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Tissue age and plant genotype affect the microbiota of apple and pear bark

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ABSTRACT

Plant tissues host complex fungal and bacterial communities, and their composition is determined by host traits such as tissue age, plant genotype and environmental conditions. Despite the importance of bark as a possible reservoir of plant pathogenic microorganisms, little is known about the associated microbial communities. In this work, we evaluated the composition of fungal and bacterial communities in the pear (Abate and Williams cultivars) and apple (Golden Delicious and Gala cultivars) bark of three/four-year-old shoots (old bark) or one-year-old shoots (young bark), using a meta-barcoding approach. The results showed that both fungal and bacterial communities are dominated by genera with ubiquitous attitudes, such as *Aureobasidium*, *Cryptococcus*, *Deinococcus* and *Hymenobacter*, indicating intense microbial migration to surrounding environments. The shoot age, plant species and plant cultivar influenced the composition of bark fungal and bacterial communities. In particular, bark communities included potential biocontrol agents that could maintain an equilibrium with potential plant pathogens. The abundance of fungal (e.g. *Alternaria*, *Penicillium*, *Rosellinia*, *Stemphylium* and *Taphrina*) and bacterial (e.g. *Curtobacterium* and *Pseudomonas*) plant pathogens was affected by bark age and host genotype, as well as those of fungal genera (e.g. *Arthrinium*, *Aureobasidium*, *Rhodotorula*, *Sporobolomyces*) and bacterial genera (e.g. *Bacillus, Brevibacillus*, *Methylobacterium*, *Sphingomonas* and *Stenotrophomonas*) with possible biocontrol and plant growth promotion properties.

1. Introduction

Plant tissues and their surfaces host endophytic and epiphytic microbial communities that can establish beneficial, detrimental or neutral associations with their host (Lodewyckx et al., 2002). These plant-associated microorganisms can affect host growth and health by interfering with regulatory pathways (Berlec, 2012), producing hormones (Schlaeppi and Bulgarelli, 2015), antagonising pathogens (Ritpitakphong et al., 2016), enhancing nutrient uptake from soil and protecting from abiotic stresses (Yang et al., 2009), eventually playing a role in the adaptation of plants to the environment (Bulgarelli et al., 2013). In addition, in grapevine the resident endophytic and epiphytic bacterial and fungal populations can also affect fruit flavour and wine quality (Barata et al., 2012; Gilbert et al., 2014). In terms of composition and abundance, plant-associated microbial communities have a complex microbial structure that is influenced by several factors, such as organ age, plant genotype, environmental conditions and agronomical practices (Bodenhausen et al., 2014; Leff et al., 2015; Vorholt, 2012; Whipps et al., 2008). In particular, leaf age affected the composition of bacterial communities on cucumbers (Suda et al., 2009) and lettuce (Williams et al., 2013), as well as fungal communities on giant dogwood (Osono and Mori, 2005) and plum (Pimenta et al., 2012). The host genotype is another important driver that influences the composition of associated microbial communities. According to Redford et al. (2010), perennial plants belonging to the same species grown in different regions showed surprisingly similar phyllosphere communities as compared with different plant species living in close proximity. Moreover, the host species is known to affect the composition of rhizosphere bacterial communities of maize, oat and barley (Berg and Smalla, 2009; Garbeva et al., 2008), as well as the phyllosphere bacterial and fungal population of *Arabidopsis thaliana* (Bodenhausen et al., 2014) and poplar (Bálint et al., 2013), respectively. Likewise, the plant cultivar is known to influence plant-associated microbial communities, such as bacterial populations of the tomato phyllosphere (Correa et al., 2007) and endophytic communities of potato roots (Manter et al., 2010). Environmental factors and agronomic practices also influence the composition of plant-associated microbial communities. For example, sugarcane- (Hamonts et al., 2017)

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and grapevine-associated microbial populations (Perazzolli et al., 2014) were affected by the growing region, and fungal communities of apple fruit varied with organic and conventional management (Abdelfattah et al., 2016).

Most studies on the composition and dynamics of plant-associated microbial communities have focused on the rhizosphere, phyllosphere and bulk soil (Berendsen et al., 2012; Bulgarelli et al., 2013; Rastogi et al., 2012; Wakelin et al., 2008), while only a few of them have studied flower (Junker et al., 2011; Shade et al., 2013), fruit (Abdelfattah et al., 2016; Martins et al., 2013) and bark microbial communities (Buck et al., 1998; Lambais et al., 2014; Leff et al., 2015). In particular, bark has been demonstrated to host many saprophytic, pathogenic and beneficial microorganisms (Buck et al., 1998; Martins et al., 2013). In addition, in grapevine it has been demonstrated that diversity and richness of bacterial species was greater in bark than in leaf and fruit (Martins et al., 2013). Bark partially shares its bacterial communities with soil (Martins et al., 2013) and leaves (Lambais et al., 2014), strengthening the hypothesis of a common origin of above-ground and underground microbial communities associated with plants (Zarraonaindia et al., 2015). Bark represents a harsh environment for microbial growth (Buck et al., 1998): it is dry and poor in nutrients, as well as rich in polymers recalcitrant to degradation, such as lignin, cellulose and hemicellulose (Valentín et al., 2010), and it is known to release secondary metabolites, such as volatile organic compounds, which can inhibit microbial growth (Pearce, 1996). Therefore, to survive on bark surfaces, microorganisms colonise microsites, such as cracks and lenticels, which represent a more favourable environment for microbial growth, because they may retain humidity and nutrients (Buck et al., 1998). Despite the potential role of bark as a reservoir of beneficial and pathogenic microorganisms (Buck et al., 1998; Martins et al., 2013), few studies have been carried out to unravel the composition of microbial communities residing on this plant tissue. The aim of this work was to compare, with a meta-barcoding approach, the composition of fungal and bacterial communities in pear bark (*Pyrus communis*; Abate and Williams cultivars) and apple bark (*Malus domestica*; Golden Delicious and Gala cultivars), comparing three/four-year-old shoots (old bark) with one-year-old shoots (young bark).

