerant, controlled population) and Diva (tolerant, clonal, in 2 different geographical locations). Quantification was achieved monthly.

Results showed that the phytoplasma titer evolution was not more rapid in susceptible plants as compared to tolerant plants. Most importantly, detection could become negative even when the plant sample had been previously tested positive, indicating the chronicity of the infection. Results concerning Diva lavender showed that disease evolution is dependant on the geographical location of the fields.

Discussion

Results showed that symptom severity was not correlated to lavender and lavandin susceptibility. Little differences in phytoplasma titer were observed between susceptible and tolerant lavenders(ins) in spring and autumn but they were not significant, only for susceptible lavenders. This might be due to temperature effect on the phytoplasma multiplication-rate.

The geographical location of the plants affected the disease development in Diva lavender, probably due to the influence of the climate on the stolbur phytoplasma multiplication, but also on the insect vector activity. Interactions between the plant and the insect vector could then be an important part of the tolerance mechanism of lavenders(ins) to stolbur phytoplasma.

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Proteomic analysis of differentially synthesized proteins in potato purple top phytoplasma-infected tomato plants

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Abstract

Potato purple top (PPT) is an emerging potato disease complex. The etiological agents of PPT are phloem-inhabiting, cell wall-less bacteria known as phytoplasmas. Tomato is an alternative host of these phytoplasmas. PPT phytoplasma-infected tomato plants exhibit symptoms including abnormal foliage development and formation of “big buds”. In the present study, two-dimensional gel electrophoresis (2-DE) was performed to compare the proteomes of healthy and Columbia Basin PPT phytoplasma-infected ‘Rutgers’ tomato. Our results showed that the levels of at least 15 host proteins were significantly altered in response to PPT phytoplasma infection. The identities of these PPT-responsive proteins and their roles in phytoplasma pathogenesis are currently being examined.

Key words: phytoplasma, potato purple top, proteomics, two-dimensional gel electrophoresis.

Introduction

Potato purple top (PPT) is a potato disease complex associated with infection by diverse phytoplasmas belonging to at least five major 16Sr groups. The agent associated with Columbia Basin PPT disease is a phytoplasma affiliated with subgroup A (16SrVI-A) of the clover proliferation group (Lee and Bottner, 2004). Tomato is an alternative host of the Columbia Basin PPT phytoplasma. One of the most striking symptoms exhibited by PPT-infected tomato plants is the formation of “big bud”, an aberrant floral structure with enlarged and fused sepals and absence of pistils, stamens, and petals (figure 1). Because phytoplasmas cannot be cultured *in vitro*, they remain one of the least characterized plant pathogens, and the underlying molecular mechanisms of their pathogenicity are poorly understood (Liefting *et al.*, 2006). To learn how tomato plants respond to PPT phytoplasma infection and develop disease symptoms, we examined global gene expression profiles at the protein level using 2-dimensional gel electrophoresis. Our results revealed that the synthesized levels of at least 15 proteins were significantly altered in Columbia Basin PPT phytoplasma-infected Rutgers tomato plants. The identities of these differentially synthesized host proteins are currently being characterized, and the results are being correlated with our tomato microarray data. Findings from the current study will provide insights into phytoplasma-host interactions in tomato and should enhance our understanding of PPT phytoplasma pathogenesis.

Materials and methods

Tomato plants (*Lycopersicon esculentum* Mill. cv. “Rutgers”) were grown in a greenhouse under 16 h light /8h dark photoperiod at 25°C and 70-80% humidity. Co-

lumbia PPT phytoplasma was graft-inoculated to healthy plants at the 8th true leaf stage. Leaf samples were collected, in triplicates, at three stages (0, 10, and 40 dpi). The leaf samples were frozen in liquid N₂ immediately and then stored at -80°C.

Protein extracts were prepared using a modified protocol of Damerval *et al.* (1986). Leaf samples were ground into powder in liquid N₂ then homogenized with 5 ml of ice-cold 10% TCA buffer. After centrifugation, the pellet was washed with 5 ml ice-cold acetone to remove the impurities (pigments and lipids) until the supernatant was colorless and the pellet turned white. The pellet was vacuum-dried and resuspended in a lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT and 1% pharmalyte). The resulting supernatant was used in subsequent experiments.

The first-dimensional separation was carried out with 17 cm IPG strips, using PROTEAN IEF Cell (Bio-Rad, CA). Equilibrated IPG strips were transferred onto 10% polyacrylamide gels and electrophoresis was performed on a Bio-Rad PROTEAN II xi Cell unit for the second-dimensional separation. The gels were stained using the Coomassie brilliant blue G250 and then viewed using Versa Doc imaging system Model 4000 (Bio-Rad). The 2d electrophoresis images and data were analyzed using the PDQuest software (Bio-Rad).

Results and discussion

IEF strips with a pH gradient of 4-7 were chosen for high-resolution first dimensional separation because, according to our pilot experiment, the pIs of most tomato leaf proteins were within this pH range. Protein profiles were reproducible among technical replicates of the same samples and among replicates from independent extractions. More than 300 protein spots were picked from each 2-DE gel by the PDQuest

software for comparison. As shown in figure 2, about 15 proteins were found to display differential accumulation patterns. Among them, seven proteins were up-regulated, and eight were down-regulated.



Figure 1. Symptoms of Columbia Basin PPT phytoplasma infection in tomato. (A): normal flowers on a healthy Rutgers tomato plant. (B): “big buds” on a phytoplasma-infected tomato plant. Due to early abortion of pistils, stamens, and petals, an abnormal floral structure, “big bud”, is formed from enlarged and fused sepals. (In colour at www.bulletinofinsectology.org)

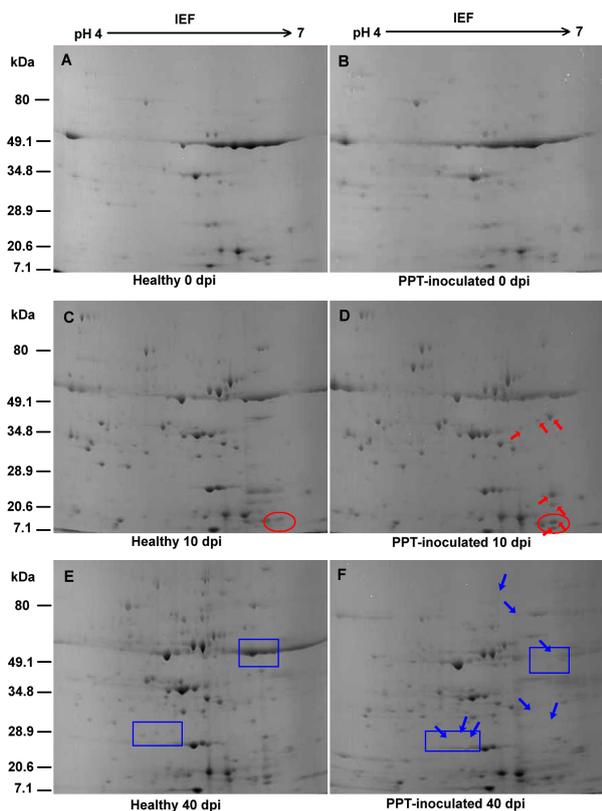


Figure 2. Representative 2-DE gels of Rutgers tomato leaf proteins. (A), (C) and (E): leaf proteins from healthy (mock-inoculated) plants. (B), (D) and (F): leaf proteins from Columbia Basin PPT phytoplasma-infected plants. Leaf samples were collected at 0, 10 and 40 dpi. Up- and down-regulated proteins are marked with red and blue arrows, respectively. Presumed PR proteins are marked by red circles. Presumed MADS-box family proteins are marked by blue rectangles. (In colour at www.bulletinofinsectology.org)

In this study, we examined host proteins that were differentially synthesized/accumulated in response to Columbia Basin PPT phytoplasma infection. A number of heavily stained protein spots with molecular weights (MW) around 14 kDa were present in samples from PPT-infected tomato plants while the corresponding spots were weakly stained in samples from mock-inoculated healthy plants at 10 dpi. Apparently, the levels of these 14 kDa proteins were much higher in infected plants than in healthy plants. Judging from their isoelectric point (pI) and MW values, these proteins could be members of a pathogenesis-related (PR) protein family. Such assumption was supported by results from our parallel tomato microarray experiments in which expressions of several PR protein genes were up-regulated in response to PPT phytoplasma infection, conceivably as part of plant basal defense against invading pathogens (van Loon and van Strien, 1999). At 40 dpi, the peak stage of flower development, a few proteins of approximately 46 kDa and 28 kDa were more heavily stained in healthy tomato plants than in PPT-infected plants. Two observations lead us suggest that down-regulation of these proteins might be responsible for flower deformation in PPT-infected plants: i) the MW values of these proteins coincide with those of MADS-box family proteins FA, DEF and TAG1, which are products of group B and group C floral homeotic genes (Theissen *et al.*, 2000), and ii) our previous RT-qPCR experiments indicated that group B and group C floral homeotic genes were down-regulated in response to PPT phytoplasma infection in ‘Rutgers’ tomato. Findings and hypotheses reported in this communication are preliminary. The identities and roles of those differentially regulated, PPT phytoplasma infection/disease-related proteins are currently being characterized, with the goal of identifying host components that are involved in phytoplasma pathogenesis.

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Effects of exogenous indole-3-acetic acid on proteomic profiles of potato purple top phytoplasma-infected tomato plants

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Abstract

Phytoplasmas are cell wall-less bacteria that cause numerous diseases in diverse crops worldwide. Phytoplasma-infected plants often exhibit symptoms suggestive of hormone disorders. Indole-3-acetic acid (IAA), a naturally-occurring auxin, is involved in multiple essential plant growth and developmental processes. It has been shown that exogenous application of IAA can effectively remit symptoms caused by phytoplasma infections. The present study was designed to learn whether exogenously applied IAA would modify phytoplasma-induced changes in host gene expression profiles at the protein level and to understand the role of IAA in phytoplasma pathogenesis.

Key words: phytoplasma, tomato, IAA, proteomics.

Introduction

Phytoplasmas are a group of small, cell wall-less bacteria responsible for numerous serious and destructive diseases in agriculturally and environmentally important plant species (Lee *et al.*, 2000). Plants infected by phytoplasmas exhibit an array of symptoms believed to result, at least in part, from hormonal imbalance. Indole-3-acetic acid (IAA) is the principal auxin that regulates growth and development of plants. Previous studies demonstrated that exogenous application of auxins on phytoplasma-infected periwinkle plants could induce symptom remission or even phytoplasma elimination (Ćurković-Perica, 2008), supporting the notion that plant hormones play a crucial role in phytoplasma pathogenesis. In the present study, we conducted a comparative proteomic analysis, using two-dimensional gel electrophoresis (2-DE), of healthy (mock-inoculated), PPT-infected, and IAA-treated/PPT-infected tomato plants. Our preliminary data revealed that, compared with healthy tomato plants, the 2-DE protein profile was altered in PPT phytoplasma-infected tomato plants. Exogenous application of IAA partially restored the normal profiles of certain PPT-responsive proteins. Findings from this study will aid us to understand the role of IAA in phytoplasma pathogenesis and the mechanism of exogenous IAA-induced phytoplasma disease symptom remission.

Materials and methods

Plant material and phytoplasma inoculum

Columbia Basin PPT phytoplasma (a member of subgroup 16SrVI-A) and its alternative host 'Rutgers' tomato were used as a pathogen-host pair. Symptomatic or healthy control shoots were grafted onto eight-week-old healthy plants in the greenhouse.

IAA application

200 ml of 0.114 mmol/L IAA solution (ethanol/water, 1/1000, v/v) was applied twice *via* root drenching. Exogenous IAA application was made at seven and nine days post-grafting inoculation. Water was withheld from the plants for two days before each application.

Protein extraction and 2-DE

Leaf samples from healthy, PPT-infected and IAA-treated/PPT-infected tomato plants were collected at 1-d and 14-d after the second IAA application. Proteins were extracted from leaves on the branch next to the graft union by a method modified from Damerval *et al.* (1986). Briefly, fresh leaf tissues (0.5 g) were triturated in a pre-cooled mortar with liquid nitrogen and homogenized with a TCA-acetone extraction buffer (10% w/v TCA in acetone containing 0.07% v/v β -mercaptoethanol). The homogenates were incubated overnight at -20°C. After centrifugation at 10,000 rpm for 1 h at 4°C in a microfuge, the pellets were washed with ice-cold acetone containing 0.07% v/v β -mercaptoethanol to remove pigments and lipids until the supernatant became colorless. Protein pellets were dried under vacuum before solubilization in 2 M thiourea, 8 M urea, and 2% CHAPS (w/v) by sonication. Insoluble tissue debris was removed by centrifugation at 13,200 rpm for 30 min. Concentrations of the protein extracts were measured using Bio-Rad Protein Assay Kit (Bio-Rad, CA).

For iso-electric focusing (IEF), 300 μ g proteins were loaded. IPG strips (17 cm, pH 4-7, Bio-Rad) were rehydrated with 200 μ L rehydration buffer (2 M thiourea, 8 M urea, 2% w/v CHAPS, 1% w/v DTT, 0.2% w/v Bio-Lyte 4-6/5-7, 0.001% w/v bromophenol blue). IEF was performed using Protean IEF Cell (Bio-Rad) at 20°C with 50 μ A/strip by applying the following program: 50 V for 12 h, 250 V for 30 min, 1000 V for 1 h, 10,000 V for 5 h, and 10,000 V for 60 kV h. After IEF, the proteins in the strips were denatured with an equilibration buffer (1.5 M tris-HCl pH 8.8, 6 M urea, 20% glycerol,

2% sodium dodecyl sulfate [SDS], 2% DTT) for 15 min and then incubated with the same buffer containing 2.5% iodoacetamide instead of DTT for an additional 15 min. The strips were transferred onto 10% polyacrylamide gels, and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a PROTEAN® II xi Cell (Bio-Rad) at 5 mA/gel for 45 min and 20 mA/gel until the bromophenol blue dye front reached the bottom of the gel. The gels were stained with Coomassie Brilliant Blue G-250 and scanned using a VersaDoc Imaging system (Bio-Rad). Stained gels were analyzed by PDQuest 2-D analysis software (Bio-Rad).

Results and discussion

Since phytoplasmas cannot be cultured in cell-free medium, these organisms remain one of the least characterized plant pathogens. The mechanisms of phytoplasma pathogenicity are particularly poorly understood. Proteomic technology provides a new approach to study interactions between phytoplasma and plant host, offering clues to a better understanding of phytoplasma pathogenesis (Ji *et al.*, 2009).

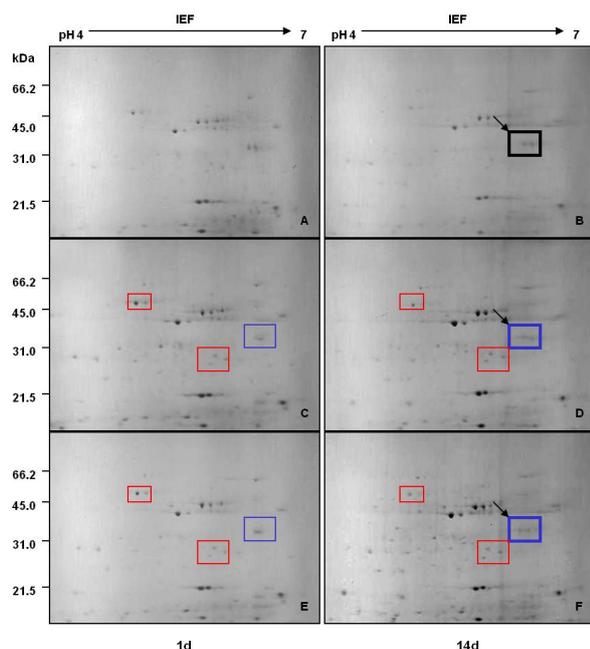


Figure 1. Representative proteomic profiles of tomato leaf tissues in response to PPT infection and IAA treatment. Proteins were extracted from leaves of healthy (A and B), PPT-infected (C and D), and IAA-treated/PPT-infected (E and F) plants. Examples of up- and down-regulated proteins are marked with red and blue rectangles, respectively. Proteins inside the heavy boxes are further discussed in figure 2. (In colour at www.bulletinofinsectology.org)

To gain insights into the effects of exogenous IAA on proteomic profiles of PPT phytoplasma-infected tomato, 2-DE gels of leaf proteins from healthy, PPT-infected,

and IAA-treated/PPT-infected tomato plants were compared (figure 1). Most of the tomato leaf proteins were concentrated in the range of pH 4-7 and molecular weight (MW) 21.5 - 66.2 kDa. With Coomassie Brilliant Blue stain, the total number of protein spots resolved was about 300 on each 2-D gel, suggesting that the proteomic approach has a great potential to unveil significant information regarding differentially synthesized proteins in PPT-infected *v.s.* IAA treated/PPT-infected plants. An analysis of the 2-D gels using the PDQuest software, with visual verification, revealed that levels of approximately 10% of the total leaf proteins were altered.

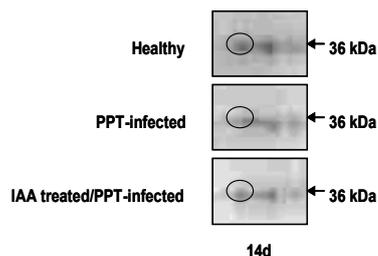


Figure 2. Close-up views of a 36 kDa protein exhibiting differential synthesis/accumulation patterns in PPT-infected *vs.* IAA-treated/PPT-infected plants.

In IAA-treated/PPT-infected plants, the levels of certain PPT-responsive proteins partially restored to levels comparable to those of healthy controls. As shown in Figure 2, a protein of approximately 36 kDa was significantly down-regulated in PPT-infected plants; however, in IAA-treated/PPT-infected plants, the synthesis/accumulation level of the protein was similar to that of healthy plants. Currently, the identities and roles of these PPT- and IAA-responsive proteins are being characterized and correlated to the data from our parallel microarray and RT-qPCR experiments. Our preliminary results have indicated that these PPT-responsive proteins may be involved in hormone homeostasis and plant basal defense against pathogen infections.

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'Bois noir' phytoplasma can be transmitted to healthy *Vitis vinifera* L. plants by rootstocks

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Abstract

The role of the rootstocks Kober 5BB, 420A and SO4 in 'bois noir' (BN) phytoplasma transmission was investigated in cuttings, named 'trionti', created by grafting a BN-infected grapevine cane (inoculum source) and a healthy grapevine bud grafted onto a healthy rootstock. Typical grapevine yellows symptoms were observed on five originally healthy grapevine sprouts, and specific real-time PCR analyses confirmed the presence of BN phytoplasmas. These findings evidenced that BN phytoplasma translocated from the infected cane to the healthy bud through the rootstock, highlighting the possible rootstock role in spreading BN phytoplasma in the nurseries.

Key words: Stolbur, *trionte*, nursery, transmission.

Introduction

'Bois noir' (BN), a disease of the grapevine yellows (GY) complex associated with stolbur phytoplasmas of group 16SrXII, was firstly reported in Veneto region in 1983 (Egger and Borgo, 1983). Nowadays, BN infects vineyards of all north-eastern Italy (Belli *et al.*, 2010), including the mother-plant vineyards, where its epidemics heavily impact on viticulture. Almost all *Vitis vinifera* L. varieties are susceptible to GY infection and show typical disease symptoms. On the other hand, rootstock varieties do not show any symptoms and are considered tolerant. Recently, GY symptoms were reported also in rootstocks, where phytoplasmas associated with 'flavescence dorée' were identified (Borgo *et al.*, 2009). As phytoplasmas infecting rootstocks could be transmitted by grafting in nurseries, a three-year research project entitled "Prevention and control of grapevine 'bois noir' in the Veneto region" studied the role of different rootstocks in the transmission of BN phytoplasma to the scion. In this work, we reported the results of the first two years.

Materials and methods

Double grafted cuttings, named 'trionti', were created by grafting a healthy Chardonnay bud grafted on a healthy rootstock (top) onto a BN-infected Chardonnay cane, used as inoculum source (bottom) (figure 1a). BN-infected or healthy Chardonnay plants and rootstocks (Kober 5BB, 420A, and SO4) used for creating the

'trionti' were selected by analyses through a real-time PCR TaqMan allelic discrimination assay for the specific detection of BN phytoplasma. Primers and probes were designed on ribosomal protein gene nucleotide sequences (data under publication). In total, 1,175 'trionti' have been grafted: 385 in 2009 and 790 in 2010 (table 1). Double grafts have been carried out at nurseries in Veneto region with a bench omega-type grafting machine. The grafted cuttings have been forced, planted, and maintained in a screen-house protected against phytoplasma vectors. GY symptoms were visually observed on sprouted cuttings in September 2009 and 2010. TaqMan real-time PCR assays were performed on DNAs extracted from leaves of sprouted cuttings for validating the visual observation and for revealing the possible presence of BN phytoplasma even in absence of symptoms.

Results

The sprouting percentages recorded have been very low: in the first year, 59 out of 385 rooted cuttings (15.3%); in the second year, 120 out of 790 (15.2%) (table 1). Leaf yellowing and rolling were observed in one 'trionte' sprouted in 2009 (rootstock SO4) and in two 'trionti' in 2010 (rootstock 420A) (table 1 and figure 1b). Molecular analyses by TaqMan real-time PCR confirmed the presence of BN phytoplasma in symptomatic 'trionti'. Further, these analyses allowed to identify BN phytoplasma also in two symptomless plants, one grafted on SO4 in 2009 and one on 420A in 2010 (table 1).

Table 1. ‘Trionte’ sprouting, GY-symptom observation and molecular detection of BN phytoplasma in the years 2009 and 2010.

Year	Rootstock	Number of ‘trionti’			
		Grafted	Sprouted	GY-symptomatic	BN-infected
2009	Kober 5BB	189	34	0	1
	SO4	105	14	1	1
	420A	91	11	0	0
	Total	385	59	1	2
2010	Kober 5BB	205	27	0	0
	SO4	300	31	0	0
	420A	285	62	2	3
	Total	790	120	2	3
Overall Total		1175	179	3	5

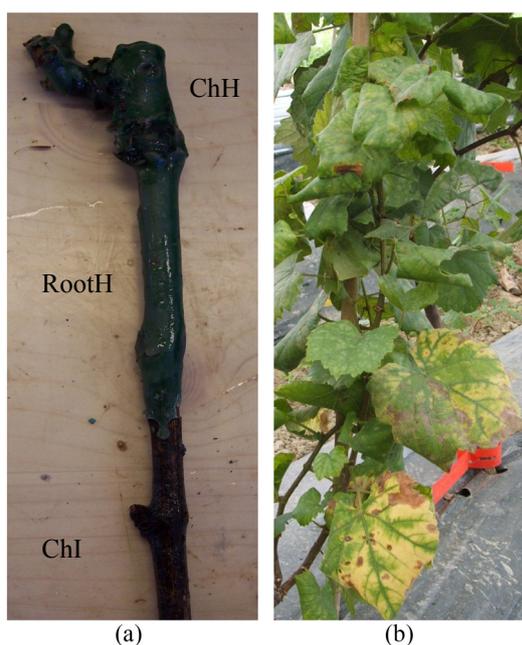


Figure 1. Double grafted cutting (‘trionte’) (a) and symptomatic plant on rootstock 420A (b). ChH: bud from healthy Chardonnay; RootH: healthy rootstock; ChI: cane from BN-infected Chardonnay. (In colour at www.bulletinofinsectology.org)

Discussion and conclusions

Obtained results suggested that the rootstocks Kober 5BB, 420A and SO4 can transmit BN phytoplasma to healthy *Vitis vinifera* L. plants. In 2011, observation and molecular analyses will be repeated on all sprouted ‘trionti’ maintained in screen-house in order to verify the possible presence of further BN phytoplasma transmission events. Interestingly, a recent BN survey in

Friuli Venezia Giulia region reported the highest number of BN infections on vines grafted on rootstocks 420A and SO4 (Ermacora *et al.*, 2011). Our results highlighted that not only FD phytoplasma (Borgo *et al.*, 2009), but also BN phytoplasma can be transmitted through different rootstocks. This evidence opens new avenues for nursery management against the spreading of economically important phytoplasma diseases.

Acknowledgements

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Preliminary proteomic analysis of pear leaves in response to pear decline phytoplasma infection

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Abstract

Pear decline phytoplasma, '*Candidatus Phytoplasma pyri*', belongs to the apple proliferation (AP) group and causes serious diseases in *Pyrus communis* fruiting cultivars in many areas around the world. It induces two types of symptoms, depending on the rootstock: 'slow' decline occurs on trees with tolerant or resistant rootstocks; 'quick' decline occurs on trees with sensitive rootstocks. The most common symptoms are leaf curl and a premature reddening and loss of foliage in the autumn. To better understand the pathogen-stress response of pear (*Pyrus communis* L.) to pear decline phytoplasma, we have initiated a comparative proteomic analysis of infected and healthy pear leaves. The proteins so far identified are mainly involved in carbohydrate metabolism and photosynthesis.

Key words: LC/ES/MSMS; host-pathogen interactions; stress response; plant pathogens.

Introduction

Phytoplasmas are small (0.2-0.8 μm), wall-less, pleomorphic prokaryotes responsible for numerous economically important plant diseases. They are characterized by a very small genome and are obligate parasites of plants and some insects that act as vectors (Lee *et al.*, 2000; Bertaccini *et al.*, 2007).

Pear decline is associated with '*Candidatus Phytoplasma pyri*' that is transmitted by pear psylla, also known as pear sucker. Two types of decline symptoms are recognized: quick decline and slow decline or leaf curl. The degree to which decline symptoms are expressed is governed by the sensitivity of the rootstock, age, and location of tree planted. In the quick decline, fruits cease to develop and both fruits and leaves wilt rapidly. This may be followed by some leaf scorching and leaf death. Trees generally die within a few weeks. In the slow decline there is a progressive weakening of the tree, which may fluctuate with severity. Terminal growth is reduced or may cease completely. Leaves are few, small, leathery and light green, with slightly up-rolled margins; they become abnormally red in autumn and drop prematurely. Although blossoming is heavy in the early stages of attack, later on, fewer flowers are produced, fruit set is reduced and fruit does not attain the normal size (Garcia-Chapa *et al.*, 2003).

Due to inability in culturing phytoplasmas *in vivo*, our knowledge about their physiology, biochemistry, and molecular biology is limited. Only recently, the introduction of molecular methods into plant mycoplasmaology have made it possible to determine the phylogenetic and taxonomic relationships between phytoplasma strains, and those between phytoplasmas and other prokaryotes (Lee *et al.*, 1998). So, these organisms remain one of the most poorly characterized plant pathogens, and the molecular mechanisms involved in their pathogenicity are still poorly understood. Following a patho-

gen attack, the host plant activates a response involving radical changes in the pattern of gene expression (Carginale *et al.*, 2004; Xianling *et al.*, 2009).

Proteomic analysis is a key molecular tool for the analysis of gene expression and has successfully identified novel protein components in plants in response to cold, salt, fungi, bacteria, and viruses. In our study, the protein profiles of healthy and diseased pear leaves were compared, and protein bands showing quantitative changes were subjected to liquid chromatography/electrospray tandem mass spectrometry (LC/ES/MSMS) for identification.

Materials and methods

Pears (*Pyrus communis* L.) were harvested in the orchard of the Fruit Tree Research Institute in Caserta, Italy. Pooled leaves from healthy and PD-infected pear samples were immediately frozen in liquid nitrogen and grinded thoroughly with a prechilled mortar and pestle. The powder obtained was suspended in 10% TCA/acetone. After centrifugation (16,000 g for 3 min), the pellets were washed first with 80% methanol/0.1 M ammonium acetate, and then with 80% acetone. Protein pellets were vacuum-dried at room temperature, and then suspended in 1:1 phenol (pH 8)/SDS buffer. After a thorough mixing (1 hr), the phenol phases were collected and precipitated by methanol/ammonium acetate. The pellets were then washed once with 100% methanol and once with 80% acetone. The resulting pellets were finally suspended in SDS sample buffer. Protein was quantified using the 2-D Quant kit, following the manufacturers' instructions.

Purified proteins from both healthy and infected samples were run on SDS-polyacrylamide gel. Gels were visualized by Coomassie or silver nitrate staining. Protein bands showing significant changes in abundance between healthy and infected samples were selected and excised for protein identification by in-gel trypsin digestion.

Table 1. Proteins identified by LC/ESMSMS.

Acc. n° NCBI and identified protein	Biological function	Acc. n° NCBI and identified protein	Biological function
gi/131385 Oxygen-evolving enhancer protein 1, chloroplastic	Photosynthesis	gi/194708200 Actin	Structural
gi/7525028 Photosystem II D2 protein	Photosynthesis	gi/115800 Chlorophyll a-b binding protein 3, chloroplastic	Photosynthesis
gi/81301580 Cytochrome f	Photosynthesis	gi/403160 Ribulose 1,5-bisphosphate carboxylase small s.u.	Photosynthesis
gi/17981607 Sorbitol 6-phosphate dehydrogenase	Carbohydrate metabolism	gi/28630975 Photosystem II D1 protein	Photosynthesis
gi/5031279 Porin	Transport	gi/27311547 Unknown protein	Unknown
gi/119905 Ferredoxin-NADP reductase, leaf isozyme, chloroplastic	Photosynthesis	gi/136057 Triosephosphate isomerase, cytosolic	Carbohydrate metabolism
gi/119656699 Photosystem II 32 KDa protein	Photosynthesis	gi/8272386 Endo-chitinase class III	Carbohydrate metabolism
gi/19184 Type 1 CP29 polypeptide	Photosynthesis	gi/16175 Adenylate translocator	Carrier
gi/2254440674 Hypothetical protein	Unknown	gi/132270 Rubber elongation factor	Metabolism

The peptide fragments obtained were then subjected to LC/ESMSMS analysis. Database searching, peptide mass fingerprinting (PMF), and MS/MS were performed using MASCOT 2.1 against the NCBI non-redundant *Viridiplantae*-specified protein sequences.

Results and discussion

Proteomic analysis represents a useful tool to gain insight into the plant host responses to stresses. To investigate the effects of PD phytoplasma on the pear protein profile SDS-PAGE on leaf proteins from infected and healthy plants showed differentially expressed protein bands that were excised from the gel and analyzed by LC/ESMSMS. The accession numbers and names of the identified proteins are listed in table 1. Among the 18 proteins identified, two were annotated as unknown, the others are involved in diverse processes including photosynthesis, carbohydrate metabolism, and metabolite transport.

These findings support the data deriving from physiological and biochemical analyses showing that infection with phytoplasmas is associated with increase in soluble carbohydrate and starch content, and decrease in the photosynthesis rate, carboxylation efficiency, and pigment content of leaves (Xianling *et al.*, 2009). In a study on gene expression profile of PD infected periwinkles, genes involved in plant defense/stress responses, protein metabolism and transport, transcriptional regulation, vesicle trafficking, and carbohydrate metabolism were identified (De Luca *et al.*, 2011). Proteomic analysis showed that the expression of many proteins changed during phytoplasma infection. These changes may alter many physiological and biochemical processes, and result in diverse and severe symptoms in infected plants.

Acknowledgements

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Evaluation of susceptibility of plum-trees to ‘*Candidatus Phytoplasma prunorum*’ using real-time PCR

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Abstract

One of the ways to control ‘*Candidatus Phytoplasma prunorum*’ infection is to find varieties of *Prunus* that are resistant or tolerant to the phytoplasma. Different real-time procedures have been recently described to quantify the number of phytoplasmas, thus, the main objective of this work was to evaluate some of these methods and apply them to estimate the concentration of the pathogen in field samples. The DNA extraction with PGB/CTAB and the amplification with two diverse real time protocols were the methods that reach amplifications at lower cycles and were the chosen methods to perform the study of susceptibility of 5 varieties of *Prunus salicina* and *P. cerasifera*. *P. cerasifera* presents the lowest percentage of infected trees. The higher percentage was obtained in the ‘Pioneer’ and ‘Fortune’ varieties despite not significant differences were obtained with the rest of varieties of *P. salicina*. The comparison of the estimated number of phytoplasmas did not reach statistical differences between the five varieties of Japanese plum due to the high intra-variety variability detected.

Key words: phytoplasma, detection, *Prunus* spp., ESFY.

Introduction

The *Prunus* species show differences in susceptibility to ‘*Candidatus Phytoplasma prunorum*’, with apricot, Japanese plum and peach trees being more susceptible than the *Prunus cerasifera* (myrabolan) and *Prunus domestica* genotypes. One of the ways to minimize the damages caused by this phytoplasma is to obtain tolerant or resistant varieties. The purpose of this work was to evaluate different methods of the real-time PCR to estimate the phytoplasma concentration in *P. cerasifera* and in different varieties of *P. salicina*.

Materials and methods

Comparison of detection methods

Previously to realize the trial of phytoplasma susceptibility, two DNA extraction methods and three PCR detection methods were assayed. For this purpose 11 infected and 5 healthy Japanese plums (*Prunus salicina*) were analyzed.

Two methods of DNA extraction were assayed after a first homogenization with PGB grinding buffer: (1) E.Z.N.A. Plant MiniPrep Kit (Omega Bio-Tek) and (2) CTAB extraction (Ahrens and Seemüller, 1992). The real-time detection methods assayed were two based on TaqMan probes (Christensen *et al.*, 2004; Hodgetts *et al.*, 2009) and one based on SYBR Green chemistry (Torres *et al.*, 2005).

Susceptibility to ‘Ca. *P. prunorum*’

For this study samples from 5 varieties of Japanese plums (15 trees of each variety) and ten trees of *P. cerasifera* (mirabolán) were taken in June 2010 (table 1).

Samples were analyzed using PGB/CTAB extraction and two amplification methods selected between those

previously carried out: Christensen *et al.* (2004) and Torres *et al.* (2005). Statistical analysis was performed using the R statistical framework (2010), release 2.11, loading additionally the ez package.

Results

Comparative of detection methods

The samples from 11 infected trees were positives with all the combinations of amplification and extraction methods, and the negative controls were also confirmed with all methods. However, the extractions with PGB/CTAB amplified at lower number of cycles with the three methods of PCR, and the most sensitive detection was obtained performing this extraction and amplifying with Christensen *et al.* (2004) or Torres *et al.* (2005), with significative differences with the rest but not between them (figure 1).

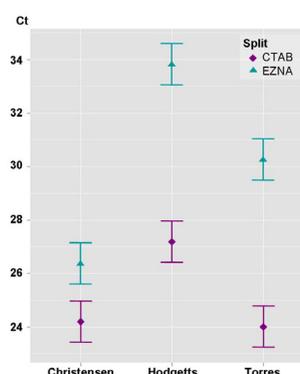


Figure 1. Multiple comparison of amplifying and extraction methods. Error bars of each factor level are the Fisher's Least Significant Difference, and plotted in order to facilitate visual post-hoc multiple comparisons.

Susceptibility to 'Ca. P. prunorum'

The phytoplasma concentration in all the samples analysed in this study was estimated by both selected methods. Results showed that in month of June the percentage of phytoplasma detection in *P. cerasifera* was lower than in *P. salicina* (table 1). Although in September a high percentage of positives were obtained (results not shown) neither of the trees of *P. cerasifera* showed symptoms. Almost all negatives of *P. salicina* are related with asymptomatic trees, 16 out of 23.

The percentage of phytoplasma detection in Japanese plum ranged from 46% to 73% (table 1). The higher percentage of infected trees was obtained in the 'Pioneer' and 'Fortune' varieties and the minor in the '606' variety, despite not significant differences were obtained.

The comparison of the phytoplasma concentration gave not significative differences between the five varieties of Japanese plum due to the high intra-varietal variability (figure 2). The same statistical result was obtained by both methods. However the estimation of the phytoplasma concentration was lower when SYBR green method was applied (data not shown).

Table 1. Proportion of infected trees, showed as positives/total (percentage), detected in *P. cerasifera* (mirabolan) and 5 varieties of *P. salicina*.

Varieties	Christensen	Torres
Pioneer	10/15 (66.7%)	11/15 (77.3%)
Autumn G.	9/15 (60.0%)	9/15 (60.0%)
Fortune	11/15 (77.3%)	10/15 (66.7%)
Anne Gold	10/15 (66.7%)	10/15 (66.7%)
606	8/15 (53.0%)	7/15 (46.7%)
Mirabolan	1/10 (10.0%)	1/10 (10.0%)

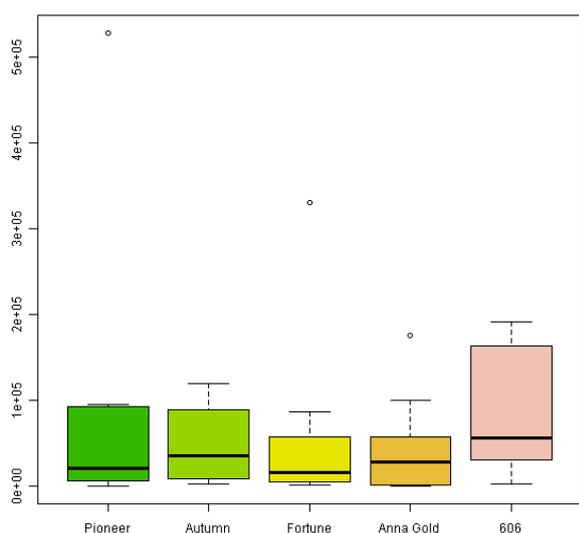


Figure 2. Multiple boxplot of phytoplasma's concentration. Results obtained with Christensen *et al.* (2004) method, in positive trees of five varieties of *Prunus salicina*, are expressed as number of copies of the gene 16S RNA per μ l of extract template.

Discussion

All the methods analyzed are suitable for the detection of 'Ca. P. prunorum'. Some combinations seem to be more sensitive, amplifying the same number of phytoplasmas at lower number of cycles. The SYBR Green method offers an efficient detection at a lower price and could be a reliable option for screening. The estimation of the phytoplasma concentration could vary depending on the real-time applied, in a future work possible matrix effects and the efficiency of the reaction should be analyzed in order to explain these differences.

P. cerasifera presents a low percentage of infected trees and not presence of symptoms. The late detection of the phytoplasma in these species indicated a late colonization of the phytoplasma in the tree. The mirabolan shows tolerance to the disease but not resistance.

The susceptibility of the varieties of Japanese plum is similar, but the range of phytoplasma concentration estimated in trees of the same variety is wide. More work is to be done in order to evaluate the severity of symptoms and the phytoplasma concentration.

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VitisCLIM, a project modelling epidemiology and economic impact of grapevine 'flavescence dorée' phytoplasma in Austrian viticulture under a climate change scenario

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Abstract

Climate warming allows invasive pests to establish in areas where they have not been recognized before. Since its introduction in the 1950's in southern France, grapevine 'flavescence dorée', a quarantine disease of grapevines, has spread significantly in Europe and has first been detected in Austria in the southeast of Styria in autumn 2009, which currently marks the Northeastern border of its extension. VitisCLIM, a project funded by the Austrian Climate and Energy Fund, started in April 2011 and aims to model the current and future potential distribution of the disease and its vector, the leafhopper *Scaphoideus titanus*, in Europe under the influence of climate change. Vine growing areas of high risk in Austria will be defined. Based on scientific literature and empiric studies, an epidemiological model will simulate the temporal and spatial dynamics of the spread of the disease and its vector. Sensitivity analysis will determine critical parameters, including different management strategies which have an impact on the dynamics of the spread. The spread model, together with the results of a survey on direct and indirect costs will then be used in Input-Output Analysis to model the potential economic impact of 'flavescence dorée' to Austrian viticulture and related economic sectors. The results of the project will be communicated to stakeholders, risk managers, policy makers.

Key words: *Scaphoideus titanus*, grapevine yellows, CLIMEX[®], climate warming, spread, spatial analysis, sensitivity analysis, economic impact assessment.

Introduction

Grapevine 'flavescence dorée' (FD) is a severe disease of grapevine. The associated organism is of quarantine concern in Europe according to EC directive 2000/29. It is transmitted by its principal vector, the nearctic leafhopper *Scaphoideus titanus*, which was introduced from North America and reported for the first time in Europe in the late 1950s in vineyards of South-West France (Schvester *et al.*, 1963). Since its first finding in Austrian vineyards in 2004 the vector of FD has spread and is now established in parts of Styria (Zeisner, 2009). FD was recorded for the first time in Austrian vineyards in 2009.

At the northern border of its range short summers prevent the further spread of *S. titanus*, since the vector has difficulties to complete its development and may therefore only form transient populations. Climate change with longer and warmer summers would consequently favour the spreading of the vector further to the north by extending the favourable developing season (Boudon-Padiou and Maixner, 2007).

By including the effect of different climate change scenarios, the project aims to investigate the establishment potential of the disease and its vector in Central Europe, particularly in Austria's main vine growing

regions in the north (Lower Austria and Burgenland). This data will be further used to develop models on the spread dynamics of GFD and on the impact to Austrian economy.

Materials and methods

Mapping establishment potential

The CLIMEX[®] software will be used to model *S. titanus*' establishment potential. Data on its current distribution will be assembled by a systematic literature search to determine the parameter setting. The vectors sensitivity to climate change will be analysed by testing different climate change scenarios based on the results of the IPCC (2007). The generated distribution maps for *S. titanus* will be combined with land use data of the vine production area in Austria and the European Union applying ArcGis.

Modelling spread dynamics

A model to simulate the dynamics of the natural spread of FD in an area will be developed. This model will be based on monitoring data of the distribution of FD and *S. titanus* in Austria and on information on population dynamics and movement behaviour of the

vector. The model will be tested using spread data from recent outbreaks and applied to high risk areas as defined during mapping of the establishment potential. Different risk management options will be incorporated in the model and evaluated with respect to their efficiency in containing the disease. Sensitivity analysis will determine critical parameters which have a major impact on the dynamics of spread.

Modelling economic impacts

The economic impact model will use Input-Output Analysis (IOA), which is a methodical instrument to record the mutually linked supply and demand structures of the sectors in an economy and to quantify the overall economic effect. It analyses direct effects and multiplier effects which are applied to different scenarios, of risk management strategies and climate change. The results of the Input-Output-Analysis may finally be compared to the costs of applied measures and activities. In this way an advanced approach of measuring impacts against inputs and costs of a plant pest will be provided.

The results of the project will be discussed in a participative dialogue with stakeholders and scientists and can be used by risk managers and policy makers as basis for their decisions.

Acknowledgements

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Epidemiology of 'bois noir' disease and molecular variability of associated phytoplasmas in organic vineyards in Tuscany (Italy)

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Abstract

The spread of 'bois noir' disease in grapevine was monitored in three organic vineyards in Tuscany (Italy). Annual incidence, infection and recovery rates were calculated to describe changes in the epidemic with time. In general symptomatic plants showed the recovery in a relatively short time, meanwhile the infection rate decreased rapidly after the second year of assessments. The temporal dynamics of infection and recovery rates were satisfactorily fitted to a power and polynomial models, respectively. Survey on the herbaceous vineyard weeds allow to identify 16SrXII phytoplasmas, polymorphisms in 16Sr and tuf genes were detected.

Key words: epidemiology, disease, molecular detection, weeds.

Introduction

Among the phytoplasmas diseases affecting grapevine 'bois noir' (BN) associated with stolbur phytoplasmas (16SrXII) is present in all grape growing areas worldwide. Its presence is often endemic but in some cases it is associated with severe epidemic, such as in several Italian regions in the last fifteen years.

The spread of 'bois noir' (BN) disease of grapevine was monitored to describe changes in the epidemic with time, and to determine the spatial pattern of the disease in selected vineyards in Tuscany (Italy) (Marchi *et al.*, 2011). Molecular analyses were also carried out to verify presence of BN phytoplasmas strains in the infected vineyards and in weeds in the same vineyards in order to verify relevance of identified polymorphisms in the BN epidemic studied.

Materials and methods

To study temporal dynamics of BN epidemics, data were collected over a 6 year period from experimental plots established within three organic vineyards of cv. Sangiovese (Cs, Fd and Fc) in the province of Florence (Italy). Vineyards Fc and Fd were established in 2001 and 2002, respectively, and are separated by a 4 meters stretch of non cultivated land. Vineyard Cs was established in 2001 in a different locality approximately 10 km apart. Bidimensional maps with locations of all symptomatic, recovered (vines with disease symptoms at least once in the previous years but asymptomatic in the current year) and healthy (disease symptoms never observed) vines were prepared from survey results for each plot and each year of assessments.

To describe and compare epidemics the following

indices were calculated: disease incidence (number of symptomatic vines divided by the total number of plants in the plot in each year), infection rate (number of newly affected plants the current year/total number of plants showing symptoms at least once in the previous period) and recovery rate (number of recovered plants the current year/total number of plants showing symptoms at least once in the previous period (Morone *et al.*, 2007).

