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**Doctoral Thesis**

# Composition, dynamics and properties of apple and pear bark microbiota under different environmental conditions and disease management strategies

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# Abstract

Plants host complex fungal and bacterial communities on and inside various organs. These microbial populations could have beneficial and detrimental effects on their host. Despite the importance of plant bark as potential reservoir of plant pathogens and beneficial microorganisms for perennial crops, no information is available on the composition of bark microbiota under different climatic and agronomic conditions. Due to its agricultural and economic relevance, apple (*Malus domestica*) was selected as model system, in order to characterise the composition and functional properties of the bark microbiota. Most of the commercially relevant apple cultivars are susceptible to several destructive diseases and require intense use of fungicides. Scab-resistant apple cultivars allow the reduction of fungicide treatments and are compatible with low-input disease management strategies. However, fungicides applied to control apple scab can also control secondary pathogens and the reduction of fungicide applications on scab-resistant cultivars may cause the outbreak of emerging pathogens hosted on bark tissues, as for example the canker agent *Diplodia seriata*. Although the risk of emerging secondary pathogens is real under low-input disease management strategies, no information is available on the composition and dynamics of bark-associated microbial communities. The main aims of this work are i) to develop metabarcoding-based approaches for the precise study of bark-associated microbial communities and to investigate proportions of plant pathogens and beneficial microorganisms associated with young (one year-old shoots) and old barks (three/four-year-old shoots), ii) to identify impacts of environmental factors (orchard location and climatic conditions), host properties (plant species, cultivar and tissue age) and agricultural practices (integrated and low-input disease management) on the bark microbiota composition and iii) to evaluate fungicide impacts against a scarcely studied canker agent of apple under controlled conditions.

A metabarcoding approach was optimized to characterise the composition of bark-associated microbial communities of two apple and two pear cultivars (Chapter II). Several potential plant pathogenic fungi (e.g. *Alternaria* spp., *Diplodia* spp., *Gibberella* spp., *Peltaster* spp., *Penicillium* spp., *Phoma* spp., *Rosellinia* spp., *Stemphylium* spp. and *Taphrina* spp.) and bacteria (e.g. *Pseudomonas* spp., *Clavibacter* spp., *Curtobacterium* spp., *Nocardia* spp.and *Pantoea* spp.) were found in presence of beneficial fungi (e.g. *Arthrinium* spp., *Aureobasidium* spp., *Cryptococcus* spp., *Rhodotorula* spp.,

*Saccharomyces* spp. and *Sporobolomyces* spp.) and bacteria (e.g. *Bacillus* spp., *Brevibacillus* spp., *Burkholderia* spp., *Deinococcus* spp., *Lactobacillus* spp., *Methylobacterium* spp. and *Sphingomonas* spp.). The composition of bark communities was affected by the tissue age, plant species and cultivar and it strongly differed between young (one year-old shoots) and old barks (three/four-year-old shoots). The optimised protocol was then applied to characterize the bark-associated communities of the scab-resistant apple cultivar Fujion under integrated and low-input disease management strategies in two different locations and consecutive seasons (Chapter III). In addition to the tissue age, orchard location and environmental conditions strongly affected fungal and bacterial richness and diversity of the bark communities. In particular, the orchard location affected the abundance of some potential plant pathogens, such as *Alternaria* spp., *Cadophora* spp., *Diaporthe* spp. *Diplodia* spp., *Phoma* spp. *Curtobacterium* spp., *Pantoea* spp. and *Ralstonia* spp. Moreover, the abundances of *Cryptococcus, Leptosphaeria, Curtobacterium* and *Pseudomonas* genera differed according to the disease management strategy, suggesting that it can shape the composition of plant-associated microbial communities. In particular, the low-input disease management strategy increased the abundances of sOTUs assigned to *Alternaria, Cryptococcus, Rhodotorula, Curtobacterium, Methylobacterium, Rathayibacter* and *Rhizobacter*, while the integrated disease management positively affected the abundances of *Filobasidium, Erwinia* and *Pseudomonas*. The functional characterization of apple secondary pathogens was then conducted on the canker agent *D. seriata* (Chapter IV). *Diplodia seriata* growth was inhibited by fungicides (captan, dithianon and fluazinam) *in vitro* although canker symptoms were not affected by dithianon on Fujion plants under greenhouse conditions.

The overall work revealed a high complexity of the bark-associated microbial communities, highlighting bark as an overwintering site and reservoir of potential plant pathogens and beneficial microorganisms. Bark-associated fungal and bacterial communities were mainly affected by tissue age, orchard location, environmental conditions, disease management strategies, plant species and cultivars, indicating strong plasticity of the bark community to the environmental and host factors. However, cavities in the bark possibly protect microorganisms form perturbing factors and probably limit the efficacy of conventional fungicides against some secondary pathogens, such as *D. seriata*. Protocols optimised in this work can be further applied for the impact estimation of innovative agronomic practices (e.g. training systems and rain covers) on pathogenic and

beneficial communities and for the precise assessment of *D. seriata* incidence under field conditions.

# Riassunto

Le piante ospitano complesse comunità fungine e batteriche sulla superficie ed all'interno dei propri tessuti. Tali popolazioni microbiche possono esercitare funzioni benefiche o deleterie nei confronti dei propri ospiti. Nonostante l'importanza della corteccia come potenziale fonte di patogeni vegetali e microorganismi benefici per le colture perenni, non esistono studi in merito alla composizione del microbioma della corteccia in relazione alle condizioni ambientali ed a diverse pratiche agronomiche. Data la sua importanza economica, il melo (*Malus domestica*) è stato selezionato come sistema modello, al fine di approfondire le conoscenze esistenti sulla composizione e le proprietà funzionali del microbioma della corteccia. La maggior parte delle varietà di melo è suscettibile a molte fitopatie, motivo per il quale è richiesto un utilizzo intenso di prodotti fungicidi. Esistono varietà di cultivar resistenti alla ticchiolatura che consentono di ridurre l'apporto di prodotti fungicidi e di attuare strategie di difesa a basso *input*. Partendo dal presupposto che i prodotti utilizzati per il controllo della ticchiolatura possano agire anche contro altri patogeni, la riduzione dei trattamenti può causare l'emergenza di patogeni secondari normalmente residenti sulla corteccia, come l'agente di cancro *Diplodia seriata*. Sebbene esista il rischio di emergenza di patogeni secondari nelle strategie di difesa a basso input, non esistono informazioni relative alla composizione e alle dinamiche delle comunità microbiche residenti sulla corteccia. Gli obiettivi principali di questo lavoro sono: i) sviluppare una metodologia basata sul metabarcoding per lo studio delle comunità microbiche residenti sulla corteccia, dei patogeni ed organismi benefici residenti su rami di un anno e su rami di tre-quattro anni; ii) determinare l'impatto di fattori ambientali (ubicazione del frutteto e condizioni climatiche), caratteristiche dell'ospite (specie vegetale, cultivar ed età del ramo) e pratiche agronomiche (difesa integrata e a basso input) sulla composizione del microbioma della corteccia; iii) valutare l'efficacia di prodotti fungicidi in condizioni controllate contro un agente di cancro del melo poco studiato.

