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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 (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 *Tru*1I and *Hpa*II 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
Alternaria sp.	39%	36%
Phoma sp.	17%	5%
Epicoccum sp.	14%	4%
Aureobasidium sp.	10%	17%
Cladosporium sp.	9%	13%
Pestalotiopsis sp.	1%	6%
Pestalotia sp.	1%	1%

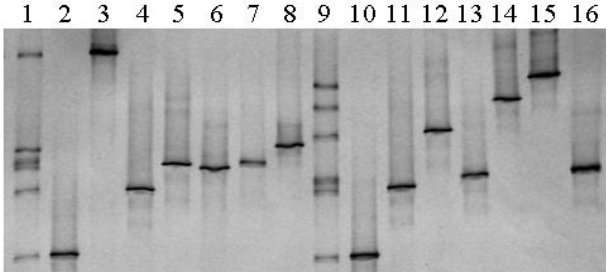

Figure 1. Example of DGGE patterns obtained from different grapevine fungal endophytes. Lanes 1 and 9: reference markers.

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 biocontrol agents acting like inducers of recovery.

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