At the end of summer 2010 all vines that were showing grapevine yellows symptoms as well as samples of the most represented dicotyledonous herbaceous species (weeds) that were present in vineyards Cs and Fd, screened for phytoplasmas presence by real time PCR (Angelini *et al.*, 2007). Samples resulted infected by stolbur were further tested by nested PCR with P1/P7 and R16F2/R2 (Deng and Hiruki, 1991; Schneider *et al.*, 1995; Lee *et al.*, 1995) primer pairs followed by RFLP analyses with *TruI*, *MboII*, *Hpy188I*, and *AluI* restriction enzymes (Fermentas, Vilnius, Lithuania). Obtained patterns were compared with those of relevant stolbur phytoplasma strains (Contaldo *et al.*, 2011) on same size amplicons.

Results

Temporal analysis of BN epidemics showed similarities between the 3 vineyards. After an initial progressive phase, in the third year of assessments (2007) it began a regressive phase that lasted until 2009. In 2010 disease incidence increased again. Changes in the annual disease incidence values over time were somehow less marked in vineyard Cs compared to vineyard Fc and Fd. The infection rate decreased rapidly over time from the maximum observed in 2006 (figure 1a). In vineyards Fc and Fd, recovery rate increased until 2009 (figure 1b) and then

decreased in 2010; meanwhile in the case of vineyard Cs increased in 2007, was nearly stationary in the two following years and then increased again in 2010. In all three vineyards a power model (Figure 1a) was appropriate to describe change in infection rate over the whole period (Fc, $R^2=0.99$; Fd, $R^2=0.99$; Cs, $R^2=0.97$) meanwhile change in recovery rate fitted well to a polynomial model (Figure 1b) although less consistently in the case of vineyard Cs (Fc, $R^2=0.96$; Fd, $R^2=0.97$; Cs, $R^2=0.88$). Real time PCR results indicated that both in grapevines and weeds only 16SrXII phytoplasmas could be detected.

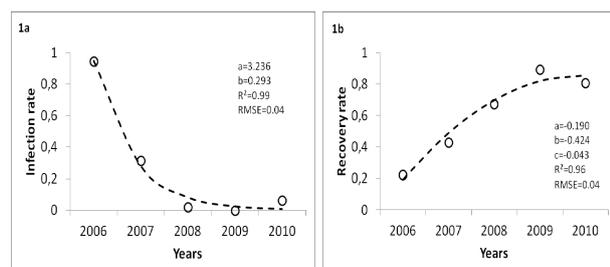


Figure 1. Relationship of the infection rate (1a) and recovery rate (1b) from 2006 to 2010 in vineyard Fc, estimated using a power model ($y=a*b^x$) and a polynomial model ($y=a+bx+cx^2$), respectively. Open circles, observed values. R^2 statistic, coefficient of determination and RMSE is the square root of mean square error. a, b and c (figure 1b) are the equation parameters.

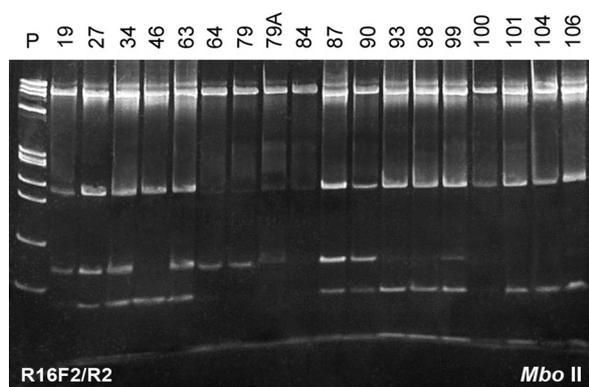


Figure 2. RFLP profiles of R16F2/R2 amplicons of weeds collected in the surveyed vineyards after MboII digest. P, marker Φ X174 HaeIII digested.

Nested PCR/RFLP analyses showed that the following 16SrXII infected weed species contained tuf-type a: *Daucus carota*, *Lactuca saligna*, *Bupleurum tenuissimum*, *Mercurialis annua*, and *Medicago lupulina*; while tuf-type b was identified in *Lactuca sp.*, *Pichiris hieracioides*, *Convolvulus spp.*, and *Cuscuta spp.*; both types were found in *Linaria vulgaris* and in grapevine samples (data not shown). Polymorphisms were detected in R16F2/R2 amplicons with MboII (figure 2), and Hpy188I, while AluI, did not show any polymorphism. In particular the MboII polymorphisms were

commonly detected in both weeds and grapevine samples, while the one with Hpy188I was only present in weeds. The latter enzyme also shows the presence of profiles compatible with interoperon heterogeneity presence or mixed phytoplasmas lineages population in both weeds and in grapevine.

Discussion

The phases of BN epidemic over time were very similar in all vineyards, suggesting that the same insect vector is present and that its interactions with the grapevine host are more variable between years than between different vineyards in the same year. Nevertheless as the spatial distance between the monitored vineyards increased, the goodness of fit of the data to the proposed models decreased, suggesting that other factor/s, strictly related to the agroecosystem, may significantly influence the shape of disease curves over time. Analyses of tuf and 16S rDNA genes of both weed and grapevines show presence of tuf-type a and b in both cases and also of possible mixed BN lineages presence. Further research is in progress to evaluate the possible relationship of these molecular findings with the evolution of BN disease in the studied agroecosystems.

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Effect of indole-3-butyric acid on the recovery of phytoplasma-infected grapevine

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Abstract

Phytoplasma-infected and healthy grapevines were treated on the leaves with indole-3-butyric acid. The effect of IBA was examined by testing the grapevine for presence of phytoplasma 16S rDNA and by measuring stress parameters before and after the treatment. Vines treated with IBA showed signs of recovery, but high incidence of natural recovery was also observed among untreated vines in the same experiment. In naturally-recovered untreated plants, higher levels of H₂O₂ were measured, while in IBA-treated plants recovery was not correlated with higher H₂O₂ levels after the treatment. Other biochemical parameter measurements, lipid peroxidation and total phenolics, did not show difference between the groups of vines tested.

Key words: IBA, phytoplasma, grapevine, hydrogen peroxide.

Introduction

Phytoplasmas cause numerous economically relevant diseases that affect hundreds of plant species including grapevine (*Vitis vinifera* L.). So far, research attempts have been made in order to understand natural-recovery phenomenon of phytoplasma-infected grapevines (Musetti *et al.*, 2007), and different methods and techniques have been applied in the attempts to eliminate phytoplasmas from their hosts (Romanazzi *et al.*, 2009). Previous research also revealed that supplement of plant growth regulator, IBA, causes recovery of phytoplasma-infected periwinkle shoots grown *in vitro* and elimination of 'Candidatus Phytoplasma asteris' reference strain HYDB (Čurković-Perica *et al.*, 2007, Čurković-Perica, 2008; Leljak-Levanić *et al.*, 2010). The aim of this research was to test the effect of IBA phytoplasma-infected vineyard-grown grapevine.

Materials and methods

In July 2008 leaves of *Vitis vinifera* L. cv. Chardonnay from the University vineyard Jazbina (Zagreb, Croatia) were taken to determine the presence of phytoplasmas in vines that exhibited symptoms of grapevine yellows. Presence of phytoplasmas was confirmed by nested PCR. Sixteen plants that were positive in 2008 were chosen for the treatment in 2009. Plants that have never showed symptoms, and in which the presence of phytoplasma was not confirmed, served as negative controls. Phytoplasma-positive grapevines were randomly divided into four different treatment groups. Vines were treated by spraying the leaves with 1 L of IBA solution at three different concentrations (0.5, 1.0, 1.5 g/L). Positive controls (four symptom-expressing, phytoplasma positive vines) were treated with water and negative controls were treated with 1.0 g IBA/L or water. All the treated groups of grapevines, including positive and

negative controls consisted of four vines. Leaf samples were collected before the treatment (May 2009) and two times after the treatment (July and August 2009), and immediately stored at -20°C. Leaf veins were cut out and used for isolation of total nucleic acid. The rest of the leaf material was ground in liquid nitrogen and used for the measurement of stress parameters.

For phytoplasma detection total nucleic acid isolation was performed according to Čurković Perica *et al.* (2007). Direct PCR, first nested and second nested PCR assays were performed using primer pairs R16F1/R0, R16F2n/R2 and R16(I)F1/R1, respectively. Nested PCRs were performed with the samples collected in July 2008 and August 2009 (table 1). Concentration of hydrogen peroxide was determined according to the method of Mukherjee and Choudhari (1983). The level of lipid peroxidation, expressed as malonyldialdehyde (MDA), was determined according to the modified method of Heath and Packer (1968). Total phenolics were determined according to the modified method of Slinkard and Singleton (1997).

Results and discussion

Škorić *et al.* (1998) showed that phytoplasma detection later in the growing season of grapevine is more accurate than in spring. Therefore, presence/absence of phytoplasma was determined in symptom-expressing and symptom-free vines, respectively, by nested PCR in July 2008. Symptoms of grapevine yellows included discoloration and necrosis of leaf veins and leaf blades, downward curling of leaves, lack or incomplete lignification of shoots, necrosis of shoots and shriveling of berries. Based on the results in 2008, vines were selected for the treatment in May 2009. Concentrations of IBA used for treatments and presence/absence of symptoms and phytoplasma in tested vines are listed in table 1.

Table 1. Presence of symptoms and phytoplasma detection on IBA-treated grapevines before and after the treatment.

vine number	treatment (g of IBA/vine)	symptoms and phytoplasma detection before the treatment	symptoms after the treatment	phytoplasma detection by PCR after the treatment
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	1.0	-	-	-
6	1.0	-	-	-
7	1.0	-	-	-
8	1.0	-	-	-
9	-	+	-	-
10	-	+	+	+
11	-	+	+	+
12	-	+	-	-
13	0.5	+	-	-
14	0.5	+	-	-
15	0.5	+	-	-
16	0.5	+	-	-
17	1.0	+	+/-	+/-
18	1.0	+	-	-
19	1.0	+	+	+
20	1.0	+	-	-
21	1.5	+	+/-	+/-
22	1.5	+	+/-	+/-
23	1.5	+	+	+
24	1.5	+	-	-

(+) = symptomatic/phytoplasma 16S rDNA detected;
 (-) = asymptomatic/absence of phytoplasma 16S rDNA;
 (+/-) = one shoot with symptoms, one asymptomatic, phytoplasma 16S rDNA not detected in asymptomatic part of the vine.

High incidence of natural recovery in the vineyard which also affected vines that served as positive untreated controls in the experiment made explanation of the results difficult; two vines although symptomatic and phytoplasma positive in 2008, were symptomless and phytoplasma-negative in summer of 2009. Those recovered untreated vines had higher H₂O₂ levels than symptomatic vines and this is consistent with finding of Musetti *et al.* (2007). High incidence of recovery was also obvious among IBA-treated vines, but recovery was not correlated with higher H₂O₂ levels in those vines. Total phenolics and MDA contents measured in tested vines correlated neither with infection nor with

recovery. MDA increased in all tested groups of vines through the growing season.

Because natural recovery was observed within the positive control group of vines treated with water and the percentage of IBA-treated completely recovered vines was not significantly higher than the percentage of naturally-recovered vines, further research is required to elucidate the possible role of IBA in grapevine recovery.

Acknowledgements

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Evaluation of the efficiency of various treatments used for sugarcane white leaf phytoplasma control

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Abstract

The objective of this study was to evaluate and compare the efficiency of various treatments used for sugarcane white leaf phytoplasma control and quantify the amount of phytoplasma after using different treatments. Different treatments were used with cane stalks to attempt to reduce the phytoplasma concentration as follows; control (no treatment), dual hot water treatment, hot water followed by cold water, and hot water followed by tetracycline HCl 500 ppm. The results showed that the control had the highest percentage of sugarcane white leaf phytoplasma at 100%, followed by the use of hot water, followed by cold water at 95.24%, dual hot water at 90.48% and hot water+tetracycline HCl 500 ppm at 71.43%. Moreover, the control had the highest quantification of the amount of SCWL phytoplasma at 158.71 copies/sample, followed by the use of hot water+cold water, dual hot water and hot water+tetracycline HCl with the amount of phytoplasma at 31.50, 10.99 and $3.72 \cdot 10^{-2}$ copies/sample, respectively. The control without any treatment had the highest cane germination at 77.5%, followed by the use of hot water followed by cold water, hot water followed by tetracycline HCl, and dual hot water with germination rates at 42.5%, 5% and 2.5% respectively. In conclusion, soaking cane stalks in hot water, followed by tetracycline HCl can reduce the percentage of SCWL phytoplasma more than other treatments whereas the cane germination rate is comparatively low.

Key words: sugarcane white leaf phytoplasma, quantification of phytoplasma, hot water, tetracycline.

Introduction

Sugarcane white leaf disease (SCWL) is a major disease of sugarcane production in Thailand, associated with phytoplasmas which transmitted to the plant by the leafhopper *Matsumuratettix hiroglyphicus* (Matsumura) and *Yamato-tettix flavovittatus* Matsumura (Hanboonsong *et al.*, 2002; 2006). Phytoplasma infected sugarcane shows the white leaf symptoms due to loss of chlorophyll. So far there is no effective methods for controlling this disease. In order to reduce or kill the phytoplasma pathogen, various treatments such as hot water, cold water and antibiotic have been recommended for cane stock treatment before planting (Moutia and Dookun, 1999). However, the quantification of the amount of pathogen reduction from the cane stalk after the treatments has not been studied. Therefore, the objective of this study was to evaluate and compare the efficiency of various treatments used for SCWL phytoplasma control and quantify the amount of SCWL phytoplasma pathogen after using different treatments.

Materials and methods

Different treatments were used with SCWL phytoplasma infected cane stalk (Khon Kaen 3 variety) to attempt to reduce or kill the SCWL phytoplasma as follows 1). Control (no treatment) 2). Treatment with hot water at 55°C for 10 min, followed by hot water at 50°C for 2 hrs. 3). Treatment with hot water at 50°C for 2 hrs., followed by cold water at 5°C for 2 hrs. 4). Soaking in hot water at 50°C for 2 hrs. followed by soaking in Tetracycline HCl 500 ppm for 2 hrs. After the treatment was completed the cane stalks were divided into 2 sets. The first set was planted into the soil to test for cane germination.

The second set was used for extraction of sugarcane DNA and detection of SCWL phytoplasma by using nested PCR with a SCWL phytoplasma 16s rRNA and 23S rRNA specific primers according to the protocol of Hanboonsong *et al.* (2006). The quantification of SCWL phytoplasma from each treatment used was also performed by real-time PCR technique.

Results

Sugarcane germination

Different treatments of hot water, cold water and antibiotic used on cane stalks at different times and temperature negatively affected cane germination. The results showed that the control without any treatment had the highest cane germination at 77.5% but they germinated to show the white leaf symptom. Next highest was from the use of hot water followed by cold water, then hot water followed by tetracycline HCl 500 ppm, and dual hot water with germination rates at 42.5%, 5% and 2.5%, respectively (figure 1). Cane stalks soaked in hot water followed by cold water germinated to show a pale green leaf but soaking cane stalks in dual hot water and hot water followed by tetracycline HCl 500 ppm germinated stalks showed a green leaf. Dual hot water treatment of cane stalks greatly reduced the germination as compared to the control.

Quantitative of sugarcane white leaf phytoplasma

The result of PCR showed that the control had the highest percent of sugarcane white leaf (SCWL) phytoplasma at 100%, followed by the use hot water followed by cold water at 95.24%, dual hot water at 90.48% and hot water followed by tetracycline HCl 500 ppm at 71.43% (table 1). Hot water followed by tetracycline HCl

500 ppm can reduce percentage of SCWL phytoplasma more than dual hot water and hot water followed by cold water at 28.57%, 9.52% and 4.76%, respectively (table 1). Moreover, the results of real-time PCR showed that the control had the highest quantification of the amount of SCWL phytoplasma at 158.71 copies/sample, followed by the use of hot water followed by cold water, dual hot water and hot water followed by tetracycline HCl with the amount of SCWL phytoplasma at 31.50, 10.99 and $3.72 \cdot 10^{-2}$ copies/sample, respectively (table 1, figure 1).

After 7 months, the shoots were cut for detection of SCWL phytoplasma. The result showed the shoots of cane stalks which were soaked in dual hot water had the highest SCWL phytoplasma at 100% followed by the use of hot water followed by tetracycline HCl 500 ppm and hot water followed by cold water, with SCWL phytoplasma rates at 75% and 69.23% respectively. But the cane stalks of the control without any treatment died within 2 months.

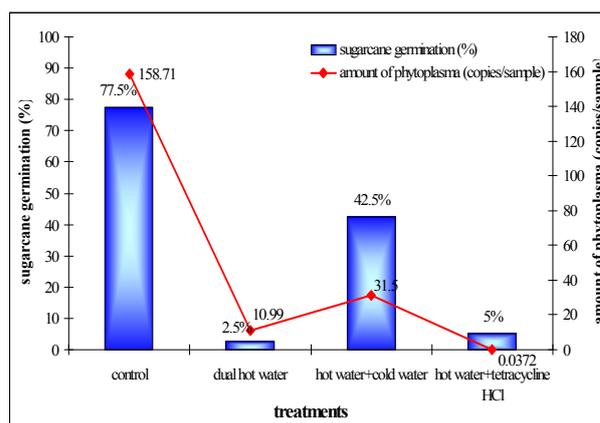


Figure 1. Germination (%) of cane stalks and amount of phytoplasma after different treatments. (In colour at www.bulletinofinsectology.org)

Table 1. Percentage of sugarcane white leaf phytoplasma in sugarcane buds from different treatments by PCR technique and the amount of phytoplasma by real-time PCR technique.

Treatments	SCWL phytoplasma (%)	Decreasing SCWL phytoplasma		Amount of phytoplasma (copies/sample)	
		T/F ¹	%	mean	range
Control	100	21/20	-	158.71	$2.44 \cdot 10^{-8}$ - 2832
Dual hot water	90.48	21/19	9.52	10.99	$1.69 \cdot 10^{-6}$ - 135.7
Hot water+old water	95.24	21/20	4.76	31.50	$8.99 \cdot 10^{-8}$ - 279.3
Hot water+tetracycline HCL 500 ppm	71.43	21/16	28.57	$3.72 \cdot 10^{-2}$	$1.86 \cdot 10^{-9}$ - 0.1803

¹ T = Total number of cane stalk, F = Number of cane stalk that found the sugarcane white leaf phytoplasma.

Discussion

Sugarcane white leaf phytoplasma spreads through sugarcane stocks and is transmitted plant to plant by the insect vectors (Hanboonsong *et al.*, 2002; Marcone, 2002). Using phytoplasma free stock through tissue culture method is currently applied by the sugarcane farmers in Thailand. It helps to reduce the disease occurrence, however, it is an expensive approach and there is insufficient tissue culture seedling for distribution during the planting season. Alternative treatments such as thermotherapy and antibiotic were introduced to sugarcane stocks for controlling sugarcane grassy shoot phytoplasma and showed positive results (Pliansinchai *et al.*, 1997). In the case of sugarcane white leaf (SCWL) phytoplasma, our results showed that soaking cane stalks in hot water at 50°C for 2 hrs followed by soaking in antibiotic tetracycline HCl for 2 hrs can reduce SCWL phytoplasma up to 28.57% more than using only hot water or with cold water. In addition the quantification of the amount of SCWL phytoplasma found that heat treatment can dramatically reduce the SCWL phytoplasma in comparison with non heat treatment. However, the cane germination after heat treatment is comparatively low. It is noticed that after 7 months of planting treated canes, the SCWL phytoplasma can be detected in all canes of heat treatment alone but only from 75% of treated cane with antibiotic. Further studies will investigate the appropriate timing for treatment use in order to increase the germination rate.

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Tolerance increase to ‘*Candidatus phytoplasma prunorum*’ in mycorrhizal plums fruit trees

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Abstract

The tolerance increase to the disease caused by ‘*Candidatus Phytoplasma prunorum*’ in Japanese plum (*Prunus salicina*) after mycorrhizal inoculation has been evaluated. Results showed that after one-year growth, non-mycorrhizal European stone fruit yellows-inoculated plants, were negatively affected by the disease infection. In non-mycorrhizal and phytoplasma inoculated plants the percentage of premature death was 30%, whereas all the mycorrhizal plants inoculated with ‘*Ca. P. prunorum*’ survived. The shoot length of mycorrhizal European stone fruit yellows-inoculated plants did not differ from the values recorded for healthy non mycorrhizal plants, indicating that early mycorrhizal inoculation increases tolerance to European stone fruit yellows disease in one-year old *Prunus salicina* cv Angeleno grafted on GF-677 rootstock.

Key words: phytoplasma control, mycorrhiza, tolerance, ESFY.

Introduction

The cultivated Japanese plum (*Prunus salicina* L.) is severely affected by the ‘*Candidatus Phytoplasma prunorum*’ associated with European stone fruit yellows disease (ESFY). This phytoplasma belongs to the apple proliferation group (Seemüller *et al.*, 1998) or 16SrX-B subgroup (Lee *et al.*, 1998), is transmitted by *Cacopsylla pruni* (Carraro *et al.*, 1998; Sabaté *et al.*, 2007). Because phytoplasmas can be persistently spread by phloem feeding insects, great efforts have been done to control the vector. The spread of the disease cannot be prevented only with the application of insecticides and other control strategies have been focused on breeding for resistant or tolerant varieties and rootstocks. Additional alternatives modifying plant physiology can increase the tolerance to the disease. In this work, the effect of mycorrhizal inoculation on European stone fruit yellows disease on *P. salicina* has been evaluated.

Materials and methods

Plant Material

Forty plants of one year-old *Prunus salicina* cv Angeleno grafted on GF-677, obtained from *in vitro* culture were used in the experiment. Twenty plants were inoculated with the arbuscular mycorrhizal fungus *Glomus intraradices*, Schenck and Smith (BEG 72). In March 2010, when plants were transplanted to 2 liter-volume individual containers, 10 g of *G. intraradices* soil inoculum were applied under the root system. Twenty plants were used as non-mycorrhizal control. Buds from 10-year-old infected *P. salicina* trees, previously determined as ESFY-positive by the PCR technique, were selected and grafted by chip budding on ten mycorrhizal and ten non-mycorrhizal plants in September 2010. The rest of the plants were used as healthy

control plants (10 healthy mycorrhizal plants and ten healthy non-mycorrhizal plants). All trees were maintained in an insect-proof greenhouse to avoid possible insect phytoplasma transmission.

Six months after ‘*Ca. P. prunorum*’ inoculation, once mycorrhizal colonization was confirmed in the roots systems of *G. intraradices* inoculated plants, trees were tested to corroborate the disease transmission. Symptoms expression, shoot lengths and stem diameter were also recorded one year later in order to evaluate the effect of mycorrhizal colonization.

DNA extraction and phytoplasma detection

DNA was isolated from approximately 1.0 g of fresh plant material, using the phytoplasma-enrichment procedure of Ahrens and Seemüller (1992). The phytoplasma detection in the ESFY-inoculated trees was carried out using the nested-PCR technique following the protocol of Garcia-Chapa *et al.*, 2003.

Results

Results showed that after one-year growth, non-mycorrhizal ESFY-inoculated plants, were negatively affected by the disease infection.

In non-mycorrhizal plants inoculated with the phytoplasma there was a high percentage of premature death (30%), whereas all mycorrhizal plants infected with ‘*Ca. P. prunorum*’ survived (table 1).

Vegetative growth of non mycorrhizal plants is lower than growth of mycorrhizal plants when they are infected by the phytoplasma, being the total shoot length of the outbreaks of 59 cm in the first ones opposite to 80 cm in the second ones (table 1). The shoot length of mycorrhizal ESFY-inoculated plants did not differ from the values recorded for healthy non mycorrhizal plants (table 1).

Table 1. Survival after inoculation with ‘*Ca. P. prunorum*’ and growth response of mycorrhizal (M+) and non mycorrhizal (M-) plants of 40 *Prunus salicina* plants cv Angeleno grafted on GF 677 rootstocks. Ten plants per treatment.

	M-		M +	
	Healthy	ESFY	Healthy	ESFY
% died after inoculation	0%	30%	0%	0%
Total shoot length (cm)	80	59	85	80

Discussion

The ESFY disease can exhibit different degrees of severity in infected trees. Some factors that could influence in the severity are the used variety, and also the graft/rootstock combination, as has been reported by Carraro and Kison (2001).

The initial and basic injury involved in graft-union pathology is the necrosis of rootstock sieve tubes. This necrosis blocks translocation, and causes the death of the fibrous roots of affected trees due to lack of carbohydrates (Lepka *et al.*, 1999).

Previous studies, have already demonstrated that the inoculation with the AM fungus *G. intraradices* increases the tolerance versus different phytoplasmas such as ‘*Candidatus Phytoplasma pyri*’ in the *Pyrus communis* OHF 333 rootstock (Garcia-Chapa *et al.*, 2004; Lingua *et al.*, 2002).

Since AM fungi colonize roots and use the host photosynthates, the failing of carbohydrate transport along the blocked phloem, and the decrease of starch in the roots due to phytoplasma infection should cause a decrease of the AM fungi growth, and the loss of the positive effects of the mycorrhizal symbiosis. However the results obtained in this work again support the hypothesis that AM fungi increase phytoplasma tolerance (Lingua *et al.* 2002). After one-year growth, ESFY-infected mycorrhizal plants did not differ from or even outgrew healthy control plants despite the presence of the disease, indicating that early mycorrhizal inoculation increases tolerance to ESFY disease in one-year old *P. salicina* cv Angeleno grafted on GF-677 rootstock.

Results obtained are encouraging. Further investigations are necessary in order to elucidate the mycorrhizal effect in ‘*Ca. P. prunorum*’ infections.

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The preservation of lime witches' broom phytoplasma in key lime by tissue culture

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Abstract

Witches' broom disease of small-fruited lime (WBDL) is a severe disease associated with phytoplasmas in the south of Iran. In this study maintenance of WBDL phytoplasma in key lime (*Citrus aurantifolia* Christm) in micropropagation is reported. After the maintenance of tissue cultures in a growth chamber, symptoms of little leaves and shortening of internodes were observed in shoots from infected plants. Micropropagated shoots that developed in tissue culture were tested for the presence of phytoplasmas using the two phytoplasma-specific primers pair P1/P7 and P3/P7. Amplification by polymerase chain reaction confirms the presence of WBDL phytoplasmas in the diseased plants 10 months after the beginning of the *in vitro* propagation. To our knowledge, this is the first report on maintenance of lime witches' broom phytoplasma in key lime shoots through tissue culture in Iran.

Key words: phytoplasma, tissue culture, key lime, PCR.

Introduction

Phytoplasmas are endocellular prokaryotes without cell wall associated with more than 600 diseases in at least 300 plant species (Kirkpatrick, 1992). Lime witches' broom (LWB) associated with '*Candidatus Phytoplasma aurantifolia*' is one of the most destructive diseases of lime in the southern Provinces of Iran (Mirzai *et al.*, 2009). Knowledge about phytoplasmas has been limited by inability to isolate them in pure culture. For scientific investigation, phytoplasmas can be maintained in living hosts (Bertaccini *et al.*, 1992, Jarausch *et al.*, 1996). Plant material collected from infected lime fields as phytoplasma source is useful for some studies but does not allow detailed study of the associated phytoplasma. Field collection and nursery or greenhouse propagation of the key lime plants is time and money requiring. Tissue culture has been used for propagation of phytoplasma diseased plants *in vitro* as a more constant source of infected material (Wongkaew and Fletcher, 2004).

The feasibility of maintenance of lime witches' broom phytoplasma in key lime shoots through tissue culture is here reported for the first time in Iran.

Materials and methods

Healthy and witches' broom diseased lime (*Citrus aurantifolia* Christm) plants were collected from key lime gardens of Jiroft in the south of Iran.

Single node shoots were excised from mother plants and surface-sterilized by soaking in a solution containing 6% bleach and 1% tween 20 for 20 min. The explants were rinsed three times in sterile distilled water.

Sterile explants were placed on agar-solidified MT medium (Murashige and Tucker, 1969) supplemented with 2.2 mg/L N⁶-benzyladenine, 1 mg/L indole-3-butyric acid and 3% sucrose. The pH was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. All explants were maintained at a temperature 24±2°C under 16 h photoperiod with light intensity of 3,000 lux. Plantlets were subcultured to fresh medium at intervals of 4 to 8 weeks for 10 months dividing the shoots into 1-cm-long segments.

WBDL phytoplasma was detected using polymerase chain reaction (PCR). Total DNA was extracted from leaves using cetyl trimethyl ammonium bromide (CTAB) extraction method (Zhang *et al.*, 1998). Two primer pairs P3/P7 and P1/P7 were used to amplify part of the 16S rRNA gene, the 16S–23S spacer region, and a portion of the 5-end region of the 23S rRNA gene from phytoplasmas.

Results

The medium successfully supported growth of all explants. Phytoplasma-diseased plants showed significantly reduced shoot height, little leaf and witches' broom symptoms when compared to the healthy control (figure 1).

The best time for subculture was every six weeks. Jar explants grew faster than the tube explants.

Amplification of two pathogen-specific DNA fragments 320 bp and 1,830 bp with two primer pairs P3/P7 and P1/P7, respectively by polymerase chain reaction (PCR) confirm the presence of WBDL phytoplasma in the diseased plants after 10 months of *in vitro* propagation (figure 2).

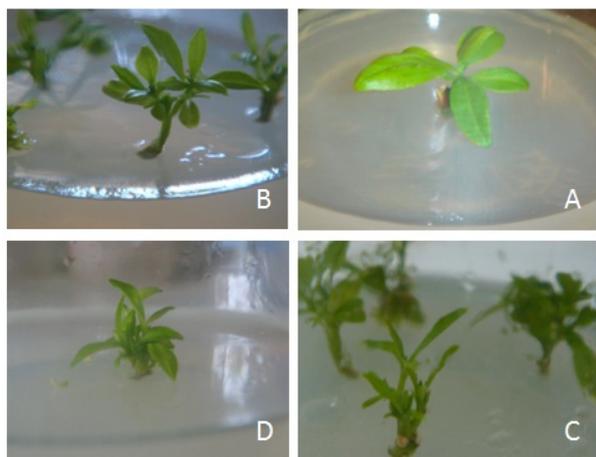


Figure 1. *C. aurantifolia* shoots in micropropagation A. Healthy shoot; B, C, D. Phytoplasma infected explants showing little leaf and witches' broom. (In colour at www.bulletinofinsectology.org)

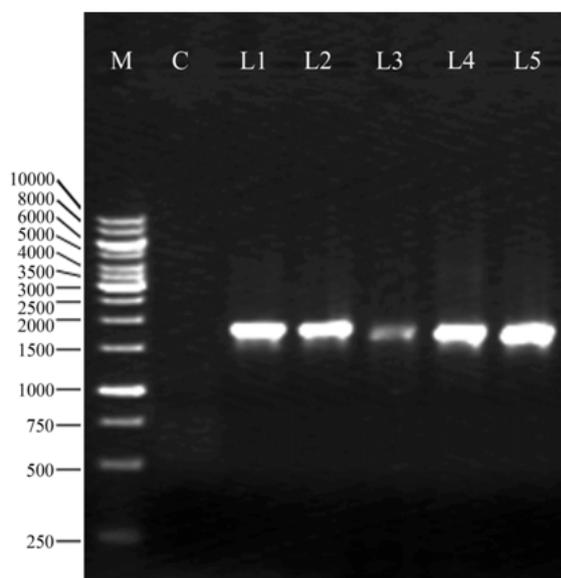


Figure 2. Agarose gel electrophoresis of PCR amplifications of a 1,830 bp fragment from symptomatic key lime shoots. C, negative control; L1, 2-months phytoplasma infected explants; L2, 4-months phytoplasma infected explants; L3, 6-months phytoplasma infected explants; L4, 8-months phytoplasma infected explants; L5, 10-months phytoplasma infected explants.

Discussion

Phytoplasma-diseased explants showed significantly reduced shoot height and leaf width compared to the

healthy control. The shoot height and leaf width were reduced by increasing interval subculture until 8 weeks, however the witches' broom symptom seems not to be correlated with phytoplasma concentration.

The maintainance of phytoplasma through tissue culture of diseased key lime can present a useful way to maintain living phytoplasma for studies of phytoplasma-plant interactions toward the understanding of phytoplasma pathogenicity and also to study possibility of their elimination by cryotherapy, or other *in vitro* techniques.

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Reliable improved molecular detection of coconut lethal yellowing phytoplasma and reduction of associated disease through field management strategies

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Abstract

The relentless spread of the fatal disease lethal yellowing throughout the coconut growing areas is having a serious economic impact on many vulnerable communities, and therefore effective management is required. Phytoplasmas from the 16SrIV group are the associated agents. Improved detection methods for this phytoplasma DNA were developed including: a multiplex direct-PCR system, PCR on 16S rRNA and hemolysin genes; a SybrGreen system based on the *GroEL* gene; and real-time PCR using TaqMan probes based on the 16S rDNA and *GroEL* gene. With these methods increased sensitivity and specificity for the detection of this phytoplasma together with quantization capability. These improved methods are part of a package for the integrated management of lethal yellowing spread that is currently tested in coconut farms in Jamaica and show that diseased incidence can be reduced.

Key words: Lethal yellowing, phytoplasmas, management, disease incidence.

Introduction

The relentless spread (Myrie *et al.*, 2006) of the fatal disease lethal yellowing (LY) throughout the coconut growing areas is having a serious economic impact on many vulnerable communities. Approximately one million two hundred thousand coconut trees were destroyed in 15 years and therefore effective management of LY spread is required. These improved methods (Black approach) are part of a package for the integrated management of LY spread that include the following strategies: strict weekly surveillance; immediate removal and destruction of LY infected trees; replanting with coconut plants selected for high yielding and LY resistance, proper weed control; and a good fertilization regime. This package is currently being tested in coconut farms in Jamaica, and the results from these tests have shown LY incidence can be reduced.

Phytoplasma from the 16SrIV group is the associated agent (Harrison *et al.*, 2002) systemically colonizing phloem tissues and leading ultimate death of coconut palm. It is therefore important to improve detection methods for LY phytoplasma DNA. Real Time and conventional PCRs with primers designed from randomly cloned phytoplasma DNA have allowed for phytoplasma detection. It was reported by Dollet *et al.* (2006) that the primer pair P1/P7 was capable of amplifying DNA region in *Bacillus sp* and other bacteria. Therefore new systems of detection are required.

Materials and methods

Seventy (70) tissue samples were collected from coconut palms with and without symptoms of LY disease. In

addition, seven samples were collected from coconut trees with different stages of the disease. Total nucleic acids were extracted employing Doyle and Doyle (1990) method. Ten insect [*Haplaxius (Myndus) crudus*] samples were also collected from coconut fields. Universal ribosomal primer pair P1/P7 (Deng and Hiruki, 1991; Smart *et al.*, 1996), LY phytoplasma group specific primer pair LY16Sr/LY16Sf (Harrison *et al.*, 2002a) HemR1(5'TCACGGAGACGACGAAGGATTAG'3)/F1(5'TTCCACCATATTCATCAACAACAA'3), *groEL*R1(5'CTTTAGGACCAAAAGGTA'3)/F1(5'GAAGAACAACAACCACTATC'3) and *groEL*R2(5'CGATAATGCTGGAGATGGGACTACT'3)/F2(5'GAACTACAGCGGCTCCTGTTGTAAT'3) were used in conventional PCRs. Real time PCR systems were used for direct detection of phytoplasma associated lethal yellowing of coconut palms. TaqMan primers (503LY16S-ANYF/503LY16S-ANYR) and probes (503LY16S-ANYM) were designed base on the 16S ribosomal RNA sequences. A SybrGreen system based on the *GroEL* gene were also developed. Comparison was made between the two PCR systems. A multiplex direct-PCR system, simultaneously amplifying two targets of interest (16S rRNA and hemolysin genes) were developed. Four farms (Nutt River Farm, Belvedere, Eco-village and Spring Garden) were selected for employing the improved method (Black's approach). Three farms (Chiquita, Needham Pen and Fair Prospect) did not used the Black's approach.

Results

Coconut lethal yellowing phytoplasma was detected by conventional PCR using the lethal yellowing group

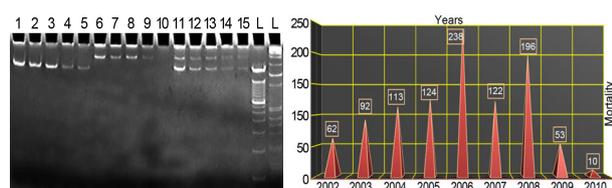
Table 1. Primers and TaqMan sequences for lethal yellowing phytoplasma used in Real Time PCR.

Name	Orientation	Sequence (5'- 3')	Amplicon length	Location
503LY16S-ANYF	Forward	GCTAAGTCCCCACCATAACGT	180 bp	16S rRNA
503LY16S-ANYR	Reverse	CGTGTCTGTGAGATGTTAGGTTAAGT	180 bp	16S rRNA
503LY16S-ANYM	Probe	FAMCCCCTGTCGTTAATTG-NFQ	180 bp	16S rRNA

Table 2. Lethal yellowing deaths at Nutts River Farm.

Years	Months												Total
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	
2007	19	9	7	9	16	8	13	9	15	4	9	4	122
2008	26	12	18	15	29	26	26	11	11	7	9	6	196
2009	4	4	5	4	5	4	5	4	3	1	5	9	53
2010	2	0	2	1	0	2	0	1	0	0	0	2	10
Total	51	25	32	29	50	40	44	25	29	12	23	21	381

specific primers, Hem primers, *groEL* primers and in real time PCR systems using TaqMan probes and primers (table 1). In conventional PCR fewer samples tested positive. Analysis of samples from coconut palms that lost their canopies for 4 months tested positive in real time PCR for LY phytoplasma. Insect samples tested positive for LY phytoplasma in real time PCR using TaqMan probes. SybrGreen system based on the *GroEL* gene have accurately detected LY phytoplasma. Multiplex direct-PCR system, have simultaneously amplified two targets of interest; 16S rRNA and hemolysin genes (figure 1). The four farms that employed the Black's approach saw a significant decline in lethal yellowing disease. The most notable reduction was on the Nutts River Farm in St. Thomas, Jamaica (figure 1). A significant decrease was noted in 2009 and 2010. The month of October had the lowest deaths of coconut trees and the month of January had the highest deaths (table 2). Farms, which had not used the Black's approach experienced a upsurge in the lethal yellowing disease (10,000 coconut palms lost in 6 years).

**Figure 1.** Left: 1-5 diseased samples amplified with P1/P7, 6-10 diseased samples amplified with Hem primers; 11-15 samples simultaneously amplified with P1/P7 and Hem primers in a single PCR. Right: lethal yellowing deaths at Nutts River Farm.

Discussion

Early detection of lethal yellowing diseased trees is an important plank of the Black's approach. Real time PCRs used in this study have shown that it has the capability to detect lethal yellowing disease tree at the onset of the first symptom. Conventional PCR is not as sensitive as real time PCR as samples that exhibit no band or faint band in the conventional PCR had given a strong positive signal when amplified in real time PCR. The amplification of

two or more genes simultaneously in the multiplex direct PCR system has contributed to the accurate identification of disease trees. It also reduces the amount of PCRs required for disease confirmation. This has also increase the efficiency the Black's approach. It is clear from the data collected that Black's approach had significantly reduced lethal yellowing disease. Farms that were not employing the Black's approach were devastated by the disease. The insect tested positive for the lethal yellowing phytoplasma does not make them a vector of the disease.

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Witches' broom disease of Mexican lime trees: disaster to be addressed before it will be too late

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Abstract

Witches' broom disease of lime (WBDL) associated with '*Candidatus Phytoplasma aurantifolia*' presence, is responsible for major losses of Mexican lime trees (*Citrus aurantifolia* L.). Iranian witches' broom disease of lime Network (IWBDLN) comprises of 10 mega projects which have been carried out since 2008 with the contribution of 15 different national institutes. Here, we will present the program and the results obtained and demonstrate how this program could contribute in controlling this destructive agent of WBDL. Furthermore, we will demonstrate how our genomics, transcriptomics, epigenomics, proteomics and metabolomics analyses of lime infected by witches' broom disease provided new insight into plant stress tolerance mechanisms. Several strategies to maximize the success of our program in controlling the disease will be presented.

Key words: Witches' broom disease of lime, '*Candidatus Phytoplasma aurantifolia*', systems biology, management.

Introduction

Witches' broom disease of lime (WBDL), associated with '*Candidatus Phytoplasma aurantifolia*', is responsible for major losses of Mexican lime trees (*Citrus aurantifolia* L.). The WBDL phytoplasma is transmitted by the leafhopper *Hishimonus phycitis* Distant (Shabani *et al.*, 2011). WBDL was first observed in Oman in 1975 leading to a 98% destruction of Mexican lime trees. The disease was then observed in UAE in 1991, in India in 1999 and in Iran in 2000. From 2000 until today, 30% of the Mexican lime trees (over half a million trees/7000 hectares) in Iran have been destroyed by WBDL.

Sadly, in 2009, the agent associated with the disease expanded its territory to other plants such as grapefruit (IWBDLN 2011, personal communications) and it is anticipated that it will pose a serious threat to other horticultural products in Iran and worldwide. Although only a small part of the globe is currently affected it is critical to take it into consideration that this disease is transferred by insects and if immediate actions are not taken, there would be a huge risk of a global epidemic.

Materials and methods

IWBDLN comprises of 10 mega projects including horticulture controlling, identification and diagnosis of host/pathogen/vector, identification of resistant Mexican lime trees, identification of substituted resistant cultivars, breeding of resistant cultivars, gardens resuscitation and replacement, education, extension and notification, finance and budget, supporting, quarantine of the intact and affected areas. These activities have been being carried out since 2008 with the collaboration of 15 national institutes.

Results

Detection of witches' broom disease of lime

A novel quantum dots FRET-based biosensor for the detection of '*Ca. P. aurantifolia*' was constructed. The quantum dots (QDs) were functionalized with a specific antibody against the '*Ca. P. aurantifolia*'. The specificity and sensitivity of the constructed nanosensor kit were found to be as high as 100%. This nano-based detection kit would facilitate early detection of the disease in order to manage and control the associated agent before the disease enters the irreversible stages of infection.

Inhibition of the pathogen using nutritional and pharmaceutical compounds

Treating the infected plants with a number of nutritional and pharmaceutical compounds such as surfactin was shown to have a significant inhibitory effect on '*Ca. P. aurantifolia*' (Askari *et al.*, 2011). Fortunately, based on the results obtained and the accomplishments made through the mega plans conducted by the Network, the destructive agent of WBDL is currently under control.

Systems biology analysis of Mexican lime trees interactions with '*Ca. P. aurantifolia*'

Although plant molecular breeding for inducing tolerance to various stresses can have a great impact on increasing crop productivity, it suffers from some drawbacks such as slow progress and shortage of information on the molecular events underlying the tolerance (Ghayeb *et al.*, 2011). Systems biology analysis of the plant/pathogen interaction provides invaluable insights into signaling pathways and molecular mechanisms underlying plant response and tolerance to disease. The advent of omics technologies has made it possible to identify a broad spectrum of genes in living systems and discover molecular mechanisms of stress tolerance.

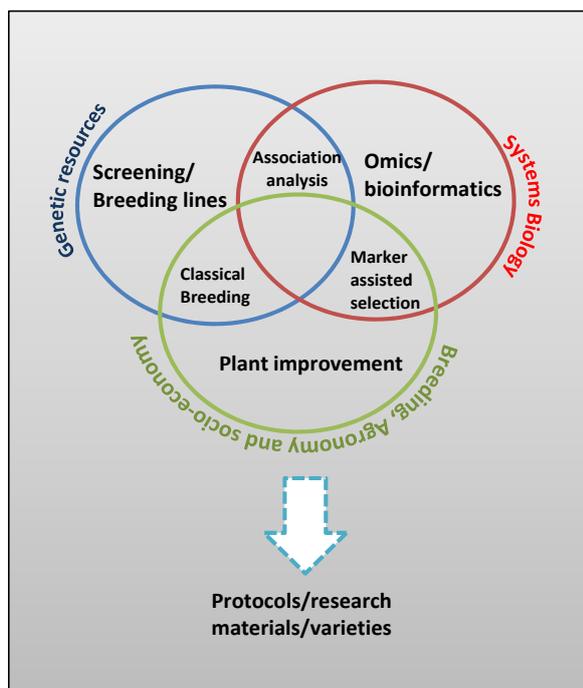


Figure 1. A strategy for developing resistant Mexican lime trees: a promising but long process.