Al fine di caratterizzare la composizione delle comunità microbiche residenti sulla corteccia di due cultivars di pero e melo, è stato ottimizzato un approccio basato sulla tecnica del metabarcoding (Capitolo II). Numerosi funghi (es. *Alternaria* spp., *Diplodia* spp., *Gibberella* spp., *Peltaster* spp., *Penicillium* spp., *Phoma* spp., *Rosellinia* spp., *Stemphylium* spp. e *Taphrina* spp.) e batteri (e.g. *Pseudomonas* spp., *Clavibacter* spp., *Curtobacterium* spp., *Nocardia* spp. e *Pantoea* spp.) potenzialmente fitopatogeni sono stati

identificati sulla corteccia, presumibilmente in equilibrio con funghi (es. *Arthrinium* spp., *Aureobasidium* spp., *Cryptococcus* spp., *Rhodotorula* spp., *Saccharomyces* spp. e *Sporobolomyces* spp.) e batteri (e.g. *Bacillus* spp., *Brevibacillus* spp., *Burkholderia* spp., *Deinococcus* spp., *Lactobacillus* spp., *Methylobacterium* spp. e *Sphingomonas* spp.) dalle proprietà benefiche nei confronti della pianta ospite. La composizione delle comunità microbiche residenti sulla corteccia è principalmente influenzata dall'età del ramo, dalla specie e varietà della pianta ospite. Sostanziali differenze sono state riscontrate in particolare tra rami di un anno e rami di tre-quattro anni.

Il protocollo ottimizzato è stato successivamente impiegato per caratterizzare le comunità microbiche residenti sulla corteccia di una varietà resistente alla ticchiolatura ('Fujion'), sottoposta a sistemi di difesa integrata e a basso input in due diverse località e in due stagioni consecutive (Capitolo III). Oltre all'età del ramo, i principali fattori che influenzano la ricchezza e la diversità delle comunità fungine e batteriche sono l'ubicazione del frutteto e le condizioni ambientali. In particolare, l'ubicazione del frutteto influenza l'abbondanza di alcuni potenziali fitopatogeni, tra cui *Alternaria* spp.,*Cadophora* spp., *Diaporthe* spp. *Diplodia* spp., *Phoma* spp. *Curtobacterium* spp., *Pantoea* spp. e *Ralstonia* spp. Inoltre, il sistema di difesa influenza l'abbondanza dei generi *Cryptococcus, Leptosphaeria, Curtobacterium* e *Pseudomonas*, suggerendo un'influenza del sistema di difesa sulla composizione del microbioma della pianta. In particolare, la strategia di difesa a basso input porta ad un aumento dell'abbondanza relativa di sOTUs (*sub-operational taxonomic units*) appartenenti ai generi *Alternaria, Cryptococcus, Rhototorula, Curtobacterium, Methylobacterium, Rathayibacter* e *Rhizobacter*, mentre la difesa integrata favorisce i generi *Filobasidium, Erwinia* e *Pseudomonas*. L'agente di cancro *D. seriata* è stato poi scelto come esempio per lo studio dell'efficacia di fungicidi commerciali sullo sviluppo di un patogeno emergente (Capitolo IV). I fungicidi captano, dithianon e fluazinam inibiscono la crescita di *D. seriata in vitro*. Tuttavia, il fungicida dithianon si è dimostrato inefficace sullo sviluppo dei sintomi di cancro su piante di 'Fujion' in serra.

Il presente lavoro di dottorato ha dimostrato che la corteccia ospita comunità microbiche altamente complesse, evidenziando anche il ruolo di quest'organo come luogo di svernamento e come fonte di potenziali patogeni e microorganismi benefici. È anche emerso che le comunità fungine e batteriche residenti sulla corteccia sono principalmente influenzate dall'età del ramo, dall'ubicazione del frutteto, dalle condizioni ambientali, dalla strategia di difesa e dalla specie e cultivar dell'ospite, indicando una grande plasticità

di tali comunità microbiche in relazione alle condizioni ambientali e alle caratteristiche della pianta ospite. La morfologia e le caratteristiche fisiche della corteccia possono giocare un ruolo nel proteggere il microbioma da fattori esterni, presumibilmente limitando l'efficacia di fungicidi convenzionali nei confronti di alcuni patogeni secondari, come *D. seriata*. I protocolli ottimizzati in questo lavoro di dottorato possono essere impiegati per stimare l'impatto di pratiche agronomiche innovative (es. nuove forme di allevamento, reti anti-pioggia) sulle componenti patogene e benefiche del microbioma e per una precisa valutazione dell'incidenza di *D. seriata* in condizioni di campo.

# Chapter I. Introduction

## 1.1 The plant microbiota

Plants host a wide variety of microorganisms, mainly belonging to fungi and bacteria (Turner *et al.*, 2013), living as epiphytes on plant surfaces and as endophytes inside plant tissues (Zilber-Rosenberg and Rosenberg, 2008). Consistently with the definition used for microbial communities associated with the human body (Zhao, 2010; Baquero and Nombela, 2012), the ensemble of microorganisms living on a plant host is defined as 'microbiota' and the 'microbiome' is the ensemble of their genes and genomes (Bulgarelli *et al.*, 2013; Mendes *et al.*, 2013) as well as the products of the microbiota and the host environment (Whiteside *et al.*, 2015). Plants offer three distinct major habitats for microbial life: the phyllosphere (which includes the surface of stem and leaves), the rhizosphere (which comprises root surface and rhizospheric soil) and the endosphere (represented by the inner plant tissues) (Berlec, 2012). Furthermore, each plant can be divided into more microenvironments, such as endorhiza (root), the antosphere (flower), the spermosphere (seeds), the carposphere (fruits), the caulosphere (stems) and the phylloplane (leaves) (Berg *et al.*, 2014; Reddy *et al.*, 2017) (Figure 1). Those habitats are characterised by very different environmental conditions, as nutrient type and availability, moisture, temperature and exposition to solar radiation (Kowalchuk *et al.*, 2010) that influence the composition of the associated microbial communities (Berg *et al.*, 2015). In particular, the phyllosphere represents a harsh habitat exposed to fluctuating environmental conditions (i.e., UV radiation, wind, rainfalls, temperature etc.) (Kowalchuk *et al.*, 2010) and with a limited nutrient availability (Vorholt, 2012). A variety of sugars and $C_1$ compounds, such as methanol, have been detected on the phyllosphere, mainly leaching from plant tissues, and their availability can vary even on different location of the same leaf (Kowalchuk *et al.*, 2010). On the other hand, rhizosphere and endosphere offer a greater protection against environmental fluctuations than the phyllosphere and are characterised by a higher nutrient availability (Kowalchuk *et al.*, 2010). Rhizosphere microbial communities can benefit from the nutrient exudates of plant roots (Berg and Smalla, 2009), from the decaying root material and simple organic molecules (Kowalchuk *et al.*, 2010). Endophytes can directly access more complex organic molecules produced by the plant (Kowalchuk *et al.*, 2010). Such nutritional and environmental differences

contribute to shape the composition of microbial communities to the extent that different plant organs host their own specific microbial communities (Berg *et al.*, 2015). Other factors affecting the composition of plant-associated microbial communities are plant age, genotype (species or cultivar) and health (Berg *et al.*, 2015), as well as agronomical practices (Leff and Fierer, 2013; Abdelfattah *et al.*, 2016), environmental conditions (Perazzolli *et al.*, 2014; Hamonts *et al.*, 2017) and organ age (Vorholt, 2012).

