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 (Rodriguez 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 ampicillin (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 TruI 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.

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).
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 1.** Numbers and percentages of morphological groups and OTUs obtained from culture-dependent and culture-independent methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Morphospecies</th>
<th>Morphological identification</th>
<th>OTUs</th>
<th>Unique OTUs</th>
<th>Shared OTUs</th>
<th>Total OTUs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture-dependent</td>
<td>21 (38%)</td>
<td>10 (18%)</td>
<td>29 (53%)</td>
<td>16 (29%)</td>
<td>13 (23%)</td>
<td>55 (100%)</td>
</tr>
<tr>
<td>Culture-independent</td>
<td>/</td>
<td>/</td>
<td>39 (74%)</td>
<td>26 (47%)</td>
<td></td>
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</tbody>
</table>

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 biocontrol agents acting like inducers of recovery.

References


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