We applied genomics, transcriptomics, epigenomics, proteomics and metabolomics approaches to analyze lime infected by witches' broom disease in order to identify new plant stress tolerance mechanisms (figure 1). The preliminary results indicated a number of candidate transcripts, microRNAs, proteins, and metabolites that might be involved in the interaction of Mexican lime trees with '*Ca. P. aurantifolia*'. We will discuss system biology based analysis of these results and novel mechanisms involved in plant pathogen interaction.

Discussion

Today, the disease has been controlled and preventive strategies have been introduced and implemented in the infected areas. However, it should be noted that its threat

is always around and being on alert globally will keep new tragedies away. Owing to a comparative analysis, several mechanisms emerged as key participants in plant response to stress. Further investigation is required to elucidate the roles of these mechanisms in the susceptibility/resistance of Mexican lime trees to disease, and to determine how strategies might be developed to incorporate these genes into molecular breeding programmes.

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Development of a molecular approach to describe the diversity of fungal endophytes in either phytoplasma infected, recovered or healthy grapevines

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Abstract

Fungal endophytes have been reported to have antagonistic effect against fungal, bacteria and other adversities affecting plants. In this work we used a “synergy” of culture-dependent and culture-independent methods that allowed to maximize the determination of grapevine fungal endophytic species. Starting from 21 morphospecies of endophytes, identified by a culture-dependent method, we were able to obtain a total of 55 OTUs by the joint application of a culture-independent method. This study also permitted to determine that seven main fungal endophyte genera represent about 82% of total grapevine endophytic fungal community. Furthermore, we set up a novel molecular fingerprinting technique, DGGE (Denaturing Gradient Gel Electrophoresis), which proved to be a rapid and reliable tool to identify the variability within grapevine fungal endophytic community. With this innovative approach we will attempt to determine and compare fungal endophytic diversity respectively in healthy, and phytoplasma infected or recovered grapevines.

Key words: isolation, ITS-RFLP, ITS-cloning, OTUs, DGGE.

Introduction

Endophytes were defined by Petrini (1991) as “All organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host”. The ecological role of these organisms is still not well determined but most of them exhibit positive effect to host plants by promoting plant growth, improving resistance to multiple stresses, protection from diseases and insects (Rodríguez *et al.*, 2008). Recently, it has been hypothesized that ‘recovery’, spontaneous remission of symptoms, could be explained through the involvement of S.A.R. (systemic acquired resistance). Endophytic microorganisms are presumed to play an active role in enhancing the host defence response against phytoplasma infection (Musetti *et al.*, 2011).

In the present work we combined culture-dependent and culture-independent methods to estimate the diversity of grapevine fungal endophytic community. We also developed a new fingerprinting molecular tool, DGGE (Denaturing Gradient Gel Electrophoresis) useful to describe and compare the fungal diversity respectively in healthy, and phytoplasma infected or recovered grapevines.

Materials and methods

In 2009, healthy grapevine leaf, node and internode tissues were randomly collected from fifteen grapevine plants cvs. Tokai and Merlot, in two organic vineyards in Friuli Venezia Giulia (FVG) region, Italy.

Fungal endophytic isolation. All tissues were surface sterilized according to Mostert *et al.* (2000) and a total of 540 small pieces obtained from these grapevine tissues were placed on PDA medium amended with am-

picillin (150 µg/ml) and streptomycin (100 µg/ml). All isolates obtained in pure culture were preliminarily grouped as morphospecies, and then sporulating fungi were identified at genus level by morphological characteristics. A DNA-dependent method was applied to all isolates, performing a DNA extraction (Martini *et al.*, 2009) followed by amplification of ITS region of fungal rRNA genes by ITS1F-ITS4 primers (Gardes and Bruns, 1993) and by restriction fragment length polymorphism (RFLP) analysis with *Tru1I* and *HpaII* endonucleases. Identical patterns were grouped into operational taxonomic units (OTUs), and one representative isolate of each OTU was randomly chosen for sequencing of ITS region and BLAST analysis.

Determination of non-culturable fungal endophytic community. Shoots of the plants described above were pooled and total genomic DNA was extracted by a CTAB procedure (Martini *et al.*, 2009), ITS region amplified and cloned using pGEM®-T Easy Vectors System (Promega, WI, USA). Six ITS libraries were constructed and 282 clones randomly selected, were grouped in OTUs by RFLP analysis described above. Primers pairs ITS1F-GC and ITS2 (Bougoure and Cairney, 2005) amplifying ITS1 region of all representative culturable endophytic isolates were used to generate amplicons suitable for DGGE analysis. PCR products were run on an 8% polyacrylamide gel in a 25-45% urea/formamide denaturant gradient using the DCode system (Bio-Rad, CA, USA). DGGE were carried out at 180 V at 60°C for 6.5 h in 1.25 x TAE buffer.

Results

A total of 236 fungal isolates, representing 44% of isolation rate, were obtained. The isolates were grouped morphologically and identified at genus level (table 1).

Table 1. Numbers and percentages of morphological groups and OTUs obtained from culture-dependent and culture-independent methods.

Method	Morphospecies	Morphological identification	OTUs	Unique OTUs	Shared OTUs	Total OTUs
Culture-dependent	21 (38%)	10 (18%)	29 (53%)	16 (29%)	13 (23%)	55 (100%)
Culture-independent	/	/	39 (74%)	26 (47%)		

Mycological analyses permitted to differentiate 21 morphospecies and the following DNA-dependent method allowed obtaining 29 OTUs (table 1).

The culture-independent method consented to discover other 26 OTUs associated to a non-culturable fraction of fungal endophytic community. From collected data, it resulted that more than 90% of isolates obtained by the culture-dependent method, belonged to seven main genera (table 2). Similarly, the same seven genera, represented the 82% of total OTUs obtained from the culture-independent method.

Table 2. Relative frequencies (%) of main fungal endophytic genera obtained respectively by isolation and cloning.

Genus	Isolation	ITS library
<i>Alternaria</i> sp.	39%	36%
<i>Phoma</i> sp.	17%	5%
<i>Epicoccum</i> sp.	14%	4%
<i>Aureobasidium</i> sp.	10%	17%
<i>Cladosporium</i> sp.	9%	13%
<i>Pestalotiopsis</i> sp.	1%	6%
<i>Pestalotia</i> sp.	1%	1%

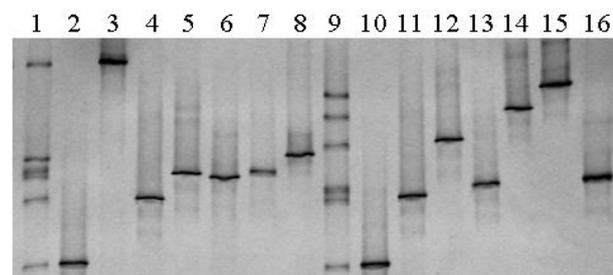


Figure 1. Example of DGGE patterns obtained from different grapevine fungal endophytes. Lanes 1 and 9: reference markers.

Differences in some of the relative frequencies of main fungal genera could be explained by the higher variability of tissues used for isolations, and by the difficulty in the culture-independent method to extract DNA from fungal endophytes that may be present predominantly by spores within plant tissues. PCR-based DGGE analysis permitted a very good discrimination among the majority of amplicons obtained from different OTU representatives derived from the culture-dependent method. Some amplicons have been chosen randomly to constitute two examples of reference markers (figure 1).

Discussion

Different tissues, collected in different localities from different grapevine cultivars grown under organic regime permitted us to obtain a great variability of fungal endophytes. Moreover the use of a synergy of different approaches, culture-dependent and culture-independent, allowed us to increase the determination of diversity of fungal endophytic community.

PCR-based DGGE analyses resulted to be a valuable culture-independent approach for the rapid and reliable identification of fungal endophytic species. Further DGGE analyses are in progress with the aim to obtain differences among fungal endophytic communities associated with healthy, recovered and phytoplasma diseased grapevines. This way, it may be possible in the future to discover fungal endophytes as potential bio-control agents acting like inducers of recovery.

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Study of stolbur phytoplasma tuber transmission in potato varieties of high starch content

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Abstract

In order to study the tuber transmission rate of stolbur phytoplasma tubers from varieties of high starch content, originating from field under high infection pressure, and their daughter plants were tested with PCR-RFLP and real-time PCR. Altogether 83.8% of the 702 tubers tested were positive for stolbur. During the three-years surveys the phytoplasma was identified in 0.5% of daughter plants. These data confirm that tuber transmission of stolbur phytoplasma also in varieties with high starch content although in a low percentage. Further studies are needed to discover factors influencing the tuber transmission ability of the stolbur phytoplasma.

Key words: potato stolbur, epidemiology, purple top, molecular detection.

Introduction

Potato stolbur phytoplasma is widespread in central and southern part of Europe causing yellows-type diseases on solanaceous crops (Cousin and Smith, 1977; EPPO/CABI, 1996). At least five phytoplasmas belonging to three different groups have been associated with potato diseases. Stolbur, potato purple top (PPT) and potato witches' broom (PWB) are the most important diseases. Stolbur disease is associated with phytoplasma belonging to 16SrXII group. Transmission of different phytoplasmas via potato tubers has been reported by only a few authors so far. Tuber transmission was proved by Paltrinieri and Bertaccini (2007) in case of stolbur phytoplasma as well as of phytoplasmas belonging to ribosomal subgroups 16SrI-B, 16SrI-C, 16SrII-D and 16SrX-A. In the USA tuber transmission was reported by Munyaneza and Crosslin (2006) in 35% of the plants grown out from tubers infected by beet leafhopper transmitted virescence (BLTVA) phytoplasma (16SrVI-A), associated with the PPT disease.

Potato varieties are categorized into groups based on common characteristics that are based on starch value. For processing varieties high starch content (17 to 20%) is required compared to the table once (10-14%) (Potato Council, 2009).

For better understanding the epidemiology of the potato stolbur, it is necessary to determine the tuber transmission rate in case of different varieties.

The objective of the present work was to verify the existence and rate of stolbur phytoplasma tuber transmission in high starch content potato varieties.

Materials and methods

Tubers of four varieties of high starch content (Lady Rosetta and varieties A, B and C) were collected from stolbur-infected field in Romania. Tubers were harvested after natural loss of foliage in August. Tubers were planted in a growing room in April 2009, in January

2010 and 2011. Visual observation of the plants was carried out regularly.

Samplings for molecular tests were performed at three times in 2009, 2010 and twice in 2011. The first test was made on the tubers (before planting out), the second test on daughter plants at four-week age and the third test at three-month age. DNA extraction from 702 tuber samples and 629 plant samples were carried out using the CTAB method (Daire *et al.*, 1997). For tubers, nested PCR was applied using primers P1/P7 (Deng and Hiruki, 1991; Smart *et al.*, 1996) and R16F2/R16R2 (Lee *et al.*, 1995) amplifying 16SrDNA. The nested products were digested with *TruI* enzyme and the RFLP profiles were compared with reference strains (EAY, 16SrI-B; MOL, 16SrXII-A provided by A. Bertaccini and AY27, 16SrI-A; CPh, 16SrI-C; PaWB, 16SrI-D; CP and PWB, 16SrVI-A from I-M. Lee).

Results and discussions

Symptomatology

Tubers. The average diameter of the tubers was 3-4 cm. Altogether 81.8% of the 702 tubers were found symptomatic, 30.9% showed hairy sprouts, 43.3% were spongy and 27.9% showed both symptoms; the percentage varied among the varieties (data not shown).

Daughter plants. The first typical phytoplasma symptoms appeared already at the four-week stage. Purple top, yellowing, stunting, uprolled crispy leaves and aerial tubers developed on one Lady Rosetta and two Variety A plants during the three-year experiment. These three plants were very small, stunted and did not develop daughter tubers (data not shown).

Molecular results

Tubers. Altogether 83.8% of the 702 tubers proved positive for stolbur (16SrXII) (table 1). Stolbur was identified in symptomatic and asymptomatic tubers as well. The severely shrunken, small tubers were negative for phytoplasma.

Table 1. Stolbur infection rate of tubers and daughter plants.

Variety	Infected tubers before planting infected/tested (%)			Infected daughter plants four-week old infected/tested (%)			Infected daughter plants three-month old infected/tested (%)	
	2009	2010	2011	2009	2010	2011	2009	2010
Lady Rosetta	86/118 (73.0)	74/110 (67.3)	92/100 (92.0)	0/104*	0/99	1/77 (1.3)	1/73* (1.4)	0/101
Variety A	/	48/54 (88.9)	90/100 (90.0)	/	1/44 (2.3)	1/80 (1.25)	/	1/52 (1.9)
Variety B	/	104/110 (94.5)	/	/	0/100	/	/	0/108
Variety C	/	94/110 (85.5)	/	/	0/66	/	/	0/107
3-year total	Lady Rosetta: 252/328 (76.8) Variety A: 138/154 (89.6) Variety B: 104/110 (94.5) Variety C: 94/110 (85.5)			Lady Rosetta: 1/280 (0.4) Variety A: 2/124 (1.6) Variety B: 0/100 (0.0) Variety C: 0/66 (0.0)			Lady Rosetta: 1/174 (0.5) Variety A: 1/52 (1.9) Variety B: 0/108 (0.0) Variety C: 0/107 (0.0)	

* = in 2009 the number of plants were decreasing due to plant death;

/ = not tested.

Daughter plants. Stolbur phytoplasma was identified only in symptomatic plants (table 1): in one of Lady Rosetta (Ct: 32.5) and in two of Variety A (Ct: 31.4, 34.2). Signal of endogenous control was measured in 99% of the tested samples (Ct values: 18.5-22.9) (data not shown).

The three-year average of infection rate, calculated on data of the four-week old daughter plants, was 0.4% for Lady Rosetta and 1.6% in case of Variety A.

The rate of tuber transmission, expressed in three-year average of the four potato varieties, was 0.5% (table 1).

These results confirm a low percentage of stolbur phytoplasma tuber transmission also in varieties with high starch content.

It can be assumed that the tuber transmission of stolbur phytoplasma may have a low epidemiological impact in the tested varieties. However the importance of stolbur tuber transmission may not be leaved out of consideration in case of other varieties. Further studies are needed to discover factors that may influence the tuber transmission ability of stolbur such as biological characters of the varieties as starch content, storage conditions or planting date.

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Phloem-specific protein expression patterns in apple and grapevine during phytoplasma infection and recovery

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Abstract

Recovery - complete remission of disease symptoms - has been reported in plants affected by phytoplasmas. The physiological basis for this phenomenon is not yet understood, but it seems associated to ultrastructural and biochemical modification of the phloem, the tissue where phytoplasmas live and spread. In this work we compared asymptomatic, phytoplasma-infected and recovered apple and grapevine leaf tissues by means of ultrastructural and gene-expression analyses, focusing on a possible role of specific phloem proteins in the plant defense-related processes. Preliminary results indicate that different occlusion mechanisms could interact in the phloem during phytoplasma symptomatic status and/or recovery.

Key words: apple, grapevine, phloem, phytoplasma, recovery.

Introduction

Recovery in grapevine and apple can occur also in single plants showing severe symptoms since several years, manifesting through a complete remission of the symptoms, associated to the disappearance of the phytoplasmas from the crown (Carraro *et al.*, 2004). The physiological basis for this phenomenon is not yet understood; it has been observed that in apple, apricot and in grapevine, recovery from phytoplasma-associated diseases was accompanied by an overproduction of hydrogen peroxide localised in the phloem tissues (Musetti *et al.*, 2004, 2005, 2007). Musetti *et al.*, (2010) reported an abnormal callose and phloem protein 2 (PP2) accumulation in the phloem of recovered apple plants associated to the up-regulation of two callose synthase- and three PP2-coding genes, supporting the hypothesis that recovered apple plants were able to develop resistance mechanisms depending on Ca^{2+} signal activities. Plugging mechanism in the phloem is carried out also by the activity of other specialized calcium-powered proteins, called Sieve Element Occlusion (SEO) proteins. Genes encoding SEO proteins are widespread among dicotyledonous plants (Rüping *et al.*, 2010). Callose deposition and protein plugging can operate in parallel: while phloem specific proteins act quickly, callose acts slowly. Specific aim of this work was to compare leaf tissues in asymptomatic (H), phytoplasma-infected (symptomatic, D) and recovered (R) apple and grapevine plants by means of ultrastructural and gene-expression analyses, focusing on a possible role of specific phloem proteins (callose synthases, PP2 and SEO) in the infection establishment and/or in the recovery from phytoplasma-associated diseases.

Materials and methods

Grapevine (*Vitis vinifera* L. cv. Chardonnay) and apple leaf samples (*Malus x domestica* cv. Florina) were

collected from H, D and R plants in late summer, when typical symptoms of the apple proliferation and the 'bois noir' infection appear in apple and grapevine, respectively. Plants were grown in experimental fields located in Friuli Venezia Giulia (North-East Italy). Total RNA was extracted from frozen leaves enriched in midribs using RNeasy Plant Mini Kit (Qiagen) with minor modifications. Total RNA was quantified spectrophotometrically (Nanodrop, ThermoScientific), treated with Turbo DNase (Ambion) and reverse transcribed using SuperScript® III Platinum® Two-Step qRT-PCR Kit (Invitrogen). Quantitative Real Time PCR analysis were performed on a DNA Engine OPTICON 2 instrument (Bio-rad) with 5 PRIME Master Mix (including SYBR Green). Genes coding grapevine callose synthases (*VvCaSy*) were identified *in silico* from grapevine genome browser, while sequences of genes coding for SEO proteins (*VvSEO*, *MdSEO*) were obtained from Rüping *et al.*, (2010). Primers were designed using Primer3 software. Ultrastructural observations were carried out on small portions of leaf midribs processed as described in Musetti *et al.* (2010), using a Philips CM 10 transmission electron microscopy (TEM), operating at 80 kV.

Results

Seven sequences from *VvCaSy* and two sequences from genes codifying for SEO proteins (*VvSEO* and *MdSEO*) were identified and considered suitable for gene expression analyses. With regards to the expression of the genes encoding *VvCaSy*, four genes among seven showed, in this preliminary effort, an expression level one order of magnitude lower than others and thus not further characterized (data not shown). Among the more expressed genes, *CaSy0* appeared to be significantly induced in D and R plants, compared to healthy ones, while *CaSy1* and *CaSy6* were not influenced by the sanitary status of the plant. Anyway, this last gene showed a slight up-regulation in R plants (figure 1).

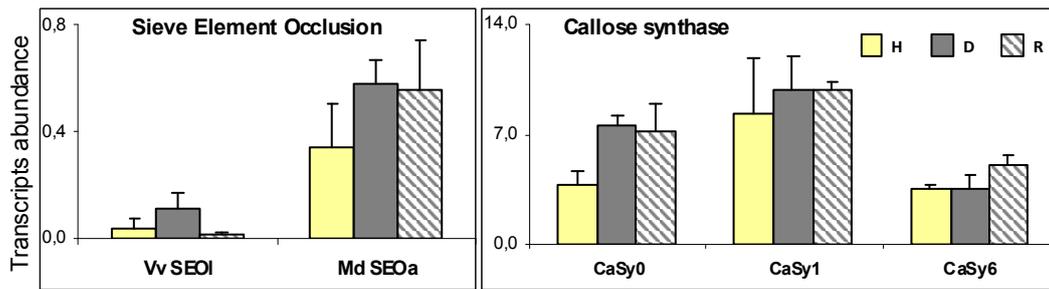


Figure 1. Relative expression levels of two genes coding for SEO protein (VvSEOI, grapevine and MdSEOa, apple) and three genes coding for grapevine CaSy. Mean expression values from three individuals for each plant group (H, D, R) plus standard errors are shown. Expression levels of the genes of interest are normalised on ubiquitin (UBQ expression level = 100) for grapevine and TEF1- α for apple.

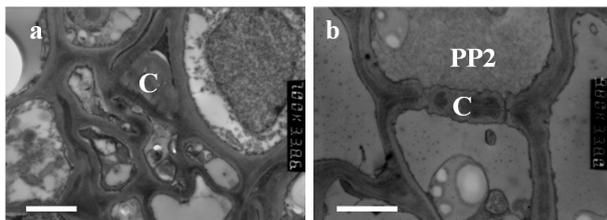


Figure 2. Micrographs of grapevine leaf tissue. (a) In the phloem of ‘bois noir’-infected leaves, callose occlusion (C) is very evident. (b) In recovered plants, PP2 plugs are accumulated in the sieve tube lumen.

TEM observations revealed callose deposition mainly in sieve plates of D grapevines, while in R ones, PP2, forming big plugs, appeared to be a common ultrastructural trait (Figure 2). As regards SEO protein expression pattern, VvSEOI and MdSEOa appeared influenced by the sanitary status of the plants, tending to have a higher transcription level in D samples (figure 1).

Discussion

Phytoplasmas colonize mostly the sieve tubes of the phloem, systemically in the plant. Upon phloem injury, sieve elements are occluded by combined callose-collar formation around sieve pores and protein plugging to prevent leakage of nutrients and pathogen invasion and spread. Identifying cellular modifications in host plants and changes of plant gene expression induced by phytoplasmas is critical for understanding how these pathogens cause diseases and consequently how plants react to their challenge, leading to recovery. In this work we performed preliminary analyses about modifications of phloem-specific protein expression patterns comparing H, D and R grapevine and apple plants. In both plant/phytoplasma associations, different occlusion mechanisms could take place in the phloem during phytoplasma symptomatic status and/or recovery. As already demonstrated in apple (Musetti *et al.*, 2010), different isoforms of callose synthases are triggered in grapevine, during phytoplasma infection (CaSy0) or

when recovery occurs (CaSy6). On the other hand, up-regulation of genes coding for SEO proteins in D plants point to their potential function in the rapid phloem occlusion mechanisms during phytoplasma spread in the host tissue.

Acknowledgements

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Preliminary results on endophytic bacterial community fluctuation during phytoplasma infection

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Abstract

In this study we investigated the influence of 'flavescence dorée' 16SrV-C/-D phytoplasmas on endophytic bacterial community by studying the seasonal fluctuation of bacterial species associated with healthy, 'flavescence dorée'-diseased and recovered plants during phytoplasma infection process. Preliminary results showed that, before phytoplasma titre inside diseased plant tissues becomes detectable, endophytic bacterial community is similar to that associated with healthy plants and differs from that associated with recovered plants.

Key words: Correspondence analysis, length heterogeneity-PCR, 'flavescence dorée'.

Introduction

Grapevine yellows (GY), a complex of phytoplasma-associated diseases, cause severe crop losses and damaged the quality of the grapes in Italy (Maixner, 2006). Recent researches, focused on fungal and bacterial endophytes associated with grapevines, suggested a possible involvement of endophytic species in the recovery phenomenon, the spontaneous remission of symptoms observed in phytoplasma-infected grapevine and fruit tree plants (Bulgari *et al.*, 2009; Musetti *et al.*, 2011). In particular, *Pantoea agglomerans*, *Curtobacterium*, *Bacillus pumilus* and *Burkholderia*, identified in association with grapevine tissues, are biocontrol agents against a broad spectrum of plant pathogens in other plants. Studies of grapevine associated bacterial endophytes and of their possible role as biocontrol agents against phytoplasmas could open new perspectives for developing novel strategies for the control of GY epidemics.

In previous work, we evidenced that endophytic bacterial richness decreases in GY-diseased and recovered grapevine plants (Bulgari *et al.*, 2010). Furthermore, we speculated that phytoplasmas can directly or indirectly determine a reorganisation of endophytic bacterial community.

In this study we investigated the influence of 'flavescence dorée' 16SrV-C/-D phytoplasmas (FD) on endophytic bacterial community by studying the seasonal fluctuation of bacterial species associated with healthy, FD-diseased and recovered plants during phytoplasma infection process.

Materials and methods

Four healthy, four FD-diseased and four recovered Barbera plants were selected and sampled each month from June to October. Leaf tissues preparation and total DNA extraction from 20 g of grapevine leaf was carried out as described (Bulgari *et al.*, 2009).

Molecular identification and FD quantification was carried out by real-time PCR on target *rplN* gene sequence (Durante *et al.*, 2009). To study endophytic bacterial structure and diversity from June to October in healthy, FD-diseased and recovered grapevine plants, total DNA was analysed by length heterogeneity-PCR (LH-PCR) (Bulgari *et al.*, 2009).

For all grapevine samples PCR amplification was run three times and three separate PCRs were also run to confirm the LH-PCR peak sizing through different PCR reactions. Statistical analyses were carried out to study the variation of endophytic bacterial composition in association to phytoplasma infection. Profiles obtained by LH-PCR analysis of healthy, diseased and recovered grapevines sampled in June were processed by correspondence analysis (CA).

The same analysis was repeated only on diseased grapevine plants collected from June to October. This analysis allowed to evaluate the influence of phytoplasmas on endophytic bacterial community composition. The statistics were performed with JMP software (JMP, version 7, SAS Institute Inc., Cary, NC, 1989-2007).

Results

No phytoplasma was detected in all grapevine plants sampled in June. On the contrary, symptomatic plants from July to October were characterized by the presence of phytoplasmas, whose concentration increased during the season reaching the highest titre in October, when the symptoms are severe. LH-PCR profiles were processed by correspondence analysis (CA), evidencing that endophytic bacterial community living in diseased plants in June was comparable to that of the healthy ones.

On the contrary the higher diversity was detected in recovered plants in comparison to diseased and healthy grapevines. Furthermore, bacterial community associated with diseased plants in June was different in comparison to the same plants sampled in the other months.

Discussion

Phytoplasma detection and quantification by real-time PCR revealed that phytoplasma titre generally increased during the season reaching the highest concentration in October. Statistical analysis of LH-PCR profiles indicated that, before phytoplasma titre inside diseased plant tissues becomes detectable, endophytic bacterial community is similar to that associated with healthy plants and differs from that associated with recovered plants. Consequently, it seems that a change in microbial composition could be determined when phytoplasmas start to replicate.

On the basis of our preliminary findings, we can speculate that endophytic bacterial community associated with grapevine plants is altered after phytoplasma infection (increase of phytoplasma titre by replication). This reorganization could be mediated by a direct competition between phytoplasmas and endophytic bacteria for colonizing a favorable niche. On the other hand, phytoplasmas can induce plant defense response leading to select some strains that are able to adapt to the new niche. In fact, plant defense responses are not activated directly, but are accelerated upon pathogen or insect attack (Frost *et al.*, 2008).

Pathogen infection can also modify endophytic bacteria quantitatively determining a change in the relative proportion of some bacterial groups (Trivedi *et al.*, 2010). Our preliminary analyses do not allow to verify this kind of influence but it could be interesting investigate them by quantitative real-time PCR.

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Predominant bacteria symbionts in the leafhopper *Matsumuratettix hiroglyphicus* - the vector of sugarcane white leaf phytoplasma

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Abstract

Sugarcane white leaf (SGWL) disease is associated with phytoplasmas and transmitted by the leafhopper *Matsumuratettix hiroglyphicus* (Matsumura). The objective of this study was to identify bacteria symbiont in this leafhopper to use for future research of symbiotic control development. The leafhoppers were collected from six different sugarcane fields in Thailand. Bacteria were identified by using PCR with a universal primer pair on 16S rRNA gene, cloning, sequencing and blast searches. Two main types of bacteria were found, the first group show 94-98% homology with the primary endosymbiont 'Candidatus Sulcia muelleri' (phylum Bacteroidetes-class Flavobacteria), which has been reported in the suborder Auchenorrhyncha. The second group was not closely matched with any sequences in the database. This group was predominant in the leafhopper and found in all specimens examined, so it was the selected bacteria. This bacterial symbiont was tested for location in the leafhopper body by specific PCR and it was prevalent in the same organs with SCWL phytoplasma, but not in salivary gland. Furthermore, this bacterial symbiont was found in all developmental stages of the insect vector including eggs, nymphs and adults of *M. hiroglyphicus*. We speculate that this bacterial symbiont might have a mutualistic relation with insect host. These results are the first report of bacterial symbionts in the leafhopper vector of SCWL disease and it is useful information for future research of symbiotic control development or for examining relationship between bacterial symbionts and leafhopper host.

Key words: sugarcane white leaf, phytoplasma, insect vector, symbiont.

Introduction

Sugarcane white leaf (SCWL) is the most serious disease of sugarcane in Thailand, and is associated with phytoplasma pathogen. Efficient methods to manage this disease have not been found except to remove the infected plant to decrease phytoplasma inoculum. This phytoplasma is transmitted by the leafhopper *Matsumuratettix hiroglyphicus* (Matsumura) (Hemiptera: Cicadellidae). This insect vector is one of the important keys for control of this disease because it is not only the vector but also the pathogen reservoir (Hanboonsong *et al.*, 2002). Although, weeds that grow in sugarcane plantations show symptoms of white leaf disease, it has been proven that they are not a reservoir for this phytoplasma (Wongkeaw *et al.*, 1997); weed management has no effect for control of this disease.

Symbiotic control has been an interesting recent approach to control pathogen transmission of insect vectors by using microorganisms that are associated with insect vectors. Symbiotic control has been investigated for the vector of 'Chagas disease' caused by a protozoa which is transmitted by a bug vector (Beard *et al.*, 2002). Also, symbiotic control has been studied to control insect vector of plant diseases such as for sharpshooter vectors of the bacterium *Xylella fastidiosa* which cause Pierce's disease in grapevine (Bextine *et al.*, 2004). Similar studies were also carried out on the leafhopper *Scaphoideus*

titanus vector of phytoplasma associated with 'flavescence dorée', yellows disease of grapevines in Europe (Marzorati *et al.*, 2006). In these studies we aimed to identify bacteria symbionts associated in *M. hiroglyphicus* by using 16S rRNA gene sequences analysis for future symbiotic control development.

Materials and methods

To identify the bacteria symbionts in leafhopper *M. hiroglyphicus* the 16S rRNA gene analysis was employed. The leafhoppers were collected by light traps from 6 different sugarcane fields in 4 provinces in the northeast region of Thailand. DNA was extracted from individual specimens by a phenol/chloroform method. PCR was performed with universal primers of 16S rRNA bacteria gene. PCR products were cloned into the plasmid vector TOPO-TA vector (Invitrogen) following the supplier's instructions. Recombinant plasmids were randomly selected for sequencing. Nucleotide sequences were compared with other 16S rRNA genes at NCBI database by Blast search. The representative sequences of predominant bacteria types were selected to analyse the phylogeny with other 16S rDNA genes. Specific primers and PCR reaction were designed to test the location of selected bacterial symbionts and also tested in all developmental stages of the leafhopper.

Results

Two main types of bacteria were found from this study. The first group show the close similarity to ‘*Candidatus Sulcia muelleri*’ (phylum Bacteroidetes, class Flavobacteria). The second group was not closely matched with any sequences in the database. It is interesting because this group was the most found sequences out of all leafhopper examined, so it was the selected bacterial symbiont. The representative sequence of this group was selected to build a phylogenetic tree and it show that this bacteria symbiont falls in proteobacteria. Specific primers and PCR reaction were designed for this bacteria symbiont to detected its location in *M. hiroglyphicus* and it was found in fat body, gut and ovary where there were also found the phytoplasma pathogen. This selected bacterial symbiont was found in all developmental stages of the insect vector including eggs, nymphs and adults. In addition to the two main types, we found *Burkholderia* sp and *Rickettsia* sp., both have been reported as found in insects.

Discussion

‘*Ca. Sulcia muelleri*’ has been reported as the primary endosymbiont of several insects including cicadas, planthoppers and leafhoppers. It lives inside a specific structure called bacteriotome (Moran *et al.*, 2005). ‘*Ca. Sulcia muelleri*’ plays an important role in hosts by synthesising essential amino acids (Wu *et al.*, 2006). So, this endosymbiont must provide nutrients for *M. hiroglyphicus*, that are not found in the phloem of sugarcane. This mutualistic relationship is the same as other insect hosts and their primary endosymbionts such as *Buchnera aphidicola* in aphids, *Wigglesworthia glossinidia* in tsetse flies, and *Carsonella ruddii* in psyllids (Gil *et al.*, 2004). The second group was not closely matched with any sequences. We propose that it’s a new bacteria symbiont. These results are useful information for future research of symbiotic control or the relationship between bacterial symbionts and the leafhopper host.

Acknowledgements

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Preliminary identification of phytoplasmas associated with grapevine yellows in Syria

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Abstract

Symptoms of grapevine yellows diseases have been observed in some grapevine growing areas near to the coast in Syria. Symptoms are similar to those associated with phytoplasmas, known as yellows diseases. In order to verify the presence of phytoplasmas using molecular techniques, leaf samples were taken from symptomatic grapevine plants that were exhibiting leaf rolling and red coloration during September 2010. Total DNA was extracted from leaf midribs of these samples and tested by nested-PCR followed by RFLP assays. Two phytoplasmas were identified in mixed infection: one related to stolbur (16SrXII) and the other tentatively related to clover proliferation group (16SrVI). To our knowledge, this is the first detection and identification of phytoplasmas infecting grapevine in Syria. Further research about final classification of the phytoplasma identified in mixed infection and about their spreading and insect vector(s) in vineyards are in progress.

Key words: grapevine yellows, stolbur phytoplasmas, 'bois noir' disease, PCR/RFLP.

Introduction

Grapevine production is of economic importance in many countries of the world including Syria, where it is a source of income and livelihood for a large number of Syrian farmers (Baghasa, 2006). In the recent years, Syria has kept its rank of 28th grape producing country in the world, accounting for 0.4% of total world production (FAOSTAT, 2004).

The grapevine is cultivated throughout Syria, although the cultivated area of grape in Syria has decreased significantly since 1997 especially in the rainfed area due to climate changes such as, frost, temperature rise and plant pests (Baghasa, 2006). Grapevine yellows (GY) are widespread diseases caused by different types of phytoplasma including 'bois noir' (BN). BN is a GY disease associated with a stolbur phytoplasma which belongs to 16SrXII ribosomal group (Lee *et al.*, 1998) and reported in Asia Minor, and in the Mediterranean and European countries (Maixner, 2006).

Since the symptoms are very similar among the different grapevine yellows diseases, the molecular investigation is needed in order to determine which phytoplasmas are associated to these diseases.

Materials and methods

In September 2010, the grapevine leaves used in this study were taken from two fields near Latakia that locates on the Mediterranean in north-west of Syria, these leaves were sun-dried and leaf veins were isolated from samples and ground in liquid nitrogen to a fine powder in order to extract the total DNA according to a chloroform/phenol procedure (Prince *et al.*, 1993).

Phytoplasmas were screened by nested PCR using P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) primer pair, followed by the primer pair R16mF1/R16mR2 (Gundersen and Lee, 1996). Final nested PCR was carried out with R16F2/R2 (Lee *et al.*, 1995). Template without nucleic acid was used as negative control. RFLP analyses were performed on the amplicons from first and second nested PCR reaction with *TruI*, *HhaI*, and *AluI* restriction endonucleases (Fermentas, Vilnius, Lithuania). After electrophoresis the restriction patterns obtained from these samples were compared with those of reference phytoplasma strains STOL (from pepper from Serbia, 16SrXII-A). Further nested PCR was carried out on P1/P7 amplicons using B5/P7 (Padovan *et al.*, 1995) followed by M1/V1731 (Martini *et al.*, 1999) using as reference strain ULW (elm yellows from France, 16SrV-A).

Results

Nested PCR results with generic phytoplasma primers B5/P7 and R16F2/R2 provided positive results from all grapevines tested. RFLP analyses on R16F2/R2 amplicons with *TruI* allow to identify phytoplasmas as stolbur or 16SrXII-A phytoplasmas (figure 1). A second RFLP profile indicated the possible presence of 16SrV, VI or VII phytoplasmas. Further analyses were carried out to identify the possible second phytoplasma detected in mixed infection. RFLP analyses with *HhaI* excluded the presence of 16SrVII group phytoplasmas (data not shown); while *AluI* suggested the presence of groups 16SrV or 16SrVI phytoplasmas. A nested PCR with 16SrV group specific primers provided negative results (figure 2) indicating the possible presence of 16SrVI phytoplasmas.

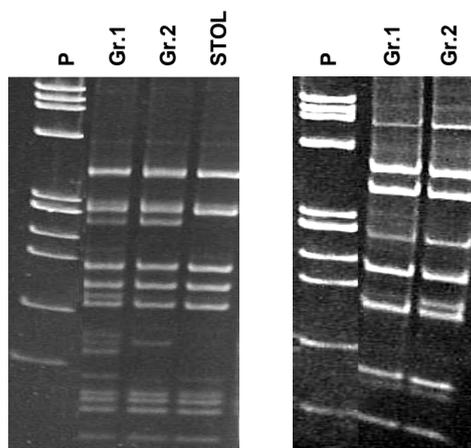


Figure 1. RFLP profiles of R16F2/R2 amplicons after *TruI* (left) and *AluI* (right) digest. Acronyms: Gr., grapevine samples; STOL, stolbur from pepper from Serbia (16SrXII-A); P, marker Φ X174 *HaeIII* digested.

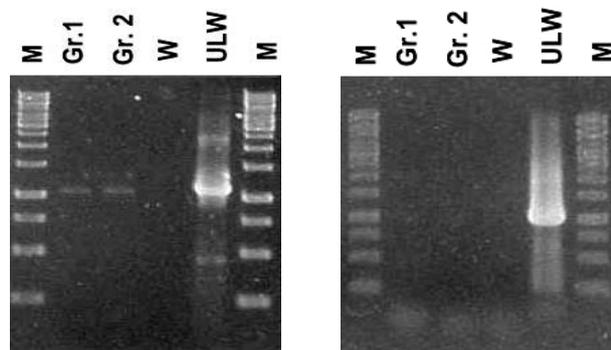


Figure 2. Nested PCR amplification with primers B5/P7 (left) and M1/V1731 (right) on grapevine samples. The lack of amplification with M1/V1731 primers indicates absence of 16SrV phytoplasmas. Acronyms: Gr., grapevine samples; ULW, elm yellows from EU (16SrV-A); M, marker 1kb DNA ladder.

Discussion

Stolbur phytoplasma is widespread in Europe in several different crops. BN disease associated with stolbur phytoplasmas has expanded geographically and increased in occurrence within the recent years (Maixner, 2006). There is no investigation up to now on the phytoplasma infections of grapevine in Syria, although phytoplasma infections have been reported in neighbouring countries to Syria (Boudon-Padieu, 2003, 2005). The finding of BN-associated phytoplasmas is in agreement with literature since it was reported in almost all grapevine growing areas worldwide.

The presence of mixed infection with 16SrVI group phytoplasmas is to be confirmed. This phytoplasma group was reported in other crops such as sesame in region near to Syria (Sertkaya *et al.*, 2007). In the future, surveys for the GY phytoplasmas will be carried out in a number of grapevine samples from different areas such as Damascus Rural, Homs, Hama, and Aleppo. In addition, further

investigations in vineyards will verify BN incidence, as well as, both insect vector and alternative hosts, considering the wide host range for BN phytoplasmas.

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Ribosomal protein gene sequences reveal a geographical differentiation between CSPWD phytoplasmas in Ghana

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Abstract

In Ghana, coconut lethal yellowing phytoplasma locally called Cape Saint Paul Wilt Disease (CSPWD) is the most damaging coconut disease. Two different *foci* of the disease can be distinguished: the first one covering the coast of the Western and Central Regions, and the second covering the coast of the Volta region. To test the hypothesis of a genetic differentiation between the CSPWD phytoplasma from the two *foci*, the partial ribosomal operon 16S, and two ribosomal protein genes (*rplV* and *rpsC*) of 14 strains were sequenced. The ribosomal protein gene sequences allowed the differentiation of the strains originating from the two different *foci* by a unique SNP, confirming a genetic differentiation.

Key words: CSPWD, phytoplasma, lethal yellowing disease, coconut, ribosomal protein genes, genetic diversity.

Introduction

In West Africa, coconut lethal yellowing is the most damaging coconut disease. It has been reported in Ghana, Nigeria and Togo (Dery *et al.*, 1997), and it is associated with a phytoplasma of the 16SrXXII group (Wei Wei *et al.*, 2007).

In Ghana, the disease is locally called Cape Saint Paul Wilt Disease (CSPWD) because it was first observed in 1932 at Cape Saint-Paul, in the eastern Volta region.

While the disease was still active in Togo and Volta region, it was further detected 350 km west at Cape Three Points in Western Region in 1964. In Central Region the disease was reported at Ayensudu in 1983. The disease was spreading and is still active in the different regions since first disease reports.

However, a disease-free gap of about 200 km between the Western-Central Regions and the Volta Region can be observed.

Because of both the history of the CSPWD and its geographical distribution in Ghana, we tested the hypothesis of a regional differentiation by sequencing the 16S rDNA gene and the 16S-23S rDNA intergenic region, as well as two ribosomal protein genes *rplV* (*rpl22*) and *rpsC* (*rps3*), which have been described as permitting a finer differentiation of phytoplasma strains (Martini *et al.*, 2007), for isolates sampled in the different regions.

Materials and methods

Two ribosomal protein genes (*rplV* and *rpsC*) were sequenced from 14 CSPWD coconut trees strains collected in Volta (6), Central (3) and Western (5) Regions in 2006 (2), 2008 (1) and 2009 (11) (figure 1, table 1). The partial ribosomal RNA operon was sequenced only for six of those isolates collected in

2009 in Volta (2), Central (2) and Western (2) Regions.

DNA extraction of all the CSPWD samples was performed using a CTAB protocol. The partial ribosomal RNA operon was amplified using the phytoplasma universal primers P1 (Deng and Hiruki, 1991) and P7 (Smart *et al.*, 1996). The *rp* gene operon was amplified using the PCR primer pair *rpLYF1* (5'-TTT AAA GAA GGT ATT ACA TGA-3') and *rpLYR1* (5'-TAA TAC CTA TAA CTC CGT G-3') designed by Marinho *et al.*, (2006). PCR conditions were 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 50°C for 1 minute and 72°C for 2 minutes and a final extension step of 72°C for 7 minutes.

The P1/P7 PCR products were directly sequenced. Products of the *rpLYF1/rpLYR1* PCR showing a clear and strong amplification were directly sequenced while PCR products showing a weak amplification were cloned into the pGEM-T Vector (Promega) and purified using QIAprep Miniprep (Qiagen) according to the manufacturer's instructions before sequencing. The sequencing was done by Beckman Coulter Genomics. The sequences were aligned by using Clustal W and analyzed by using DnaDist under Bioedit version 7.0.9.0 program software.

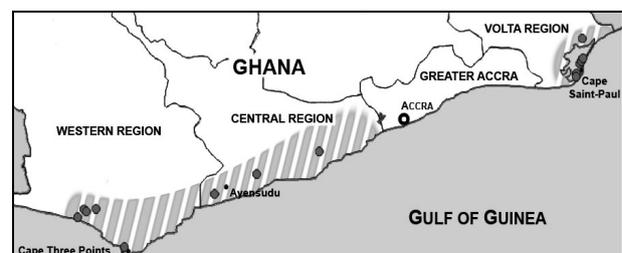


Figure 1. Geographical distribution of the 14 samples analyzed in this study and schematization of the two distinct CSPWD foci in Ghana.

Table 1. Sequences *rplV-rpsC* from nucleotide positions 340 to 351 for the 14 samples presented in this study (*sample sequenced also for P1/P7), their region of origin (VR: Volta Region, CR: Central Region, WR: Western Region) and the sampling year.

Sample Name	Region	Sampling Year	<i>rp</i> sequence (340-351)			
GH06-TET	VR	2006	ACG	TCA	CAT	AAT
GH06-WOE	VR	2006	C..	...
GH09-001*	VR	2009	C..	...
GH09-006	VR	2009	C..	...
GH09-015*	VR	2009	C..	...
GH09-022	VR	2009	C..	...
GH09-051	CR	2009	ACG	TCA	AAT	AAT
GH09-069*	CR	2009	A..	...
GH09-090*	CR	2009	A..	...
GH08-127	WR	2008	A..	...
GH09-102	WR	2009	A..	...
GH09-111*	WR	2009	A..	...
GH09-121*	WR	2009	A..	...
GH09-125	WR	2009	A..	...

Results

The primer pair P1/P7 amplified a 1,756 bp fragment. The six P1/P7 amplified sequences from the three different geographic regions were 100% identical to each other.

Amplification of the two ribosomal protein genes *rplV* and *rpsC* using rpLYF1/rpLYR1 produced a band of 983 bp. A total of seven mutations were observed between the sequences of the rpLYF1/rpLYR1 region with a similarity between 99.53 and 100%. Six of the mutations were present in only one strain. The last mutation, at nucleotide position 346, was common to all the strains from the same region of origin.