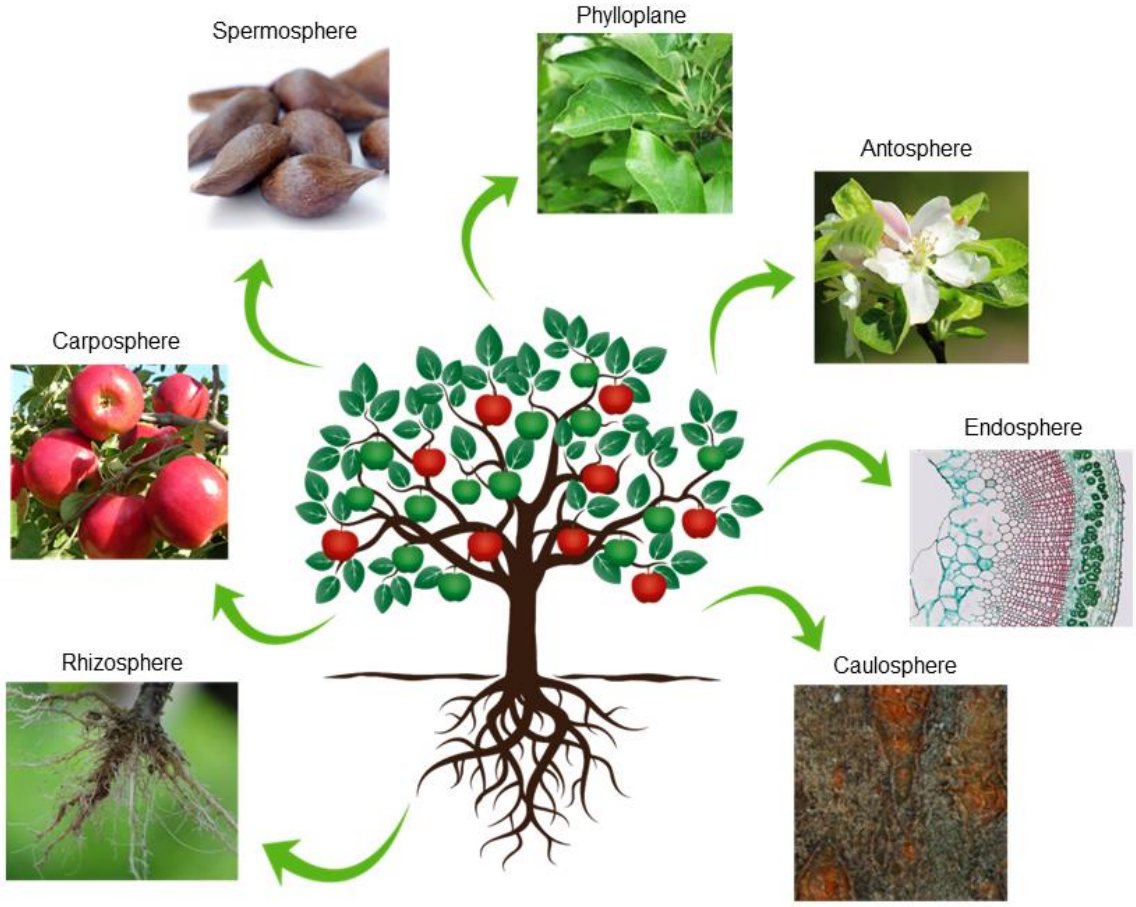



**Figure 1.** The plant habitats hosting microbial life. Image sources: Arrigoni *et al.* (2018); www.wikipedia.org**;** www.commons.wikimedia.org **;** www.agric.wa.gov.au**.**

Despite the presence of plant-associated microbial communities on every plant tissue (Vandenkoornhuyse *et al.*, 2015), most studies about their composition and dynamics have focused on the phylloplane, rhizosphere (Berg *et al.*, 2014) and endosphere (Berg *et al.*, 2015). Nonetheless, few studies have focused on other plant microenvironments, such as bark (Buck *et al.*, 1998; Lambais *et al.*, 2014; Leff *et al.*, 2015), flowers (Junker *et al.*, 2011; Tadych *et al.*, 2012; Shade *et al.*, 2013a), fruit (Abdelfattah *et al.*, 2016; Shen *et al.*, 2018) and seeds (Links *et al.*, 2014; Nelson, 2018). The importance of rhizosphere is due to its involvement in plant nutrition and health, while

the large and exposed area of the phyllosphere is directly in contact with air microbiota and airborne pathogens (Berg *et al.*, 2015). However, fruit crops are deciduous plants and they defoliate in autumn (Dixon and Aldous, 2014). On the contrary, bark is a perennial organ present for the whole lifespan of the plant and it is exposed to environmental conditions and agronomic practices for a longer period than leaves. Moreover, bark hosts saprophytic, pathogenic and beneficial microorganisms (Buck *et al.*, 1998; Martins *et al.*, 2013) and to harbor a greater species richness and diversity than leaves and fruits (Martins *et al.*, 2013). Although the potential role of bark as a reservoir of beneficial and pathogenic microorganisms, few studies have been carried out to unravel the composition of microbial communities residing on this plant tissue and no information is available on environmental and agronomic impacts on bark-associated microbial populations.