2. Materials and methods

2.1. Sample collection and isolation of bark microorganisms

In order to minimise the influence of environmental conditions and plant physiological state on plant-associated microbial communities, samples were collected at the same time during the dormancy stage (12 January 2016) from plants of the same age, grown in the same environmental conditions and managed with identical agronomical practices in an experimental orchard planted in 2011 in San Michele all'Adige (northern Italy; latitude, N46.190723; longitude, E11.135518; altitude, 228 m). The experimental orchard was managed according to standard agronomic practices. In particular, the applied fungicides included dithianon, pyrimetanil, ziram, iprodione, penconazole, boscalid and copper-based products, and the last treatment (copper hydroxide) was applied in November 2015. The daily mean temperature ranged from −2.1 to 5.4 °C, with mean relative humidity of 88.2% in the week before sample collection.

Bark samples (curls 20 mm long, 5 mm wide and 1 mm thick) were collected in triplicate (named from 1 to 3) from randomly chosen bark of three/four-year-old shoots (old bark) or one-year-old shoots (young bark) of Abate and Williams pear cultivars and Golden Delicious (Golden) and Gala apple cultivars. Samples were collected in the orchard following a split-plot sampling design, where the first factor was bark age and the additional factors were plant species and cultivar. Each sample consisted of a pool of 30 bark curls (corresponding to 0.5 g) collected from five plants. Bark samples were collected using a fire-sterilised scalpel, kept in ice and ground into sterile stainless steel jars with 2.5 ml of a cold (4 °C) sterile isotonic solution (0.85% NaCl) using a mixer-mill disruptor (MM 400, Retsch, Germany) at 25 Hz for 45 s. The viability of culturable fungi and bacteria was assessed using the classic plating method, as described by Cappelletti et al. (2016), on potato dextrose agar media (Oxoid, Basingstoke, United Kingdom) supplemented with 2.5% lactic acid, and on nutrient agar (Oxoid) supplemented with 100 mg/l of cyclohexymide, respectively. Plates were kept at room temperature and the number of colony forming units (CFUs) per gram of bark fresh weight (CFUs/g) was determined after seven and five days, for fungi and bacteria, respectively. The remaining ground samples were stored at −20 °C in 500 μl aliquots until DNA extraction.

Bark surfaces were observed using a Nikon SMZ800 stereoscope with Nikon C-W10XA/22 oculars and an external source of white light. Images were captured using a Nikon Digital Sight DS-Fi1 digital camera with 10× magnification.

2.2. Genomic DNA extraction, amplicon library preparation and sequencing

DNA was extracted from the bark samples using the FastDNA spin kit for soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. For the identification of fungi, the internal transcribed spacer 2 (ITS2) was amplified using the primer ITS3 forward (5′-CATCGATGAAGAACGCAG-3′) (Tedersoo et al., 2014) and ITS4 reverse (5′-TCCTSSSCTTATTGATATGC-3′), modified from Tedersoo et al. (2014). For the identification of bacteria, the V5-V7 region of 16S rDNA was amplified using the primer 799 forward (5′-AACMGGATTAGATACCCKG-3′) (Chelius and Triplett, 2001) and 1175 reverse (5′-ACGTCRTCCCCDCCTTCCT-3′) (Bonder et al., 2012). The forward and reverse primers included the specific overhang Illumina adapters for amplicon library construction (5′-TCGTCGGCAGCG TCAGATGTGTATAAGAGACAG-3′ and 5′-GTCTCGTGGGCTCGGAGATG TGTATAAGAGACAG-3′, respectively).

Amplicons were obtained from 3 ng of DNA using the FastStart High-Fidelity PCR system (Roche, Branford, CT, USA) with 0.25 mM of each deoxynucleoside triphosphate (dNTP), 0.25 mg bovine serum albumin (BSA), 4% dimethyl sulfoxide, 0.3 μM of each primer and 2.5 U of FastStart High Fidelity DNA polymerase (Roche) in a final volume of 50 μl. The thermal cycling profile consisted of a denaturation step at 95 °C for 5 min, followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 1 min and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 10 min 16S amplicons were purified by agarose gel separation, followed by the NucleoSpin Gel and PCR Clean-up purification kit (Macherey-Nagel, Düren, Germany) in order to eliminate contaminants. Subsequently, dual indices and Illumina sequencing adapters Nextera XT Index Primer (Illumina) were attached to ITS and 16S amplicons by seven PCR cycles according to the 16S Metagenomic Sequencing Library Preparation kit (Illumina). After purification by the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA), libraries were analysed on a Typestation 2200 platform (Agilent Technologies, Santa Clara, CA, USA) and quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) by the Synergy2 microplate reader (BioTek, Winooski, VT, USA). All the libraries were pooled in an equimolar amount in a final amplicon library, analysed on a Typestation 2200 platform (Agilent Technologies) and sequenced on an Illumina MiSeq (PE300) platform with MiSeq Control software (version 2.5.0.5) and Real-Time Analysis software (version 1.18.54.0). Sequences were deposited at the Sequence Read Archive of NCBI (http://www.ncbi.nlm.nih.gov/sra) under the accession number SRP125675 and BioProject number PRJNA419865.

2.3. Bioinformatic analysis

Amplicon read data were processed as previously described (Mitter et al., 2017). SILVA 123 (Quast et al., 2012) and UNITE 7.1 (Abarenkov

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