At this position, an adenosine (A) is present for strains originating from Western and Central regions, while a cytosine (C) can be observed for the strains from Volta region, independent of the year they were collected (table 1).

Discussion

Sequencing of the ribosomal protein genes *rplV-rpsC* reveals a geographical differentiation of the CSPWD phytoplasma in Ghana by a unique SNP at nucleotide position 346, whereas the partial ribosomal RNA operon

failed to detect genetic variability. This geographical differentiation suggests an independent evolution of the two CSPWD *foci* without exchange of phytoplasma strains between them.

However, the number of samples sequenced in this study is small and analysis of a larger number of strains using the restriction enzyme *Tsp45I*, which is able to recognize the SNP will be necessary to confirm the results.

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Epidemic of lethal yellowing disease affecting *Phoenix dactylifera* and *Sabal mexicana* in Central Mexico

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Abstract

Phytoplasmas are nonculturable pleomorphic prokaryotes associated to plant diseases worldwide. Lethal yellowing of coconut palms disease has high economic impact of the palm population affected in several countries. *Phoenix dactylifera* and *Sabal mexicana* plants showed symptoms of lethal decline in central Mexico at locations that were approximately 500 km from the coast and altitudes ~ 1700 masl which are unusual for putative lethal yellowing. We identified two strains that fit within the group 16SrI, subgroup D. This is the first report of the presence of the LY phytoplasmas far from the coasts and at extreme high altitudes. The vector *Myndus crudus* is only present in warm areas due to its high susceptibility to low temperatures. Our findings could indicate the presence of a new unknown vector or that a new biotype of *M. crudus* is spreading in central Mexico.

Key words: mollicutes, PCR, detection, vector.

Introduction

Phytoplasmas are pleomorphic prokaryotes associated to plant diseases worldwide. Infected plants show abnormal growth with excessive bud proliferation, yellow stems, yellow mosaics and unusual colorations. These pathogens induce hormonal disorders on their hosts but they normally do not cause premature death of affected plants. An exception to this however, is the lethal yellowing (LY) of coconut palms. LY appeared in Mexico in 1971 devastating palm plantations from the States of Yucatán, Chiapas and Campeche (Martínez-Soriano *et al.*, 1994) and recently in the Pacific coasts of Oaxaca and Guerrero. This disease has high economic impact with up to 68% of the palm population being affected. In 2009 *Phoenix dactylifera* and *Sabal mexicana* plants showed symptoms of lethal decline in central Mexico at locations that were approximately 500 km from the coast and altitudes ~ 1700 m asl which are unusual for putative LY. Symptoms observed were fruit drop as well as flower necrosis. These were followed by appearance and chronological progression of foliar yellowing. First younger leaves turn reddish-brown to dark brown, finally inducing death of apical meristem.

Materials and methods

DNA extraction

Plant samples of *Phoenix dactylifera* and *Sabal mexicana* were taken at the municipalities of Abasolo, Salamanca and Irapuato, State of Guanajuato in Mexico. Total DNA was extracted as described by Lopez and Larkins (1993).

PCR assays

Universal primer pairs targeting the 16S rRNA gene in the initial reaction primers R16mF2 and R16mR1

(Gundersen and Lee, 1996) were used. Then sequential nested PCRs with the primers R16F2/R2 (Lee *et al.*, 1993) were performed. PCR was performed in a 25 µl total volume of reaction, containing approximately 50 ng of genomic DNA, 2 mM MgCl₂, 10 pmol of each primer, 1X of PCR buffer solution, 200 mM of dNTPs and 2.5 units of Platinum®*Taq*DNA polymerase (Invitrogen). PCR rounds were as conventional programs. This amplification products were used to made 1:20 dilutions as DNA template for second amplification reaction. All products were visualized in 1% agarose gels by staining with ethidium bromide.

Cloning and nucleotide sequencing

RFLP and DNA sequence analysis were conducted for molecular characterization. PCR products were purified with Pure Link PCR purification kit and inserted into pCR TOPO TA cloning 2.1 vector (Invitrogen) used to transform *E. coli* DHα cells. Plasmids were sequenced using an ABI PRISM 377 PERKIN-ELMER DNA sequencer.

Sequence alignment and phylogenetic analysis

Restriction DNA patterns of 16S rDNA amplicons were generated using the iPhyClassifier software. DNA sequences were analysed by NCBI Blastn algorithm.

Results

Nucleotide sequences were determined for 16S rDNA fragments amplified after nested PCRs were deposited at the GenBank database (accession numbers JF431249 and JF431250). Sequence similarity among the 16S rDNAs of *Sabal mexicana* strain and others reported for the States of Guerrero (Mexico) and Florida (USA) were 99% identity of 98% coverage. The *Phoenix dactylifera* strain showed 99% identity of 98% coverage

with Texas Phoenix (USA) and Yucatán (Mexico) strains. On the basis of RFLP patterns, these two strains of the lethal yellowing phytoplasmas fit within the group 16SrI, subgroup D (Lee *et al.*, 1998).

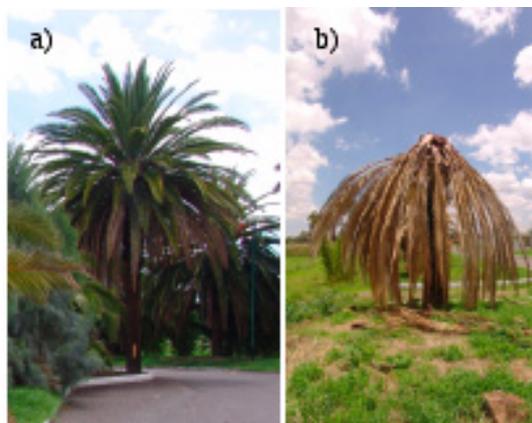


Figure 1. Palms showing coconut lethal yellowing phytoplasma associated diseases. a) Symptomless, b) lethal yellowing in terminal stage. (In colour at www.bulletinofinsectology.org)



Figure 2. Presence of coconut yellowing phytoplasma far from Mexican coasts. (In colour at www.bulletinofinsectology.org)

Discussion

This is the first report worldwide of the presence of the LY phytoplasmas far from the coasts and at extreme high altitudes. The vector *Myndus crudus* is only present in warm areas due to its high susceptibility to low temperatures. Our findings could indicate the presence of a new unknown vector or that a new biotype of *M. crudus* is spreading in central Mexico.

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First outbreaks of grapevine ‘flavescence dorée’ in Austrian viticulture

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Abstract

A long-term survey on the presence of phytoplasmas in Austrian viticulture was conducted from 2004 to 2010. ‘Boir noir’ was widespread in all Austrian vine growing areas, no other phytoplasmas could be identified until 2009. The first finding of grapevine ‘flavescence dorée’ (FD) was in Southeast Styria in 2009. FD was identified in *Vitis vinifera* (cv. “Müller Thurgau” and “Sämling 88”), in an American hybrid (cv. Isabella) and phytoplasmas of the same subgroup were also detected in *Clematis vitalba*. This first disease outbreak affected four different vineyards. Incidence of few newly-affected grapevines and clematis plants were observed within the infected area, however about 0.5 ha vine growing area has been uprooted until 2010. A second outbreak of FD was detected in the region of South Styria. It was an isolate disease outbreak on two single grapevines (cv. Sämling 88) and FD-related phytoplasmas were also detected in three plants of *C. vitalba*. The ribosomal group affiliation of all characterized samples from both regions indicated that the phytoplasmas belonged to the ribosomal subgroup 16SrV-C. To prevent further spreading of this quarantine pathogen *focus* zones and a buffer zones were designated in Southeast Styria and in South Styria.

Key words: Viticulture, grapevine yellows, ‘flavescence dorée’, *Scaphoideus titanus*.

Introduction

In Europe grapevine ‘flavescence dorée’ (FD) phytoplasma is listed as a quarantine pathogen (Council Directive 2000/29/EC, Annex II/AII). Its vector, the nearctic leafhopper *Scaphoideus titanus*, was introduced into Europe from North America. FD and *S. titanus* have spread from France to the eastern and southern vine-growing areas of Europe. *S. titanus* was first recorded in Austria in 2004, since then it has invaded the southeastern parts of Austria (Zeisner, 2009). The phytoplasma associated with FD belong to two strains in ribosomal group 16SrV (FD-C/16SrV-C, and FD-D/16SrV-D) (Martini *et al.*, 1999; Angelini *et al.*, 2001). The wild plant *Clematis vitalba*, was determined as possible host for 16SrV-C phytoplasmas (Filippin *et al.*, 2009). A national survey was conducted to broadly estimate the occurrence of phytoplasmas in Austrian viticulture.

Materials and methods

The surveys were carried out on 82 locations by sampling leaves and shoots from grapevines and clematis plants over a 7-year period (2004-2010). The study sites were located in Lower Austria, Vienna, Burgenland, Styria and the vine growing areas of Carinthia. The nucleic acid extraction of the phytoplasmas from plant samples was done according to Dolye and Doyle (1990) method. A real-time PCR assay was used for screening (Angelini *et al.*, 2007). The identification of the phytoplasma and the group affiliation was performed according the EPPO Diagnostic Protocol (EP7/79).

Results

All samples collected between 2004 to 2008 were infected by stolbur phytoplasmas (16SrXII) associated with ‘bois noir’ (BN) disease.

The first finding of FD was in the vine growing region of Southeast Styria in 2009. The phytoplasma was detected in the cultivars Isabella, Müller Thurgau and Sämling 88. In the vicinity of the infected vineyards a *C. vitalba* plant displaying leaf rolling and reddening was found to harbour also 16SrV-C phytoplasmas (figure 1).

This first disease outbreak affected four different vineyards. Due to the high disease presence about 0.5 ha vine growing area has been uprooted until 2010. In the established *focus* zone of this area, enhanced surveillance activities revealed only a few newly infected grapevines in 2010.

Several asymptomatic and symptomatic clematis plants at the border of the vineyards and outskirts of nearby forests were positive to the presence of phytoplasmas of the same ribosomal subgroup. In the investigated vineyards ‘boir noir’ was also present. Mixed BN and FD infections were also detected in same cases.

FD appears to be restricted to Southeast Styria, but subsequent surveys detected a second isolated disease outbreak in South Styria in 2010. Contrary to the first finding, the disease incidence was very low in that area. The phytoplasma could be detected only in two single grapevines (cv. Sämling 88) and in two clematis plants.

The ribosomal group affiliation of all characterized samples from both regions revealed that all FD strains belonged to the ribosomal subgroup 16SrV-C.



Figure 1. Reddening symptoms on *C. vitalba*, including downward leaf rolling of leaves.
(In colour at www.bulletinofinsectology.org)

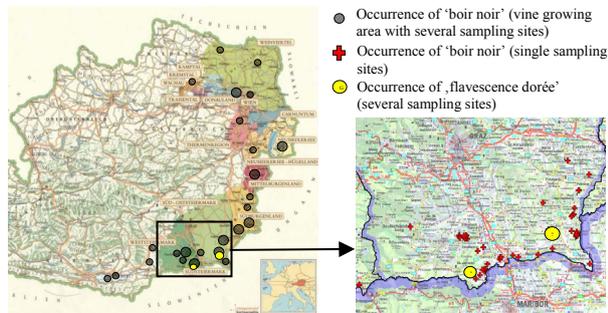


Figure 2. Current distribution of phytoplasmas in Austrian viticulture. Austrian vine growing areas are coloured.
(In colour at www.bulletinofinsectology.org)

Discussion

During the last decade, Austrian vine growers have been faced an increasing occurrence of BN disease. Besides the challenge of combating this non regulated disease of grapevine, there was a high risk of introduction of the quarantine disease FD to Austrian vine growing areas (Steffek *et al.*, 2007). Despite increased monitoring activities in the preceding years, the dimension of the first disease outbreak in Southeast Styria was considerable, and assumed to be contributed to the abundance of the vector in this area. A *focus* zone (1 km around the outbreak) and a *buffer* zone (5 km around the outbreak) were designated in this area to prevent further spreading of this pest. This outbreak was brought under control with uprooting of all diseased grapevines and *Clematis* plants and the combat of the vector. The vector has been established in this area since 2004. A regulation was issued to control *S. titanus* since 2009. The control has been mandatory not only for the vine growing areas of Southeast Styria but also parts of South Styria. In contrary to the first outbreak, the disease incidence in South Styria is limited to few plants, which can be contributed to the very low abundance of *S. titanus* in this area. Only

few individuals of the vector were caught in this area and there is no established population. This disease outbreak support the hypothesis that clematis or other wild plants may serve as a reservoir host for FD. Transmission experiments showed that *Dictyophara europaea* is able to transmit the FD-related phytoplasma from clematis to grapevine (Filippin *et al.*, 2009). Other leafhoppers, such as *Orientalis ishidae* are potential vectors (Mehle *et al.*, 2010). The parallel occurrence of BN and FD in the same vine growing areas may also enhance the risk that individual grapevines are hot spots for new outbreaks.

The results of this study lead to the conclusion that phytoplasmas threaten the Austrian viticulture; well-designed prospective trails may increase our understanding of control points and strategies.

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Identification of different phytoplasmas infecting grapevine in Turkey

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Abstract

Grapevines with severe redness and inward curling of leaves were collected from the main viticulture production areas, Aegean, Central Anatolia and Western Anatolian regions of Turkey. Collected samples were subjected to nucleic acid extraction followed by nested PCR/RFLP analyses that allow to identify different phytoplasmas in symptomatic grapevines. The majority of samples were infected with phytoplasmas belonging to 16SrXII group and referable to the 'bois noir' phytoplasmas, while in some samples 16SrIX or 16SrI-B phytoplasmas were identified. The 16SrIX phytoplasmas are firstly reported in grapevine.

Key words: Grapevine, PCR/RFLP analyses, phytoplasma identification, 'bois noir'.

Introduction

Turkey is one of the nations native to grapevine in the Middle East, where table and vine grape varieties have been grown in Thrace, Central Anatolia, Mediterranean, Aegean and Eastern Anatolia regions of Turkey. The country is the 4th and 6th in the world, respectively, for grapevine cultivation and production of table and wine grape. Grapevine is affected worldwide by several phytoplasma diseases named grapevine yellows. Phytoplasmas belonging to different ribosomal groups were identified such as aster yellows (16SrI group), elm yellows (16SrV group) and stolbur (16SrXII group), together with 16SrII, 16SrIII, 16SrVII and 16SrX groups in different countries (Varga *et al.*, 2000, Boudon-Padieu, 2005, Gajardo *et al.*, 2009, Duduk *et al.*, 2004, Milkus *et al.*, 2005). Two of these phytoplasmas are associated with specific diseases such as 'flavescence dorée' (16SrV-C and V-D subgroups) and 'bois noir' (subgroup 16SrXII-A). Grapevines with severe redness and inward curling of leaves were observed in the main viticulture production areas of Turkey therefore surveys were carried out to verify phytoplasmas presence and identity.

Materials and methods

The main viticulture production areas, Aegean, Central Anatolia and Western Anatolian parts of Turkey, were surveyed in the summer of 2009 and 167 leaf samples were collected from symptomatic plants. Severe redness and inward curling of leaves were the major symptoms of the collected plants. Nucleic acid was extracted from midribs according to a chloroform/phenol protocol (Prince *et al.*, 1993). The phytoplasma strains stolbur (STOL, ribosomal subgroup 16SrXII-A), aster yellows (PRIVA, ribosomal subgroup 16SrI-B) and Naxos (ribosomal subgroup 16SrIX-C) maintained in collection in periwinkle were employed as reference strains in restriction fragment length polymorphism (RFLP) analy-

ses. Direct PCR with ribosomal P1/P7 universal primer pair, followed by nested PCR with R16F2n/R2 (Gundersen and Lee, 1996), and R16(I)F1/R1 and R16(V)F1/R1 (Lee *et al.*, 1994) primer pairs were carried out. R16(I)F1/R1 products were subjected to RFLP analysis with *TruI* and *HhaI*. One uncloned R16F2/R2 amplicon was purified using Qiagen PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced in both directions with R16F2 and R16R2 primers, using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK) for molecular characterization. The obtained sequence was aligned by using Clustal W and BioEdit (Hall, 1999) softwares and deposited in GenBank.

Results

R16F2n/R2 amplicons of the expected size were obtained for half of the samples tested. The majority of positive samples exhibited *TruI* RFLP pattern indistinguishable from those of 16SrXII ribosomal group. These phytoplasmas are also referred to as stolbur phytoplasmas, and reported to be associated in grapevine to 'bois noir' disease. Phytoplasmas belonging to aster yellows group (16SrI-B) were also identified by RFLP analyses on amplicons obtained with primer R16(I)F1/R1 in a few cases with *TruI* (figure 1) and *HhaI* (data not shown). In some of the symptomatic samples 16SrIX phytoplasmas were identified (data not shown). One of the latter samples showing 16SrIX phytoplasma infection was employed for sequencing, and the obtained sequence of 1,063 bp was deposited in the GeneBank under ID HQ714331. This sequence showed a 99% identity with that of pigeon pea witches' broom phytoplasma and other phytoplasma members of group 16SrIX, including the strains related to 'Candidatus Phytoplasma phoenicium' present in the Genbank. Comparison of the obtained sequence with those available in GenBank for 16SrIX group phytoplasmas allow to verify that the sequence show five mismatch with other sequences.

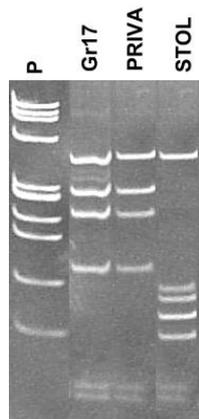


Figure 1. RFLP profiles of R16(I)F1/R1 amplicons after *TruI* digest. Acronyms: Gr., grapevine sample; PRIVA, primula yellows from Germany (16SrI-B); STOL, stolbur from pepper from Serbia (16SrXII-A); P, marker Φ X174 *Hae*III digested.

Discussion

'Bois noir' disease is widespread and occurs from Spain to Ukraine and from Germany and Northern France to Lebanon and Israel (Maixner, 2011). The disease was also recently reported in Turkey (Canik *et al.*, 2011). Stolbur group-related grapevine phytoplasmas have been also recently been reported from Iran (Karimi *et al.*, 2009) and China (Duduk *et al.*, 2010). Aster yellows phytoplasmas were reported in grapevine in several countries after the first finding in Italy (Alma *et al.*, 1996). The 16SrIX group phytoplasmas are severely infecting plants in different regions, especially in those bordering Turkey (Choueiri *et al.*, 2001; Abou-Jawdah *et al.*, 2002) so their first identification indicates the susceptibility of the species to this pathogen and the urgent need to further verify its presence in grapevine to prevent any further epidemic. Work on finer classification of the identified phytoplasmas, as well as their further detection in grapevine and in other host species and in potential insect vectors, is in progress.

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The occurrence of '*Candidatus phytoplasma rhamni*' in *Rhamnus cathartica* L. without symptoms

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Abstract

Common buckthorn, *Rhamnus cathartica* L., is a small tree or shrub of Eurasian origin that has become invasive in North America. A survey was conducted during 2010 to evaluate occurrence of buckthorn witches' broom (BWB) disease symptoms induced by '*Candidatus Phytoplasma rhamni*' in the native range of *R. cathartica*. The distribution of phytoplasma-infected trees was identified over a large expanse of Europe, from south-west Switzerland to north-east Serbia. Although 25% of analyzed buckthorn trees were infected with phytoplasma (34 out of 133 plants), all plants were symptomless, indicating a commensal relationship between the phytoplasma and its plant host without negative effects which would lead to disease development.

Key words: common buckthorn, distribution, PCR, phytoplasma, *Rhamnus cathartica*, symptoms.

Introduction

Common buckthorn, *Rhamnus cathartica* L., is a shrub or small tree native to much of Europe and western Asia that has become invasive in North America. *R. cathartica* was introduced to North America as an ornamental shrub in the earlier 1800's and is now naturalized throughout the Upper Midwestern and North-eastern United States and the maritime provinces of Canada.

In 1964 research using native range surveys in biological control programs resulted in over 30 specialized arthropod species recorded on *R. cathartica* in Europe, including nine sap sucking species: six Hemiptera and three Acari (Gassmann *et al.*, 2008). Among these, three are psyllids: *Trichoermes walkeri* (Foerster) and *Triozia rhamni* (Schrank) of the family Triozidae and *Cacopsylla rhamnicola* (Scott) belonging to the family Psyllidae. Psyllids are phloem-feeding insects and have potential to acquire and transmit the phloem-limited pathogens such as phytoplasmas. This ability represents a possible threat to the native plants, if a phloem-feeding insect is introduced as a biocontrol agent in a new geographic area without previous experimentation of its capability to act as a possible vector. A new program was initiated in 2001 taking into consideration increasing concerns over potential non-target impacts of biological control agents and greater demands for high levels of agent specificity (Louda *et al.*, 1997; Pemberton, 2000).

In its native range *R. cathartica* is found to be naturally infected with a phytoplasma - a wall-less, non-culturable, phloem-limited, insect-transmitted plant pathogen of the class *Mollicutes*. A lethal witches' broom disease of *R. cathartica* induced by buckthorn witches' broom BWB phytoplasma ('*Candidatus Phytoplasma rhamni*', 16SrX-E subgroup) was observed for the first time in the Rhine Valley in south-western Germany in the 1990's (Mäurer and Seemüller, 1996). The

disease is characterized by brush-like witches' brooms, leaf distortion, phloem necrosis, off-season growth and overall decline of the plant. The main goal of this study was to determine occurrence of '*Ca. P. rhamni*' in the native range of *R. cathartica*.

Materials and methods

A survey was conducted during July and August of 2010 in the buckthorn growing habitats of western and south-eastern Europe. Altogether, 26 localities in five countries were surveyed and overall 133 *R. cathartica* samples were collected (table 1). Characteristic symptoms of witches' broom, which would indicate phytoplasma presence, were not observed on any of the surveyed buckthorn sites. In some localities discrete leaf yellowing and/or small leaves were present on few trees and these were sampled separately and treated as possibly symptomatic. All other sampled *R. cathartica* trees were symptomless.

Total nucleic acids from plant midribs and petioles were extracted using a previously reported CTAB protocol (Angelini *et al.*, 2001). Phytoplasmas were detected by polymerase chain reaction (PCR) amplification of 16S rRNA gene using the universal phytoplasma and group specific primer pairs. Amplification was performed in nested PCR with P1/P7 primers followed by R16F2n/R2 universal primer pair according to Lee *et al.* (1998) or R16(X)F1/R1 primers specific for amplification of 16SrX group and related phytoplasmas (Lee *et al.*, 1995). Amplicons obtained with R16F2n/R2 primers were subjected to RFLP analyzes with *Mse*I, *Alu*I and *Hpa*II endonucleases (Lee *et al.*, 1998). '*Ca. P. rhamni*' DNA isolated from naturally infected *R. cathartica* located between Neuhofen and Ludwigshafen in Rhineland-Palatinate (type locality of '*Ca. P. rhamni*'; provided by Bernd Schneider) was used as a reference positive control in all reactions.

Table 1. Geographic origin and number of *R. cathartica* samples analyzed with PCR results on ‘*Ca. P. rhamni*’ presence.

Country	Canton/Region	PCR results	
		positive	analyzed
Switzerland	Geneva	0	7
Switzerland	Vaud	4	5
Switzerland	Fribourg	5	5
Switzerland	Jura	5	18
Germany	Hesse	3	25
Austria	Niederösterreich	9	25
Austria	Burgenland	2	5
Serbia	South Banat	1	22
Serbia	Braničevo	1	5
Serbia	Bor	4	9
Serbia	Zaječar	0	5
Montenegro	Kolašin	0	2
TOTAL		34	133

Results

‘*Ca. P. rhamni*’ was detected in 25% of *R. cathartica* samples, at several sites in all countries surveyed, except for Montenegro (table 1). We have not observed the witches’ broom disease symptoms as described by Mäurer and Seemüller (1996), at any locality, and at present we cannot associate the presence of the phytoplasma with any particular symptoms in buckthorn.

Discussion

Trees and shrubs of common buckthorn were found to be infected with ‘*Ca. P. rhamni*’ at almost all surveyed localities confirming previous reports of host association of this phytoplasma with *R. cathartica*. However, the symptoms of witches’ broom were not observed. Absence of symptoms on all phytoplasma infected trees could be an indication of commensal relationship between the phytoplasma and its plant host without negative effects which would lead to a disease development. Plants with this kind of symptomless presence of phytoplasma are considered to be a wild reservoir of the pathogen, since they are not affected by its presence, which can be the case with ‘*Ca. P. rhamni*’ and the common buckthorn.

However, occurrence of phytoplasma-infected trees over a wide geographic area within the native range of *R. cathartica* is raising concerns about possible infection of this weedy shrub in its naturalized range in North America, which needs to be tested. Considering that this phytoplasma has been described as associated with witches’ broom disease of *Rhamnus*, its influence on *R. cathartica* and other *Rhamnus* species needs to be tested in controlled conditions.

Considering possible vectors of the phytoplasma, the importance of psyllids in the phytoplasma transmission has been recognized only recently, and more comprehensive research on their role as vectors has been elucidated in the past few years (reviewed in Jarausch and

Jarausch, 2010). All confirmed and recognized psyllid vectors today belong to a single genus – *Cacopsylla*, and they are transmitting apple proliferation group phytoplasmas (AP, 16SrX) on apple, stonefruit and pear trees. ‘*Ca. P. rhamni*’ has the closest phylogenetic relatedness to the phytoplasmas of the AP group and could be expected to be transmitted by the psyllids. This enforces the need for the assessment of the potential role of *C. rhamnicola* as a vector, elucidation of ‘*Ca. P. rhamni*’ epidemiology, as well as a host-plant specificity of ‘*Ca. P. rhamni*’ to *R. cathartica* and its congeners.

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First report of maize redness disease in Hungary

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Abstract

During 2010, several maize production areas in Hungary were surveyed for the occurrence of maize redness (MR) disease symptoms associated with stolbur phytoplasma, as well as for the presence of the known vector of the disease, a planthopper *Reptalus panzeri* (Low). Incidence of maize plants with symptoms of reddening was low in all surveyed areas. Altogether, 25 symptomatic maize plants were collected at 9 localities and tested for phytoplasma presence. In addition, from one locality specimens of cixiids *R. panzeri* and *Hyalesthes obsoletus* Signoret were collected and PCR analyzed. Presence of stolbur phytoplasma in MR symptomatic maize plants and stolbur-infected *R. panzeri* was identified at the single locality Monorierdő in central Hungary. This finding represents the first report of MR presence in Hungary.

Key words: corn, *Hyalesthes obsoletus*, maize reddening, *Reptalus panzeri*, stolbur.

Introduction

Maize redness (MR) is a severe disease of maize associated with stolbur phytoplasma (16SrXII-A) which is transmitted to maize by the cixiid planthopper *Reptalus panzeri* (Low) (Jović *et al.*, 2007). Disease is characterized by midrib, leaf and stalk reddening, followed by reddening and desiccation of the whole-plant and abnormal ear development. Although MR has been reported from Serbia, Romania and Bulgaria for more than 50 years, the associated agent, a vector and the epidemiological cycle of the disease have only recently been determined (Jović *et al.*, 2009). MR can cause significant yield losses in the years of epiphytotic appearance, as was recorded during the 2002/03 outbreak in and around the South Banat district of Serbia.

Recent reports of maize with reddening symptoms in Italy (Calari *et al.*, 2010), as well as possible host-shift of *R. panzeri* to maize as a preferable host plant (Jović *et al.*, 2010) are suggesting an ongoing, and in the future expected spread of the disease and of its insect vector. Previously reported presence of *R. panzeri* in Hungarian vineyards and their natural infection with stolbur phytoplasma (Palermo *et al.*, 2004), in addition to economic importance of maize production, increased the need for a survey of MR presence in the territory of Hungary. Here we present the first results of this survey.

Materials and methods

During August and September of 2010 selected maize fields in certain production areas of Hungary were surveyed for the occurrence of reddening symptoms on maize. Plants expressing symptoms such as midrib, leaf and stalk reddening were collected from nine sampling sites in south, southwest, northwest and central Hungary (figure 1). On all surveyed localities only individual

plants were symptomatic, and no presence of epiphytotic appearance of the symptoms was recorded. During July *R. panzeri* and *Hyalesthes obsoletus* Signoret cixiids were collected from a single maize field in the vicinity of village Monorierdő in central Hungary, which was later sampled for MR symptomatic maize plants. Insects were collected by sweep nets in the vicinity of maize fields.

DNA was extracted from fresh leaf midribs and adventitious roots of MR symptomatic maize plants and individual insects according to previously reported CTAB protocols (Daire *et al.*, 1997; Clair *et al.*, 2003). Phytoplasma identification was conducted using a nested PCR on the 16S rRNA gene with primer pairs P1/P7 and R16F2n/R16R2 (Lee *et al.*, 1998). The obtained nested-PCR products were digested with *TruI* restriction enzyme and RFLP profiles were compared with reference strains (Rep9 and WRp5 MR from J. Jović; EAY, 16Sr-B, MOL, 16SrXII-A from A. Bertaccini and AY27, 16SrI-A, CPh, 16SrI-C, PaWB, 16SrI-D from I-M. Lee). In parallel, all samples were tested with TaqMan real-time PCR amplifying phytoplasma nonribosomal *map* gene of the 16SrXII-A subgroup, applying plant endogenous control (EC) (Pelletier *et al.*, 2009) with slight modification of probe labelling and PCR conditions.

Results and discussion

Only in three out of 25 symptomatic maize plants, collected from nine sampling sites in Hungary, was the stolbur phytoplasma (16SrXII-A) identified by PCR/RFLP based analysis of the 16S rRNA gene. All three stolbur infected corn samples originated from the same locality (Monorierdő) in central Hungary (figure 1) where the potential planthopper vectors were identified. In the case of analyzed insects, two out of six *R. panzeri*

and three out of eight *H. obsoletus* specimens tested positive for the presence of stolbur phytoplasma. The results were confirmed by real-time PCR. The Ct values were measured in all the stolbur positive samples ranging from 31.1-33.8. Amplification curves of EC were observed in each sample with Ct values between 18.2-22.5.

None of the other maize samples, collected at other localities (figure 1) were positive for any phytoplasma. Although these plants were symptomatic, absence of phytoplasma was not surprising, taking into account that reddening of maize can also occur as a consequence of different biotic and/or abiotic factors, as well as the absence of the MR vector *R. panzeri*. These results lead to the conclusion that presence of MR disease can be identified only when a three-parameter identification of symptoms, phytoplasma and vector is performed.



Figure 1. Map of maize sampling sites in Hungary.

Since the first records of MR appearance in the South Banat district of Serbia in 1957 (Marić and Kosovac, 1959) it has been noted that the disease has periodical variations in symptom intensity and incidence. Due to specificities of the disease epidemiological cycle and the insect vector life cycle (Jović *et al.*, 2009), environmental factors play a significant role both in the intensity and incidence of the disease. More severe disease and higher incidence of symptoms are associated with warm springs and summers which facilitates earlier emergence of the vector adults and consequently earlier infection of maize with the stolbur phytoplasma. Thus, if the vector and stolbur phytoplasma as agent associated with the disease are present in a certain geographic area, risk of epiphytotic appearance of MR is also present, whenever favorable environmental conditions are.

Based on these results we conclude that stolbur phytoplasma associated with maize redness disease, and the identified vector of the disease *R. panzeri* are present in corn in Hungary. Presence of the stolbur-infected vector of MR *R. panzeri* is in agreement with previously determined role of this cixiid as a major vector of MR in the South Banat district of Serbia (Jović *et al.*, 2009). However, the role of *H. obsoletus* in MR epidemiology

in Hungary is yet to be studied, since this cixiid was not reported to play a significant role in MR epidemiology in Serbia.

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Molecular characterization of 'bois noir' phytoplasma populations from North-Eastern Italy

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Abstract

Genetic diversity of 'bois noir' phytoplasma populations in two vineyards of Verona province (Veneto region, North-Eastern Italy) was investigated by the use of multilocus analysis of gene sequences amplified in the polymerase chain reaction. Based on results from restriction fragment length polymorphism profiling and nucleotide sequence alignments of *16S rRNA*, *tuf*, *hlyC*, *trxA-truB*, *cbiQ-glyA*, and *rplS-csdB* genes, 'bois noir' phytoplasma strains were grouped in five SNP lineages. The data suggest that strain composition of BN phytoplasma populations may be modified by ecological relationships in vineyards.

Key words: multiple gene analysis, PCR, RFLP, stolbur.

Introduction

'Bois noir' (BN) is a grapevine yellows disease associated with infection by stolbur phytoplasma. Its biological complexity, indicated by the existence of numerous plant hosts and diverse insect vectors, has stimulated studies of molecular markers useful for researching genetic diversity among BN phytoplasma strains. Recently, an impressive diversity of BN phytoplasma strains was revealed by characterization of *vmp1* and *secY* genes (Filippin *et al.*, 2009; Pacifico *et al.*, 2009).

In the present work, we explored the genetic diversity of BN phytoplasma populations in two vineyards of North-Eastern Italy by use of nucleotide sequence and RFLP analyses of *16S rRNA*, *tuf*, *hlyC*, *trxA-truB*, *cbiQ-glyA*, and *rplS-csdB* genes amplified in polymerase chain reaction (PCR) assays.

Materials and methods

Leaf samples were collected from 20 symptomatic grapevine plants in two vineyards [San Pietro di Lavagno (1) and Ronco all'Adige (2)] of Veneto region, North-Eastern Italy (table 1).

Nested-PCR assays were carried out for amplifying the genes *16S rRNA* [primers R16F2n/R2 (Gundersen and Lee, 1996)], *tuf* [primers fTufAY/rTufAY (Schneider *et al.*, 1997)], *hlyC*, *trxA-truB*, *cbiQ-glyA*, and *rplS-csdB* [designed on the basis of phytoplasma sequences deposited in GenBank by Cimerman *et al.* (2006)].

PCR products were digested by using restriction enzymes having recognition sites that included SNPs previously identified (Quaglino *et al.*, 2009), distinguishing

among BN phytoplasma strains. The enzymes used (and gene analyzed) were: *AluI*, *BfaI*, *BstUI*, and *MseI* (*16S rDNA*); *HpaII* (*tuf*); *SspI* (*hlyC*), *BsaHI* (*trxA-truB*), *Hpy188I* (*cbiQ-glyA*), and *HpyCH4V* (*rplS-csdB*).

BN phytoplasma SNP genetic lineages were identified by comparisons of collective RFLP patterns.

Results and discussion

RFLP patterns of 16S rDNA amplicons indicated that all plants were infected by BN phytoplasma strains of subgroup 16SrXII-A.

For each of the other gene amplicons it was possible to identify two distinct RFLP profiles, previously reported also for BN phytoplasma strains in Lombardy region, north-western Italy (Quaglino *et al.*, 2010). Strain types based on RFLP patterns of gene *tuf* were consistent with those based on the gene *hlyC*. RFLP patterns of the other genes were not consistent with *tuf-hlyC* strain types.

Based on collective RFLP patterns from all the analyzed genes, BN phytoplasma strains were grouped in five SNP genetic lineages, named BN4, BN6, BN8, BN9, and BN10 (table 1). Lineages BN4 and BN6 were identified also within BN phytoplasma populations in North-Western Italy (Quaglino *et al.*, 2010); lineages BN8, BN9 and BN10 were identified exclusively in North-Eastern Italy. Lineages BN8 (1 strain) and BN9 (one strain) were identified only in the vineyard of San Pietro di Lavagno, while lineage BN10 (five strains) were observed only in the vineyard of Ronco all'Adige. Lineages BN6 (11 strains) and BN4 (two strains) were present in both vineyards (table 1).

Table 1. SNP lineages in multiple genetic *loci* of BN phytoplasma strains from vineyards in north-eastern Italy.

Strain	Vineyard	Grapevine cultivar	16S rDNA	<i>Tuf</i> -type	<i>hlyC</i> -type	<i>cbiQ-glyA</i> *	<i>trxA-truB</i> *	<i>rplS-csdB</i> *	Lineage of strain
			<i>Mse</i> I	<i>Hpa</i> II	<i>Ssp</i> I	<i>Hpy</i> 188I	<i>Bsa</i> HI	<i>Hpy</i> CH4V	
VR456	1	Chardonnay	XII-A	tuf-a	hlyC-a	B	A	A	BN8
VR460			XII-A	tuf-a	hlyC-a	B	B	A	BN6
VR461			XII-A	tuf-a	hlyC-a	B	B	A	BN6
VR462			XII-A	tuf-b	hlyC-b	A	B	A	BN9
VR464			XII-A	tuf-a	hlyC-a	B	B	A	BN6
VR466			XII-A	tuf-a	hlyC-a	B	B	A	BN6
VR475			XII-A	tuf-a	hlyC-a	B	B	A	BN6
VR477			XII-A	tuf-a	hlyC-a	B	B	A	BN6
VR481			XII-A	tuf-a	hlyC-a	B	B	A	BN6
VR501			XII-A	tuf-b	hlyC-b	A	B	B	BN4
VR502	2	Chardonnay	XII-A	tuf-a	hlyC-a	B	A	A	BN10
VR503			XII-A	tuf-a	hlyC-a	B	A	A	BN10
VR507			XII-A	tuf-a	hlyC-a	B	B	A	BN6
VR509			XII-A	tuf-b	hlyC-b	A	B	B	BN4
VR510			XII-A	tuf-a	hlyC-a	B	B	A	BN6
VR511			XII-A	tuf-a	hlyC-a	B	B	A	BN6
VR512			XII-A	tuf-a	hlyC-a	B	A	A	BN10
VR513			XII-A	tuf-a	hlyC-a	B	A	A	BN10
VR514			XII-A	tuf-a	hlyC-a	B	A	A	BN10
VR516			XII-A	tuf-a	hlyC-a	B	B	A	BN6

*, identical letter = identical profile.

Conclusions

Presence of certain lineages in one and not another vineyard is possibly explained by differences in the ecology of vineyards and/or surrounding areas that influence the composition of BN phytoplasma populations through strain selection. Future research, involving larger sample sizes, will focus on genetic diversity of BN phytoplasma populations from several geographic regions, as well as on BN lineages in grapevines, insects, and weeds in vineyards. The new knowledge should aid understanding of BN epidemics and open new avenues for developing innovative approaches for BN disease management.

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Current status of phytoplasma diseases of medicinal and nutraceutical plants in Southern Italy

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Abstract

In Southern Italy, several medicinal and nutraceutical plant species are affected by yellows, witches' broom and decline diseases, which severely impair productivity and phytochemical content of affected plants. This brief review, which is a mix of newly and previously published information summarizes the current knowledge about the mentioned diseases of a number of selected plant species. Emphasis is given on description of symptoms, taxonomic position of the associated phytoplasmas and changes induced by phytoplasmal infections in the composition of secondary metabolites of affected plants.

Key words: secondary metabolites, 16Sr group, aster yellows, elm yellows, stolbur, spartium witches' brooms, white leaf.

Introduction

In modern societies, there is an increasing interest in the use of medicinal and nutraceutical plants due to their role in promoting human health. In southern Italy, several plant species which have medicinal and nutraceutical properties are known to be affected by yellows, witches' broom and decline diseases. These diseases severely impair productivity and phytochemical content of affected plants. The present brief review, which is a mix of newly and previously published information summarizes the current knowledge about the mentioned diseases of a number of selected plant species.

Phytoplasma diseases of medicinal and nutraceutical plants

Spartium junceum (Spanish broom) is severely affected by spartium witches' broom (SpaWB) disease. The most characteristic symptoms of the disease are pronounced witches' brooms, shortened internodes, off-season growth and death of the plants. SpaWB is associated with two genetically different phytoplasmas which induce the same symptoms. These agents are (i) '*Candidatus* Phytoplasma spartii', a member of the 16SrX group and (ii) a phytoplasma that belongs to the elm yellows (EY) group or 16SrV group, subgroup 16SrV-C (Marccone *et al.*, 1996, 2004a; Lee *et al.*, 2004a). Most of the diseased plants are dually infected with the two phytoplasmas of which, one is predominant and readily detectable by direct polymerase chain reaction (PCR) assays, whereas the other occurs at a very low titre and can be detected only by the highly sensitive nested PCR (Marccone *et al.*, 1996). A '*Ca. P. spartii*'-related strain is also known to occur in *Sarothamnus scoparius* (syn. *Cytisus scoparius*) affected by the *S. scoparius* witches' broom disease (Marccone *et al.*, 2004a). Recent work has shown that the yield of volatile fraction, *i.e.*, essential oils, extracted from flowers of SpaWB-affected Spanish broom plants is lower than that of healthy plants (Mancini *et al.*, 2010a). Also, substantial amounts of

sesquiterpenes and a marked decrease in the amount of *n*-alkanes and aliphatic compounds are known to occur in the volatile fraction from flowers of diseased plants. Sesquiterpenes could not be detected in the volatile fraction of healthy plants (Mancini *et al.*, 2010a). Great differences between diseased and healthy Spanish broom plants were also identified in the alkaloid compounds (Mancini *et al.*, 2010b). The alkaloid content was considerably higher in samples of diseased plants than in those of healthy plants. Seven different alkaloids were identified only in diseased plants. These compounds included *N*-methylcytisine, its isomer, *N*-formylcytisine and a hydroxy-substituted derivative of sparteine. Four alkaloids including hydroxy-derivatives of cytisine and anagryne were shared by both healthy and diseased plants. All identified alkaloids were quinolizidine alkaloids (Mancini *et al.*, 2010b). Collective data indicated that changes in the composition of secondary metabolites of SpaWB-affected Spanish broom plants can be related to the role of phytoplasma infections in triggering plant defense responses (Mancini *et al.*, 2010a, 2010b).

Eucalyptus spp. (eucalypt) are affected by eucalyptus little leaf. The symptoms include abnormally minute leaves, yellowing, shortened internodes and proliferation of axillary shoots. Phytoplasmas of 16SrV and aster yellows (AY) (=16SrI group) groups, subgroups 16SrI-B and 16SrI-C, have been identified in the affected plants (Seemüller *et al.*, 1998; Camele *et al.*, 1999).

Myrtus communis (myrtle) plants showing symptoms of yellowing, small leaves and witches' brooms have been observed in Apulia, Molise and Basilicata regions. These plants proved to be infected by a subgroup 16SrI-B phytoplasma (Camele *et al.*, 1999). Myrtle plants exhibiting the mentioned symptoms are also common in several areas of the Campania region (C. Marccone, unpublished observations).

Rubus fruticosus (wild blackberry) is affected by rubus stunt (RuS), a disease causing stunting, small leaves and proliferation of axillary shoots. The RuS agent is a member of the 16SrV group, subgroup 16SrV-E, recently described as '*Ca. P. rubi*' (Seemüller *et al.*, 1998; Malembic-Maher *et al.*, 2010).

Taraxacum officinale (dandelion), *Cichorium intybus* (common chicory) and *Picris echioides* (bristly ox-tongue) showing symptoms of yellowing, proliferation of slender secondary shoots, small leaves and phyllody are infected by phytoplasmas of the 16SrII group, subgroup 16SrII-E. However, *P. echioides* is also known to harbour a 16SrIX group phytoplasma (Marccone *et al.*, 2001).

Medicago sativa (alfalfa) has been reported to be affected by a witches' broom disease, the Italian alfalfa witches' broom, in the Basilicata region. Main symptoms of the disease are witches' brooms, yellowing and small leaves. The causal agent was identified as a member of the 16SrII phytoplasma group (Seemüller *et al.*, 1998).

Brassica spp. (*Brassica oleracea* var. *capitata*, *italica*, *palmifolia*, *rapifera* and *botrytis*), *Raphanus raphanistrum* (wild radish), *Allium cepa* (onion), *Catharanthus roseus* (periwinkle), *Calendula officinalis* (pot marigold), *Papaver rhoeas* (corn poppy), *Primula* sp. (primrose), showing symptoms of virescence, phyllody, yellowing, upright growth habit, little leaves, and general stunting, *Daucus carota* (carrot) with cluster of dwarfed, chlorotic, upright adventitious shoots, *Portulaca oleracea* (purslane) with spindling upright growth, small leaves, and chlorosis, and *Plantago lanceolata* (English plantain) which grows poorly with narrow and chlorotic leaves, are widespread in southern Italy. Diseased plants of the mentioned species are known to harbor the aster yellows phytoplasma 'Ca. P. asteris', 16SrI group, subgroup 16SrI-B (Marccone *et al.*, 2000, 2001; Lee *et al.*, 2004b).