## 1.2 Functions of the plant microbiota

Plants cannot be seen as standalone organisms, as they host a wide variety of microbial communities inside their tissues and on their surfaces (Vandenkoornhuyse *et al.*, 2015). They are rather considered as meta-organisms or holobionts, where microbial communities provide several functions essential for host fitness (Mendes *et al.*, 2013; Berg *et al.*, 2015) and, in return, plants provide microorganisms with organic carbon fixed through the photosynthesis (Mendes *et al.*, 2013). In the holobiont, microorganisms establish complex interactions with the plant host and inside the microbial community (Vorholt, 2012). The beneficial, detrimental or neutral outcomes of such interactions deeply influence plant growth and survival, hence affecting host fitness (Turner *et al.*, 2013; Vandenkoornhuyse *et al.*, 2015). A notable example about the importance of plant-associated microorganisms is represented by mycorrhizal fungi (Turner *et al.*, 2013). They colonise the roots of the majority of the terrestrial plants where they enhance the uptake of mineral nutrients, improve root growth and function and protect plants from pathogens (Borowicz, 2001). They are also thought to having played a fundamental role to the evolution of terrestrial plants about 700 million years ago (Heckman *et al.*, 2001). Likewise, other non-mycorrhizal members of the plant microbiota can affect the plant nutritional status by providing nutrients, increasing their bioavailability or enhancing their uptake (Müller *et al.*, 2016) (Figure 2). For instance, nitrogen-fixing bacteria were found in rhizosphere, phyllosphere and endosphere (Müller *et al.*, 2016) and other rhizosphere-colonising bacteria were shown to enhance phosphorus and iron uptake from soil

(Bulgarelli *et al.*, 2013; Mendes *et al.*, 2013). Plant-associated microorganisms can also help plants by producing plant hormones as auxins that are able to modify the architecture of the root system, hence enhancing nutrient uptake (Müller *et al.*, 2016) (Schlaeppi and Bulgarelli, 2015) (Figure 2). Plants like mosses and orchids are not able to initiate the germination process without the presence of microorganisms that are vertically inherited through the seed (Berg *et al.*, 2015). Plant microbiota can also promote plant resistance to abiotic stresses, such as salinity, drought, heavy metal contamination and extreme temperatures (Berg *et al.*, 2014; Müller *et al.*, 2016) and protects plants from pathogens (Turner *et al.*, 2013) (Figure 2). The main mechanisms by which plant-associated microorganisms protect plants against phytopathogens include competition for niches and nutrients, parasitism, production of lytic enzymes, antibiosis, induction of systemic resistance in the plant host and suppression of pathogen virulence (Mendes *et al.*, 2013; Müller *et al.*, 2016).

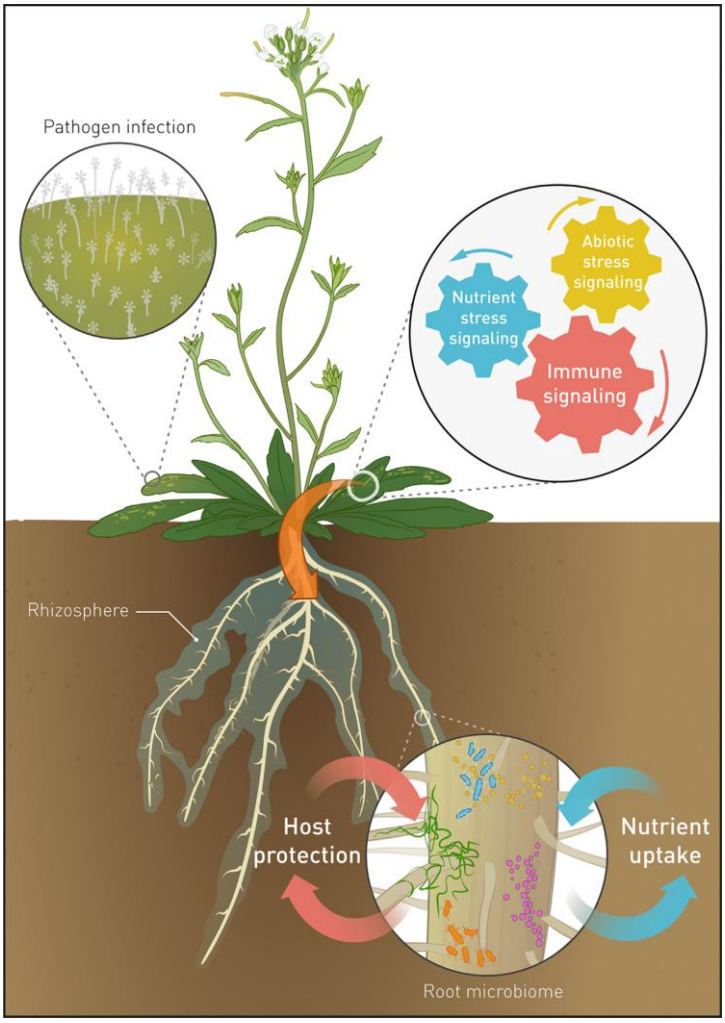



**Figure 2.** Some of the multiple functions of plant microbiota (Bakker *et al.*, 2018). Another function not mentioned in the figure is the promotion of seed germination (Berg *et al.*, 2015).

The plant microbiota also includes pathogenic microorganisms. Namely, fungi belonging to the family Botryosphaeriaceae have been found on woody plants worldwide, particularly on branches, stems, leaves, twigs of woody plants and culms of grasses (Chethana *et al.*, 2016). Botryosphaeriaceae species, like *D. seriata*, *D. pinea*, *Botryosphaeria dothidea*, *Neofusicoccum australe* and *Lasiodiplodia theobromae*, cause dieback, cankers, shoot blights, leaf spots, fruit and seed rots on a wide variety of plant hosts, apple included (Chethana *et al.*, 2016). Since they have been isolated from several healthy plant tissues, they are recognised as endophytes, often representing a prominent component of endophytic communities of many plant species (Slippers and Wingfield, 2007). Several endophytic fungal pathogens were also isolated from the wood of different grapevine cultivars (Casieri *et al.*, 2009), from banana leaves (Photita *et al.*, 2004), *Arabidopsis thaliana* (Junker *et al.*, 2012) and cranberry flowers (Tadych *et al.*, 2012). A pathogenic interaction between endophytic pathogens and their host happens in case of

stressful conditions for the plant and favourable ones for the endophyte, underlying the thin border between endophyte and latent pathogen (Slippers and Wingfield, 2007; Junker *et al.*, 2012). Likewise, several potential plant pathogens have been found on different plant organs such as fruits (Shade *et al.*, 2013a; Gomba *et al.*, 2017; Vepštaitė-Monstavičė *et al.*, 2018), leaves (Rastogi *et al.*, 2012; Vorholt, 2012) and roots (Arfi *et al.*, 2012; Chaparro *et al.*, 2014). However, bark is a potential overwintering site of pathogens for perennial plants and it is a dangerous source of secondary pathogens (Brown-Rytlewski and McManus, 2000; Beer *et al.*, 2015). Despite the importance of bark as potential reservoir of plant pathogens, no information is available on the composition of bark microbiota under different climatic and agronomic conditions. In order to investigate the composition and functional properties of the bark microbiota, apple was selected as model system due to its agricultural and economic relevance worldwide.

