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Long- term grapevine cultivation and agro-environment affect rhizosphere microbiome rather than plant age

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ABSTRACT

Single plant replacement is one of the most commonly adopted practices to overcome vineyard crop decline associated with grapevine trunk diseases. A study was undertaken in long-term cultivated vineyards of an important wine producing district in northern Italy to investigate whether rhizosphere microbiome differed between adult and young replaced plants.

Fungal and bacterial communities in adult and young replaced plants were investigated in ten vineyards across four geographic areas within the district. General and specific fungal and bacterial primers were used for PCR-DGGE and qPCR analysis. Moreover, quali-quantitative evaluation of *Cylindrocarpon*-like fungi, agents of foot rot belonging to the disease complex involved in vineyard decline, was performed with specific primers using nested PCR.

Fungal and bacterial communities did not differ between adult and young plants, conversely they differed in geographic areas. While *Pseudomonas* and *Bacillus* communities varied mostly according to geographic origin, *Ascomycota* (microfungi) and *Basidiomycota* (white-rot fungi) also varied between the vineyards within each area. Furthermore, *Cylindrocarpon*-like species showed an ubiquitous occurrence, regardless plant age.

Results show that long-term plant growth legacy overcomes plant age in shaping rhizosphere microbiome. Although composition of rhizosphere microbiome does not seem to represent a bio-indicator of vineyard decline according to findings of this study, the site-dependent variation of microbiome indicates that part of the complex phenomenon of crop decline may be due to a variable interaction between a series of soil biotic components and physiological state of grapevines.

1. Introduction

Specialized perennial tree and shrub crops are frequently affected by a progressive reduction of plant vigor and health which is generally defined as “crop decline”. Severity of this complex disease can vary from vigor and yield reduction up to plant stunting or dieback (Mazzola and Manici, 2012; Ogawa and English, 1991). In most cases, the biotic origin of this phenomenon has been demonstrated (Browne et al., 2006; Manici et al., 2013), though plant physiological state and abiotic stress can mediate severity (Berger et al., 2007). Nevertheless the main cause remains elusive so much so that a multiple series of pathogens is often quoted as responsible, and fully-effective techniques for controlling the disease have yet to be identified (Mazzola and Manici, 2012). On the other hand, an unspecified fruit tree crop decline has been increasing over recent decades due to an increase of abiotic stress, extreme events and land use (Gramaje et al., 2016; Parker and Warmund, 2011).

One of the most emblematic cases of economic damage worldwide is the decline of vineyards associated to Esca syndrome or grapevine trunk disease (Bertsch et al., 2013; Larignon et al., 2009; Mugnai et al., 1999). This disease has shown a progressive trend over the past two-three decades and has reached dramatic proportions in the main vine producing countries such as France, where 12% of vineyard areas is currently affected; Italy, where up to 50% of cumulated esca disease was estimated in some vineyards in 2006; as well as Argentina, Austria and California (Bertsch et al., 2013; Hofstetter et al., 2012; Surico et al., 2006; Van den Boch et al., 2011). A series of fungal species such as *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp., and various white rot fungi, have been associated with this chronic wood disease (Bruno and Sparapano, 2007; Cloete et al., 2014; Kuntzmann et al., 2015), while many other fungal species, or complex of species, have been associated to several other co-occurring diseases such as *Cylindrocarpon*-like fungi and *Eutypa lata* associated to black-foot disease and eutypiosis respectively (Halleen et al., 2007; Mugnai et al., 1999; Petit and Gubler, 2005; Úrbez-Torres et al., 2009). Nevertheless, since the

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primary causal agents and the co-causal agents of grapevine trunk diseases have not yet been fully elucidated, a number of fungal agents has been progressively associated to this phenomenon which is often reported as “complex” (González and Tello, 2011; Hofstetter et al., 2012; Riou et al., 2016).

Grapevine trunk diseases appear in adult plants that are over seven years old (Martin and Cobos, 2007; Mugnai et al., 1999) and disease progression is gradual. Therefore, since a fully effective protocol for controlling the disease does not exist, when it appears in vineyards, single plant replacement of dead grapevine plants and hard pruning down to the healthy wood are the most applied practices to counteract the phenomenon and to avoid replanting whole grapevines in vineyards of specialized growing areas (Becker and Oberhofer, 2009; Hofstetter et al., 2012; OIV, 2016). Several studies indicate that plants have a strong effect on rhizosphere microbial communities composition, especially bacteria, through root exudates composition (Smalla et al., 2001). This has been reported to be linked to a series of environmental and agronomic factors among which plant species, soil type, management and history seem to prevail (Haichar et al., 2008; Kowalchuk et al., 2002; Lupwayi et al., 1998); also plant age and physiological state of plants have been found to affect rhizosphere bacterial composition (Cavaglieri et al., 2009; Lupwayi et al., 1998; Marques et al., 2014). If on one hand a number of phytopathological studies concerning fungal endophytes associated with Esca symptomatic and asymptomatic plant tissues and nursery plants has been performed (Bertsch et al., 2009; Hofstetter et al., 2012), of the other side little investigation has been carried out on soil microbiome associated with vine roots (rhizosphere) in adult and young replaced plants which escape disease in vineyards showing Esca disease or a more general progressive health decline. Based on the abovementioned considerations, a study was undertaken to evaluate whether young transplanted grapevines could affect the rhizosphere microflora differently from adult ones.