Capsicum annuum (red pepper), *Lycopersicon esculentum* (tomato) and *Nicotiana tabacum* (tobacco) showing typical stolbur symptoms, *Apium graveolens* (celery) with yellowing and stunting, *Convolvulus arvensis* (field bindweed) showing yellowing, stunting and/or proliferating and erect growth habit, and *Vitis vinifera* (grapevine) with typical grapevine yellows symptoms, are all infected by subgroup 16SrXII-A phytoplasmas (Marccone *et al.*, 2001).

Cynodon dactylon (Bermuda grass) and *Digitaria sanguinalis* (crab grass) with white leaf symptoms are infected by 'Ca. P. cynodontis' (16SrXIV group) (Marccone *et al.*, 2004b).

Concluding remarks

This brief review reflects the advances made during the last two decades in detection, molecular characterization and identification of phytoplasmas associated with yellows, witches' broom and decline diseases of medicinal and nutraceutical plants in southern Italy. However, there is still very little known about several other aspects of the mentioned diseases including disease management, phytoplasma insect vector relationships and role of phytoplasmas in eliciting secondary metabolites which can be pharmaceutically important.

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Identification of phytoplasma belonging to X-disease group in cherry in Chile

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Abstract

During summer 2006 sweet cherry (*Prunus avium* L.) trees, exhibiting disease symptoms suggestive of possible phytoplasma infection were observed in the Libertador General Bernardo O'Higgins region of Chile. Branches were collected from ten plants (five with and five without symptoms) and nucleic acid extracted from leaf midribs and phloem scrapings was used for phytoplasmas testing. Nested polymerase chain reaction (PCR) amplification allows the detection of phytoplasmas in samples from plants that showed symptoms, but not in those from asymptomatic ones. Restriction fragment length polymorphism, cloning, and sequencing allowed identification of phytoplasmas into ribosomal subgroup 16SrIII-J.

Key words: cherry, nested-PCR, phytoplasmas, RFLP, sequencing.

Introduction

Production of sweet cherry (*Prunus avium* L.) in Chile is concentrated in three regions: Libertador General Bernard O'Higgins (VI), Maule (VII) and Bío-Bío (VIII) and the fruit is for fresh consumption or for processing.

In one orchard located in the VI region a decay, low vigor and even death of plants in the variety Basler Langstieler (for processing), especially when grafted onto the rootstock Colt, were observed. The symptoms appear on plants of at least three years: the vigor reduction has been moderate to strong, and in most cases, removing the bark, phloem necrosis was observed in trunk or branches. No fungal or bacterial isolation was obtained.

Through the observation of symptoms phytoplasma presence was suspected and molecular analyses were carried out both in healthy and symptomatic plants to verify the presence of these prokaryotes.

Materials and methods

Samples were collected in summer 2006 from five symptomatic and five asymptomatic plants and consist of branches and leaves from which phloem and midribs respectively, were quickly separated, immediately frozen in liquid nitrogen and stored at -80°C.

Total nucleic acids were extracted from 1 g of mixture of main leaf midribs and phloem tissues (Prince *et al.*, 1993), dissolved in Tris-EDTA pH 8.0 buffer, and maintained at 4°C; 20 ng/μl of nucleic acid were used for amplification.

After direct polymerase chain reaction (PCR) with primer pair P1/P7, nested PCR with R16F2n/R2 primers (Gundersen and Lee, 1996) was performed. PCR and

nested PCR reactions were carried out following published protocol (Schaff *et al.*, 1992). Identification of detected phytoplasma was done using restriction fragment length polymorphism (RFLP) analyses on ribosomal DNA amplified with primer pair R16F2n/R2, with *TruI* and *HhaI* (Fermentas, Vilnius, Lithuania) restriction enzymes.

Selected R16F2n/R2 amplicons were purified using Concert Rapid PCR Purification System (Life Technologies, Gaithersburg, MD, USA). DNA fragments were ligated into T/A cloning vector pTZ57R/T following the instructions of the InsT/Aclone PCR Product Cloning Kit (Fermentas, Vilnius, Lithuania). Putative recombinant clones were analysed by colony-PCR, sometimes in combination with the insert-specific primers. Amplicons obtained from three colonies per cloned fragment were subjected to RFLP analyses, as described above. Selected fragments from cloned DNAs were sequenced in both directions using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). The sequences were then aligned with BLAST engine for local alignment (version Blast N 2.2.12).

Results and discussion

Positive results were obtained only after nested amplification on P1/P7 amplicons. Cloned fragments of R16F2n/R2 amplicons were sequenced and subjected to RFLP analysis that allowed the assignment of the phytoplasma to the ribosomal subgroups 16SrIII-J (related to X-disease group) (figure 1). This phytoplasma was identified in all plants with symptoms, but not in the asymptomatic ones. In all cases there was no sequence difference among the three cloned R16F2n/R2 fragments from the same sample (1,244 bp each).

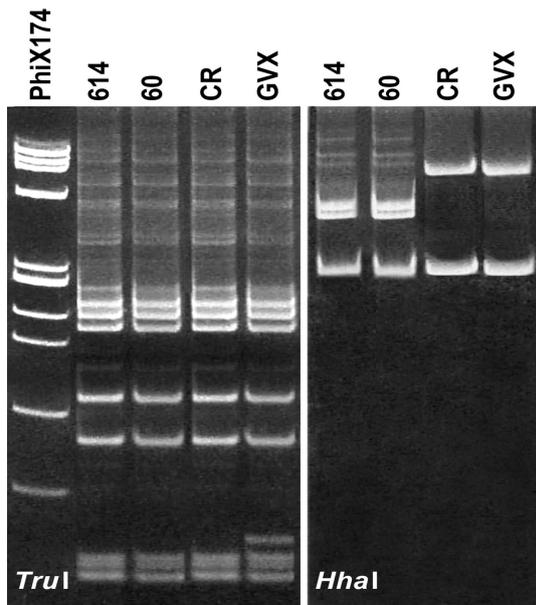


Figure 1. RFLP analysis of 16S rDNA amplified in nested-PCR with primer pair R16F2n/R2, from infected cherry sample 614, after digestion with *TruI* and *HhaI*. Controls: 60, 16SrIII-J; CR, *Crepis biennis* yellows (16SrIII-B); GVX, Green Valley X-disease (16SrIII-A). PhiX174: marker Φ X174 *HaeIII* digested.

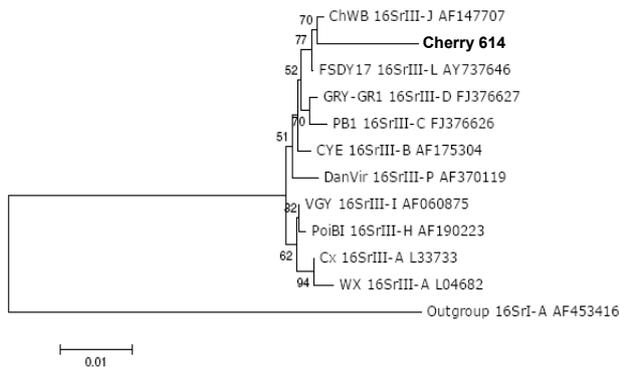


Figure 2. Phenetic tree constructed using neighbor-joining method with 16S rDNA region sequences from the strain # 614 from Chilean cherry and related phytoplasmas. Bar represents a phenetic distance of 1%.

The comparison of nucleotide sequences of 16S rDNA and the percentages of similarity showed the close correlation of Chilean isolates with those from X-disease group (figure 2). Phytoplasma 16S rRNA gene sequences were retrieved from the NCBI's nucleotide sequence database. The strain EF514210, corresponding to Delphinium phytoplasma from the UK, showed 99% similarity with the cherry strain #614 in the 16S rRNA gene. The same occurs with the strains AF147706 and AF147707, corresponding to chayote witches' broom phytoplasmas (16SrIII-J) from Brazil (Montano *et al.*, 2000), and strain AF495657 chinaberry yellows phytoplasma from Bolivia (Harrison *et al.*, 2003).

Phytoplasmas belonging to ribosomal group 16SrIII are widespread in South America. Reports were made from Brazil, Colombia and Bolivia (Wheeler *et al.*, 2005). In Chile and Argentina phytoplasma of the 16SrIII ribosomal group had been detected in sugar beet (Castro *et al.*, 2000). This suggests that transmission to fruit trees in Chile may have occurred by polyphagous insect vector species. However it would be appropriate to verify whether cases of incompatibility scion/rootstock found often in Chile in cherry plants, whose production is for fresh consumption (Cruz, 2005), could be related to the presence of phytoplasma belonging to ribosomal subgroup 16SrIII-J.

The same samples used for phytoplasma detection have been analyzed for cherry viruses (data not shown) and the same viruses (*Prunus necrotic ringspot virus* and *Prune dwarf virus*) were detected in symptomatic and asymptomatic plants. This suggests that the phytoplasmas belonging to the ribosomal subgroup 16SrIII-J are responsible for the described disease in the cherry variety Basler Langstieler in Chile.

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First report of witches' broom disease of tomato associated with phytoplasmas in Saudi Arabia

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Abstract

Tomato is a very important crop in Saudi Arabia grown in both open fields and in greenhouses. Leaf samples of tomato plants showing witches' broom symptoms were tested by nested PCR assays followed by sequencing. A DNA fragment of the expected size was amplified from symptomatic and not from symptomless plants. Phylogenetic analysis of the 16S rDNA sequence clustered the tomato witches' broom phytoplasma detected in Saudi Arabia within the peanut witches' broom (16SrII) group ('*Candidatus* Phytoplasma aurantifolia'). To our knowledge, this is first detection of a witches' broom phytoplasma associated with tomato in Saudi Arabia.

Key words: Tomato, phytoplasmas, witches' broom, sequencing.

Introduction

Tomato (*Solanum lycopersicum*) is a very important crop in Saudi Arabia; grown in open fields and greenhouses. The cultivated area is estimated about 15,127 ha, with a year production in 2009 of more than 0.5 million tons. Recently witches' broom and stunting symptoms disease were observed in tomato growing areas, therefore molecular analyses were carried out in order to identify the phytoplasma possibly associated with the disease.

Materials and methods

In 2010 leaf samples of tomato plants showing witches' broom symptoms were collected from different locations in Alhasa, Saudi Arabia. Samples with and without symptoms were analyzed for the presence of phytoplasmas using 16SrDNA PCR assays. Total nucleic acids were extracted from leaves using the DNeasy Plant Mini kit (QIAGEN) and were used as template in a nested PCR reaction using primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by primers R16F2/R16R2 (Lee *et al.*, 1995).

A PCR product obtained from P1/P7 primers was purified and directly sequenced.

Results and discussion

A DNA fragment of the expected size (1.2 kb) was amplified by nested PCR from all the symptomatic plants tested. No amplification was obtained from symptomless plants.

The 1,753 bp sequence obtained from the sequencing of a P1/P7 amplicon was deposited in GenBank (accession no. HM584815). Phylogenetic analysis of this

16S rDNA sequence showed that the tomato witches' broom phytoplasma clustered with the peanut witches' broom (16SrII) group members ('*Candidatus* Phytoplasma aurantifolia'). In particular the strain showed a significant sequence identity with the *Catharanthus roseus* phytoplasma II strain, a member of the 16SrII-E (EU096500) collected in United Arab Emirates.

The 99% homology was found with scaevola witches' broom, tomato big bud, and alfalfa witches' broom phytoplasmas described in Oman (Khan *et al.*, 2002; Al-Zadjali *et al.*, 2007.). The 99% similarity with the obtained sequence was also found with peanut witches' broom (EF193356), subgroup 16SrII-A (Lee *et al.*, 1998) and sweet potato witches' broom (DQ452417) subgroup 16SrII-D; a 98% homology was obtained with faba bean phyllody phytoplasma (HQ589188).

To our knowledge, this is first detection of witches' broom phytoplasma associated with tomato in Saudi Arabia and also the first detection of 16SrII phytoplasmas in tomato indicating the wide ability of this phytoplasmas to infect both herbaceous and woody host also in Arabian countries.

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First report of stolbur phytoplasma infecting celery in Serbia

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Abstract

During field survey conducted in 2010 in Serbia, symptoms of foliar reddening were observed on celery on the locality Padinska Skela, in the vicinity of Belgrade. Leaf samples from six symptomatic and two asymptomatic plants were collected and tested for phytoplasma presence detection.

Nested polymerase chain reaction analyzes using universal primer pairs P1P7 followed by R16F2n/R2 identified presence of phytoplasmas in all symptomatic plants, while asymptomatic plants were tested negative. Restriction analysis of amplified 16Sr DNA fragments with enzyme *MseI* identified in all positive samples the same pattern as a reference strain of stolbur phytoplasma belonging to the 16SrXII-A ribosomal subgroup. Molecular differentiation of stolbur phytoplasma detected in celery was performed by amplification and RFLP analysis of the elongation factor Tu (*tuf* gene). Digestion of *tuf* gene indicated presence of *tuf*-type b of stolbur phytoplasma in all symptomatic celery plants. This is the first record of stolbur phytoplasma in association with celery expressing symptoms of foliar reddening in Serbia.

Key words: *Apium graveolens*, PCR, RFLP, Serbia, stolbur phytoplasma, *tuf* gene.

Introduction

The stolbur phytoplasma belonging to 16SrXII-A ribosomal subgroup is widely distributed in Europe, associated with severe diseases on many cultivated plants (grapevine, maize, solanaceous crops, potato, carrots, sugarbeet, strawberry).

In celery crops (*Apium graveolens* L.), stolbur phytoplasma infection has been reported in Italy (Carraro *et al.*, 2008), Hungary (Vicizian, 2002) and in Czech Republic (Navratil *et al.*, 2009). Celery was determined as highly susceptible to stolbur phytoplasma infection (Fialova *et al.*, 2009), with symptoms consisting of diffuse yellowing and/or reddening of the leaves and stunting.

In Serbia, stolbur phytoplasma has been associated with maize redness disease of maize (Duduk and Bertaccini, 2006; Jović *et al.*, 2007) and 'bois noir' in grapevine (see in Cvrković, 2010) causing severe yield losses with tendency of growing epidemics, but never in association with celery.

Primary goal of this study was to identify and characterize phytoplasmas in association with celery showing symptoms of foliar reddening.

Materials and methods

In September 2010, a total of six samples of celery with reddish discoloration of leaves (figure 1) were collected on locality Padinska Skela (near Belgrade) and analyzed for phytoplasma presence. In addition, two symptomless plants were collected and used as negative controls.

Nucleic acids were extracted from fresh leaf midribs using CTAB protocol according to Angelini *et al.* (2001). Phytoplasma identification was conducted

through nested PCR amplification of 16S ribosomal RNA gene according to Lee *et al.* (1998), with the universal primer pairs P1/P7 and R16F2n/R2. Restriction fragment length polymorphism (RFLP) analysis of the amplified phytoplasmas 16S rRNA gene fragments was performed with *MseI* enzyme. RFLP profiles of phytoplasma identified in celery were compared with a reference phytoplasma strains.

Molecular differentiation of stolbur phytoplasma detected in celery was performed by amplification and restriction digestion of the elongation factor Tu - *tuf* gene. Amplification was conducted in nested PCR with fTuf1/rTuf1 and fTufAY/rTufAY primers followed by digestion with *HpaII* restriction enzyme, according to Langer and Maixner (2004).



Figure 1. Stolbur infected celery with symptoms of foliar reddening.

(In colour at www.bulletinofinsectology.org)

Results and discussion

Nested PCR analysis with 16S rRNA universal primers detected the presence of phytoplasmas in all celery plants which exhibited symptoms of foliar reddening. All asymptomatic plants tested were negative. Restriction analysis of PCR products with endonuclease *Mse*I showed in all samples the same pattern as the one of the reference strain of the stolbur phytoplasma belonging to the 16SrXII-A subgroup (figure 2A). The *tuf* gene was amplified in all symptomatic samples. Digestion with *Hpa*II endonuclease determined presence of *tuf*-type b stolbur phytoplasma (figure 2B). Identification of stolbur in infected plants represents the first record of this phytoplasma in celery crops in Serbia.

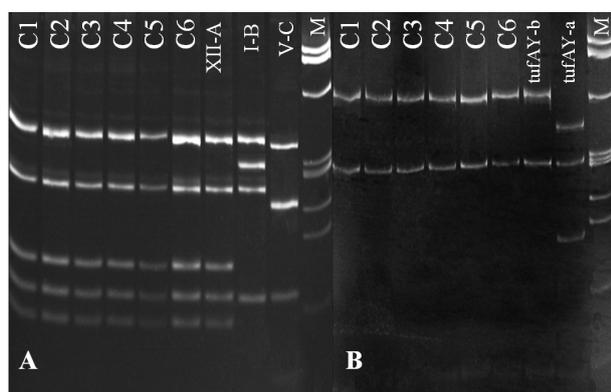


Figure 2. RFLP profiles of: (A) 16S rRNA fragments amplified by nested PCR with primer pairs P1/P7 and R16F2n/R2, followed by digestion with *Mse*I; (B) *tuf* gene fragments amplified with primers fTuf1/rTuf1 and fTufAY/rTufAY and digested with *Hpa*II; C1-C6: stolbur infected celery, XII-A (16SrXII-A, from naturally infected maize from South Banat region, Serbia), I-B (16SrI-B, provided by E. Angelini, Italy), V-C (16SrV-C, FD-C from naturally infected field-growing grapevine from Nišavski region, Serbia), tufAY-b: stolbur type tufAY-b from the Mosel region of Germany (provided by M. Maixner, Germany); tufAY-a: stolbur tuf-type a, from Middle-Rhine region of Germany (provided by M. Maixner, Germany); M: molecular weight marker ϕ X174/*Hae*III digested (Fermentas).

Stolbur phytoplasma mostly originates from the naturally infected plants, from which it is transmitted to cultivated plants by polyphagous planthoppers of the Cixiidae family. It is known that celery is a very susceptible host to stolbur phytoplasma infection (Fialova *et al.*, 2009), which implicates that cultivation of this crop can be seriously compromised when the pathogen occurring in natural reservoirs is transmitted by active vectors to cultivated plants.

Celery is important vegetable crop in Serbia, thus, it is of particular importance, besides incidence and impact of the disease, to study the epidemiology of stolbur

appearance in correlation with movement of potential vectors from wild plants to vegetable crops during the growing season. These studies are key points for elucidating the epidemiological cycle of stolbur disease in association with celery and relevant point for further pest management strategy of this phytoplasma.

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A strain of phytoplasma related to 16SrII group in *Picris hieracioides* L. in Serbia

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Abstract

During epidemiological survey for phytoplasmas in association with agricultural crops in Serbia, a new species of common weed has been detected to harbor phytoplasmas in Serbia. In 2010, a total of 38 samples of *Picris hieracioides* (Asteraceae), commonly known as hawkweed oxtongue, were sampled from vineyards in Jasenovik (near Niš, South Serbia) and analyzed for phytoplasma presence. Nested polymerase chain reaction analysis using primers specific to the phytoplasma 16SrDNA gene showed six samples of *Picris hieracioides* to be positive. Digestion of amplified 16SrDNA fragments with endonuclease *MseI* identified the same pattern as the one of a reference strain of tomato big bud belonging to the 16SrII ribosomal group. Sequence obtained from the PCR product associated with infected *P. hieracioides* was submitted to BLAST analysis which showed a 99% similarity with reference strain of *Picris echioides* phyllody from Italy, belonging to 16SrII-E subgroup. This is the first report of phytoplasma related to 16SrII group infecting *Picris hieracioides*, as well as, the first record on the presence of this group of phytoplasmas in Serbia and South East Europe.

Key words: *Picris hieracioides*, 16SrII, phytoplasma, Serbia, PCR.

Introduction

Phytoplasmas belonging to the peanut witches' broom group (16SrII) have been recorded from weeds and cultivated plants worldwide, causing in significant losses in lime, carrots, alfalfa, potato, and ornamentals. Phytoplasmas of the 16SrII group have been found in the Middle East (Khan *et al.*, 2007), Mediterranean region (Tolu *et al.*, 2006), Australia (Aryamanesh *et al.*, 2011), Mexico (Hernandez-Perez *et al.*, 2009), Israel (Sobolev *et al.*, 2007), and Indonesia (Harling *et al.*, 2009).

In Europe phytoplasmas of the 16SrII group have so far been detected in several weed species, and also cultivated plants (Tolu *et al.*, 2006; Davino *et al.*, 2007; Parrella *et al.*, 2008). In Sardinia (Italy), phytoplasmas belonging to the 16SrII group have been identified in association with *Calendula arvensis* L., *Solanum nigrum* L. and *Chenopodium* spp., and in central and southern Italy infecting *Picris echioides* L. (Tolu *et al.*, 2006).

The main goal of this study was identification and characterization of phytoplasmas associated with *Picris hieracioides* L., (hawkweed oxtongue), a common weed of the family Asteraceae in vineyards in Serbia.

Materials and methods

In July 2010, 38 plants of *P. hieracioides* were collected from vineyards in Jasenovik (near Niš, South Serbia) and analyzed for the presence of phytoplasmas. Weeds were sampled randomly and showed no typical symptoms of phytoplasma infection. Plants were collected with roots, which were later sliced, prepared into 0.2-1.0 gram aliquots, and stored at -20°C until DNA extraction.

Total nucleic acids from *P. hieracioides* plants were extracted using the CTAB protocol described by

Angelini *et al.* (2001). Phytoplasmas presence was detected by amplifying the 16S ribosomal RNA gene by nested PCR with universal primer pairs P1/P7 (Deng and Hiruki, 1991; Smart *et al.*, 1996) and R16F2n/R2 (Gundersen and Lee, 1996), followed by RFLP analysis with *MseI* restriction enzyme. In order to obtain longer fragments for sequencing, 16S rRNA amplicons were obtained in nested PCR assay with the universal primers P1A/P7A with reaction conditions according to Lee *et al.* (2004). Samples of *P. hieracioides* with the same RFLP pattern as a reference phytoplasma strains belonging to 16SrII group were sequenced (BMR Service, Italy) and submitted to BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Alignment and comparison of phytoplasma sequences were carried out using ClustalW program integrated in MEGA4 software (Tamura *et al.*, 2007).

Results and discussion

Specific 16S rRNA fragments of phytoplasmas were amplified from six out of 38 analyzed samples of *P. hieracioides*. Restriction analysis of PCR products with endonuclease *MseI* showed in all infected plants presence of the same pattern as a reference strain of tomato big bud (TBB) belonging to 16SrII-D subgroup (figure 1). One of the 16S rRNA amplicons was sequenced and submitted to the National Center of Biotechnology Information with the accession number JF799094. BLAST analysis of the 1,447 bp sequence obtained from the Serbian *P. hieracioides* phytoplasma determined it to be 99% identical to reference strain of *Picris echioides* phyllody from Italy (PEP) (Acc. No. Y16393) belonging to 16SrII-E subgroup. Alignment and comparison of 16SrRNA sequences of reference

PEP and Serbian *P. hieracioides* strain showed pairwise distance of 0.6%.

P. hieracioides is a common weed in vineyards in Serbia, however, this plant species has not previously been detected in association with phytoplasma diseases. This is the first report of a phytoplasma from the 16SrII group infecting *P. hieracioides*, as well as the first record of the presence of this group of phytoplasmas in Serbia and South East Europe.

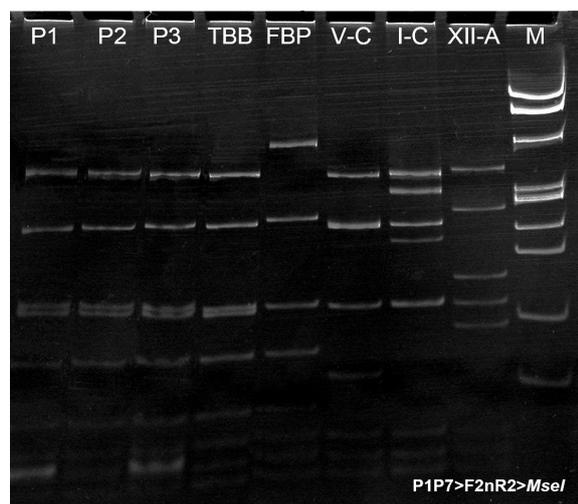


Figure 1. RFLP profiles of 1,447 bp fragment of 16S rRNA gene of 16SrII phytoplasma strain infecting *P. hieracioides* in Serbian vineyard (P1-P3) and reference phytoplasma strains amplified by nested PCR with primers P1/P7 followed by R16F2n/R2 and digested with *MseI* endonuclease: TBB- tomato big bud (16SrII-D), and FBP- faba bean phyllody (16SrII-C) (provided by A. Bertaccini, Italy); V-C (16SrV-C; FD-C from naturally infected field-growing grapevine from Nišavski region, Serbia); I-C (16SrI-C, provided by Elisabeth Boudon-Padieu, France); XII-A (16SrXII-A, maize redness from naturally infected maize from South Banat region, Serbia); M: molecular weight marker ϕ X174/*HaeIII* digested (Fermentas, Vilnius, Lithuania).

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Geographical distribution of 'flavescence dorée' phytoplasmas in Croatian grapevines

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Abstract

Seven Croatian grapevine samples found positive for the presence of 'flavescence dorée' (FD) phytoplasmas in 2010 survey confirm the first findings of FD phytoplasma from previous year, as well as the existence of new grapevine yellows heavily affected narrow area west and southwest of Zagreb along the border with Slovenia. Two new locations 100 km east and 60 km north-east of Zagreb, respectively, were found to be affected, widening the potential FD-risk zone and placing the capital in its centre. Grapevine varieties infected with FD-related phytoplasmas encompassed widely known Pinot Noir and Riesling, but also indigenous red Plemenka Crvena and Ružica Crvena, as well as white variety Škrlet. Preliminary molecular characterization results suggest the variability of 16SrV-C identified phytoplasmas, especially from the locations west and southwest of Zagreb.

Key words: 'flavescence dorée', 16SrV-C, PCR, RFLP.

Introduction

The occurrence of 'flavescence dorée' (FD) phytoplasma vector *Scaphoideus titanus* Ball (Budinščak *et al.*, 2005) in Croatia, as well as the occurrence of FD and/or *S. titanus* in the surrounding countries stimulated the efforts to identify the most heavily grapevine yellows (GY) affected areas in Croatia (Šeruga Musić *et al.*, 2009). Although it had been expected that these areas would have been under the greatest FD infection pressure, FD-related phytoplasmas were detected in a new GY foci located west and southwest of Zagreb (Šeruga Musić *et al.*, 2010).

This study aims to continue investigating grapevine phytoplasma diversity in the country, including the wider Zagreb area with confirmed FD-related phytoplasma occurrence.

Materials and methods

Leaf veins from mature grapevines were sampled and kept on CaCl₂ at 4°C for phytoplasma identification. Besides 40 symptomatic grapevines from the 2009 survey (Šeruga Musić *et al.*, 2010), 55 grapevines from 2010 survey were included in this study. In addition, 29 *S. titanus* and 5 weed samples were analyzed for phytoplasma presence in 2010.

DNA was extracted (Mikec *et al.*, 2006) and phytoplasma 16S rRNA gene was amplified by using generic primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) in direct PCR, followed by a nested PCR using primer pair R16F2n/R2 (Gundersen and Lee, 1996). Phytoplasma ribosomal group affiliation was determined by restriction fragment length polymorphism (RFLP) analysis of the nested PCR products with enzyme *Tru*II (Fermentas, Vilnius, Lithuania).

P1/P7 amplicons from samples harbouring phytoplasmas belonging to the ribosomal group 16SrV were submitted to additional nested PCRs primed by group-

specific primers R16(V)F1/R1 (Lee *et al.*, 1994) and by 16Sr758f/M23Sr (Gibb *et al.*, 1995; Padovan *et al.*, 1995, respectively). The latter amplicons were digested with *Taq*I (Fermentas, Vilnius, Lithuania) for the RFLP analysis.

Results

Out of 40 grapevines showing typical GY symptoms collected throughout the country for phytoplasma analyses in 2009, 21 were positive for their presence. The majority of samples (19) harboured 'bois noir' (BN) agents, while for the first time two samples from the vicinity of Zagreb were found infected with phytoplasmas belonging to the ribosomal subgroup 16SrV-C (Šeruga Musić *et al.*, 2010). The FD phytoplasmas still constituted only a portion of positive samples (9.5%). They were identified from red varieties Pinot Noir (Vivodina) and Plemenka Crvena (Brezje) geographically located in a narrow region west and southwest of Zagreb. These samples came from the highly infected vineyards in a smaller winegrowing region previously not determined to be under high GY infection pressure (Šeruga Musić *et al.*, 2009).

In the batch of 55 grapevine samples from 2010, one sample of indigenous white variety Škrlet not analyzed in 2009 survey was also tested. The 25 positive samples detected reveal smaller percentage of infected samples in the 2010 (45.5%) compared to 2009 (52.5%). Yet, the proportion of grapevine samples positive for FD phytoplasmas increased to 28.0% (7 samples). These samples included Škrlet sampled in 2009 from Voloder (about 100 km east of Zagreb), Riesling and Ružica Crvena (indigenous red variety) from Brckovljani, a village about 60 km northeast of Zagreb. The remaining 4 FD phytoplasma positive grapevines were found in the same area as Plemenka Crvena and Pinot Noir from 2009 (figure 1). None of the weeds or *S. titanus* tested in 2010 was found phytoplasma positive in this study.

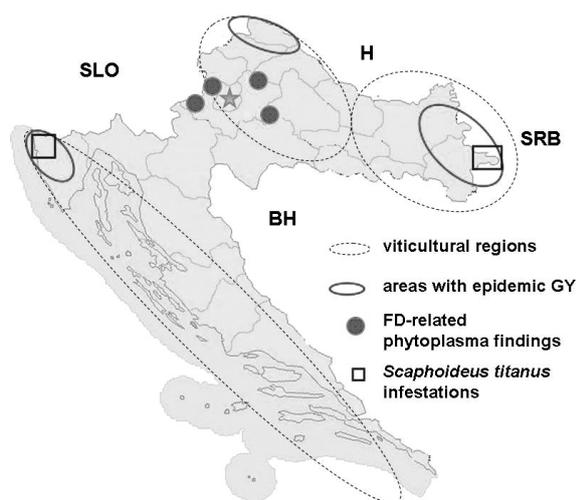


Figure 1. Geographical distribution of grapevines infected with FD phytoplasmas in 2009-2010 in relation to the most heavily GY affected areas in Croatia. The position of Zagreb is marked with a star.

Discussion

BN and FD phytoplasmas are the main GY agents in Euro-Mediterranean area. BN phytoplasmas belong to the ribosomal subgroup 16SrXII-A and they are still the most widespread in the Croatian winegrowing regions (Šeruga Musić *et al.*, 2009; 2010). FD phytoplasmas belong to the ribosomal subgroups 16SrV-C and 16SrV-D and despite the finding of principal FD vector in Croatia six years ago (Budinišćak *et al.*, 2005), no FD phytoplasmas had been detected before 2009 (Šeruga Musić *et al.*, 2010). Interestingly, those phytoplasmas were detected in heavily symptomatic vineyards West (Brezje) and Southwest (Vivodina) of Zagreb in a narrow winegrowing area along the Croatian-Slovenian border. Fifteen *S. titanus* from these two locations were examined in 2010, but none was positive for phytoplasma presence. Nonetheless these initial negative results for the vectors in this area, this highly probable route of natural phytoplasma transmission and it should be further investigated. The finding of FD phytoplasmas in the Škrlet sample from Voloder in year 2009 widens the potential FD-risk area to almost 100 km East of Zagreb, while new FD grapevine positive samples from Brckovljani close this circle to about 60 km northeast placing Zagreb in its centre.

The sequences of the FD phytoplasmas from 2009 revealed affiliation of Vivodina phytoplasma to 16SrV-C ribosomal subgroup, while the other one from Brezje revealed some variability (Šeruga Musić *et al.*, 2010). The nested PCR experiments with 16Sr758f/M23Sr primers, also suggested the presence of variability since this primer pair could not amplify the DNA from this

sample. Similar difficulties were encountered with samples from the same area collected in 2010. Further analyses are needed to assess the molecular variability and epidemic potential of FD phytoplasmas from Croatian grapevines.

Acknowledgements

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Grapevine yellows in Bosnia and Herzegovina: surveys to identify phytoplasmas in grapevine, weeds and insect vectors

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Abstract

Presence and diffusion of grapevine yellows phytoplasmas was investigated in 2008 and 2010 in twelve vineyards located in two viticultural areas of Srpska region of Bosnia and Herzegovina. From the same vineyards, grapevine samples from different cultivars and weeds were collected for molecular analyses. In addition, some potential insect vectors were collected in the vineyards of these regions. 'Bois noir' phytoplasma presence was confirmed to be associated with grapevine yellows, while in one *Clematis vitalba* sample phytoplasma from 16SrV-C subgroup was identified. RFLP analysis of the *tuf* gene indicated the presence of the *tuf*-type b of stolbur phytoplasma in the 'bois noir'-infected samples. From collected hemipteran insects *Dictyophara europaea* and *Reptalus cuspidatus* were identified. Molecular analyses did not show phytoplasma presence in the tested insect samples of these species.

Key words: nested-PCR, RFLP analyses, grapevine yellows, 'bois noir', elm yellows phytoplasma group.

Introduction

Investigations on grapevine phytoplasmas associated with yellows diseases (GY) in Bosnia and Herzegovina (B&H) were done through several surveys in 2006, 2008 (Delic *et al.*, 2007; Delic and Lolic, 2010), and in 2010. Aim of the present work was to determine the presence of 'flavescence dorée' (FD) and 'bois noir' (BN) phytoplasmas in grapevine and verify the presence of alternative species that could host the phytoplasmas associated with these diseases as well as to collect potential insect vectors. Furthermore, polymorphisms studies BN strains detected in (B&H) were performed.

Materials and methods

Surveys of vineyards for GY disease presence in grapevine and in alternative host species were conducted in Srpska region of Bosnia and Herzegovina in 2008 and 2010. In the last year the survey was extended for collection potential insect vectors of GY. The monitored area was East Herzegovina (Trebinje, Popovo polje, Mokro polje, Dubljani, Dobromani, Arandelovo) the most important viticulture region of the Srpska. Several vineyards of the western part of the region (Bistrica, Doboje, Gradiška) were also subjected to surveys. Symptomatic samples were collected from 34 different grapevine cultivars (table 1). Weed samples such as bindweed (*Convolvulus arvensis*), *Clematis vitalba*, foxtail (*Setaria* spp.), dogwood (*Cornus* spp.) and vitex (*Vitex agnus castus*) were collected in the same vineyards and in the surrounding meadows. Hemipteran species including leafhoppers and planthoppers were collected by sweep net from *Clematis*, bindweed, vitex and grapevine plants and from

grasses in the meadows. Insects were collected from 3 sites in the East Herzegovina region and from 1 site in western part of the region during July and August 2010.

DNAs were extracted from the leaf midribs of plant samples using the DNeasy Plant Mini kit protocol (Qiagen, USA) with slight modifications. Identified insects were subjected to the DNA extraction following a method described by Maixner *et al.* (2006). DNA samples were then first tested with P1/P7 primer in direct PCR followed with R16F2n/R16R2 in nested-PCR: The latter amplicons were restricted with *Mbo*II and *Hpy*188I enzymes. All phytoplasma positive samples from grapevine were tested in nested PCR with Tuf1f/r/TufAYf/r, and TufINT1f/TufINT4r primer pairs (Botti *et al.*, 2005) followed by RFLP analyses with *Hpa*II to determine the *tuf*-type. All samples were checked also with FD specific primer pairs FD9f2/FD9r in direct PCR and FD9f3/FD9r2 in nested PCR.

Table 1. List of tested grapevine cultivars.

Surveys areas	Grapevine variety
East Herzegovina	Vranac, Smederevka, Kardinal, Afus Ali, Merlo, Cabernet
	Chardonnay, Muskat Galia, Nero, Čabski biser, Kraljica Konisen, Trollinger, Muskat Tolinger, Müller Thurgau, Guledei Wie, Merlo, Frankovka, Italian
West Herzegovina	Riesling, Chardonnay, Lasta, Beogradsko rano, Afus Ali, Gročanka, Demi kan, Kardinal, Ribijer, Black Burgundy, Sauvignon, Chardonnay, Petra, Carmen, Muscat Italia, Muscat Hamburg, Rhine
	Riesling, Palatine, Panonija, Trollinger

Table 2. Phytoplasma detection 2008/2010 samples.

Type of samples	Total	BN	FD	year
grapevine	71	35	0	2008
weeds ¹	14	0	0	2008
grapevine	33	5	0	2010
weeds ²	34	0	1	2010

¹*C. vitalba* 7 samples; bindweed 6 samples, foxtail 1 sample; ²*C. vitalba* 9 samples; bindweed 10 samples, vitex 1 sample; dogwood 4 samples.

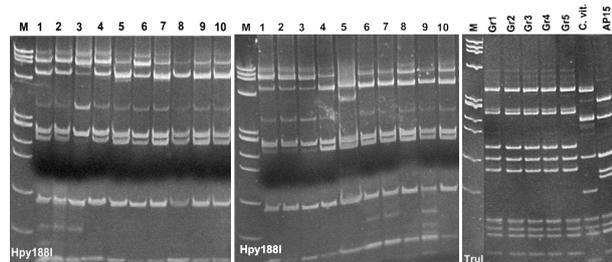


Figure 1. RFLP on R16F2/R2 amplicons. Selected (1 to 10) BN infected grapevine samples. BN (Gr); *C. vitalba* (*C. vit.*), apple proliferation (AP15). M, marker PhiX174 *Hae*III digested. Enzymes: *Hpy188I*, *TruI*.

Results

The presence of BN phytoplasma was detected in 40 out of 57 tested samples from East Herzegovina (Trebinje, Popovo polje, Mokro polje, Dubljani, Dobromani) as well as in two out of 47 tested samples from the western part of the region (Gradiška) (table 2). Restriction profiles obtained on R16F2/R2 amplicons showed some polymorphism that indicates possible presence of different BN strains (figure 1); RFLP analyses of tuf amplicons showed the presence of tuf-type b in all BN positive samples. Molecular analyses of weed samples showed presence of a phytoplasma from the 16SrV-C subgroup in one *C. vitalba* plant collected in August 2010 in Arandelovo (East Herzegovina) (table 2). Among collected hemipteran species in samples from East Herzegovina (Petrovo polje, Dubljani, Arandelovo) *D. europea* was mainly present while, from insects collected in western part of the region (Bistrica) *R. cuspidatus* was identified; nonetheless, phytoplasmas were not detected in the tested specimens of *D. europea* and *R. cuspidatus*.

Discussion

The present work demonstrated that BN phytoplasma is widespread in the main viticulture centres of the inspected regions. Results showed that several cultivars (Vranac, Smederevka, Kardinal, Afus Ali, Merlo, Cabernet, Frankovka) are BN-infected. East Herzegovina is very close with the border of Montenegro where BN infected grapevines were also reported (Radonjić *et al.*, 2009). On the other hand, a phytoplasma belonging to the 16SrV-C subgroup was for the first time detected in the country in a weed sample. FD on grapevine still has not been reported in B&H but it was confirmed to occur

in neighbouring countries such as Croatia (Šeruga Musić *et al.*, 2011), Serbia (Duduk *et al.*, 2004) and Slovenia (Hren *et al.*, 2007) together with its vector *Scaphoideus titanus* Ball. and potential vectors such as *Dictyophara europaea* L. (Filippin *et al.*, 2009). *S. titanus* was only found in East Herzegovina vineyards (Delic *et al.*, 2007).

In order to get clearer view of GY incidence, an extensive epidemiological study should be done monitoring Western Herzegovina vineyards, searching alternative host plants and performing vector transmission trials.

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Alternate hosts of alfalfa witches' broom phytoplasma and winter hosts of its vector *Orosius albicinctus* in Yazd-Iran

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Abstract

Alfalfa witches' broom (AWB) vectored by *Orosius albicinctus* leafhopper is one of the most important alfalfa diseases in Iran especially Yazd province. During 2007-2009, overwintering hosts of *O. albicinctus* and weed hosts of phytoplasmal agent of AWP phytoplasma were evaluated. Based on the results of this study, *O. albicinctus* leafhopper were collected on *Cynodon dactylon*, *ordeum murinum*, *Digitaria sanguinalis*, *Echinochloa crus-galli*, *Lolium temulentum*, *Setaria viridis*, *Tamarix ramosissima* and *Seidlitzia rosmarinus* during winter season and these plants are reported as overwintering hosts of this phytoplasma vector. Direct PCR using P1/P7 and nested PCR using P1/P7 and R16F2n/R16R2 primer pairs showed phytoplasma infection of *Prosopis farcta* and *Cardaria draba*. Restriction fragment length polymorphism analysis of nested PCR products and indirect ELISA test using AWP polyclonal antibody identified peanut witches broom group member phytoplasmas in *Prosopis farcta* and *Cardaria draba* which were identical to each other and to Yazd AWP phytoplasma.

Key words: *Prosopis farcta*, *Cardaria draba*, 16SrII group, Yazd, Iran.

Introduction

Alfalfa witches' broom (AWB), vectored by *Orosius albicinctus* D. leafhopper is one of the most important and destructive diseases of alfalfa in Iran, especially in the Yazd province (Salehi *et al.*, 1995). Knowledge of alternate hosts and vector is important in order to study the epidemiology and to proceed control measures for AWP disease. The aims of this study were to identify herbaceous plant species that in nature harbour the phytoplasma disease agent of AWP or acts as the winter hosts of *O. albicinctus*.

Materials and methods

During surveys in 2007-2009, winter hosts of *O. albicinctus* and weed hosts of phytoplasmas associated with AWP disease were evaluated. Samples were taken from 40 weed species with suspicious symptoms in 10 infected alfalfa fields or adjacent areas in Mehriz, Ardakan and Abarkouh regions in Yazd province. Total DNA was extracted from 1 g of midrib tissue of weed samples following the protocol described by Zhang *et al.*, (1998). Total DNA samples were subjected to direct PCR using P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) and nested PCR using primer pairs P1/P7 (first step) and R16F2n/R16R2 (Gundersen and Lee, 1996) (second step). Primer pair P1/P7 amplifies a 1800-bp fragment of the ribosomal operon which includes the 16S rRNA gene, the 16S-23S intergenic spacer region (SR), and a portion of the 5' region of the 23S rRNA gene whereas primer pair R16F2n/R16R2 amplifies 1200 bp of the 16S rRNA gene. Each 25 µl PCR reaction mix contained 100 ng of total DNA, 2.5 µl 10X PCR buffer, 0.8 U Taq polymerase, 0.2 mM

dNTPs, 1.5 mM MgCl₂ and 0.4 µM of each primer. PCR was performed for 35 cycles using the following conditions: 1 minute (2 minutes for the first cycle) denaturation step at 94 °C, 2 minutes for annealing at 55°C and 3 minutes (10 minutes for the last cycle) at 72°C for primer extension. For identification of associated phytoplasmas, nested PCR products (1,200 bp) were digested with *AluI*, *HinfI*, *MseI*, and *RsaI* restriction enzymes and digestion profiles were compared with those of known phytoplasmas. Furthermore, ELISA test using polyclonal antibody prepared against alfalfa witches' broom from Yazd (Esmailzadeh-Hosseini *et al.*, 2003) was used for serological detection of phytoplasmas in suspected weeds. In order to identify winter hosts of *O. albicinctus*, specimens were collected by D-Vac aspiration from plant species and weeds in and around the 15 AWP affected alfalfa fields and sorted by their gross morphology.

Results and discussion

From different species of herbaceous plants, *Prosopis farcta* (Banks & Soland.) Macbr and *Cardaria draba* (L.) Desv. showed phytoplasma-type symptoms (figures 1 and 2). *Prosopis farcta* and *Cardaria draba* proved to be positive in direct and nested PCR and PCR products of expected size (1,800 and 1,200 bp, respectively) were amplified. Five of 13 samples of *Prosopis farcta* and 3 of 10 samples of *Cardaria draba* tested were positive. Restriction fragments length polymorphism (RFLP) analysis of nested PCR products (1,200 bp of 16SrRNA) indicated that phytoplasmas associated with *Prosopis farcta* and *Cardaria draba* are similar to each other and with Yazd AWP agent, a peanut witches' broom group related phytoplasma (Lee *et al.*, 1998). Elisa test showed serological relationship of *Prosopis farcta* and



Figure 1. Small laves, shortened internodes, proliferation of axillary buds and bushy growing habit in *Prosopis farcta* in Esfandabad (Abarkouh, Yazd province). (In colour at www.bulletinofinsectology.org)



Figure 2. Dwarfing, virescence, phyllody and infertile flowers in *Cardaria draba* in Banadak Sadat (Mehriz, Yazd province). (In colour at www.bulletinofinsectology.org)

Cardaria draba phytoplasmas with Yazd AWB phytoplasma (YAWBP). RFLP analysis and serological relationship indicated that *Prosopis farcta* and *Cardaria draba* are hosts of YAWBP. *O. albicinctus* leafhoppers were collected on *Cynodon dactylon* L. (Pers.), *Hordeum murinum* L., *Digitaria sanguinalis* (L.) Scop., *Echinochloa crus-galli* (L.) P. Beauv., *Lolium temulentum* (L.), *Setaria viridis* (L.) P. Beauv., *Tamarix ramosissima* Ledeb and *Seidlitzia rosmarinus* (Ehrh.) Bge. during winter season and these plants are reported as overwintering hosts of the phytoplasma vector. In consequence, on the basis of this study eradication of weed species growing next to alfalfa farms is recommended.