## 1.3 Economic relevance and history of apple

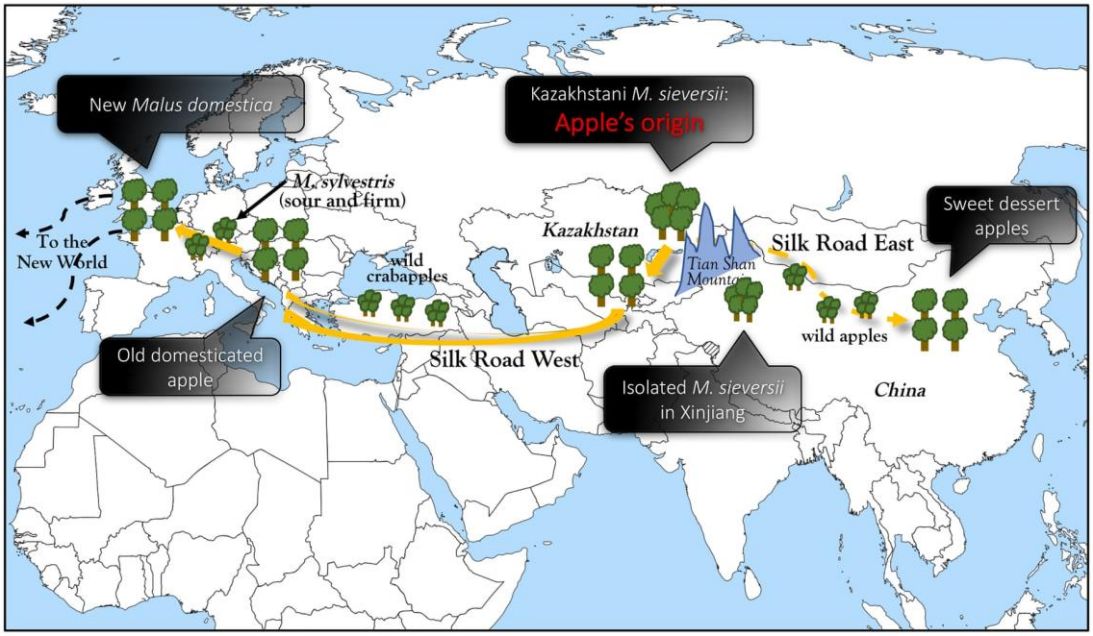



**Figure 3.** Map showing the spread of apple from the Tian Shan mountains in Kazakhstan to Europe and China along the silk road and later to the New World. (https://www.theguardian.com/science/2017/aug/15/geneticists-trace-humble-apples-exotic-lineage-all-the-way-to-the-silk-road)

The origins of domesticated apple (*Malus domestica*) trace back to the west side of Tian Shan Mountains, in Kazakhstan, where the progenitor *M. sieversii* were domesticated

for 4000-10000 years (Figure 3) (Cornille *et al.*, 2012; Duan *et al.*, 2017). As early as 5000-8000 years ago, merchants began to transport apples from Asia to Europe through the Silk Road, allowing the hybridization with wild *Malus* species such as *M. sylvestris*, *M. baccata* and *M. orientalis* (Gladieux *et al.*, 2008; Duan *et al.*, 2017). The domesticated apple started to be grown in Israel around 3000 years ago, to be later introduced by Romans and Greeks in Europe and North Africa (Harris *et al.*, 2002). In the last 500 years, Europeans were responsible for spreading the domesticated apple to North America and Australia (Carisse and Dewdney, 2002). So far, thousands of varieties have been selected in Europe, Asia and America (Harris *et al.*, 2002) and apple is grown in all the temperate regions, representing one of the most important fruit crops worldwide (Gladieux *et al.*, 2008).

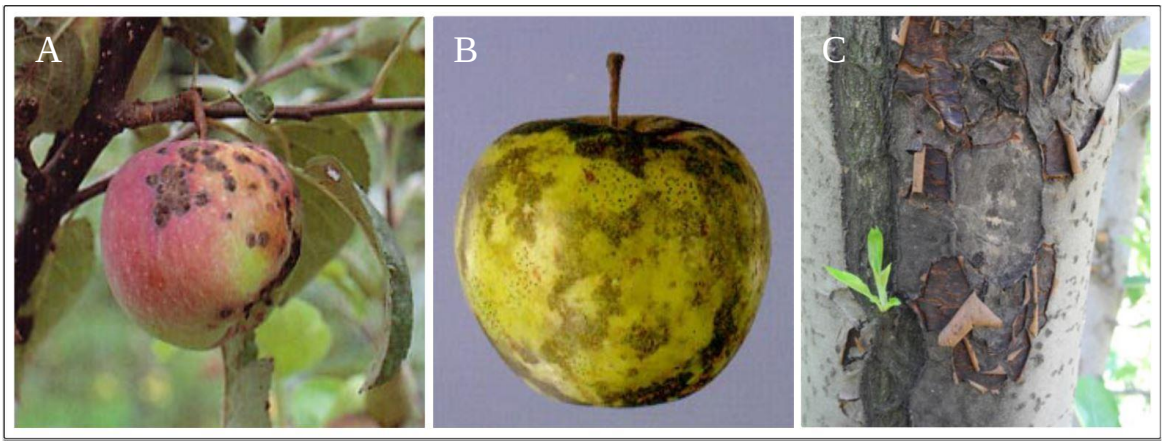



**Figure 4.** Fruit symptoms of the main disease apple scab (A) and of the secondary diseases flyspeck and sooty blotch (B). Symptoms of the canker agent *Diplodia spp.* on apple trunk (C). Adapted from Aylor (1998), Williamson and Sutton (2000) and Arzanlou and Bakhshi (2012).

Apple orchards cover an area of 5,3 million hectares worldwide with a production of 89.3 million tonnes (data from 2016, http://www.fao.org/faostat). Apples are produced in almost all the European countries and they represent the most important fruit sector in terms of economic relevance, with 12.7 million tonnes harvested in 2016 (Forti and Henrard, 2016). However, most of the commercially relevant apple cultivars are susceptible to a large spectrum of destructive diseases such as apple scab (Figure 4A), powdery mildew and fireblight (Jones and Aldwinckle, 1990; MacHardy, 2000) and to other secondary diseases such as sooty blotch, flyspeck (Figure 4B), rust, cankers (Figure 4C) and fruit rots (Jones and Aldwinckle, 1990; MacHardy, 2000). Moreover, postharvest diseases like blue mold, gray mold, alternaria rot and bull's eye rot can cause important damages to apples during storage (Jones and Aldwinckle, 1990). Among all, apple scab is considered the most important disease threatening apple production (Gladieux *et al.*, 2008)

and the major target of the fungicide programs applied in apple orchards (MacHardy, 2000).