2. Materials and methods

2.1. Vineyard locations and sampling

This study was conducted in the ‘Prosecco di Conegliano-Valdobbiadene’ wine district across 15 municipalities in the Veneto Region. The area is primarily hilly, extending for approximately 35 and 25 km in the East–West, and North–South directions respectively, centered at 45.95N e 12.17E. Elevation ranges from 50 to 500 m a.s.l., and the majority of vineyards is grown between 100 and 300 m a.s.l., covering a surface of about 9000 ha. The area is characterized by a warm temperate climate according to the Köppen and Geiger climate zones (http://koeppen-geiger.vu-wien.ac.at/alps.htm). In the period 1994–2016, the average total annual rainfall was 1438.9 mm. November and February are months with respectively the highest (176.3 mm) and the lowest (75.1) mean precipitation. The warmest month is July, with a mean temperature of 23.2 °C while that of the coldest one (January) is 3.7 °C (averages of six weather stations distributed in the area for the period 1994–2016) (Supplementary material Fig. S1). This is one of the most intensive wine producing areas of northern Italy with a surface of 24,792 ha, where esca decline generally appears ten to twelve years after transplant and plant replacement is the most applied control option. The latter is an efficient way to counteract worldwide yield and quality losses caused by trunk diseases and to limit dissemination of the disease (Bruno and Sparapano, 2007; OIV, 2016).

Soil sampling was carried out in four areas of the wine district Prosecco (Interactive map viewer online KML file). Each area selected for sampling was located within 10 km from the others and represented four different agro-environments (Table 1). The soil carbon content varied from 1.2 to 2.4% in three out of four areas; in one area only was soil carbon content lower (0.7–1.2%) (Table 1).

Based on the observation that symptoms of trunk diseases appear often in over 7-year-old vineyards (Díaz and Latorre, 2013), a total of ten vineyards over twelve years in age were selected according to the following criteria: i) esca decline symptoms observed with variable severity in the last 4–6 years; ii) plant replacements in the last 3–6 years (Table 1). At least two vineyards per area were selected regardless of the cultivars, which were amongst the main ones grown locally such as Glera, Merlot and three others, as reported in Table 1. The rootstocks were those commonly adopted in the ‘Prosecco’ wine district: Kober 5BB, Millardet et de Grasset 420A and Selection Oppenheim (SO4). In all cases, vineyards had high-density planting, varying from 2220 to 2500 plants ha^{-1}. Counter-espalier is the most widely adopted growing system with a permanent inter-row vegetative ground cover and periodic tillage or chemical weed control on the planted rows. Agronomic management of the vineyards was generally the same, except for small differences due to different properties.

Rhizosphere soil samples were collected in mid-July 2015 at a depth of 0–25 cm under the canopy of two adult and two replaced plants in each vineyard (Table 1). Adult plants either showed typical symptoms of Esca disease or were selected in plots showing high disease frequency in the last years. Soil samples adhering to roots were taken and mixed to obtain a homogeneous sample of 2 kg per treatment in each vineyard. A soil subsample of 100 g was randomly taken from each original rhizosphere soil sample; it was, air dried at room temperature, sieved through a 2 mm mesh, and stored in 50 ml sterile vials at −80 °C until processed. DNA extraction was performed in two samples per treatment.

2.2. DNA isolation and PCR-DGGE analyses

Total soil DNA was extracted from two 0.25 g samples of rhizosphere soil (dry weight) using PowerSoil DNA Isolation kit according to manufacturer’s instructions (MoBio Laboratories, Carlsbad, CA, USA) and stored at −20 °C until use. Total soil DNA quantification and purity control (ratio of absorbance at 260/280 nm around 1.8) was performed using Infinite 200 NanoQuant (Trading AG, Switzerland). Amplification prior to DGGE (Denaturing Gradient Gel Electrophoresis) analysis was performed using a nested PCR approach. A first PCR reaction was carried out for DNA amplification of total fungi, ascomycetes and basidiomycetes using the following primer pairs: ITS1F/ITS4, ITS1F/ITS4A and ITS1F/ITS4B respectively (Table 2). The first PCR reactions were carried out using a TGradient Thermal Cycler (Biometra, Goettingen, Germany) in 25 µl reaction volumes containing 1 µl of total soil DNA, 10 pmol of each primer, 1.88 mM $MgCl_2$, 1× buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 200 µM dNTPs mix and 1.25 U Taq polymerase (Invitrogen, Carlsbad, CA, USA). Cycling parameters were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s; 55 °C for 30 s; 72 °C for 30 s, and final extension at 72 °C for 10 min. Amplicons obtained from the first reactions were used as template (1 µl) for a nested PCR with the primer pair ITS1F-GC/ITS2 for fungi in general (Table 2), using the same conditions described above. A 40-nucleotide GC clamp was inserted on the 5′ end of the forward primer ITS1F to prevent complete melting of PCR products during DGGE runs (Muyzer et al., 1993). A negative control without template DNA was included in every PCR run.

A double gradient DGGE gel was prepared by using both a 6–8% acrylamide porous gradient and a 40–60% urea/formamide denaturing gradient (Cremonesi et al., 1997). These conditions permitted to obtain optimal separation of DGGE bands. DGGE analysis was performed with a D-code system (Bio-Rad Laboratories, Hercules, CA, USA). PCR products (200–250 ng) were loaded on DGGE gel and electrophoresis was run in 1× TAE buffer at a constant voltage of 60 V at 60 °C for 16 h. Following electrophoresis, gel was stained with GelRedTM (Biotium) at 10,000× dilution in 1× TAE for 30 min, washed in dH2O for 20 min, and photographed using an Alpha Image UV illuminator (Alpha Innotech, San Leandro, CA, USA). DGGE analysis was repeated three