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Hibiscus witches' broom disease associated with different phytoplasma taxa in Brazil

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Abstract

Stolbur group phytoplasmas were detected in symptomatic hibiscus plants from Brazil showing witches' broom symptoms while in other symptomatic samples of the same locations '*Candidatus Phytoplasma brasiliense*' was identified. This finding confirms that diverse phytoplasmas can be associated with this disease in hibiscus. This is the first report of identification of a 16SrXII phytoplasma in this genus worldwide.

Key words: Brazil, '*Candidatus Phytoplasma brasiliense*', China rose, stolbur phytoplasma.

Introduction

Hibiscus rosa-sinensis (Malvaceae) make an excellent foundation planting, being widely used in landscaping (Lorenzi, 2008). In Brazil, due to hibiscus witches broom disease, the adoption of hibiscus plants is under restriction, mainly in the state of Rio de Janeiro. Symptoms of the disease are characteristic of witches' broom syndrome, such as leaf yellowing, short internodes, proliferation of shoots, and in some cases, premature flower dropping. Diseased plants may die in case of severe infection (Vicente *et al.*, 1974, Kitajima, 1994). The identity of the phytoplasma associated with hibiscus witches' broom disease in Brazil was demonstrated and the phytoplasma, designated '*Candidatus Phytoplasma brasiliense*', was affiliated to the group 16SrXV, subgroup A (Montano *et al.*, 2001).

To evaluate epidemiological spreading of this disease in hibiscus further research to verify the phytoplasma presence in symptomatic plants, from different areas in Brazil were carried out.

Materials and methods

Eight hibiscus samples exhibiting shoot proliferation, short internodes and reduced leaf size (figure 1) were collected in two locations, in the quarter of Barra da Tijuca, in the city of Rio de Janeiro, Brazil. Six of the samples (1 to 6) were collected in the location known as Peninsula, and two of them (7 and 8) in Barra Shopping Mall. One gram of phloem tissue scraped from twigs of symptomatic plant was submitted to nucleic acid extraction with a chloroform/phenol protocol (Prince *et al.*, 1993). Nucleic acid was employed at the concentration of 20 ng in direct PCR with universal primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995), followed by nested PCR with primers F1/B6 (Davis and Lee, 1993; Padovan *et al.*, 1995), and by a second nested PCR with R16F2/R2 or R16(I)F1/R1 (Lee *et al.*, 1995).



Figure 1. *Hibiscus rosa-sinensis* with proliferation, short internodes and reduced leaf size.

RFLP analyses were carried out and with *TruI*, *TaqI* and *AluI* (Fermentas, Vilnius, Lithuania) restriction enzymes and patterns were compared with phytoplasma reference strains (Bertaccini *et al.*, 2000). Amplicons obtained after R16(I)F1/R1 were further selected and purified with "PCR Clean-Up" (Macherey–Nagel, Germany) for sequencing. The obtained sequences were aligned by using Clustal W and BioEdit (Hall, 1999) softwares, and compared with those of phytoplasma sequences deposited in GenBank.

Results

On the basis of phytoplasma-specific DNA amplification in PCR phytoplasmas were detected in all symptomatic hibiscus plants. In particular RFLP analyses with *AluI*, *TaqI* and *TruI* restriction enzymes on R16F2/R2 fragments allowed identification of phytoplasmas belonging to ribosomal group 16SrXV only in the Peninsula 4 sample (figure 2). Further nested PCR with R16(I)F1/R1 allowed detection of phytoplasma presence in all symptomatic samples, and RFLP analyses with *TruI* indicated a 16SrXII phytoplasma in the majority of samples. However a profile different from all those already reported, was detected in samples Peninsula 4 and 5 (figure 2).

Sequencing of selected amplicons from each profiles confirmed the presence of 'Ca. P. brasiliense' in the samples with different RFLP profiles, while the sequencing of samples having the 16SrXII profile was not readable with several double peaks indicating possible presence of mixed phytoplasma infection.

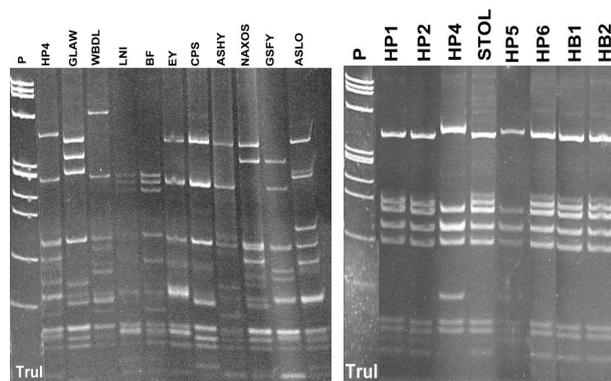


Figure 2. RFLP profiles of R16F2/R2 (left) and R16(I)F1/R1 (right) amplicons after *TruI* digest. Acronyms: HP, Hibiscus Peninsula; HB, Hibiscus Barra; GLAW, gladiolus witches' broom (16SrI-B); WBDL, witches' broom disease of lime (16SrII-B); LNI, plum leptonecrosis (16SrII-B); BF, peach X disease (16SrIII-A); EY, elm yellows (16SrV-A); CPS, crotalaria phyllody (16SrVI-C); ASHY, ash yellows (16SrVII-A); Naxos, periwinkle virescence from Sicily (16SrIX-C); GSFY, German stone fruit yellows (16SrX-B); ASLO, aster yellows from Slovenia (16SrXII-A); STOL, stolbur from pepper from Serbia (16SrXII-A); P, marker Φ X174 *Hae*III digested.

Discussion

Detection of stolbur group phytoplasmas in symptomatic hibiscus plants from Brazil indicates that witches' broom symptoms can be associated with the presence of a phytoplasma other than 'Ca. P. brasiliense'. The work carried out also indicated that the primers 16Sr(I)F1/R1 are more general than reported amplifying also the group 16SrXV, besides groups 16SrI, -II, and -XII. The finding of phytoplasmas distinct from 16SrXV-A, as previously reported to be associated with hibiscus witches' broom (Montano *et al.*, 2001), confirms that diverse phytoplasmas can be associated with this disease in hibiscus, and this is a common feature for phytoplasma-associated diseases worldwide. Moreover, this is the first report of identification of a 16SrXII phytoplasma in this genus worldwide; there is only a report of an unidentified phytoplasma in Australia associated with a witches broom disease of *Hibiscus heterophyllus* (Hiruki, 1987).

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Phytoplasmas in Brazil: an update

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Abstract

In Brazil a number of plant species are affected by phytoplasma diseases, comprising wild and cultivated crops. Amongst the recently botanical species reported as natural phytoplasma host is sweet orange (*Citrus sinensis*) with huanglongbing symptoms. The majority of phytoplasmas identified in Brazil belongs to groups 16SrI and 16SrIII.

Key words: yellows diseases; witches' broom; classification; phytoplasmas; phytoplasmas.

Introduction

Phytoplasma diseases have been described in association with wild and economically important plant species in Brazil. Classification of associated phytoplasmas is of utmost importance to identify insect vectors and alternate plant hosts, enabling the establishment of disease control. In recent years new taxa have been identified associated with different plant hosts (table 1). Remarkable is the identification of a phytoplasma related to the group 16SrIX in citrus plants

with huanglongbing disease symptoms (Teixeira *et al.*, 2008); the leafhopper *Scaphytopius marginelineatus* was found the natural vector of the phytoplasma (Marques *et al.*, 2010). The finding of a phytoplasma in citrus, an important income crop to Brazil, may represent a push to general phytoplasma research in the country.

The majority of phytoplasmas reported in Brazil belongs to groups 16SrI and 16SrIII, following the worldwide distribution of phytoplasmas representative to these groups.

Table 1. Phytoplasma classification on the basis of RFLP analyses of 16S rRNA gene.

16S rRNA group	Host plants	Reference
16SrI	<i>Bougainvillea spectabilis</i> , <i>Erigeron</i> (<i>Conyza</i>) <i>bonariensis</i> , <i>Elaeis guineensis</i> , <i>Passiflora edulis</i> f. <i>flavicarpa</i> , <i>Saccharum</i> sp. (I-B), <i>Vitis vinifera</i> (I-B), <i>Zea mays</i> (I-B)	Davis <i>et al.</i> , 1994, Bedendo <i>et al.</i> , 1998; Bianchini and Bedendo, 2000; Neroni <i>et al.</i> , 2006, Silva <i>et al.</i> , 2006, Silva <i>et al.</i> , 2009, Montano <i>et al.</i> , 2007
16SrII	<i>Catharanthus roseus</i>	Barros <i>et al.</i> , 1998
16SrIII	<i>Begonia</i> sp., <i>Brassica oleraceae</i> var. <i>botrytis</i> , <i>Brassica oleraceae</i> var. <i>capitata</i> , <i>Brassica oleraceae</i> var. <i>italica</i> , <i>Catharanthus roseus</i> , <i>Crotalaria juncea</i> (III-B), <i>Cucurbita moschata</i> (III-J), <i>Cucurbita pepo</i> , <i>Euphorbia pulcherrima</i> , <i>Helychrisum bracteatum</i> , <i>Leonurus sibiricus</i> , <i>Luffa cylindrica</i> , <i>Lycopersicon esculentum</i> , <i>Malus domestica</i> , <i>Manihot esculenta</i> (III-B), <i>Momordica charantia</i> (III-J), <i>Sechium edule</i> (III-J), <i>Sicana odorifera</i> , <i>Solanum melongena</i> (III-B), <i>Melia azedarach</i> (III-B), <i>Solidago microglossa</i> , <i>Vitis vinifera</i>	Barros <i>et al.</i> , 1998, Amaral Mello <i>et al.</i> , 2006, Neroni <i>et al.</i> , 2006, Ribeiro and Bedendo, 2006, Montano <i>et al.</i> , 2000, Montano <i>et al.</i> , 2006, Amaral Mello <i>et al.</i> , 2007, Montano <i>et al.</i> , 2007, Ribeiro <i>et al.</i> , 2007a, Ribeiro <i>et al.</i> , 2007b, Rapussi and Bedendo, 2008, Melo <i>et al.</i> , 2009, Duarte <i>et al.</i> , 2009, Ekstein <i>et al.</i> , 2010, Flôres <i>et al.</i> , 2011a; 2011b
16SrV	<i>Crotalaria juncea</i>	Amaral Mello <i>et al.</i> , 2004
16SrVII	<i>Erigeron</i> (<i>Conyza</i>) <i>bonariensis</i> (VII-B)	Barros <i>et al.</i> , 2002
16SrIX	<i>Catharanthus roseus</i> , <i>Citrus sinensis</i> , <i>Crotalaria juncea</i>	Barros <i>et al.</i> , 1998, Teixeira <i>et al.</i> 2008; Wulff <i>et al.</i> , 2009
16SrXII	<i>Hibiscus rosa-sinensis</i>	Montano <i>et al.</i> , 2011a
16SrXIII	<i>Turnera ulmifolia</i>	Montano <i>et al.</i> , 2011b
16SrXV	<i>Catharanthus roseus</i> (XV-A), <i>Hibiscus rosa-sinensis</i> (XV-A), <i>Sida</i> sp.	Montano <i>et al.</i> , 2001a, 2001b, Eckstein <i>et al.</i> , 2011

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The importance of psyllids (Hemiptera Psyllidae) as vectors of phytoplasmas in pome and stone fruit trees in Austria

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Abstract

To study the occurrence and distribution of psyllids as potential vectors of European stone fruit yellows (ESFY), pear decline (PD) and apple proliferation (AP) a survey was conducted in Austrian orchards during the last years. Specimens were collected by using the beating tray method in apricot, pear and apple tree orchards. The obtained psyllids were analysed by PCR and RFLP assays for the presence of phytoplasmas. Molecular analyses showed few infections of *Cacopsylla pruni* with 'Candidatus Phytoplasma prunorum' and few individuals of *Cacopsylla pyricola*, *C. pyri* and *C. pyrisuga* were carrier of 'Candidatus Phytoplasma pyri'. The data presented in this study are a preliminary report because analyses of apple psyllids are still in progress.

Key words: *Cacopsylla* spp., European stone fruit yellows, pear decline, apple proliferation.

Introduction

European stone fruit yellows (ESFY), pear decline (PD) and apple proliferation (AP) are quarantine diseases associated with phytoplasmas ('Candidatus Phytoplasma prunorum', 'Ca. P. pyri' and 'Ca. P. mali'), which are responsible for great economic losses in fruit production (Seemüller and Schneider, 2004). The spread of these phytoplasmas is due to infected planting material or insect vectors, especially psyllids (Hemiptera Psyllidae). The occurrence of these diseases has been described in Austria by Richter (1999), Spornberger *et al.* (2006), Steffek and Altenburger (2008). A literature review revealed a lack of data on potential vectors in Austria. The aim of this study was to gain more information on the occurrence of these potential vectors and the phytoplasma infection status of psyllids from pome and stone fruit trees in Austria.

Materials and methods

Investigation sites were located in the Eastern part of Austria - 10 apricot orchards in Lower Austria, Burgenland and Vienna, 5 pear orchards and 3 apple orchards in Lower Austria. Samples were collected by using the beating tray method with 100 beats respectively 100 branches (trees) per sampling date and orchard. Psyllid captures were done in the period from March to July, on apricot trees in 2005 and 2006, on pear and apple trees in 2009 and 2010. Collected psyllids were identified according to Ossiannilsson (1992) and Burckhardt and Jarausch (2007).

Molecular analyses for phytoplasma infection of psyllids were carried out with 1 to 8 individuals per sample taken for testing: CTAB-method for the DNA-extraction of psyllids (Maixner *et al.*, 1995), qualitative PCR for the detection of phytoplasmas in the psyllid samples using universal primers fU5/rU3 (Lorenz *et al.*, 1995) and then for nested PCR using phytoplasma specific primer pairs P1/P7 primer (Deng and Hiruki, 1991; Schneider

et al., 1995) and f01/r01 primer (Lorenz *et al.*, 1995) respectively. RFLP assays using restriction enzymes *SspI* and *RsaI* were applied to discriminate among the three fruit tree phytoplasmas AP, PD and ESFY (Tedeschi *et al.*, 2009).

Results

The plum psyllid *Cacopsylla pruni* was found in all investigated apricot orchards. The first report of *C. pruni* on apricot trees in Austria was mentioned in Lethmayer and Hausdorf (2005). Interesting was the high number of *Cacopsylla melanoneura* on apricot trees at some investigation sites which was due to hawthorn hedges near the apricot orchards. The three pear psyllid species *Cacopsylla pyricola*, *Cacopsylla pyri*, *Cacopsylla pyrisuga* and the hawthorn psyllid *C. melanoneura* were the main species on pear trees. *C. pyricola* was the most abundant species. On apple trees the psyllids *C. melanoneura* and *Cacopsylla picta* were mainly captured.

Molecular analyses of *C. pruni* showed five positive samples (with 30 individuals in total) out of 37 tested samples (with 142 individuals in total) with 'Ca. P. prunorum'. These samples originated from four sites in Lower Austria. All individual of *C. melanoneura* caught on the apricot and pear trees tested negative for phytoplasmas. All three pear psyllid species, *C. pyricola*, *C. pyri* and *C. pyrisuga*, were found infected with 'Ca. P. pyri' comprising 16 positive samples (with 48 individuals in total) out of 33 tested samples (with 118 individuals in total). Positive samples were obtained at three pear sites in Lower Austria. First analyses showed that the all psyllids collected on apple were infected with 'Ca. P. mali'. An overview of the molecular analyses is given in table 1.

Other studies have already confirmed that the psyllid species which were tested positive in our study are vectors of the respective phytoplasmas (reviewed by Jarausch and Jarausch, 2010).

Table 1. Results of the RFLP analyses of the *Cacopsylla* samples taken in fruit tree orchards in Austria.

date	orchard/ fruit tree	psyllid species	positive (+)/negative (-) tested for		
			ESFY	PD	AP
2005	apricot	<i>C. pruni</i>	+	-	-
2006	apricot	<i>C. pruni</i>	-	-	-
2006	apricot	<i>C. melanoneura</i>	-	-	-
2009	pear	<i>C. pyricola</i>	-	+	-
2009	pear	<i>C. pyri</i>	-	+	-
2009	pear	<i>C. pyrisuga</i>	-	+	-
2009	pear	<i>C. melanoneura</i>	-	-	-
2009	apple	<i>C. melanoneura</i>	-	-	-
2010	apple	<i>C. melanoneura</i>	-	-	-
2010	apple	<i>C. picta</i>	-	-	-

C. pyrisuga has been found infected with 'Ca. P. pyri' (Kucerova *et al.*, 2007) but its ability of transmission is still not verified (Jarausch and Jarausch, 2010). Due to the geographical position of Austria it is interesting which of the two psyllid species found on apple can be identified as main vector for transmission of AP in Austria. First investigations did not indicate a particular vector capacity for 'Ca. P. mali' by *C. melanoneura* or *C. picta*. However, due to the low number of investigated apple psyllids further analyses are necessary to clarify the vector role and therefore this issue is still in progress.

Discussion

One of the main phytosanitary measures for preventing phytoplasma diseases spread is the control of their vectors. The use of insecticides in sustainable production methods is restricted. Therefore, knowledge on the vectors (mainly psyllids), their distribution and biology is strongly needed for control strategies, especially for new approaches on integrated control strategies.

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'Candidatus Phytoplasma mali' infected *Cacopsylla picta* found in apple orchards in South-Western Finland

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Abstract

Apple orchards in four localities in southwestern Finland were surveyed in May 2009 and 2010 to monitor the occurrence of *Cacopsylla picta* (Förster) and *C. melanoneura* (Förster), vectors of apple proliferation phytoplasma. Both psyllid species were present in apple orchards, but *C. picta* prevailed. The number of individuals varied considerably in 2009-2010. The total number of captured overwintering *Cacopsylla* species from the same orchards was 395 in 2009 and 45 in 2010. Individuals of *C. picta* infected with 'Candidatus Phytoplasma mali' were found from single orchards in Lohja and Parainen. In 2010 the incidence of 'Ca. P. mali' in *C. picta* individuals collected from apples (*Malus domestica*) was 11.4%.

Key words: apple proliferation, *Malus*, psyllids, PCR, RFLP.

Introduction

Apple proliferation (AP) phytoplasma, classified as 'Candidatus Phytoplasma mali' (Seemüller and Schneider, 2004) was first described in Italy in the 1950s (Rui *et al.*, 1950) and currently causes severe epidemics and economic losses in many pome fruit growing areas in Europe, including Italy and Germany (Carraro *et al.*, 2008; Jarausch *et al.*, 2004).

The pathogen is transmitted through infected grafting material, via natural root grafts (Ciccotti *et al.*, 2007) and sap-sucking insects. *Cacopsylla picta* (Förster) has proved to be the most important vector of 'Ca. P. mali' in Germany and neighbouring regions (Jarausch *et al.*, 2007), whereas *C. melanoneura* (Förster) was reported as the main vector of 'Ca. P. mali' in Northwestern Italy (Tedeschi *et al.*, 2002).

C. picta has been known to occur on apple in Southern Finland (Ossiannilsson, 1992) and *C. melanoneura* was found in Finland in the 1990s (Albrecht *et al.*, 2003). In the 2000s *C. melanoneura* spread northwards to a greater extent than *C. picta* (Mattila and Söderman, 2011). *C. melanoneura* occurs mainly on hawthorn, but has been found also on apple and pear (Ossiannilsson, 1992). Both psyllid species are presumed to overwinter on conifers in Fennoscandia (Ossiannilsson, 1992).

Apple cultivation has been predicted to expand in Finland in the future as a result of global warming (Kaukoranta *et al.*, 2010). The main area for commercial apple cultivation in Finland is in the south and southwestern parts of Finland, and in the Åland Islands. Apple proliferation phytoplasma has not been surveyed or reported either from apple trees or insects in Finland. The aim of the present study was to confirm the occurrence of *C. picta* and *C. melanoneura* in apple orchards in southern Finland and to determine the incidence of 'Ca. P. mali' in *C. picta*.

Materials and methods

In May 2009, 18 psyllid samples were collected in Southwestern Finland (Parainen, Piikkiö, Lohja, Jokioinen) from apple (*Malus domestica*) in commercial orchards, research stations and in a few home gardens.

In May 2010, 19 samples were collected in the same orchards. Overwintering adult psyllids were collected using a beating net with an opening of 0.25 m². In commercial orchards, one branch from each of 33 randomly selected apple trees was beaten for each sample. Insects were separated, identified and counted by examining male and female terminalia (Ossiannilsson, 1992).

Specimens were stored in 95% ethanol until DNA extraction. In some of the later samples, 1st-3rd nymph stages of *Cacopsylla mali* (Schmidb.) were also present. *C. mali* overwinters as eggs and is the most common psyllid on apple in Finland (Mattila and Söderman, 2011).

Total DNA from adult *Cacopsylla* species was extracted from all individual psyllids from single specimens in 2010, whereas DNA from a third of the psyllids collected in 2009 was extracted from 26 batches of five individuals.

DNA extraction was done using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Apple proliferation phytoplasma was detected in the DNA extracts through the use of PCR amplification with AP-specific primers AP5/AP4 (Jarausch *et al.*, 1994).

DNA from AP phytoplasma positive samples was analysed further for subtypes by PCR/RFLP using primers AP13/AP10 (Jarausch *et al.*, 2000) and amplicons were digested using enzymes *HincI* and *RcaI* (New England BioLabs) following the instructions of the manufacturer.

Results

In 2009 a total of 395 *Cacopsylla* adults were collected in the four localities: 294 were identified as *C. picta* and 101 as *C. melanoneura*. In 2010 a total of 45 *Cacopsylla* spp. were collected from the same localities and orchards. Of the 45 identified individuals 44 were *C. picta* and one was *C. melanoneura*.

In 2009, *C. picta* was present in all collected samples, the numbers varying from 1 to 41 per sample, 56% of the 294 were females. *C. melanoneura* was found in half of the samples, 1 to 46 per sample, 65% of the 101 were females. In 2010, *C. picta* was found in 12 samples, 1 to 16 per sample, and 82% were females. *C. melanoneura* was present in only one sample as a single female.

'*Ca. P. mali*' was detected in four groups of the selected 26 groups of *C. picta* samples (4/26) collected in 2009. The characteristic amplicon (483 bp) was amplified from insects using specific primers AP5/AP4. All the positive samples were from one orchard in Parainen.

'*Ca. P. mali*' was detected in five of 45 individuals collected in 2010, all from the same orchard (Lohja). The occurrence of '*Ca. P. mali*' in the specimens of *C. picta* in that orchard was 20.8% (5/24), whereas the incidence in all *C. picta* specimens collected in 2010 was 11.4% (5/44).

AP13/AP10 amplicons and RFLP analyses confirmed that the '*Ca. P. mali*' subtypes present in Finnish orchards were AP and AT-2.

Discussion

Results of this preliminary survey confirmed the incidence of both psyllid vector species of '*Ca. P. mali*', *C. picta* and *C. melanoneura*, in Finnish apple orchards. The number of individuals of both species varied in 2009-2010, but *C. picta* prevailed in both years. It is interesting that the reduction in numbers of *C. melanoneura* after the hard winter in 2010 was relatively much higher compared with that for *C. picta*. As *C. melanoneura* is considered to be a newcomer in Finland (Vänninen *et al.*, 2011), it may be present here at its northernmost border.

Occurrence of '*Ca. P. mali*' in *C. picta* was determined in samples collected in two localities. One of these orchards (Parainen) represented organic farming, where numerous imported foreign apple varieties were planted. The other orchard (Lohja) represented an old conventional orchard. Observation of abnormal symptoms in apple orchards have not been reported by growers or advisors. '*Ca. P. mali*' infected apple trees have yet to be found and therefore any conclusions based on the present distribution of '*Ca. P. mali*' in Finnish apple orchards are premature.

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Investigation on the apple proliferation epidemics in the orchards of the Pelion Mountain and preliminary observations on possible phytoplasma vectors

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Abstract

The apple producing region in the Pelion Mountain (Magnesia prefecture, Thessaly, Greece) is suffering for more than 10 years from the apple proliferation disease. The orchards are basically planted with cv 'Starking Delicious', are grafted on seedling rootstocks and are older than 40 years. The presence of the '*Candidatus Phytoplasma mali*' was proven by PCR/RFLP analyses as well as by sequencing. '*Ca. P. pyri*' was also detected in at least three different orchards. First attempts to identify the vectors were carried out by monitoring the insect populations in 8 different orchards. One species of psyllids morphologically identified as *Cacopsylla pulchella* (Low) was the most present insect in the orchards all along the two-month monitoring (May/June). *Cacopsylla mali* (Schmidberger) was also present in the orchards but in lower numbers. The monitoring is continued and still in progress.

Key words: apple proliferation, psyllid vector, molecular identification, phytoplasmas.

Introduction

The apple producing region in the Pelion Mountain (Magnesia prefecture, Thessaly, Greece) is suffering for more than 10 years from the apple proliferation disease. The disease was firstly described in Greece in late eighties (Rumbos, 1986) and the '*Candidatus Phytoplasma mali*' was recently detected and identified by molecular methods in infected material (Rumbou *et al.*, 2007).

The infected orchards are basically planted with cv Starking Delicious, are grafted on seedling rootstocks and are older than 40 years. Approximately 90% of the apple trees are infected and the yield losses range from 20-40% in the lower elevations to 70-100% in the higher elevations, where apple orchards neighbour beech forest. The last five years and because of the extended epidemics, infected trees are massively removed and replaced with new trees grafted on dwarf rootstocks and planted in palmettos. However, the pathogen has been detected also to those newly planted trees (Rumbou *et al.*, 2010a), therefore survey was undertaken to verify the disease presence as well as to identify the insect vector of the disease.

Materials and methods

Samples from apple trees grown in different orchards in the Pelion Mountain and exhibiting apple proliferation symptoms were collected from September to November 2009 and were preserved at -80°C or -20°C. Screening of symptomatic apple material was carried out with total DNA extraction from 24 leaf and root samples from diseased apple trees. DNA extraction was performed in Julius Kühn Institute (Institute for Plant Protection in

Fruit Crops and Viticulture, Dossenheim, Germany) according to a modified protocol following Doyle and Doyle (1990). PCR amplification with P1/P7 was followed by nested PCR with the universal phytoplasma primers fU5/rU3 or with the fruit tree-specific primers fO1/rO1 (Lorenz *et al.*, 1995). The PCR products were cloned and sequenced. A second round of PCR amplifications was performed in Max-Planck Institute (Institute for Molecular Genetics, Berlin) with universal primers fU5/rU3. Sequencing was carried out using ABI3730XL capillary systems (ABI 3730xl) and resulted in 20-fold coverage. Sequence quality assessment and assembly were performed using MIRA assembly program and GAP4.

A first attempt to identify the vectors of the pathogen was done in 2006 by monitoring the insect populations in eight different orchards for the flying season.

Results

Positive results were obtained mainly from root samples, in particular nine samples originating from eight different orchards were identified as '*Ca. P. mali*', while three samples originating from three different orchards were identified as '*Ca. P. pyri*'. Two samples from the same orchard were found to be infected by '*Ca. P. pyri*' and '*Ca. P. mali*', respectively (Rumbou *et al.*, 2010b).

From the insects trapped, two species of psyllids and one of Jassidae, possible phytoplasma vectors, were identified. From these one species of psyllids, morphologically identified as *Cacopsylla pulchella* (Low), was the most frequently trapped insect in the orchards with 50-1,660 adults/8 traps and flying mainly from 17/5-1/6, with the exception of an orchard in high altitude where

they were present until the 21/6. A non-identified species of Jassidae was the second insect for number of individuals (18-278 adults/8 traps) and present during the two-month monitoring (26/4-21/6). *Cacopsylla mali* (Schmidberger) was also present in the orchards but in lower numbers (1-59 adults/total traps).

Discussion

The presence of the '*Candidatus Phytoplasma mali*' was firstly proven by molecular means in 2005. Lately, the first sequences from local strains were obtained with the use of phytoplasma universal and 16SrX-group specific primer pairs revealing that, apart from '*Ca. P. mali*' also '*Ca. P. pyri*', the agent associated with pear decline, is present in at least three different orchards in the surveyed region.

This year the insect monitoring is repeated and the identification will be done with more accurate tools. The detection of phytoplasma in the insects' body remains to be shown before the transmission trials will be initiated.

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Survey of psyllid vectors of fruit tree phytoplasmas in Bulgaria: a preliminary report

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Abstract

The spread and the frequency of individuals from the psyllid genus *Cacopsylla* has been investigated in spring 2011 in four fruit tree orchards in three different regions of Bulgaria. Insects were captured and identified morphologically for species determination. Phytoplasma infection in psyllid samples was analysed by universal and specific PCR. All psyllid species described as vectors of fruit tree phytoplasmas were present in the investigated areas. Four individuals of *Cacopsylla pruni* from two different regions were carrying 'Candidatus Phytoplasma prunorum', the agent of European stone fruit yellows (ESFY). This is the first ESFY detection in these regions in Bulgaria.

Key words: 'Candidatus Phytoplasma prunorum', European stone fruit yellows, psyllid vectors, PCR detection.

Introduction

A few species of the psyllid genus *Cacopsylla* (Hemiptera Psyllidae) have been demonstrated to be vectors of European fruit tree phytoplasmas: 'Candidatus Phytoplasma mali' associated with apple proliferation (AP), 'Candidatus Phytoplasma prunorum', the agent of European stone fruit yellows (ESFY) and 'Candidatus Phytoplasma pyri', the agent of pear decline (PD) (Seemüller and Schneider, 2004). Two psyllids, *Cacopsylla picta* (Foerster) and *Cacopsylla melanoneura* (Foerster) are recognised vectors of 'Ca. P. mali'. The psyllid *Cacopsylla pruni* Scopoli was described as vector of 'Ca. P. prunorum' whereas three psyllid species are recognised or presumed vectors of 'Ca. P. pyri': *Cacopsylla pyri* (L.), *Cacopsylla pyricola* (Foerster) and *Cacopsylla pyrisuga* (Foerster) (reviewed by Jarausch and Jarausch, 2010).

ESFY and PD have been first detected in Bulgaria near Plovdiv (Topchiiska *et al.*, 2000). But so far no report exists concerning cases of infected psyllid species in Bulgaria. AP has been reported to occur in Bulgaria since long time (www.eppo.org); however the *Cacopsylla* species vectoring fruit tree phytoplasmas were described in Bulgaria before they have been identified as phytoplasma vectors (Harizanov, 1966a; 1966b; 1982). However almost nothing is known about the incidence and the spread of these quarantine diseases in Bulgaria. Therefore, the aim of the present work was to gain first information on the spread and frequency of psyllid vector species and to determine their natural infection status in selected fruit tree orchards in Bulgaria.

Materials and methods

A survey was conducted in spring 2011 in four fruit tree orchards in three different regions in Bulgaria. Insects were caught using sweep-netting. Captured psyllids were frozen at -20°C and psyllid species identification was

done using different determination keys (Hodkinson and White, 1979; Ossiannilsson, 1992; Burckhardt and Jarausch, 2007). DNA was extracted from single psyllid individuals with a CTAB-based protocol as described by Maixner *et al.* (1995).

PCR amplification of phytoplasma DNA was achieved with universal ribosomal primers fU5/P7 (Lorenz *et al.*, 1995; Schneider *et al.*, 1995). For specific PCR of positive *C. pruni*, ESFY-specific non-ribosomal primers ECA1/ECA2 were applied (Jarausch *et al.*, 1998).

Results and discussion

In the surveyed fruit tree orchards all known and putative psyllid vectors of fruit tree phytoplasmas were identified: *C. pruni*, *C. picta*, *C. melanoneura*, *C. pyri*, *C. pyrisuga* and *C. pyricola*. They were captured in three different region: Dupnica, Sofia (Gorni Lozen, Vrajdebna), and Plovdiv. (figure 1, table 1).

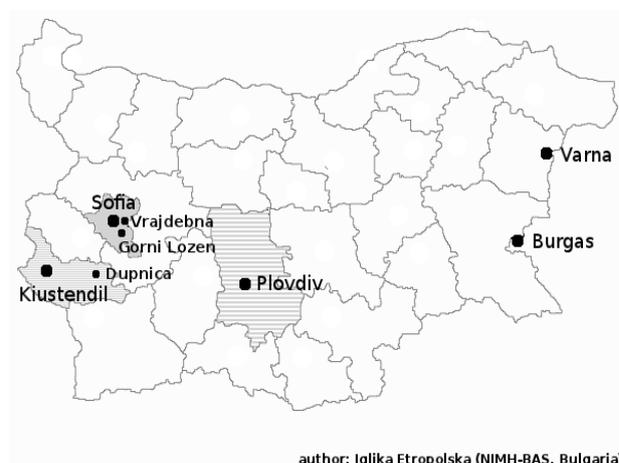


Figure 1. Location of fruit tree orchards surveyed in spring 2011 in Bulgaria.

Table 1. Number of individuals of *Cacopsylla* species captured in fruit tree orchards and results of phytoplasma detection by PCR in individual insects.

Psyllid species	Phytoplasma	Region			
		Dupnica	Gorni Lozen	Vrajdebna	Plovdiv
<i>Cacopsylla pruni</i>	'Ca. P. prunorum'	3 / 94 (3.2%)*	1 / 16 (6.3%)	nt	nt
<i>Cacopsylla picta</i>	'Ca. P. mali'	0 / 3	0 / 0	nt	0 / 1
<i>Cacopsylla melanoneura</i>	'Ca. P. mali'	0 / 23	0 / 1	nt	0 / 11
<i>Cacopsylla pyri</i>	'Ca. P. pyri'	0 / 0	0 / 0	0 / 181	nt
<i>Cacopsylla pyricola</i>	'Ca. P. pyri'	0 / 2	0 / 0	0 / 1	nt
<i>Cacopsylla pyrisuga</i>	'Ca. P. pyri'	0 / 84	0 / 6	0 / 1	nt

* PCR positive versus total number of individuals tested; nt = not tested.

In total, 440 individuals of the six *Cacopsylla* species were collected in the different fruit tree orchards and individually analysed for phytoplasma presence. Among the different known and putative vector species, only 4 individuals of *C. pruni* were found to be infected by phytoplasmas with universal ribosomal primers. The specific PCR revealed the presence of 'Ca. P. prunorum' in all four phytoplasma-infected *C. pruni* insects. Interestingly, the infected specimens originated from two different collection sites. This is the first report of ESFY detection in these regions.

Discussion

Psyllid species described in Bulgaria by Harizanov (1966a, 1966b, 1982) were from different regions from those investigated in the present study. In all surveyed orchards an important number of psyllid vector species was found; the collected specimens showed variations in presence and abundance at the different sites. In two of the investigated pear orchards (Dupnica and Gorni Lozen, table 1) only *C. pyrisuga* and *C. pyricola* were present while at the location of Vrajdebna *C. pyri* was the only pear psyllid species found. Despite abundances of *C. pyri* and *C. pyrisuga*, none of the individuals captured during this preliminary survey was phytoplasma infected. The detection of 'Ca. P. prunorum' in its vector species in two different regions indicates a broader distribution of ESFY in Bulgaria as known so far. The future investigations will aim to monitor regularly insects and plants in these regions and other fruit tree growing areas in Bulgaria for possible phytoplasma infection.

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Effects of possible repellents on feeding and survival of *Cacopsylla pruni* (Scopoli)

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Abstract

During the last years *Cacopsylla pruni* has become a major concern in Austria because of its vectoring of Stone fruit yellows in apricots. Products based on kaolin, paraffin oil, orange oil and extract of fennel oil as active ingredients were tested for their ability to repel the disease vector *C. pruni* from landing and feeding on *Prunus armeniaca*. In free choice experiments all products showed significant repellency to adults 24 h after start of the tests. After 72 h, however, fennel extract oil and orange oil had lost their effect as compared with the untreated control, whereas plants treated with kaolin or paraffin oil were barely colonized.

In no choice experiments kaolin and paraffin oil significantly influenced feeding behaviour. All tested products significantly reduced the number of surviving insects in comparison to water treated controls.

Key words: *Cacopsylla pruni*, kaolin, paraffin oil, repellent.

Introduction

Wachau is one of the most important apricot growing areas in Austria. Apricot gardens dominate the banks of the river Danube. The river valley is adjoined by areas of higher altitude covered by forests. In European Union law the apricot produced there are designed as "Wachauer Qualitätsmarille" a product with protected designation of origin (PDO). Fruits are produced mainly of the local cultivar 'Klosterneuburger Marille' (a clone of 'Hungarian Best') and the rootstocks are apricot seedlings, myrobalans and other plums. In addition to its commercial value, apricot farming in the Wachau is very important for tourism. The Wachau was inscribed as "Wachau Cultural Landscape" in the UNESCO List of World Heritage Sites in recognition of its architectural and agricultural history. The spring landscape is characterized by wide expanses of flowering apricot trees.

During the last decade European stone fruit yellows caused by 'Candidatus Phytoplasma prunorum' has become a major concern in the Wachau area. Due to the high susceptibility of the predominant cultivar, the small scale structured apricot gardens and the close vicinity of conifers as putative overwintering hosts for the vector *Cacopsylla pruni* (Carraro *et al.*, 1998) disease incidences are high. Tree losses are a daily occurrence. It is well established that apricot cultivars differ in regard to their disease sensitivity and disease incidence might also depend on a grower effect (Thébaud *et al.*, 2006). In the case of the 'Wachauer Qualitätsmarille', however, a switch to less sensitive cultivars is not in accordance with the PDO status. The small scale structured apricot gardens are a part of the protected landscape and should therefore be kept as they are. All these factors make the development of control strategies very difficult.

Previous studies point out that overwintered adult *C. pruni* returning to apricot orchards in spring are the most efficient disease vectors (Thébaud *et al.*, 2009). The aim of our present work is the development of strategies to repel the vector *Cacopsylla pruni* from

landing and feeding on apricot plants. Therefore, we investigated the repellency to *C. pruni* of commercially available products (based on kaolin, mineral oil and plant extracts) under experimental conditions.

Materials and methods

Cacopsylla pruni adults used in this study were field collected on *Prunus spinosa* in March and April 2011. One year old *Prunus armeniaca* (cv. 'Klosterneuburger Marille') grafts in pots and excised *P. armeniaca* branches (cv. 'Klosterneuburger Marille') inserted into water were used for the experiments. The plants were maintained under outdoor conditions but protected from rain.

Concentrations and manufacturers of the products evaluated in this study are indicated in table 1. Deionized water was used for untreated controls. The apricot grafts and excised branches were sprayed to run off with the tested products and allowed to air dry before placement of insects.

In free choice assays excised branches treated with the mentioned products were simultaneously placed into insect proof cages (40x35x40 cm). 100 insects were introduced into the cages and allowed to freely move around in the cage. Numbers of adults present on each branch and on the cage surface were counted daily. Eggs present on 5 randomly selected leaves per treatment were also counted.

In no-choice assays insects were kept in cylindrical cages (diameter 9 cm, height 25 cm) on potted grafts and excised branches. 10 individuals were introduced into each cage. Numbers of feeding and surviving adults were counted daily. Numbers off eggs were also recorded.

All experiments were repeated four times. Statistical analyses (ANOVA, least significant difference test) were performed by aid of the statistics program SPSS 12.0 (SPSS, Chicago, Illinois, USA).

Table 1. Plant protection products and concentrations used in this study.

Product name	Active ingredient	Concentration	Manufacturer
Cutisan	Kaolin	5% (w/v)	Biofa, Münsingen, Germany
Prev-B2	Orange oil, fatty alcohol ethoxylate	0.4% (v/v)	Biofa, Münsingen, Germany
Nu Film 17*	Pine oil	0.15 (v/v)	Löffler, Leopoldsdorf, Austria
Promanal	Paraffin oil	2% (v/v)	Proagro, Abenberg, Germany
HF Pilzvorsorge	Extract of fennel oil	0.4% (v/v)	Biofa, Münsingen, Germany

*, only used in combination with Cutisan.

Results

In free choice experiments all tested products significantly influenced host plant choice of *C. pruni* adults 24 h after start of the experiment. On average of 72% of adults had landed on untreated controls, 8% on plants treated with fennel oil extract, 20% on plants treated with orange oil. No insects were observed on kaolin or paraffin oil treated plants. After 72 h, however, fennel extract oil and orange oil had lost their repellency as compared with the untreated control, whereas only few individuals were present on plants treated with kaolin or paraffin oil.

In no choice experiments the active ingredients kaolin and paraffin oil significantly influenced feeding behaviour. One day after treatment on average of 53% of insects fed on water treated control plants but only 10% on paraffin oil treated plants and 12.5% on kaolin treated plants. All tested products significantly reduced the number of surviving insects in comparison to water treated controls. In the control treatments on average of 83% of insects were still alive after an observation period of 6 days. On plants treated with kaolin in combination with pine oil 22.5% of insect were still alive after this period, on plants treated with kaolin alone 19%, on fennel oil extract treated plants 53%, on orange oil treated plants 57% and on paraffin oil treated plants 21%.

Discussion

In the present study especially kaolin (Cutisan with and without addition of Nu Film) and paraffin oil (Promanal) had a prominent repellent effect on *C. pruni* adults both in free-choice and in no-choice experiments. Therefore, they might have a great potential for the use in integrated pest management programs targeted against *C. pruni*. Our experiments, however, were conducted under experimental conditions excluding rainfall. Thus, the repellent effects of these products to *C. pruni* must be verified under field conditions. Kaolin was successfully used in laboratory and field experiments against the European pear sucker, *Cacopsylla pyri* (L.) significantly reducing the population density of this

polyvoltine species (Daniel *et al.*, 2005; Erler and Cetin, 2007). These experiments show that the kaolin particle film is stable under field conditions. In the mentioned experiments with *C. pyri*, however, attention was mainly paid on the effects of kaolin on oviposition and development of nymphs. In case of the univoltine *C. pruni* repellency to incoming adults as the most effective vectors would be required. Thus, long term experiments proving the effect of these products not only on vector density but also on disease incidence are desirable. Besides this, more products should be screened for their ability to repel *C. pruni* from landing and feeding on apricot trees.

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The use of *Spiroplasma melliferum* as a model organism to study the antagonistic activity of grapevine endophytes against phytoplasma

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Abstract

The objective of the research was to isolate from grapevines endophytes with antagonistic activity against phytoplasmas. In order to overcome the inability of grow phytoplasmas *in vitro*, the antagonistic activities of endophytes isolated from various grapevines on *Spiroplasma melliferum*, a phylogenetic close and cultivable Mollicute as a model organism was tested. No correlation was found so far between the inhibitory activity and the different plant sources, i.e. healthy, recovered or infected vines.

Key words: phytoplasmas, *Spiroplasma melliferum*, endophytes, recovery.

Introduction

The recovery phenomenon and the various degrees of susceptibility of grapevine cultivars to yellows disease may indicate a possible involvement of endophytes in the mechanism of plant resistance.

The objective of the research project was to isolate from grapevines endophytes with antagonistic activity against phytoplasmas. However, the inability to cultivate phytoplasmas on artificial medium prevent from a direct trial. In order to overcome this problem, the antagonistic activities of endophytes isolated from various grapevines on *Spiroplasma melliferum*, a phylogenetic close and cultivable Mollicute as a model organism was tested.

Materials and methods

Isolates from infected, recovered and healthy Cabernet-Sauvignon vines and from deserted vines were isolated in the early and late summer by placing sterilized discs of canes on nutrient agar and on PDA. The percentage of discs from which fungi or bacteria developed was recorded (table 1).