### 1.4 Apple scab

Apple scab, caused by *Venturia inaequalis*, is one of the most important apple diseases worldwide, especially in temperate climates with cool and moisty springs (Bowen *et al.*, 2011). It represents an example of invasive pathogenic fungus: it emerged in Central Asia and it was transported to Europe together with its plant host, from where it spread worldwide with the expansion of apple growing (Gladieux *et al.*, 2008). The first report of apple scab in orchards was in 1819 but the painter Michelangelo 'Caravaggio' Merisi


**Figure 5.** Caravaggio's painting 'Supper at Emmaus' (A) and detail of the basket containing apples with scab symptoms (B). Adapted from MacHardy *et al.* (2001).

already painted scabbed apples in his opera 'Supper at Emmaus' in 1601-1602 (Figure 5) (MacHardy *et al.*, 2001). Nowadays, *V. inaequalis* is well established in all the apple growing areas, where it mainly attacks fruits and leaves causing important losses in terms of fruit quality and yield. In cases of insufficient control, losses can reach 70% of the production (Belete and Boyraz, 2017). The fungus overwinters as pseudothecia formed in the leaf litter following the sexual reproduction (Bowen *et al.*, 2011) and spring rainfalls cause the release of mature ascospores which are disseminated by wind and cause primary infections (Figure 6A) (Jones and Aldwinckle, 1990). Symptoms appear in spring as

circular light-coloured spots on leaves (Figure 6B) that become curled and deformed, leading to severe defoliation in susceptible cultivars (Jones and Aldwinckle, 1990). As spots become olive-coloured due to the presence of conidiophores, multiple secondary infections are initiated by conidia, transported by rain and wind (Jones and Aldwinckle, 1990). Symptoms on young fruits (Figure 6C) are similar to those on leaves and can lead to premature fruitlets fall (Ogawa and English, 1991) and subsequent *V. inaequalis* infections cause brown and cracked lesions and make fruits unmarketable (Jones and Aldwinckle, 1990; Ogawa and English, 1991).

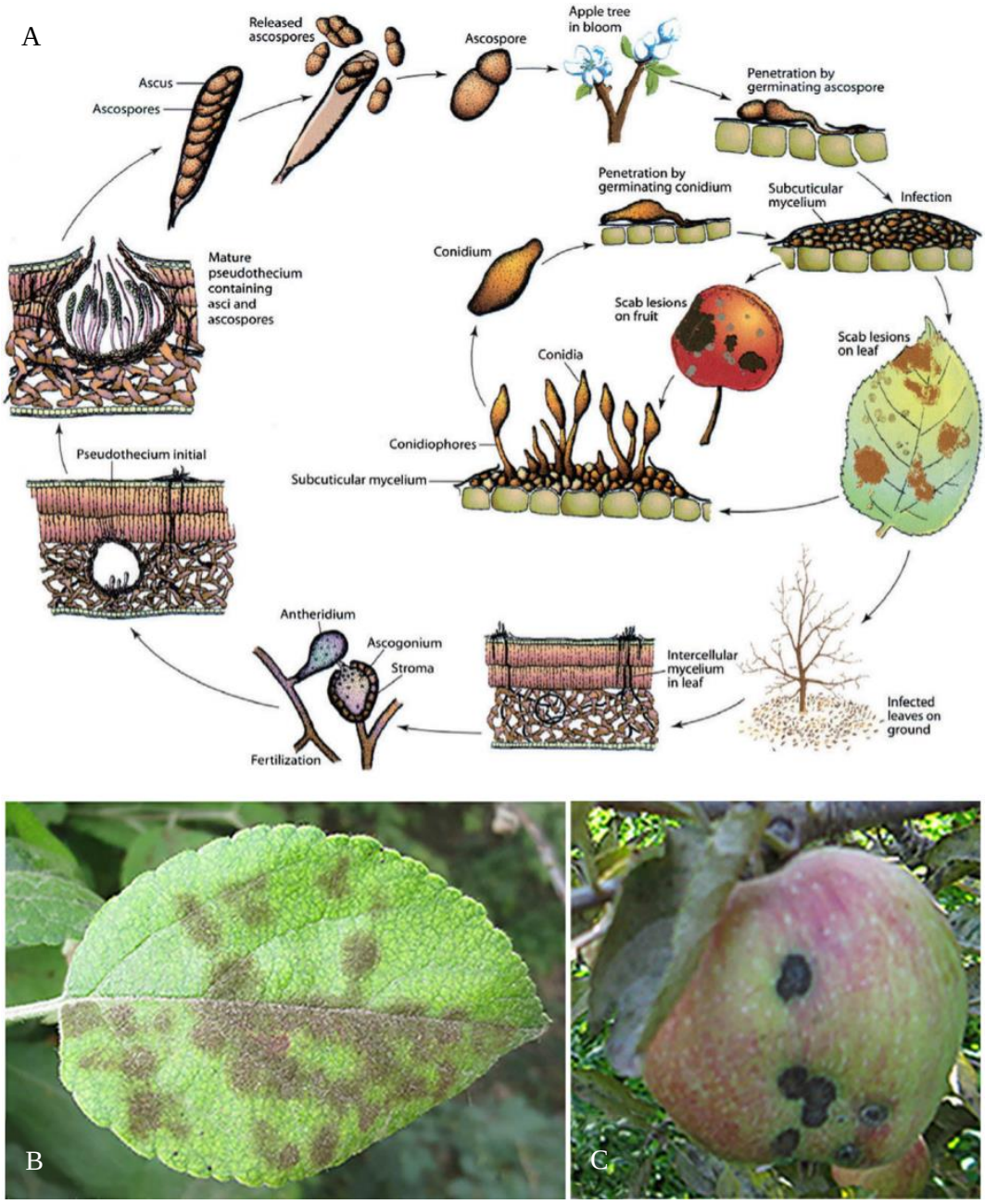



**Figure 6.** Life cycle of *Venturia inaequalis* (A) and symptoms on leaves (B) and fruits (C), adapted from Agrios (2005) and Ebrahimi *et al.* (2016).

## 1.5 Disease management of apple scab

The disease management of apple orchards is mainly directed to control the apple scab that is the main disease of apple and it is responsible for the biggest part of the fungicides applied in apple production (MacHardy, 2000). At the same time, fungicides applied to control apple scab can have side effects against other secondary pathogens, such as the canker agent *Diplodia seriata* (Brown-Rytlewski and McManus, 2000; Beer *et al.*,

2015), sooty blotch and flyspeck (Weber *et al.*, 2016a). Nowadays the apple scab management is conducted following the principles of Integrated Pest Management (IPM) (MacHardy, 2000; Beckerman *et al.*, 2015). IPM combines different crop disease managements with the monitoring of pathogen's life cycle in order to reduce the amount of pesticides applied, balancing agricultural production with the protection of human health and environment (Barzman *et al.*, 2014; Beckerman *et al.*, 2015). Such measures include cultural, biological and chemical strategies that aim at reducing the level of scab ascospores in the leaf litter as a preventive measure and to limit the ascosporic and conidial infections during the productive season (Belete and Boyraz, 2017). The use of disease forecasting models and weather monitoring tools (MacHardy, 2000), such as the software Adem (Berrie and Xu, 2003), RIMpro (Trapman and Polfliet, 1997) and A-scab (Rossi *et al.*, 2007) allow farmers to forecast favourable conditions for scab infections and to precisely target plant protection measures (Bowen *et al.*, 2011). Although apple IPM programs mainly rely on chemical-based strategies, cultural and biological approaches can replace some fungicide applications through the use of alternative practices to control the pathogen, with a positive impact on the IPM program (MacHardy, 2000).

### 1.5.1 Chemical control of apple scab

The majority of commercially relevant apple cultivars is susceptible to apple scab (Holb, 2007). Since the solely cultural management approaches are not enough to achieve a satisfactory disease control (Peil *et al.*, 2018), apple scab is mainly controlled with the application of fungicides on susceptible cultivars (Belete and Boyraz, 2017). Frequent fungicides treatments are required during the productive season (spring-summer) in most of the apple growing regions for conventional, integrated and organic production systems (Holb *et al.*, 2006; Bowen *et al.*, 2011). Spring treatments are targeted to early scab infections produced by ascospores with the aim to limit the later leaf and fruit infections, while summer fungicide sprays are directed to secondary infections (Belete and Boyraz, 2017). Under favourable climatic conditions, 20-30 fungicide treatments may be needed to achieve a satisfactory control of the disease (Soriano *et al.*, 2009). For this reason, anti-scab fungicides represent the most expensive part of the apple disease management, reaching up to 90% of the annual fungicide costs (Holb *et al.*, 2006) and they represent up to the 80% of the total amount of fungicide applied in apple orchards (Didelot *et al.*, 2016).

The intensive use of fungicides was responsible for the development of fungicide resistance in *V. inaequalis* with a consequent reduction on disease control efficacy (Bowen *et al.*, 2011; Chapman *et al.*, 2011). Fungicide resistance has been shown for dodine (Szkolnik and Gilpatrick, 1969), methyl benzimidazole carbamates (Jones and Walker, 1976), demethylation inhibitors (Kunz *et al.*, 1997), quinone-oxydase inhibitors (Köller *et al.*, 2004) and succinate dehydrogenase inhibitors (Toffolatti *et al.*, 2016). Strategies to delay the development of fungicide resistance in *V. inaequalis* are based on restricting the number of applications of each fungicide class per season and alternating or mixing fungicides of different classes (Bowen *et al.*, 2011; Belete and Boyraz, 2017). The massive use of fungicides has an important impact on human health and the environment, causing pollution of soils, water bodies, atmosphere and food products (Zhang *et al.*, 2011). In particular, fungicide residues have been detected in soils (Li *et al.*, 2005) and ambient air (Coscollà *et al.*, 2010) of apple growing areas and on fresh apples (Baker *et al.*, 2002; Li *et al.*, 2005). Fungicides are also demonstrated to negatively affect human health, being reported to cause allergic dermatitis (Chowdhury *et al.*, 2001) and correlated with neurological disorders (Costa *et al.*, 2008) and prostate cancer (Band *et al.*, 2011). Fungicides are also reported to have toxic effects on beneficial insects and mites, with negative consequences on the natural control of pests in apple orchards (Trapman, 2006).

The increasing concern about fungicides residues (Simon *et al.*, 2011) and their impact on human health and the environment (Fantke *et al.*, 2012) led several countries to direct regulations to reduce their application (Hillocks, 2012; Skevas *et al.*, 2013). Promoting the use of IPM to minimize polluting inputs in plant disease management (Meissle *et al.*, 2010), the EU also encourages the use of preventive and non-chemical control methods, according the IPM principles (Barzman *et al.*, 2015). In apple production, such methods include cultural strategies and the use of biocontrol agents and of scab-resistant apple cultivars (Belete and Boyraz, 2017).

### 1.5.2 Cultural and biological control methods against apple scab

Cultural control methods are mainly preventive and based on agronomic practices such as the removal of fungal inoculum sources (i.e. crab apple trees), use of proper training systems (e.g. centrifugal training) and planting patterns that allow air circulation in the canopy and pruning trees adequately (Belete and Boyraz, 2017). In addition, sanitary practices that reduce the ascospore inoculum can be applied to the leaf litter. They include

autumn urea sprays, which suppress the sexual reproduction of the fungus (Sutton *et al.*, 2000), the use of earthworms to promote leaf litter breakdown (Holb *et al.*, 2006) and leaf shredding, burning and mulching (Gomez *et al.*, 2007). Such practices allow a reduction of scabbed fruits of about 40-80% as compared with the control plots (Sutton *et al.*, 2000; Gomez *et al.*, 2007; Belete and Boyraz, 2017), but they may result expensive and not compatible with some commercial operations (Belete and Boyraz, 2017).

Biological methods rely on the use of biocontrol agents to control or suppress *V. inaequalis*. Namely, the application of *Microsphaeropsis ochracea* and *Athelia bombacina* in late summer or autumn are shown to reduce the level of spring ascospores of about 60-99% and 84%, respectively, compared to the untreated plots (Carisse and Rolland, 2004). Other fungi has been identified as potential biocontrol agents against *V. inaequalis*, such as *Chaetomium globosum*, *Aureobasidium botrytis*, *Cladosporium spp.* and *Trichoderma longibrachiatum* (Palani and Lalithakumari, 1999; Fiss *et al.*, 2003). However, the exclusive use of biocontrol agents does not provide a satisfactory control of apple scab at commercially acceptable levels, therefore they should be compatible with the application of fungicides and appropriated agronomic practices (Carisse and Dewdney, 2002; Belete and Boyraz, 2017).

### 1.5.3 Scab-resistant apple cultivars

The interest on scab-resistant apple cultivars dates back to the end of 19$^{th}$ Century (Williams and Kuc, 1969). In Germany, the studies of Aderhold (1902) highlighted the presence of 11 markedly resistant cultivars among 160 tested, including the Russian cultivar Antonovka. The resistant phenotype of this cultivar was later attributed to several 'Antonovka-related' genes, globally identified as *Va* or *Rvi10* (Gessler *et al.*, 2006; Masny, 2017). Studies about sources of scab resistance continued for the next decades and they led to the identification of resistance in the Asiatic species *Malus floribunda* 821 (Hough, 1944). The resistance gene from *M. floribunda* 821, known as *Vf* or *Rvi6* (Masny, 2017), became the most frequently used source of scab resistance in apple breeding programs (Gessler and Pertot, 2012). Indeed, in almost all the commercial resistant cultivars, such as Prima, Priscilla and Jonafree, the resistance is due to the presence of the *Vf* gene (Carisse and Dewdney, 2002) (Gessler and Pertot, 2012). However, symptoms of apple scab on *Vf* resistant cultivars have been observed in scab-resistant *Malus* collections since 1984 and ten years later they were observed in a commercial orchard in The Netherlands (Gessler