To test the antagonistic activity of the isolated endophytes, the different isolates were grown in a modified spiroplasma broth for 10 days (Trachtenberg and Gilad, 2001). After centrifugation (4,000 rpm; 30 min) and filtration, spiroplasma cells were added to either 50% diluted or 100% of the supernatant to make an initial concentration of ca. 10^6 cells/ml and incubated at 29°C for five days. Spiroplasma growing in fresh modified broth served as a positive control and the inhibitory effect of 0.5 µg/ml oxy-tetracycline was used as a reference. To monitor spiroplasma development in the filtrates we inoculated fresh spiroplasma medium with 1 µl of the incubated filtrate. The fresh medium was supplemented with phenol red as a color marker for cells growth (Trachtenberg and Gilad, 2001). Spiroplasma cell growth causes a pH decrease that changes the color of the medium from red to yellow. The time required to color change is correlated with the initial spiroplasma concentration and was therefore used as a quantitative parameter for the inhibitory effect of the filtrates. Inhibition index was defined as the ratio between the number of days to color change in the filtrate and the time required in the positive control. The inhibition index of 0.5 µg/ml oxy-tetracycline was 2. Thus, an isolate was considered inhibitory if the inhibition index of its filtrate was ≥ 2 (figure 1A and B).

Table 1. Percent of discs with endophytic fungi or bacteria from four collection dates. Discs were cut from deserted grapevines and from healthy, recovered 1y, recovered 2y and infected Cabernet-Sauvignon grapevines.

Date of isolation	Deserted		Healthy		Recovered 2y		Recovered 1y		Infected	
	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria
5.2009	1.7	45.6	0.0	6.0	1.9	20.0	2.9	20.9	0.8	10.0
7.2009	35.8	7.8	10.4	2.7	4.9	2.8	5.9	0.8	9.5	1.7
5.2010	22	0.6	1.2	0.7	2.5	6.5	2	0.8	1.0	4.0
8.2010	69.3	3.7	8.0	2.0	6.3	9.3	16.7	13.3	24.7	3.3
Average	34.5	6.9	4.9	2.87	3.9	9.7	6.9	9.5	9.0	4.8

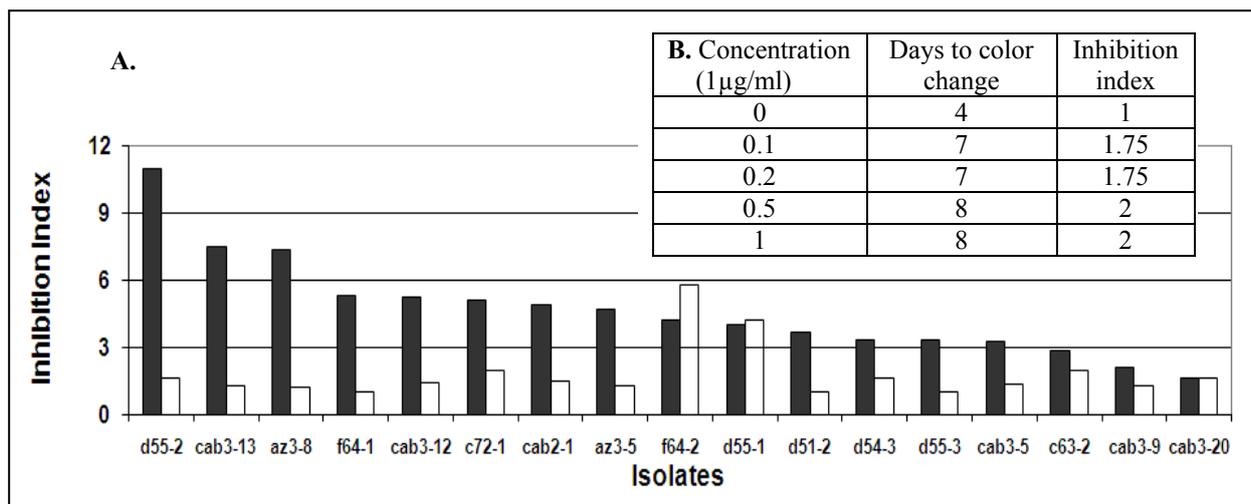


Figure 1. Inhibition index for Spiroplasma of different isolates (Mean of 2-15 replications).

A. In: ■ -100% filtrate; □ -50% diluted filtrate.

B. Inhibition index of oxy-tetracycline.

Results and discussion

In general, the highest number of populated discs was found in deserted vines (34.5% fungi, 6.9 bacteria) in contrast to healthy vines with the lowest percentage of isolates (avg. 4%). Endophytes developed from 4.8-9.7% of the discs cut from canes of recovered and infected vines. In the first date of collection we isolated more bacteria than fungi while in the other three dates the number of fungal isolates was higher.

Using this method, one fungus and several bacteria (out of 300 tested isolates) showed a relatively high inhibition activity against Spiroplasma (figure 1). Growing in 100% filtrates caused 2-10 folds inhibition of Spiroplasma cell growth relative to the positive control and was similar or higher compared to inhibition activity of 0.5 µg/ml oxy-tetracycline. However, the inhibitory effect of the diluted (50%) filtrate was much lower. No correlation was found so far between the inhibitory activity and the different plant sources, i.e. healthy, recovered or infected vines. This study shows that Spiroplasma can serve as an initial model system to test the

effect of different compounds on phytoplasma development. In further studies the filtrates will be tested on phytoplasma in nurse culture *in vitro*.

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'*Candidatus Phytoplasma phoenicium*'-related strains infecting almond, peach and nectarine in Lebanon

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Abstract

Genetic diversity among '*Candidatus Phytoplasma phoenicium*'-related strains infecting almond, peach and nectarine plants in diverse geographic regions of Lebanon was investigated by virtual restriction fragment length polymorphism (RFLP) analysis of 16S rDNA nucleotide sequences. Calculation of virtual restriction similarity coefficients indicates the presence of two new subgroups, -F and -G, in group 16SrIX. Obtained results open new opportunities for in-depth studies on the distribution of '*Ca. P. phoenicium*' strains in plant hosts and insect vector populations from different geographic areas of Lebanon.

Key words: single nucleotide polymorphisms, genetic diversity, phytoplasma classification, restriction fragment length polymorphism.

Introduction

'*Candidatus Phytoplasma phoenicium*' strains, belonging to subgroups 16SrIX-B and -D, are associated with a lethal devastating disease of almond trees (almond witches broom, AlmWB) in Lebanon (Abou-Jawdah *et al.*, 2002). By the year 2002, more than 100,000 almond trees had died by AlmWB in Lebanon; in 2009, '*Ca. P. phoenicium*' was identified also in association with a severe disease of peach and nectarine in southern Lebanon (Abou-Jawdah *et al.*, 2009).

The rapid spread of '*Ca. P. phoenicium*' over large geographical areas in North Lebanon suggested the presence of an efficient vector (Abou-Jawdah *et al.*, 2009). However, this vector has not been identified yet. In order to have a better understanding of the disease epidemiology and achieve an effective disease management, a development project financed by Italian Co-operation is being implemented by AVSI (Association of Volunteers in International Service) Foundation in Lebanon. In the present study, data on genetic diversity among '*Ca. P. phoenicium*' strains infecting almond, nectarine and peach plants from diverse Lebanese regions are reported.

Materials and methods

Leaf samples were collected in 15 orchards from 24 plants (table 1) showing symptoms such as witches' broom, phyllody, virescence and chromatic alterations. Total DNA was extracted from 100 mg of leaf veins and used for phytoplasma detection by 16S rDNA amplification in nested PCRs primed by phytoplasma-universal primer pairs P1/P7 and R16F2n/R16R2 (Gundersen and Lee, 1996).

Amplicons from nested PCRs were sequenced, assembled, and compared with the GenBank database with the aim of searching possible identity. A total of 37 16S rDNA sequences of phytoplasma group 16SrIX (13 from GenBank and 24 obtained in this work), plus sequences from phytoplasma strains representative of known 16Sr subgroups, were analyzed through an automated *in silico* restriction assay, as described by Wei *et al.* (2007).

Results and discussion

Primer pair R16F2n/R16R2 primed amplification of DNA from templates derived from all samples studied. Phytoplasma strains identified here shared a 99-100% of sequence identity with '*Ca. P. phoenicium*' (accession number AF515636). Visualization and comparison of virtual gel plotted images revealed three different RFLP patterns (table 1). One pattern, indistinguishable from that characteristic of strains classified in the subgroup 16SrIX-D, was exhibited by DNAs from 15 '*Ca. P. phoenicium*' strains (figure 1). The remaining two virtual RFLP patterns differed from that of the previously described subgroup IX-D (figure 1), and shared similarity coefficients of 93 to 97%, confirming their affiliation with group 16SrIX. Actual RFLP analyses confirmed the recognition of two new subgroups in group 16SrIX. Prior to this work, five subgroups had been described in the group 16SrIX; the results of this study add two new, confirmed by real RFLP subgroups -F (two strains) and -G (seven strains) from almond, nectarine and peach plant hosts. The data evidenced extensive diversity of '*Ca. P. phoenicium*' in Lebanon, particularly in Sarada regions, where three 16SrIX subgroups (-D, -F, and -G) co-exist and infect nectarine plants.

Table 1. ‘*Ca. P. phoenicium*’ strains, belonging to distinct 16SrIX subgroups, in orchards of Lebanon regions.

Strain	Origin	Orchard No.	Host	Subgroup 16SrIX
SarN1-2	Sarada	1	nectarine	-G
SarN5	Sarada	1	nectarine	-F
SarN8-1	Sarada	2	nectarine	-D
SarN9-7	Sarada	1	nectarine	-D
SarN10-8	Sarada	3	nectarine	-D
SarP10(297)	Sarada	4	peach	-D
MarN13-1	Marjayoun	5	nectarine	-D
MarN14-1	Marjayoun	6	nectarine	-D
MarN27-2	Marjayoun	7	nectarine	-F
MarN28-1	Marjayoun	7	nectarine	-D
FegA1-1	Feghal	8	almond	-G
FegA11-4	Feghal	9	almond	-D
FegA13-1	Feghal	9	almond	-G
FegA16-4	Feghal	8	almond	-D
FegA18-1	Feghal	10	almond	-G
FegP1-2	Feghal	11	peach	-D
FegP2-6	Feghal	11	peach	-D
FegP3-1	Feghal	11	peach	-G
FegPL3-1	Feghal	11	almond	-D
FegA3	Feghal	12	almond	-G
FegA4	Feghal	13	almond	-G
KKN18-1	Kerbet Kanafar	14	nectarine	-D
KKN19-1	Kerbet Kanafar	14	nectarine	-D
KKN29-1	Kerbet Kanafar	15	nectarine	-D

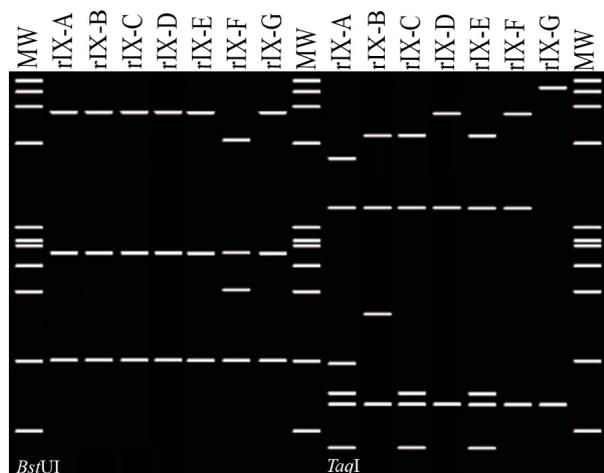


Figure 1. Virtual R16F2/nR2 RFLP patterns by key enzymes *Bst*UI and *Taq*I for distinguishing among 16SrIX subgroups.

Conclusions

The broad genetic diversity among ‘*Ca. P. phoenicium*’-related strains suggests possible influence of different ecological and/or climatic niches on phytoplasma population composition. In particular, it would be interesting to investigate whether particular ‘*Ca. P. phoenicium*’ subgroup(s) could be correlated with certain biological properties and different species of insect vector. These investigations will be crucial for a better understanding of the disease epidemiology and achieving an effective disease management.

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Monitoring *Scaphoideus titanus* for IPM purposes: results of a pilot-project in Piedmont (NW Italy)

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Abstract

A pilot-project was conducted in Piedmont to determine if insecticidal sprays against *Scaphoideus titanus*, the vector of grapevine 'flavescence dorée' disease, can be decreased in case of low populations of the leafhopper. Field samplings included a sequential sampling of nymphs on leaves and yellow sticky traps for capturing adults in vineyards subject to different pest management strategies. Population levels of *S. titanus* were lower in conventional than in organic vineyards, the latter being always more variable, suggesting some problems in the distribution of the active ingredient. The vineyards that were under the proposed threshold received just one spray, however no increase in the presence of *S. titanus* was observed. As a result, provided a suitable sampling plan, it is possible to reduce insecticidal sprays against this leafhopper vector.

Key words: grapevine, 'flavescence dorée', leafhopper vector, mandatory phytosanitary procedures.

Introduction

The nearctic leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) is the main vector for 'flavescence dorée' (FD) phytoplasmas (16SrV, subgroups C and D) (Boudon-Padieu, 2003). In Italy, the control of *S. titanus* and FD is mandatory, and include insecticidal treatments and removal of infected plants. Usually, at least two-three sprays are necessary in conventional and organic farming, respectively. However, if no or little FD occurs, they may be dropped to one, provided the population levels of the leafhopper are low enough, in order to preserve non-target organisms. This research consists in a pilot-project for targeting the efforts in controlling *S. titanus* in grapevine growing areas of Piedmont (North-Western Italy).

Materials and methods

Data were collected in 2009-2010 in 22 vineyards situated in 12 districts of the Cuneo Province (44.38271-44.56752 °N; 7.85674-7.98295 °E), and subject to traditional or organic pest management against *S. titanus*: active ingredients used included thiamethoxam (TH), organophosphates (OP), etofenprox (E), pyrethroids (P), and natural pyrethrum (NP). All the vineyards were of cv "Dolcetto", except one (cv "Chardonnay"), and their size range was 0.5-0.9 ha. Plants with FD symptoms were detected only in one of them (no. 12) and were up to 2% of the total.

Nymphs of *S. titanus* were sampled at the middle of June, before insecticidal sprays, with direct observations on the leaves following a sequential sampling plan with a fixed precision level of 0.75 (Lessio and Alma, 2006): counts were interrupted either when the stop was reached, or if the pest density was lower than 0.02 nymphs per 5 leaves per plant (no more than one nymph

on 137 plants). Adults were sampled with yellow sticky traps, 3 per vineyard, placed at the beginning of July and changed every 10 days until the middle of October. In Piedmont, the thresholds for dropping to one insecticidal spray are < 0.02 nymphs, and ≤ 2 adults on 3 traps/ha. Data were analysed with a hierarchical cluster method, using the level of nymphs and adults as variables, in order to find a trend in clustering of vineyards subject to different PM strategies.

Results

During 2009, nymphs and adults of *S. titanus* were found in 15 and 18 vineyards out of 22, respectively; three vineyards were under the threshold of 0.02 nymphs and 2 adults. In 2010, no nymphs were found in 5 vineyards, whereas adults were captured in different numbers in all vineyards; 2 vineyards were under the threshold. The highest presence of both nymphs and adults was recorded in the same vineyard (no. 5), that was subject to organic farming, in both years. The cluster analysis showed the presence of 5 and 6 groups in 2009 and 2010, respectively (table 1, figure 1). Cluster 1 was the most represented, and included vineyards with low populations of nymphs and adults. As a result, NP-treated vineyards were almost evenly distributed within different clusters, indicating a great variability in the effectiveness of this active ingredient, probably depending on the accuracy of distribution (e.g. sprays during day or evening, pH of the solution, etc.) or on the date of application. In 2009, the majority of vineyards (13) were in cluster 1, and adopted a PM strategy based on TH + OP. In 2010, PM strategies were more heterogeneous due to the fact that many farmers applied just one spray, provided low population levels of the vector were detected; however 16 vineyards were in cluster 1 (figure 1).

Table 1. Results of hierarchical cluster analysis of vineyards with different levels of *S. titanus* nymphs (mean density calculated with sequential sampling plan) and adults (mean captures per 3 traps).

Cluster	2009			2010		
	No. vineyards	Nymphs (mean)	Adults (mean)	No. vineyards	Nymphs (mean)	Adults (mean)
1	16	0.06	3.19	13	0.08	4.57
2	3	0.05	26.67	4	0.03	12
3	1	0.65	91	1	0.33	39
4	1	0.11	55	1	0.11	32
5	1	1	475	1	0.71	91
6	-	-	-	1	0.71	216

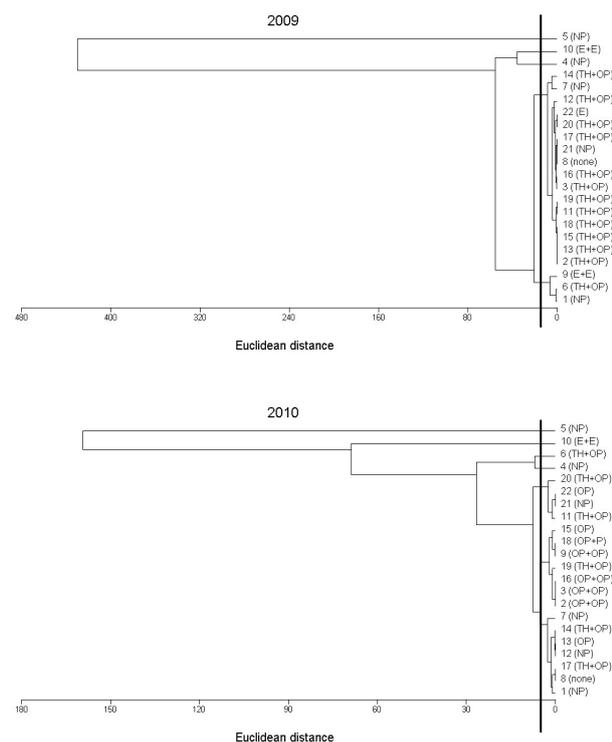


Figure 1. Dendrogram showing the vineyards subject to different PM strategies (see text for details) and falling into different clusters of *S. titanus* density. The vertical line indicates the points of dendrogram cutting.

Discussion

This pilot-project showed how a correct monitoring of the leafhopper vector, *S. titanus*, is important for targeting pest management strategies. When populations at the nymphal stage are very low (<0.02), one spray is enough to prevent infestation and therefore disease outbreaks if no FD is present within the vineyard. However, monitoring with traps permits to shift to 2 sprays within the season in case of an increase of adults' captures. The reduction of spraying should also have a benefit for honeybees and foraging insects, especially given the high toxicity of certain active ingredients Iwasa *et al.*, 2004). The reduction cannot be applied in the case of organic pest management, as pyrethrum has

no or little persistence and must be repeated at least 3 times during the season, however it has in any case a low environmental impact. Another risk factor is the presence of wild grapevine close to the vineyards, which can host both *S. titanus* and 16SrV phytoplasmas (Lessio *et al.*, 2007; Lessio *et al.*, 2011). In this case, decreasing insecticidal sprays should be considered carefully.

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On the distribution of 'Candidatus Phytoplasma pyri' in the European Union based on a systematic literature review approach

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Abstract

The present study was conducted within the framework of a European Food Safety Authority (EFSA)-funded project (Prima phacie). The objective was to determine the current status of 'Ca. P. pyri' in the fruit growing areas of the European Union (EU) based on a systematic literature review approach (SLR). The results show that 'Ca. P. pyri' occurs in 15 out of the 27 EU countries, including the most important pear production areas in the EU. 'Ca. P. pyri' is not reported from ten pear producing countries, only two of which declared the absence on the basis of official surveys. In the eight others, official surveys for pest freedom are not available, thus contributing to the uncertain pest status in those countries. Within the Prima phacie project, the results of the SLR are used when analyzing the risk of entry, establishment, spread and impact of 'Ca. P. pyri' in regions where it is not present. Furthermore, the results are used to test the effectiveness of different options in reducing the risk of introduction and spread.

Key words: Pear decline, pest risk assessment, literature search.

Introduction

Prima phacie is a European Food Safety Authority (EFSA)-funded project to improve methodology in pest risk assessment and in the evaluation of the effectiveness of risk reduction options (MacLeod *et al.*, 2010). Risk assessment methods being developed within the project are tested by using ten case study pests, among them 'Candidatus Phytoplasma pyri', which is associated with pear decline (PD) disease. This is a destructive disease of pear (Jarausch and Jarausch, 2010), which is currently regulated within the European Plant Health Directive 2000/29 EC. Obtaining up to date information on the present distribution of a pest, by countries and areas within countries, is essential in pest risk assessments. The objective of this study was to determine the current status (presence/absence) of 'Ca. P. pyri' in the fruit growing areas of the EU. The information obtained is based on a systematic literature review (SLR), including results of a questionnaire sent to the country representatives of EFSA's scientific network for risk assessment in plant health.

Materials and methods

Following the principles of the EFSA Guidance on application of systematic review methodology (EFSA, 2010), the key steps to conduct a SLR were applied: a clearly formulated question was developed *a priori* ("What is the distribution of 'Ca. P. pyri' in the fruit growing areas of the EU?"), search terms were defined and combined (Pear decline OR Phytoplasma pyri OR Parry's disease OR *Cacopsylla pyri* OR *Psylla pyri* OR *Cacopsylla pyricola* OR *Psylla pyricola* OR *Cacopsylla*

pyrisuga OR *Psylla pyrisuga* = Set 1; Set 1 AND occur*, Set 1 AND distribute*, Set 1 AND presen*, Set 1 AND spread, Set 1 AND monitor*, Set 1 AND survey) to search for articles in scientific abstracting databases (AGRICOLA, Agris and CAB Abstracts, searched on 08.04.2010; Web of Science, searched on 21.06.2010). The searches were not restricted concerning language and were traced back to the first findings of PD in the early 20th century.

In addition, a hand search was conducted, because not all relevant literature was expected to be included within electronic databases. For this purpose the following sources were used: EPPO Reporting Service (back to 1967, accessed 26.06.2010), EPPO-PQR (version 4.6; 07-2007), meeting reports (e.g. COST Action FA0807, Sitges, Spain, February 2010). Furthermore, in 2010 a questionnaire on the current status (the presence or confirmed absence) of 'Ca. P. pyri' in different fruit growing areas was sent to the delegates of EFSA's scientific network for risk assessment in plant health.

In a two step screening procedure all abstracts from databases and hand searches were checked for relevance to the question by two reviewers. In the first step, abstracts were filtered out which do not address the two predefined criteria. 1. Does the abstract describe the distribution of the phytoplasma diseases and/or the vector? 2. Does the abstract describe primary research (as opposed to a review?). For all abstracts, meeting these eligibility criteria, full text papers were obtained from library services and evaluated in a second stage by using the following question: Does the paper clearly specify the fruit growing region for which the results are applicable (e. g. region Emilia-Romagna, province of Parma, municipalities x and y; as opposed to "Northern Italy").

Results

Searches with the key word combinations in the databases resulted in 477 abstracts (after removal of duplicates). 50 (10.5%) of them have been selected for the second stage screening. The main reason for papers not to be included was that the distribution of PD in fruit growing areas of the EU was not addressed, as the majority of these papers focused on diagnosis, biology or management of the phytoplasma and/or its vector. In the second stage, 24 full papers (out of 50) were selected to be included in the SLR. The main reason for discarding information was that more recent data for a specific fruit growing area was available. Furthermore, the results of 17 questionnaires (Austria, Belgium, Bulgaria, Czech Republic, Denmark, Estonia, Finland, Germany, Italy, Latvia, Malta, Poland, Portugal, Slovenia, UK + questionnaires from pest experts in Greece and Cyprus), 9 citations in the EPPO Reporting Service and 5 papers through hand searching were included in the SLR.

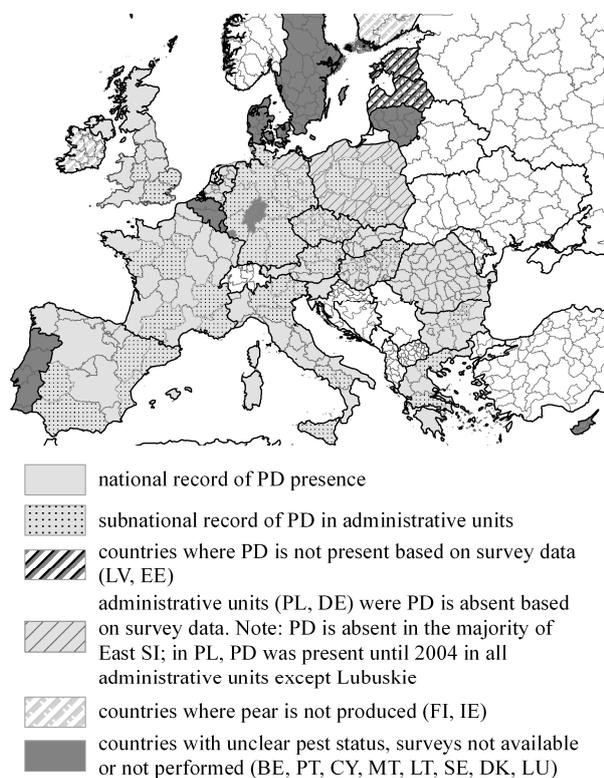


Figure 1. Status of ‘*Ca. P. pyri*’ in the EU.

The results of the SLR are shown in figure 1. ‘*Ca. P. pyri*’ is a native European species, which is very widespread and has been reported in 15 out of the 27 EU countries, including the largest pear production areas in Italy and Spain. In Poland and Germany, PD is officially absent in few administrative units.

‘*Ca. P. pyri*’ is not reported from ten pear producing countries. Of these countries only Latvia and Estonia declared the absence based on official surveys. Portugal and Belgium, two major pear producing countries are cur-

rently surveying the pest status. In Denmark and Cyprus, the disease is assumed to be absent; however, surveys have not been undertaken so far. No data were available from Lithuania, Sweden, Malta and Luxembourg, but these countries have only limited area of pear production.

Discussion

The study applies a SLR to assess the distribution of ‘*Ca. P. pyri*’ in the EU. The methodology of SLR was found particularly useful to answer a specific question in the pest risk assessments of ‘*Ca. P. pyri*’ as it presents an exhaustive, transparent and unbiased way of collecting, reporting and analyzing data. The results show that this pest is widespread in the EU; however, in a few countries/regions the disease appears to be absent. Within the Prima phacie project the results of the SLR are used when analyzing the risk of ‘*Ca. P. pyri*’ entering new areas. Entry comprises different elements, such as the association of the pest and the host plant at the place of origin, the volume of trade of the commodity, the survival of the pest along the pathway and the transfer to a suitable host. By testing different risk assessment methods, particular emphasis is given to the introduction and impact of ‘*Ca. P. pyri*’ in regions where PD has not been reported. Moreover, the effectiveness of risk reduction options on the introduction and spread is assessed.

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Response of apple proliferation-resistant *Malus sieboldii* hybrids to multiple infections with latent apple viruses

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Abstract

Apple proliferation (AP) is the most important phytoplasma-associated disease affecting apple in Europe. The failure in controlling this disease by standard means strongly increased the importance of adopting resistant genotypes. About 6000 seedlings were obtained from a breeding programme crossing *M. sieboldii*, donor of resistance to AP, with standard apple rootstocks (M9 mainly) as donor of agronomic value. Resistance screening showed that the trait was inherited to the progenies and trials are in progress to test the agronomic value of these genotypes. In an additional trial, the response of AP-resistant genotypes to a superinfection with different latent apple viruses was investigated. For this, *M. sieboldii*-derived first and second generation hybrids were analysed. In summer, three repetitions for each genotype were inoculated with *apple chlorotic leaf spot* (ACLSV), *apple stem grooving* (ASGV) and *apple stem pitting* (ASPV) virus. The two following springs after infection, the presence of the viruses was assessed by ELISA test and virus-specific symptom recording on young leaves. In parallel, the reaction of the plants to infections with Trentino strains of '*Candidatus Phytoplasma mali*' was evaluated. AP-susceptible *Malus x domestica* genotypes were considered as controls. The results confirmed an incidence of the viral infections on *Malus sieboldii* as it was reported in the past. However, the *M. sieboldii* hybrids showed a high variability of response ranging from no viral symptoms to severe symptoms. Nevertheless, highly phytoplasma-resistant genotypes which showed no presence of viral superinfections could be identified in these experiments.

Key words: '*Candidatus Phytoplasma mali*', *apple stem grooving* virus, *apple stem pitting* virus, resistance screening, breeding.

Introduction

Apple proliferation (AP) is one of the most important phytoplasma diseases in Europe that causes considerable economic losses. It is transmitted by grafting, insect vectors and root bridges (Ciccotti *et al.*, 2007). The failure in controlling this disease by standard means strongly increased the importance of adopting resistant genotypes. Previous work indicated that, due to the colonization behavior of the associated agent, the disease can be controlled by the use of resistant rootstocks (Seemüller *et al.*, 1984). While extensive screening revealed no satisfactory resistance in established rootstocks (Kartte and Seemüller, 1991), substantial levels of resistance were identified in experimental rootstocks derived from crosses of the apomictic species *Malus sieboldii* and genotypes of *M. x domestica* and *M. x purpurea* (Bisognin *et al.*, 2008a and b; Seemüller *et al.*, 2008).

As these experimental rootstocks had poor agronomic values, a breeding programme was started ten years ago in order to develop commercial AP-resistant apple rootstocks exploiting the natural resistance found in *Malus sieboldii* (Bisognin *et al.*, 2009). Resistance screening showed that the trait was inherited by the progenies and trials are in progress to test the agronomic value of these genotypes (Jarausch *et al.*, 2010). Moreover, some apomictic rootstocks budded with a virus-contaminated scion source revealed great differences in susceptibility to such viruses that include *apple chlorotic leaf spot virus* (ACLSV), *apple stem pitting virus* (ASPV) and *apple stem grooving virus* (ASGV) (Seemüller *et al.*, 2008). In the present study, the response of different *Malus sieboldii* hybrids to infection with three different

latent viruses was investigated and compared with phytoplasma resistance of these genotypes to two Trentino strains of '*Candidatus Phytoplasma mali*'.

Materials and methods

Healthy one-year-old micropropagated plants of *M. sieboldii*-derived first and second generation hybrids, *M. sieboldii*, 4551, D2212, H0909, H0801 o.p., Gi 477/4 o.p., C1907 o.p., 4551 o.p. (Ciccotti *et al.*, 2008) and selected hybrids obtained from the crosses 4551xM9, D2212xM9, H0909xM9 and M9xD2212 (for details see Bisognin *et al.*, 2009), were inoculated in pots *ex vitro* during summer 2008. Some AP-susceptible genotypes were taken as control.

In a first experiment three replicates for each genotype were separately inoculated by chip budding with *apple chlorotic leaf spot* (ACLSV), *apple stem grooving* (ASGV) and *apple stem pitting* (ASPV) virus. In a second experiment three replicates for each genotype were contemporary inoculated with the three viruses to evaluate the reaction of the plants to superinfection. Trials were conducted in an insect-proof greenhouse. In spring 2010 ELISA test was used to evaluate the presence of the viruses and symptoms were recorded on young leaves. Symptom incidence of the viruses was evaluated as follows: 0 = no symptoms, x = low incidence, xx = moderate incidence, xxx = high incidence.

The same genotypes were evaluated in a parallel experiment for AP resistance. *Ex vitro* plants were inoculated by grafting with phytoplasma infected scions with two '*Ca. P. mali*' strains PM6 and PM11 isolated in Trentino, Northern Italy. Three repetitions for each

genotype-strain combination were performed. The second autumn after inoculation, phytoplasma infection was evaluated and expressed by a disease index based on incidence of specific symptoms such as enlarged stipules, witches brooms, foliar reddening, stunting (index values ranged from 0 = no symptoms to 4 = high presence of symptoms). In the same period 'Ca. P. mali' concentration in the roots was also evaluated by real time quantitative PCR (data not shown).

Results and discussion

In the first experiment single infections with the latent apple viruses ACLSV, ASGV and ASPV were difficult to evaluate as more than 50% of the plants were not infected as assessed by ELISA. In contrast, the multiple infections of the *M. sieboldii* hybrids with all three viruses yielded an incidence of the viral symptoms ranging from no to severe symptoms. Indeed, sensitivity of apomictic rootstocks to latent apple viruses was already observed by Schmidt (1988) as stunting and chlorosis. Seemüller *et al.* (2008) observed a poor development and stunting of *M. sieboldii* and 4,551 seedlings inoculated accidentally with both, phytoplasma and latent viruses. Our results showed that plants of *M. sieboldii* and 4,551 selections were slightly to moderate affected by the multiple presence of viruses alone. In contrast, D2212 which was less affected in the work of Seemüller *et al.* (2008) showed no symptoms of virus infections and behaved as the tolerant *M. x domestica* control M9. The same was observed for plants of the apomictic selections like C1907 and Gi 477/4 which were originally derived from open pollination. Interestingly, the sensitivity to latent apple viruses was expressed very heterogeneously in the progeny of the crosses made with D2212, H0909 and 4551. The progeny genotypes showed either no viral symptoms or were much more severely affected as the parental *M. sieboldii*-derived genotypes.

The objective of the work to find a rootstock resistant to AP and tolerant to the latent apple viruses was achieved by apomictic genotypes like D2212 and C1907 o.p. as well as by some selected progeny of the breeding programme as tested here. These genotypes showed no or only mild symptoms upon inoculation with the Trentino strains of 'Ca. P. mali' and exhibited no viral symptoms after multiple infections.

These findings should be confirmed in further trials in which breeding genotypes will be used as rootstocks of commercial varieties in order to follow the influence of virus infections also in the production of the plants. After this step the response to infection will be completely understood.

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Breeding apple proliferation-resistant rootstocks: durability of resistance and pomological evaluation

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Abstract

To develop apple proliferation (AP) resistant rootstocks, a breeding program was initiated in 2001 employing apomictic *Malus sieboldii* and *M. sieboldii*-derived hybrids as donors of the resistance trait and mainly standard apple rootstock *Malus x domestica* cv. M9 as donor of agronomic values. Examination of the experimentally inoculated progeny of seven crossings made in 2001 and 2002 over a period of seven to eight years showed that inheritance of resistance differs considerably among the parental lines by yielding between 10 to 55% of resistant offspring. Resistant rootstocks were characterized by poor host properties for the AP phytoplasma. This is evidenced by lower phytoplasma titers in resistant genotypes than in standard stock M9 and by preventing detectable phytoplasma development in the top grafted susceptible cv. Golden Delicious. At the end of the observation period, 80% of the root samples collected from resistant rootstocks tested PCR-negative. Size and productivity of trees grown on resistant rootstocks varied over a wide range. A preliminary pomological evaluation was done on the cv. Golden Delicious which has been grafted as infected scion on the progeny genotypes to test. Comparisons with trees grown on M9 rootstocks indicated that there are genotypes among the offspring examined that fulfill the requirements of commercial apple growing.

Key words: Apple proliferation, apple rootstocks, *Malus sieboldii*, phytoplasma titer, resistance breeding.

Introduction

Apple proliferation (AP) is induced by the wall-less bacterium 'Candidatus Phytoplasma mali' and is widespread in several major fruit-growing areas in Europe. AP is of considerable economic importance due to its negative effect on tree productivity and fruit quality.

The disease is difficult to control. Because the recommended measures are often not satisfactory, the most promising approach to control AP appears the use of resistant plants. Previous work indicated that, due to the seasonal fluctuation of 'Ca. P. mali' between stem and roots of infected apple trees, growing the scion cultivars on resistant rootstocks can prevent the disease (Schaper and Seemüller, 1982; Seemüller *et al.*, 1984).

However, examination of many established and experimental rootstocks, which were mostly based on *Malus x domestica*, and a large number of other *Malus* taxa revealed that there is no satisfactory resistance in these groups (Kartte and Seemüller, 1991; Seemüller *et al.*, 1992). Suitable resistance was observed in some experimental rootstock selections derived from the apomictic species *M. sieboldii* (Bisognin *et al.*, 2008; Seemüller *et al.*, 2008).

Because trees on the resistant genotypes were more vigorous and less productive than trees on standard stock M9, a breeding program was initiated to develop resistant rootstocks with satisfactory pomological properties by using *M. sieboldii*-based parental lines from the above screening as donors of resistance and M9 and other dwarfing stocks as donors of pomological values (Bisognin *et al.*, 2008; Bisognin *et al.*, 2009; Jarausch *et al.*, 2007; Jarausch *et al.*, 2010). Here we present results

of long-term observations on level and durability of resistance, the relationship of phytoplasma concentration to resistance, and on vigor and productivity of trees grown on selected offspring.

Materials and methods

In 2001 and 2002 the following crossings were made that resulted in a substantial number of offspring (see Bisognin *et al.*, 2009 for details): 4551 (Laxton's Superb x *M. sieboldii*) x M9; 4608 (*M. purpurea* 'Eleyi' x *M. sieboldii*) x M9; H0909 [(Laxton's Superb x *M. sieboldii*) x M9] x P22; H0909 x M9; *M. sieboldii* x M9; D2212 [(Laxton's Superb x *M. sieboldii*) x o.p.] x M9; M9 x D2212.

All progenies were grown in pots in the greenhouse. Sets of 5 to 6 locus-specific simple sequence repeats (SSR) markers were employed to distinguish sexually derived seedlings from apomictically derived seedlings (Bisognin *et al.*, 2009). In July, preferentially recombinant seedlings, for comparison also a representative number of motherlike (apomictic) plants, were graft-inoculated with cv. Golden Delicious infected with severe strains of 'Ca. P. mali'. The following spring, inoculated plants were transplanted to the nursery where they were observed for 2 to 3 years for symptom expression on Golden Delicious. All trees on recombinant seedlings that never developed symptoms or only temporarily mild symptoms such as foliar reddening or enlarged stipules were considered to be resistant and were transplanted for further evaluation under commercial growing conditions. In addition, some trees on

motherlike seedlings were also transplanted. Symptom development and yield were recorded annually for 4 to 5 years. Vigor as expressed by trunk diameter in 40 cm height was determined in fall of 2010.

Quantitative real-time PCR was performed to determine presence and concentration of 'Ca. P. mali' in inoculated trees as described (Bisognin *et al.*, 2008).

Results and discussion

From the crossings made, approximately 3,000 offspring were obtained. SSR genotyping revealed that the majority of them derived from apomixis. Some 750 seedlings were inoculated of which 535 were recombinant.

Screening during the nursery phase revealed considerable differences in the inheritance of AP resistance of the various apomictic parents used. The best donors of this trait were selections 4608 and D2212. Crossings of these genotypes with M9 yielded 60 to 70% recombinant offspring classified as resistant. In the progenies of the other crossings resistance ranged between 20 and 30%.

At the end of the nursery growing phase, 207 trees on recombinant rootstocks and 47 trees on motherlike rootstocks were selected and transplanted for further evaluation. In the following field observation period, 70 to 80% of the trees on both recombinant and motherlike rootstocks continued to show excellent resistance properties whereas the remaining trees showed, mostly temporarily, mild to moderate symptoms. Only the trees on rootstocks derived from selection H0909 depicted lower values on the persistence of resistance, being in the range of 40%. In contrast to selected trees from resistant parents, all transplanted control trees on M9 rootstocks showed permanently moderate to severe AP symptoms.

Quantitative RT-PCR showed that the phytoplasma titer in *M. sieboldii*-derived resistant rootstocks is usually in the range from 10^4 to 10^6 cells/g phloem. In the roots of M9 stock the titer was 10 to 1,000 times higher. This is confirming previous findings (Bisognin *et al.*, 2008). Accordingly, the phytoplasma titer in roots of severely affected progeny genotypes was found to be about 10 times higher than in offspring that was not or only slightly affected. Furthermore, there is indication that the phytoplasma infection in resistant roots is eliminated or reduced to an undetectable level. Eight years post inoculation, 80% of the root samples collected from resistant stocks tested PCR-negative.

At the end of the observation period the selected trees on recombinant rootstocks differed considerably in size. Most of them were too vigorous for the commercial growing of culinary apples. However, in all progenies recombinant stocks were identified that mediate satisfactory dwarfism to the scion cultivar. E.g., more than 10% of the trees on resistant genotypes of the 4608 x M9 progeny were similar in size to trees on M9.

The correlation of productivity and vigor known from established rootstocks also applies for the selected resistant stocks. Regarding trees on resistant stocks derived

from the most successful crossing 4608 x M9, the cumulative yield ranged from 1.6 to 5.0 kg/cm² cross section of the trunk. Comparison showed that the average yield of trees on dwarfing stocks was 3.7 kg/cm² whereas that of trees on stocks too vigorous for commercial growing was only 2.4 kg/cm².

Promising genotypes are currently multiplied by micropropagation for further agronomic evaluation.

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First data obtained by shotgun proteomics from *Nicotiana occidentalis* infected by 'Candidatus Phytoplasma mali'

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Abstract

The protein content of *Nicotiana occidentalis* infected by the non-cultivable phytopathogenic mollicute 'Candidatus Phytoplasma mali' strain AT was determined by shotgun proteomics. 102 out of 497 predicted phytoplasma proteins were identified as expressed in shoot tissue. In addition, 940 proteins of *N. occidentalis* were detected. Results demonstrate the successful application of LTQ Orbitrap XL ETD™ mass spectrometer in detecting phytoplasma-specific proteins in protein mixtures. A high portion of proteins with unknown function was identified beside prominent proteins involved in translation. Several of the proteins with unknown function contain a signal peptide suggesting a potential pathogen-host interaction.

Key words: phytoplasma, apple proliferation, proteomics.

Introduction

'Candidatus Phytoplasma mali' is the agent associated with apple proliferation disease which represents one of the most economically important phytoplasma diseases in Europe. Infection results in impaired fruit quality and productivity of the apple trees. 'Ca. P. mali' belongs together with 'Ca. P. pyri', 'Ca. P. prunorum' and few other phytoplasmas to the apple proliferation group, which forms a distinct major subclade within the phytoplasmas (Seemüller and Schneider, 2004). The genome sequence of 'Ca. P. mali' strain AT was determined and highlighted an unusual linear chromosome organisation and a low G+C content of 21.4% (Kube *et al.*, 2008). The condensed genome with a size of 602 kb encodes 497 protein coding genes.

This study aims to evaluate the application of shotgun proteomics to 'Ca. P. mali' strain AT infected plant material and thereby provide additional information and evidence for the expression of the predicted proteins. The shotgun proteomics approach was successfully used for the identification of proteins assigned to the mulberry dwarf phytoplasma by comparing mass spectra with the proteins of all *Mollicutes* (Ji *et al.*, 2009). Due to the complete genome sequence of 'Ca. P. mali' a more stringent assignment is possible. Here we present the first experimental derived proteome data of this study.

Materials and methods

Protein isolation from plant material

Nicotiana occidentalis greenhouse plants were inoculated with 'Ca. P. mali' strain AT as previously described (Kube *et al.*, 2008) at the Julius Kühn Institute Dossenheim. One plant showing distinct symptoms of infection such as little leaves and witches' broom was

chosen for the initial experiment. Proteins were isolated from shoots by two different methods in parallel. First approach (I) started by freezing the tissue in liquid nitrogen followed by TissueLyser treatment (Qiagen), suspension in SDS lysis buffer containing protease inhibitors (unpublished) and sonification. For the second approach (II), shoots were disrupted in meshbags (extraction Bags, Bioreba) and proteins isolated using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) according to the manufacturer's instruction. Proteins were separated in 12.5% SDS-PAGE gel and stained by Coomassie G250 for visualisation. After destaining in 20% methanol, 7.5% acetic acid gel lanes were cut into 16 slices. Reduction and alkylation was performed. Proteins were digested within the gel slices with trypsin (Roche Diagnostics) for 16 h at 37°C in humidified atmosphere. Peptides were extracted and vacuum dried. Afterwards, samples were resuspended in 5% acetonitrile with 2% formic acid.

LC-MS/MS measurement

LC-MS/MS was performed on LTQ Orbitrap XL ETD™ mass spectrometer (Thermo Fisher Scientific) equipped with a nanospray ion source (Thermo Fisher Scientific) and an Agilent 1200 Series HPLC- System (Agilent Technologies). The System was fitted with a self-packed C18 RP column (0.15 mm × 150 mm *PicoFrit*, New Objective; *ReproSil- Pur C18- AQ* Dr. Maisch). Buffers A (2% acetonitrile + 0.1% formic acid) and B (98% acetonitrile + 0.1% formic acid) served as mobile phase and the peptides were eluted via a gradient of 2.7% to 90% buffer B over a period of 150 min with a flow rate of 0.2 µl/min. Full-scan mass spectra were detected with the Orbitrap mass spectrometer. The ten most intense peptides were selected for CID MS/MS scans in the linear ion trap. Each sample has been injected two times.

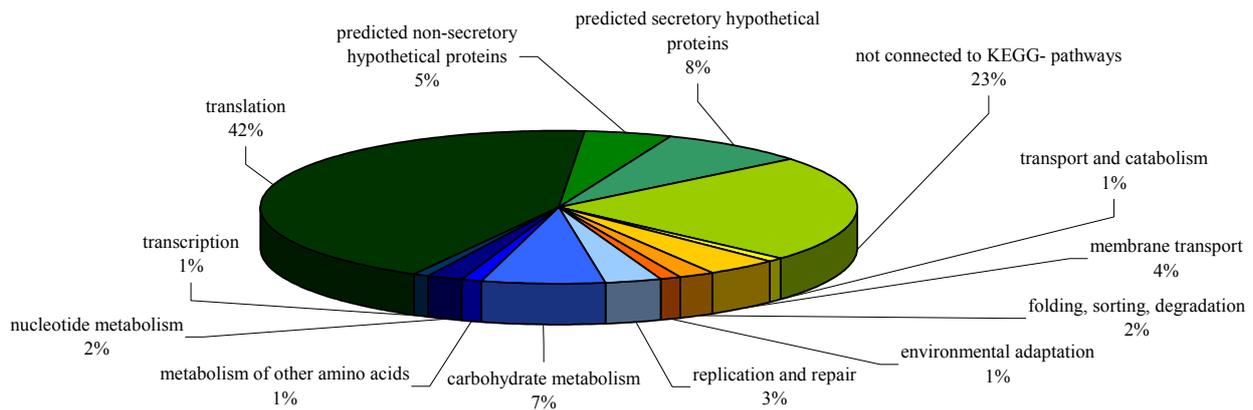


Figure 1. Functional categories of the 102 identified ‘*Ca. P. mali*’ strain AT proteins according to their connection to KEGG pathways. Proteins with assigned function but no connection to KEGG were grouped in “not connected to KEGG pathways” whereas those with no assigned function were grouped in “predicted secretory/non-secretory hypothetical proteins”.
(In colour at www.bulletinofinsectology.org)

Protein identification

Protein identification has been carried out using the Andromeda search engine (Cox *et al.*, 2011) in the MaxQuant V1.1.1.25 environment (Cox *et al.*, 2008) with a FDR at peptide and protein level of 1% and a maximum mass deviation for the fragment ions of 0.5 Da. A target-decoy database was constructed comprising 9002 *N. occidentalis* (<http://www.ncbi.nlm.nih.gov/>) and 497 ‘*Ca. P. mali*’ protein sequences (CU469464). In addition, 13,080 open reading frames (ORFs) with a minimum length of 20 amino acids were calculated from the “*Ca. P. mali*” strain AT genome sequence and also implemented in the database. A set of common contaminant proteins (<http://www.maxquant.org/>) like keratins has been included. A maximum of three missed cleavages was allowed for the protease trypsin. Oxidation of methionine and acetylation of the N-terminus have been applied as variable modifications. Carbamidomethylation was set as fixed modification. A protein hit has been considered as valid, if the protein was identified by at least two peptides, of which one had to be unique.

Results and discussion

Within these first experiments 102 proteins of ‘*Ca. P. mali*’ and 940 *N. occidentalis* were identified. While the plant derived proteins are still under investigation, a preliminary analysis of the ‘*Ca. P. mali*’ proteins was performed. Genes assigned to the KEGG (Kanehisa *et al.*, 2010) based functional category translation (figure 1) dominate the overall number of proteins. The nearly complete set of ribosomal proteins dominates this category. However, proteins of unknown function represent the third largest group of the identified proteins. A high portion of predicted secretory proteins without assigned function is remarkable and indicates the lack of information in phytoplasmas. As supposed the immunodominant membrane protein Imp was identified.

First results clearly indicate that the proteomic shotgun approach is successful applicable for the identification of expressed ‘*Ca. P. mali*’ proteins in plants. Analysis of the plant-derived proteins and of additional tissue from other *N. occidentalis* plants representing biological replicates is in progress.

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TAL effectors from *Xanthomonas*: design of a programmable DNA-binding specificity

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Abstract

Xanthomonas spp. are Gram-negative bacteria with powerful molecular weapons to attack their plant hosts. Key for pathogenicity of *Xanthomonas* is a type III secretion system that injects a cocktail of effector proteins into plant cells to function as potent virulence factors. TAL (transcription activator-like) effectors from *Xanthomonas* function as transcriptional activators of plant genes in the plant nucleus. They contain a central domain of tandem, near-identical 34 amino-acid repeats. Each repeat recognizes a single base pair in a contiguous DNA sequence and two adjacent hypervariable amino acids per repeat specify the base that is bound. This modular DNA-binding code allows a simple reprogramming of DNA-binding specificity, a feature with high potential for biotechnology. We developed a method called "Golden TAL Technology" that allows a flexible assembly of TAL proteins with a designed order of repeats.

Key words: Transcription factor, AvrBs3, zinc-finger proteins, golden gate cloning, TALE.

Introduction

Pathogenicity of plant pathogenic *Xanthomonas* relies on the delivery of virulence proteins, so called effectors, into target plant cells. Transport of effectors is facilitated by a specialized type III secretion system (Hrp) which spans both bacterial membranes and employs a hollow exterior conduit (the Hrp-pilus) that traverses the plant cell wall and delivers effectors across the plant plasma membrane via a translocon (Büttner and Bonas, 2010).

Typically, *Xanthomonas* strains harbor approx. 30 different effectors. In most cases their molecular activities are still unknown. TAL (transcription activator-like) effectors form a large and important effector family that is nearly exclusively found in *Xanthomonas* (Boch and Bonas, 2010). They function as transcription factors of plant genes and several TAL effectors induce expression of sugar exporters (Chen *et al.*, 2010).

TAL effectors contain a central domain of tandem near-identical 34-amino acid repeats (2 to 34). Each repeat binds to one DNA base pair in a contiguous sequence. Specificity of the repeats is determined by two hypervariable amino acids per repeat. Rearranging the repeats yields novel and predictable DNA-binding specificities (Boch *et al.*, 2009; Moscou and Bogdanove, 2009). TAL-nuclease fusions have been used to specifically edit the human genome (Miller *et al.* 2010).

Materials and methods

Golden TAL Technology

Four key TAL repeat types (NI, HD, NN, NG; table 1) were chosen that specifically recognize the four bases of the DNA. Individual TAL repeats were cloned with flanking *BpiI* sites. One to six repeats are ligated into an

assembly vector replacing a *lacZ* selection marker by *BpiI* cut-ligation (step I). Several repeat assemblies are combined together with fragments encoding N- and C-terminus of the TAL effector, respectively, into a target expression vector by *BsaI* cut-ligation (step II).

Cut-ligations were set up with 50-100 ng of each plasmid, 1 μ l restriction enzyme (*BpiI* or *BsaI*), 2 μ l ATP (10 mM), 2 μ l restriction buffer no. 4 (NEB), 1 μ l T4-ligase (5 u/ μ l) in a 20 μ l reaction. Reactions were incubated for 1 h at 37°C followed by 20 min. at 70°C to inactivate the enzymes. For further details please see (Geißler *et al.*, 2011).

Table 1. DNA-specificity of individual TAL effector repeats.

TAL repeats ^a	DNA specificity ^b
NI	A
HD	C
NN	G/A
NK	G
IG	T
NG	T
HG	T

^a According to amino acids 12 and 13 per repeat.

^b Only upper strand base is shown.

Results

Construction of designer TAL effectors

TAL effector repeats are highly repetitive on protein as well as DNA level which complicates cloning approaches. Our initially constructed artificial TAL effectors with novel repeat arrangements were based on

a random assembly of individual modules of four key repeats encoding a specificity for the four DNA bases, respectively (table 1). This cloning was possible, because exactly one restriction site for the type IIs restriction enzyme *Esp3I* is naturally present in each TAL repeat. Type IIs restriction enzymes have separate recognition and restriction sites, which lead to non-palindromic overhangs and a linear unidirectional ligation of TAL repeat fragments (Boch *et al.*, 2009).

To construct TAL effectors with a given order of repeats and, therefore, a programmed DNA-specificity, it was essential to align TAL effector repeats in a more controlled fashion. We improved our initial construction method and modified a method termed golden gate cloning (Engler *et al.*, 2008). In essence, golden gate cloning describes the assembly of a series of fragments containing specific overhangs that have been generated with flanking type IIs restriction enzymes. The fragments are designed such that correct ligation products lack the applied restriction site and are enriched in the reaction.

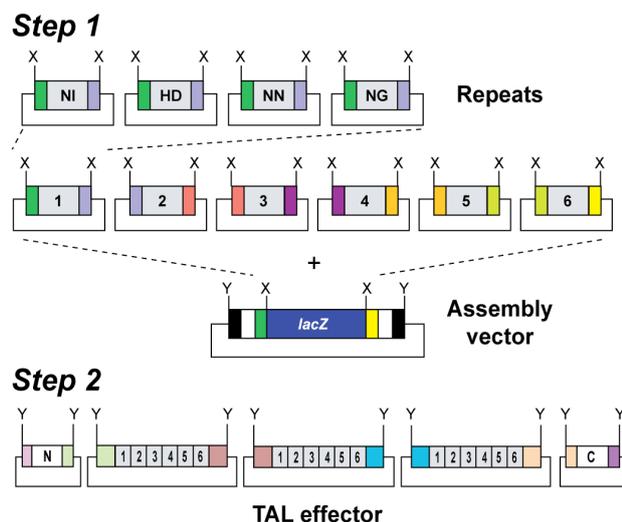


Figure 1. The golden TAL technology. TAL effectors with a designed repeat composition were assembled in two cut-ligation steps. X, *BpiI*; Y, *BsaI*. Same colors indicate matching overhangs. (In colour at www.bulletinofinsectology.org)

TAL proteins with a desired order of repeats were constructed using a two-step assembly technique. First, a library of four key repeats with specific flanking type IIs restriction sites was constructed. Up to six repeats were combined into an assembly vector (figure 1). Second, several assembly vectors were combined with fragments encoding N- and C-terminal regions of TAL effectors into an expression vector (Geißler *et al.*, 2011).

Our setup allows a very flexible generation of TAL effectors with any designed order of repeats. Generation of programmable DNA-binding specificities is now easily possible.

Discussion

The controlled assembly of TAL effectors with a designed repeat composition is key to use these proteins as programmable DNA-targeting devices. Our golden TAL technology allows an easy assembly of TAL effectors with designed repeats. In addition, it is flexible to incorporate N- and C-terminal variations, as well as different tags as modules in the cut-ligation reaction. This should greatly facilitate the adoption of this technique also for the generation of fusion proteins (e.g. TAL-nucleases) for biotechnology.

Several groups have recently developed similar techniques. The tools that are currently generated will enable a broad applicability of the TAL technology for different biological fields.

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European interlaboratory comparison and validation of detection methods for '*Candidatus Phytoplasma mali*', '*Candidatus Phytoplasma prunorum*' and '*Candidatus Phytoplasma pyri*': preliminary results

THE EUPHRESCO FRUITPHYTOINTERLAB GROUP

Abstract

A working group was established in the frame of EUPHRESCO Phytosanitary ERA-NET Project aimed to compare and validate diagnostic protocols for the detection of '*Candidatus Phytoplasma prunorum*', '*Ca. P. mali*' and '*Ca. P. pyri*' in fruit trees. Four molecular protocols were submitted to interlaboratory trials starting from extracted DNAs prepared in four laboratories. The tested molecular protocols consisted in universal and group-specific real time and conventional nested PCR assays. A good agreement among laboratories was obtained and high values of diagnostic sensitivity were revealed for all tested protocols. The preliminary analysis of the results also highlighted some diagnostic specificity problems that require further investigations.

Key words: ringtest, '*Candidatus Phytoplasma prunorum*', '*Candidatus P. mali*' and '*Candidatus P. pyri*'.

Introduction

In the competitiveness of agricultural products the phytosanitary quality is of increasing importance and harmonized protocols had taken an active role in the agricultural food chain.

In this context a working group was established in the frame of EUPHRESCO Phytosanitary ERA-NET Project aimed to compare and validate diagnostic protocols for the detection of '*Candidatus Phytoplasma prunorum*', '*Ca. P. mali*' and '*Ca. P. pyri*' in fruit trees. These phytoplasmas are agents of relevant diseases inducing severe crop losses in fruit trees and particularly, the last two phytoplasmas are included in the EPPO A2 List of quarantine pests.

Protocols based on conventional and real time PCR for the detection of above mentioned phytoplasmas were selected and submitted to ringtest trials performed in 22 European laboratories. Validation data (diagnostic sensitivity and specificity) were calculated and statistically analysed according with the UNI CEI EN ISO/IEC 17025.

In the present paper the preliminary results of the collaborative studies are reported.

Materials and methods

Design of the study

The ringtest program was scheduled from April 2010, for exchange of procedures, to February 2011 for laboratories trials and statistical analysis.

The 22 participant laboratories (table 1) analysed an identical series of 30 blind samples following the provided working protocols. The involvement of each participant laboratory in the ringtest is reported in table 2. In the analysis of the results all the participant laboratories are reported with anonymous number.

Table 1. List of participants involved in the interlaboratory trials.

Institution	Contact person	Location
AGES	Helga Reizenzein	AT
CRA-W	Stephen Steyer	BE
ILVO	Kris de Jonghe	BE
CLPQ	Zhelju Avramov	BG
ACW	Santiago Schaerer	CH
State Phytosan. Admin.	Gabriela Schlesingerova, Hana Orsagova	CZ
JKI	Bernd Schneider	DE
Aarhus Univ.	Mogens Nicolaisen	DK
DAR	Ester Torres, Joan Bech	ES
IRTA	Assumpcio Batlle, Amparo Laviña	ES
UPVLC	Isabel Font	ES
CRA-PAV	Graziella Pasquini, Luca Ferretti	IT
Phytosanitary Lab. Lombardy	Marica Calvi	IT
University of Bologna	Samanta Paltrinieri, Assunta Bertaccini	IT
BIOFORSK	Dag-Ragnar Blystad, Sonja Sletner Klemsdal	NO
PPS	Linda Kox, Jeanette Teunisse, Bart van de Vossenber	NL
Main Inspect. of Plant Health	Ewa Hennig, Justyna Moszczynska	PL
INRB/L-INIA	Esmeraldina Nascimento, Agostinho de Sousa, Eugenia Andrade	PT
UKSUP	Lubomir Horvath, Michaela Hudecoba	SK
NIB	Marina Dermastia, Natasa Mehle	SI
GDAR	Nursen Ustun, Aydan Kaya	TR
FERA	Adrian Fox, Anna Skelton	GB

Table 2. Numbers of laboratories involved in each tested protocol.

Number of labs involved	Nested PCR	Specific qPCR (Nikolic <i>et al.</i> , 2010)	Universal qPCR (Christensen <i>et al.</i> , 2004)	Universal qPCR (Hodgett <i>et al.</i> , 2009)	Plasmid
6	X	X	X	X	X
8	X	X	X	X	
2	X	X	X		
4	X				
1	X		X	X	
1	X	X		X	

To avoid manipulation of living quarantine organisms, the samples to be tested were constituted by extracted DNAs. Standards of a cloned P1/P7 fragments from ‘*Ca. P. mali*’ and ‘*Ca. P. pyri*’ in concentration from 10^7 to 10^1 were also included in the trials.

In order to standardize the experiments Taq DNA polymerase and real time master mix brand were specified and Taqman probes were supplied by the organizer.

Samples

A series of 30 samples, target (symptomatic and asymptomatic infected plants) and non-target (healthy plants and closely related bacteria) have been selected to perform all experiments (table 3).

Table 3. List of tested samples and their origin.

N°	Specie	Sanitary status	Origin
1	apple	healthy	JKI
2	apple	‘ <i>Ca. P. mali</i> ’	JKI
3	apple	‘ <i>Ca. P. mali</i> ’	JKI
4	apricot	‘ <i>Ca. P. prunorum</i> ’	CRA-PAV
5	apricot	‘ <i>Ca. P. prunorum</i> ’	CRA-PAV
6	plum	healthy	DAAM
7	pear	healthy	JKI
8	plum	healthy	DAAM
9	pear	‘ <i>Ca. P. pyri</i> ’	JKI
10	extracted DNA	bacteria	FERA
11	extracted DNA	bacteria	FERA
12	apple	‘ <i>Ca. P. mali</i> ’	JKI
13	pear	‘ <i>Ca. P. pyri</i> ’	JKI
14	extracted DNA	bacteria	FERA
15	apple	‘ <i>Ca. P. mali</i> ’	JKI
16	apple	‘ <i>Ca. P. mali</i> ’	JKI
17	extracted DNA	bacteria	FERA
18	pear	‘ <i>Ca. P. pyri</i> ’	JKI
19	apple	healthy	JKI
20	pear	‘ <i>Ca. P. pyri</i> ’	JKI
21	plum	healthy	DAAM
22	pear	‘ <i>Ca. P. pyri</i> ’	JKI
23	extracted DNA	bacteria	FERA
24	extracted DNA	bacteria	FERA
25	pear	healthy	JKI
26	apricot	‘ <i>Ca. P. prunorum</i> ’	CRA-PAV
27	pear	healthy	JKI
28	apricot	‘ <i>Ca. P. prunorum</i> ’	CRA-PAV
29	pear	healthy	JKI
30	plum	‘ <i>Ca. P. prunorum</i> ’	DAAM

From each tested sample the total DNA was extracted from midribs using CTAB protocol (Doyle and Doyle, 1990) in four laboratories (DAAM, JKI, CRA-PAV, FERA) and sent to each partner as dried DNA. All the laboratories were asked to re-suspend it in 500 µl of distilled water.

In all tested protocol undiluted and tenfold diluted extracted DNA from each sample was used as template.

Tested protocols. Four molecular protocols were submitted to interlaboratory trials:

1. AP group specific nested PCR (nested PCR);
2. real time for the specific detection of ‘*Ca. P. mali*’, ‘*Ca. P. pyri*’ and ‘*Ca. P. prunorum*’ (Nikolic *et al.*, 2010) (specific qPCR);
3. real time PCR for the universal detection of phytoplasmas (Christensen *et al.*, 2004) (universal qPCR-C);
4. real time PCR for the universal detection of phytoplasmas (Hodgett *et al.*, 2009) (universal qPCR-H).

Nested PCR (1)

The protocol is based on a direct PCR using the universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995), followed by a group specific nested PCR performed with the primer pairs fO1/rO1 (Lorentz *et al.*, 1995). Direct and nested PCR were performed in a 25 µl mixture containing: 1X Green GoTaq reaction buffer, 200 µM dNTPs (Promega), 0.4 µM of each primer, 0.625 U of GoTaq DNA polymerase (Promega), 1 µl of extracted DNA for direct PCR and 1 µl of P1/P7 amplicons diluted 1:30 for nested PCR (for 50 µl mixture: 2 µl DNA/diluted PCR product).

The cycling parameters included an initial denaturation step at 94°C for 2 min, followed by 36 and 38 cycles, for direct and nested PCR, respectively: 1 min at 94°C (denaturation), 1 min at 55°C and 50°C, for direct and nested PCR, respectively (annealing), 2 min at 72°C (extension) and a final extension step at 72°C for 8 min.

Specific qPCR (2)

The protocol consisted in a TaqMan real time PCR (qPCR) employing three specific FAM-MGB probes (AP-P, ESFY-P and PD-P) in separate reactions, using chemicals and amplification conditions reported in Nikolic *et al.* (2010).

Universal qPCR-C (3)

The protocol consisted in a TaqMan qPCR employing a FAM-TAMRA phytoplasma universal probe using chemicals and amplification conditions reported in Christensen *et al.* (2004).

Universal qPCR-H (4)

The protocol consisted in a TaqMan qPCR employing a VIC-TAMRA phytoplasma universal probe using chemicals and amplification conditions reported in Hodgetts *et al.* (2009). All the participants included their own positive and negative template controls. By qPCR, all samples were tested in duplicate. COX or human 18S rRNA (Applied Biosystem) was used as endogenous quality control of DNA extraction. qPCR were performed in 10 or 25 µl reactions.

Processing of the result data

The participants were asked to provide only '+' or '-' results for nested-PCR. Ct values for each replicate were asked for qPCR protocols, specifying threshold and baseline (manual or automatic).

The following parameters were calculated, using the R statistical framework (2010), to analyze the result:

- Agreement between laboratories - measured by calculation of the Kappa coefficient (Fleiss *et al.*, 2003) and interpreted as reported in Landis and Koch (1977). The output included also significance tests for the kappa index (the null hypothesis is a zero kappa value).
- Diagnostic sensitivity (SE) - an estimation of the ability of the method to detect the target.
- $SE = 100 \times TP / (TP + FN)$ (table 4);
- Diagnostic specificity (SP) - an estimation of the ability of the method not to detect the non-target.
- $SP = 100 \times TN / (FP + TN)$ (table 4).

If a laboratory had more than 10% of no-coincident replicates or more than 10% of unexpected differences between undiluted and diluted sample for one method, all the results obtained were omitted in the statistical analysis.

Table 4. Parameters for calculation of diagnostic specificity and sensitivity.

TP – true positive	positive detected from positive expected
FN - false negative	negative detected from positive expected
FP - false positive	positive detected from negative expected
TN - true negative	negative detected from negative expected

Results

All the participant laboratories carried out analysis on all DNA samples according to provided protocols. Results obtained with endogenous controls included in real time PCR protocols confirmed the good quality of all extracted DNAs. Analytical sensitivity obtained from standards of the cloned P1/P7 fragments from '*Ca. P. mali*' and '*Ca. P. pyri*' is reported in table 5. No relevant differences were observed among the protocols.

Agreement among laboratories. Fleiss' kappa index showed a 'almost perfect' agreement for all tested protocols (table 6), and p-values were almost 0. Diagnostic sensitivity and diagnostic specificity. SE and SP were calculated for each protocol and for each laboratory and mean values are reported in table 6. SE values resulted high for all protocols, ranging from 99.3% to 100%, whereas the specificity ranged from 93.8% to 99.7%.

Table 5. Analytical sensitivity of tested protocols calculated using serial dilutions of P1/P7 cloned fragment from '*Ca. P. mali*' and '*Ca. P. pyri*'. The analysis was not performed for universal qPCR-H, that amplify a region different from 16S-23S.

Laboratories	Nested-PCR		Specific qPCR		Universal qPCR-C	
	' <i>Ca. P. mali</i> '	' <i>Ca. P. pyri</i> '	' <i>Ca. P. mali</i> '	' <i>Ca. P. pyri</i> '	' <i>Ca. P. mali</i> '	' <i>Ca. P. pyri</i> '
1	n.t.	n.t.	10 ¹	10 ¹	10 ¹	10 ²
8	n.t.	n.t.	10 ¹	10 ¹	10 ¹	10 ¹
11	n.t.	n.t.	n.t.	10 ¹	10 ¹	10 ¹
12	10 ³	10 ¹	n.t.	n.t.	10 ¹	10 ²
13	n.t.	n.t.	10 ¹	10 ¹	10 ¹	10 ¹
16	10 ¹	10 ¹	n.t.	n.t.	n.t.	n.t.

Table 6. Diagnostic sensitivity (SE), Diagnostic specificity (SP) and agreement values calculated by the Fleiss' kappa index for each tested protocol.

Method	Number of laboratories considered	Mean		Kappa index*
		SE	SP	
Nested-PCR	20	99.3	97.7	0.940
Universal real time-C	10	100.0	96.0	0.926
Universal real time-H	12	99.4	97.2	0.945
Specific qPCR – ' <i>Ca. P. pyri</i> '	12	100.0	99.7	0.980
Specific qPCR – ' <i>Ca. P. mali</i> '	12	100.0	98.7	0.924
Specific qPCR – ' <i>Ca. P. prunorum</i> '	13	100.0	93.8	0.840

* Kappa values interpreting: <0 poor agreement; 0.00-0.20 Slight agreement; 0.21-0.40 Fair agreement; 0.41-0.60 Moderate agreement; 0.61-0.80 Substantial agreement; 0.81-1.00 Almost perfect agreement (Landis and Koch, 1977).

Discussion

The results obtained in the interlaboratory trials showed that all four tested protocols resulted sensitive. The robustness of the protocols was also supported by the agreement levels for the different participants using different thermo cyclers.

Nevertheless, diagnostic specificity values resulted affected by some unexpected results that open important questions and make necessary further investigations. Particularly, some non-target samples (plants assumed as healthy and phytoplasma related bacteria) gave positive results in different experiments and laboratories. In case of related bacteria the positive reactions can be explained as 'laboratory contamination', whereas in case of samples assumed as negative this result indicates the necessity to establish if the samples are 'true negative' or if they have a low titre of phytoplasma, detectable only by highly sensitive techniques.

Finally, in this ringtest the DNA extraction step have not been taken into consideration because living quarantine pathogens have to be manipulated as reported in CE 95/44 directive. Therefore the results are only related to the reliability of the amplification procedures, even if the nucleic acid extraction should be considered a critic step in phytoplasmas detection.

All the questions and problems derived from the preliminary analysis of the results have induced the participants to implement the experiments and further trials are in progress.

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Highlights on some EPPO activities in plant quarantine

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Abstract

One of the aims of EPPO is to help its member countries to prevent entry or spread of dangerous pests (plant quarantine). The Organization has therefore been given the task of identifying pests which may present a risk (early warning), evaluating their risk for the region and making proposals on the phytosanitary measures which can be taken against them (Pest Risk Analysis). To perform these activities, much information on pests presenting a risk to the EPPO region is required and is collected by the Organization and made available to its member countries. In addition, as identifying the pests is important EPPO has established since 1998, a work programme in the area of diagnostics to harmonize procedures across the region. The different activities conducted in the framework of this programme are presented.

Key words: pests, emergence, alert systems, diagnostics, accreditation.

Emerging plant diseases

Human societies have throughout their histories faced the emergence of new plant diseases which damaged crops or the environment. In plant pathology, the classical example remains the disastrous consequences of the introduction of potato late blight which caused famine in Ireland in the 1840s and now causes problems in potato production worldwide. In more recent history, many new plant diseases have emerged in different parts of the world, and this phenomenon seems to have accelerated. Although there is no agreed definition of what is an emerging plant disease, it can correspond to an already known disease whose incidence or geographical distribution is notably increasing but it may also be caused by newly described pathogens. The causes of plant disease emergence are multiple and quite complex, but it is generally accepted that human activities (e.g. trade of plants, accidental introduction of vectors, modifications of agricultural practices or land use) play an important role.

In the European and Mediterranean region, agriculture is an economically important sector covering a large variety of plants which are subject to an ever increasing trade and at the same time potentially threatened by a wide range of pests and diseases. Therefore, it is essential for Plant Protection Services to avoid the introduction and spread of new pests via commercial exchanges. Over the years, EPPO has made recommendations to its fifty member countries on phytosanitary measures which should be implemented to avoid the introduction of damaging pathogens (e.g. *Xanthomonas citri* pv. *citri*, *Liberibacter* species associated with citrus huanglongbing which are currently emerging in the Americas) or to prevent further spread of diseases which already occur in the region (e.g. Citrus tristeza virus, Plum pox virus). However, these existing phytosanitary measures can be challenged by the emergence of new diseases. In the EPPO strategy, it is felt essential to assess the risks associated with emerging diseases and, whenever appropriate, to propose management measures (i.e. restrictions on trade) against them. EPPO has

elaborated a Pest Risk Analysis (PRA) scheme which will be presented. When new diseases are emerging, it is also important to provide early warning to Plant Protection Services so that they can put into place import inspections and surveillance programmes on their territories. Since 1998, EPPO has set up an Alert List on its website (www.eppo.org) to provide data on emerging diseases (e.g. stolbur phytoplasmas, *Chalara fraxinea*, *Fusarium oxysporum* f. sp. lactucae, *Phytophthora kernoviae*, *Pseudomonas syringae* pv. *actinidiae*, viroids of solanaceous plants, new tomato viruses). Some of these emerging pathogens may later be submitted to a PRA and eventually be recommended for regulation as quarantine pests. When a quarantine status is considered appropriate for an emerging pathogen, EPPO Standards can also be developed in order to provide guidance on diagnostics, certification schemes, eradication and containment programmes.

Diagnostics

Since 1998, EPPO has established a work programme in the area of diagnostics to harmonize procedures across the region. The different activities conducted in the framework of this programme are presented.

Diagnostic protocols

In 1998, a programme was initiated to develop diagnostic protocols for as many as possible of the pests of the EPPO A1 and A2 lists (Zlof *et al.*, 2000). The preparation of protocols involves close collaboration between different Panels composed of diagnostic experts: the Panels on Diagnostics (coordination role), on Bacterial Diseases, on Nematodes, on Certification of Fruit Crops and the European Mycological Network. Each first draft is prepared by an individual expert according to a common format and should contain all the information necessary to detect and positively identify a particular pathogen or pest. The draft is then reviewed by relevant Panels. 92 diagnostic protocols for specific pests and 3 horizontal standards have now been

approved as (see www.eppo.org). 15 protocols are in different stages of preparation.

A survey on the use of the protocols was conducted in 2008 on a selection of 58 protocols in all disciplines of plant health diagnosis (Petter and Suffert, 2010). Laboratories registered in the EPPO database on Diagnostic Expertise (see below) were asked to indicate the number of samples that they tested in 2007 and which test they used. From this survey it can be concluded that many of the tests for detection mentioned in EPPO diagnostic protocols are widely used in laboratories in the EPPO Region.

Accreditation and quality management

In 2003, a separate Panel was created to develop standards on quality assurance (two standards have been developed so far OEPP/EPPO, 2007 and 2010). A joint informative document between EPPO and EA (European Co-operation for Accreditation, the European network of nationally recognised accreditation bodies) states that “EA will recommend that assessors from Accreditation Bodies take note of EPPO documents when evaluating plant pest diagnostic laboratories”. It is also envisaged to create a database where validation data from laboratories could be shared between EPPO countries. EPPO also organized two workshops on quality assurance in 2007 and 2009, to allow experts to share their experience on quality assurance and accreditation.

EPPO database on diagnostic expertise

In 2004, EPPO Council stressed that the implementation of phytosanitary regulations for quarantine pests was jeopardized by decreasing knowledge in plant protection. The Panel on Diagnostics proposed that an inventory should be made of the available expertise on diagnostics in Europe. The database on Diagnostic Expertise was created (Roy *et al.*, 2010) to allow identification of experts who can provide diagnosis of regulated species and those who can help in the identification of

new or unusual species. EPPO member countries were contacted and as of May 2010, 70 laboratories from 25 countries have provided data corresponding to more than 500 experts. These results are available in a searchable database on the EPPO website. The database can also help national accreditation bodies identify auditors for pest diagnostic laboratories for accreditation.

The EPPO Secretariat considers that these initiatives and future plans will aid the optimization of diagnostic activities in laboratories in the EPPO region.

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Difficulties with conventional phytoplasma diagnostic using PCR/RFLP analyses

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Abstract

Polymerase chain reaction (PCR) with subsequent restriction fragment length polymorphism (RFLP) analysis is often used for phytoplasma identification and classification. Although these techniques are very sensitive and specific, in some cases, nonspecific reactions, false positives and negatives results, as well as unusual or illegible profiles after RFLP analyses, amplification of plant host's DNA or other difficulties occurred. Experiences with suitability of positive and negative controls integration in PCR, evaluation of critical samples and other difficulties in phytoplasma PCR/RFLP identification are reported.

Key words: DNA extraction, PCR, primers, RFLP, positive and negative controls, critical samples.

Introduction

The 'Candidatus Phytoplasma' taxon comprises prokaryotic wall-less pathogens of the class *Mollicutes* that inhabit plant phloem and insects. Polymerase chain reaction (PCR) with primers from sequencing of randomly cloned phytoplasma DNA, from 16S rRNA, from ribosomal protein gene sequences, from SecY and Tuf genes, and from membrane associated protein genes opened paths for phytoplasma finer identification and classification. Restriction fragments length polymorphism (RFLP) analysis together with the sequencing of 16Sr phytoplasma genes was the first step on this way enabling the construction of phylogenetic trees of many micro-organisms especially in the *Mollicutes* taxon (Bertaccini, 2007). However, sequence similarity of phytoplasma with hosts plants or other micro-organisms genes, their low concentration and uneven distribution as well as presence of phenolic substances and other inhibitors, especially when the extraction is performed from woody plants can make their detection difficult. Experiences with phytoplasma detection and identification using PCR/RFLP analyses to show several difficulties and their resolution is demonstrated and discussed.

Materials and methods

The nucleic acid extraction was performed from the following phytoplasma reference strains in *Catharanthus roseus* kindly provided by A. Bertaccini: peanut witches' broom, PnWB (16SrII-A), peach X-disease, CX (16SrIII-A), German stone fruit yellows 1, GSFY/1 (16SrX-B), German stone fruit yellows 2, GSFY/2 (16SrX-B), Molière disease, MOL (16SrXII-A). Phytoplasma strains: aster yellows, AY (16SrI-B, host: *Calistephus chinensis*), clover phyllody, CPh (16SrI-C, host: *C. roseus*), clover yellow edge, CYE (16SrIII-B, host: *Trifolium pratense*), apple proliferation, AP (16SrX-A, host: *Malus x domestica* 'Matčino'), pear decline, PD (16SrX-C, host: *Pyrus communis*) previously identified

in our lab, and tissues from 18 healthy *C. roseus* plants were also used for DNA extraction. A phenol/chloroform method, a CTAB method and commercially available kits were tested. PCR assay was carried out with different primer pairs combination. To amplify region that includes the 16S rRNA gene, the spacer region, and the start of 23S rRNA gene of the phytoplasma genome, the primer pairs P1/P7 and P1A/P7A were used in direct PCR. PCR products were diluted with sterile distilled water (1: 29) prior to amplification by nested PCR using P1A/P7A, F1/B6, R16(I)F1/R, fU2/P7, fU5/rU3, 16R758F/16R1232R, F1/R0, Pc399/Pc1694, R16F2n/R2 and F1/B6, R162n/R2 primer pairs, respectively. Double nested PCR was carried out by several ways with subsequent primer pairs combinations: P1/P7-P1A/P7A-R16F2n/R2, P1/P7-F1/B6-R16F2n/R2, P1/P7-R16F2/R2-R16(I)F1/R1, P1/P7-F1/B6-16R758F/16R1232R, and P1/P7-F1/B6-fU5/rU3. About 20 ng of each DNA preparation in water were added to the PCR mix (Schaff *et al.*, 1992) in a final reaction mixture volume of 25 µl. The DNA was amplified by 35 cycles in a MJ Research thermocycler (Watertown, MA, USA). To reduce handling errors, in some cases PCR reactions were repeated up to 6 times. Approximately 200 ng of DNA of each positive PCR product from positive controls and DNA originating from 5 asymptomatic *C. roseus* plants, which revealed often positive signals in PCR, were separately digested from R16F2n/R2 amplicons. Digestions were carried out with 2.5 U of *MseI*, *AluI*, *HhaI* and *RsaI* restriction enzymes. Restriction patterns obtained were compared with positive controls and with those described in the literature (Lee *et al.*, 1998) after electrophoresis through an 8% polyacrylamide gel in 1x TBE buffer followed by staining with ethidium bromide and visualization under an UV transilluminator.

Results

DNA extracted by phenol/chloroform or CTAB techniques diluted in distilled water reacted positively also

after 5 years of storage in refrigerator. DNA extracted by used-friendly and quick commercial kits showed lower concentration and also low bands intensity under UV transilluminator after one month of storage in the same conditions as above or in conditions recommended by manufacturer. Some of these positives samples did not work in PCR or produced bands of nonspecific length after one year of storage.

Primers P1/P7-R16F2n/R2 and P1A/P7A-R16F2n/R2 amplified specifically DNA of all positive controls in nested PCR. Highly specific and sensitive was also the double nested PCR using P1/P7-F1/B6-R16F2n/R2 primer combination; no product was obtained by amplification of DNA from all 18 healthy *C. roseus* plants as well as from water. RFLP profiles with *Mse*I, *Alu*I, *Hha*I and *Rsa*I were in agreement with literature (Lee *et al.*, 1998). However the DNA from 5 asymptomatic out of 18 *C. roseus* examined, give amplification with some other primers combination. False positives were obtained sporadically using primer pairs combination P1/P7-fU5/P7, P1/P7-fU5/rU3 and exceptionally P1/P7-P1A/P7A. DNA amplicons from 5 healthy *C. roseus* plants, which gave positive reactions up to 9 primer combinations, were choose for RFLP. RFLP with all four endonucleases employed showed R16F2n/R2 patterns different from those characteristic for phytoplasmas. After repeated digestion, a very weak profile corresponding to ribosomal subgroup 16SrI-B was observed in one sample, with *Mse*I. The sequencing of this amplicon (1,500 bp) confirmed no phytoplasmas (data not shown).

Discussion

Though PCR/RFLP analyses are routine techniques for phytoplasma detection and identification, their still meet some difficulties, at least with some primers: several primer pairs and their combination are recommended (Heinrich *et al.*, 2001). Moreover, in some papers, non-specific PCR amplifications are mentioned. For example, Siddique *et al.* (2001) described after PCR amplification with P1/P6 primers besides the band of expected size, additional bands of different sizes. The same was observed with primer pairs Pc399/Pc1694, P1/U3 and M1/P7 in our analyses. According to Heinrich *et al.* (2001), some primers can induce dimers, bands of non-specific sizes. In these cases, false positives can be expected. In our hands, nested PCR with primer combination P1/P7-16R758F/16R1232R amplified products not only from all positive controls and asymptomatic *C. roseus* plants, but also with water used as template or when only master mix and primers were used for PCR amplifications. Similar reactions were observed using P1/P7-Pc399/Pc1694 and P1/P7-fU5/rU3 primer pairs in nested PCR. In the contrary, the same DNA samples amplified for example with P1/P7-P1A/P7A, P1A/P7A-R16F2n/R2 or P1/P7-P1A/P7A-R16F2n/R2, P1/P7-F1/B6-R16F2n/R2 never reacted with DNA from healthy *C. roseus* plants or with water controls.

According to our knowledge, it seems that in the case of phytoplasma positive samples, the primers preferentially amplified phytoplasma sequence of expected sizes, exceptionally, also additional bands could be observed. In the case of DNA isolated from healthy plants, some primers can react probably with sequences of plant genome or dimers and false positives could be observed. That is one of the reasons for including DNA originating from corresponding healthy plants and also water controls in PCR assays. In some cases, no visible products were obtained not only from healthy controls, but also from phytoplasma positive samples. This could be caused by inhibitor presence. In this case, higher dilution of DNA is advised (Heinrich *et al.*, 2001). The PCR alone is not sufficient enough for phytoplasma detection. Subsequent confirmation of phytoplasma presence and its identification must be accomplished at least by RFLP analyses using at least two or more endonucleases. In the case of critical samples, different primer pair combination, RFLP with more enzymes and also sequencing should be used for elucidation of phytoplasma presence.

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Sharing information and collections on phytoplasmas: from QBOL to QBANK

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Abstract

A total of 154 phytoplasma strains from 15 ribosomal groups were employed for barcode sequences production. Besides strains in periwinkle, 36 strains in natural infected plants such as napier grass, grapevine, plum, jujube, apple, pear, spartium, pine tree, hibiscus and erigeron were employed. Barcode sequences were produced for 16SrDNA, tuf and SecA gene for 36 phytoplasma strains, while for 54 strains 16SrDNA sequences were obtained, and for 118 and 89 strains respectively the tuf and secA barcode. All obtained sequences and protocol for extraction and PCR amplification will be available in Qbank.

Key words: phytoplasmas, collection, barcode, detection, quarantine.

Introduction

The increased international trade of plant material is increasing also exchanges of possible quarantine pathogens and a quick and reliable system for their identification is of the utmost importance.

DNA barcoding is a generic diagnostic method that uses short standardised genetic markers to aid species identification. The first genetic marker to be described as a “barcode” was the mitochondrial cytochrome *c* oxidase I (COI) gene which is used for species identification in the animal kingdom (Herbert *et al.* 2003). Among quality standards requirements for “barcodes” sequences are that the sequence data must be obtained from fully identified and vouchered specimens from a known origin with a unique identifier; sequence data must be at least 500 bp long and must be associated with trace files for the forward and reverse sequencing runs, and of forward and reverse primers used; primer sequences must be trimmed from the barcode sequence data (Hanner, 2009).

DNA barcoding protocols are under development and validation within the Quarantine organisms Barcoding of Life (QBOL) project financed by 7th framework program of the European Union. Using the developed DNA barcoding protocols, sequence data of EU regulated plant pests and phylogenetically related species was generated and will be made publicly available using the Q-Bank database.

The list of phytoplasma diseases that are of EU quarantine are summarized in table 1. It is important to underline that the quarantine list a number of diseases but it is well known that different phytoplasmas are associated to the same disease in diverse parts of the world. Therefore, phytoplasmas listed in table 1 were selected

as main representatives of those associated with the same disease worldwide. Based on this knowledge and in order to provide the most useful and robust tool possible a number of phytoplasma strains belonging to the majority of described taxons were barcoded and will be deposited in the QBank.

Materials and methods

Fresh or frozen phytoplasma infected plant material from periwinkle or from natural plant hosts was used after a chloroform/phenol extraction of total nucleic acid (Prince *et al.*, 1993). This DNA was then employed for sequencing the marker regions selected for reliable identification of quarantine phytoplasmas; for all markers a 400 - 600 bp fragment is suggested for the use of the barcode system.

A total of 154 phytoplasma strains from 15 ribosomal groups were employed for barcode sequencing. Besides strains in periwinkle also 36 strains in natural infected plants such as napier grass, grapevine, plum, jujube, apple, pear, spartium, pine tree, hibiscus and erigeron were employed. Strains belongs to the official collection of micropropagated phytoplasmas (Bertaccini, 2010), or were collected or provided by colleagues listed below. Specific protocol and primers are in validation phase in order to provide a quick and reliable tool for identification of quarantine phytoplasmas. The selected barcode regions are within the 16Sr DNA, tuf and SecA genes. The produced barcode sequences will be uploaded in the publicly available database that is in preparation (Qbank) where protocols for nucleic acid extraction and primers for amplification will also be available after the end of the validation process.

Table 1. List of EU quarantine phytoplasma-associated diseases.

Name of the disease	' <i>Candidatus</i> Phytoplasma'	16Sr DNA grouping of phytoplasmas Qbol target*	Other 16Sr DNA phytoplasmas associated with the disease*
Elm phloem necrosis	' <i>Ca. P. ulmi</i> '	16SrV-A	None
Peach rosette		16SrIII	16SrI
Peach X disease		16SrIII-A	None
Peach yellows		16SrIII	16SrI-B/16SrXII
Strawberry witches' broom		16SrI-C	' <i>Ca. P. fragariae</i> ', 16SrI-B, 16SrXII
Apple proliferation	' <i>Ca. P. mali</i> '	16SrX-A	None
Apricot chlorotic leafroll	' <i>Ca. P. prunorum</i> '	16SrX-B	None
Pear decline	' <i>Ca. P. pyri</i> '	16SrX-C	None
Palm lethal yellowing		16SrIV	16SrI/16SrXXII
Witches' broom on <i>Citrus</i>	' <i>Ca. P. aurantifolia</i> '	16SrII-B	16SrVI, 16SrIX
Grapevine flavescence doré		16SrV-C/-D	None
Potato stolbur		16SrXII-A	16SrI-A, 16SrI-C, 16SrII
potato purple top wilt	' <i>Ca. P. americanum</i> '	16SrXVIII-A	16SrVI

* Groups and subgroups are according with Lee *et al.*, 1998 and as in Bertaccini and Duduk, 2009.

Results

For a total of 26 phytoplasma strains all the three barcode sequences were produced, while for 54 strains 16SrDNA sequences were obtained, and for 118 and 89 strains, respectively, *tuf* and *secA* barcode sequences were obtained.

For the naturally infected plants, 4 strains were sequenced in the 16S rDNA, while 74 sequences were obtained for *tuf* gene and 54 sequences for the *secA* gene. The project is still in progress and more sequences are under production.

Discussion

The three barcode sequences employed in the project will allow to unambiguously detect and identify quarantine phytoplasmas in a short time and with no need of specific phytoplasma expertise in the laboratory. From the research carried out, the *tuf* gene fragment was shown to be able to differentiate the majority of phytoplasmas enclosing those in the EU quarantine list (table 1) and together with the 16S rDNA appears to be very helpful in barcoding of phytoplasmas. Further work is in progress toward increasing number of sequences to be deposited in the Qbank.

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