and Pertot, 2012). Scab lesions were caused by a '*Vf* -breaking' scab pathotype or race that overcame plant resistance given by the *Vf* gene (Gessler and Pertot, 2012). The evolution of *Vf*–breaking *V. inaequalis* races highlights the need for the characterization of other sources of resistance (Padmarasu *et al.*, 2014), such as *Vbj*/*Rv11* from *M. baccata jackii*, *Vb*/*Rvi12* from the Hansen's *baccata*, *Vr*-DW, *Vh2*/*Rvi2* (also known as *Vr* or *Vr*-A) and *Vh4*/*Rvi4* (also known as *Vx* or *Vr1*) from *M. pumila*, *Vr2*/*Rvi15* from the scab-resistant selection GMAL 2473, *Vm*/*Rvi5* from *M. micromalus* and *M. atrosanguinea* 804, *Vd*/*Rvi13* from the old apple cultivar 'Durello di Forlì' (Gessler *et al.*, 2006; Soufflet-Freslon *et al.*, 2008; Jha *et al.*, 2009). Likewise, the so-called 'ephemeral genes' are known to give resistance to just a part of the scab population, like *Vg*/*Rvi1* from the cultivar Golden Delicious (resistance to scab race 7), *Vfh*/*Rvi7* from *M. floribunda* 821 (resistance to scab race 1), *Vh8*/*Rvi8* from *M. sieversii* (resistance to scab race 8) (Gessler *et al.*, 2006; Soufflet-Freslon *et al.*, 2008; Jha *et al.*, 2009). The interaction between *V. inaequalis* and a plant carrying one or more $V_x$/$Rvi_n$ genes results in a resistance reaction (Gessler *et al.*, 2006; Bowen *et al.*, 2011). Different resistance reactions appear on plant leaves depending on the $V_x$/$Rvi_n$ gene carried by the plant and on the pathogen race involved (Figure 7) (Bowen *et al.*, 2011).

Breeding for resistance to apple scab is regarded as the most effective method in apple disease management in terms of reduction of fungicide applications (Belete and Boyraz, 2017). It has been demonstrated that the use of scab-resistant apple cultivars is compatible with low-input and organic disease management strategies and it allowed a reduction of 44-63% of fungicide treatments as compared with the susceptible cultivars (Ellis *et al.*, 1998; Simon *et al.*, 2011; Didelot *et al.*, 2016). Such reduction of fungicide applications results in positive outcomes for management costs and for environmental and health safety, offering the possibility of a more sustainable apple cultivation onwards (Didelot *et al.*, 2016). However, fungicides applied to control apple scab can have side effects and potentially act against other secondary pathogens, such as *D. seriata* (Brown-Rytlewski and McManus, 2000; Beer *et al.*, 2015), sooty blotch and flyspeck disease complex (Weber *et al.*, 2016a) to which scab resistant cultivars are susceptible (Ellis *et al.*, 1998; Trapman and Jansonius, 2008). Indeed, secondary pathogens, such as the canker and fruit rot agent *D. seriata*, are demonstrated to cause severe damages in organic apple orchards, where the use of fungicides is limited (Trapman *et al.*, 2008). Therefore, the introduction of scab-resistant apple cultivars and the consequent reduction in fungicide sprays may cause the outbreak of emerging pathogens (Warner, 1991; Ellis *et al.*, 1998).

Plant pathogenic fungi and bacteria are normally part of microbial communities (Martins *et al.*, 2013; Shen *et al.*, 2018; Vepštaitė-Monstavičė *et al.*, 2018) and they maintain an equilibrium with the other members of the community on which the outcome of the pathogen infection depends (Raaijmakers *et al.*, 2009). Thus, it is important to evaluate the impact of low-input disease management strategies from a microbial community point of view, considering pathogenic, beneficial and neutral microorganisms. Although the risk of emerging secondary pathogens is real under low-input disease managements of apple scab-resistant cultivars (Ellis *et al.*, 1998), no information is available on the effect of disease management strategies on population dynamics of bark-associated fungal and bacterial communities.

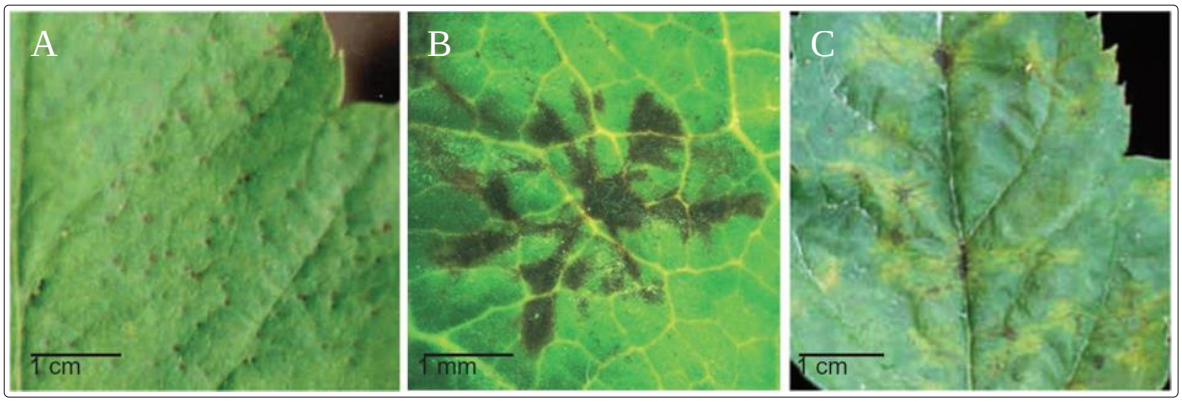



**Figure 7.** Scab resistance reactions on apple leaves conditioned by *Vh4/Rvi4* (A), *Vh2/Rvi2* (B) and *Vf/Rvi6* (C) genes (Bowen *et al.*, 2011).

## 1.6 Secondary diseases of apple

In addition to apple scab, which represents one of the main pathogens of apple worldwide (Bowen *et al.*, 2011), apples are exposed to a wide range of other diseases. With the increase of the apple growing areas and the spread of susceptible commercial varieties, new diseases appeared and the already existing ones became more destructive (Verma and Sharma, 1999). Such diseases can occur on various parts of the plant, such as leaves, roots, stems, branches, shoots and fruits and they can also cause important product losses during the storage (Jones and Aldwinckle, 1990). Fungal secondary diseases such as cankers, flyspeck, sooty blotch, powdery mildew and post-harvest rots represent a serious problem in organic and low-input orchards, where the use of fungicides is limited (Ellis *et*

*al.*, 1998; Swezey, 2000; Trapman *et al.*, 2008) and on scab-resistant apple varieties, which are susceptible to a wide range of secondary diseases (Ellis *et al.*, 1998).

Among secondary diseases, cankers occur in all the apple growing areas around the world (Verma and Sharma, 1999) and cause branch girdling, shoot blight and dieback, eventually leading the diseased plant to death (Jones and Aldwinckle, 1990). The causal agents are represented by different species of fungi which can also attack fruits and leaves, causing fruit rots and leaf spots (Verma and Sharma, 1999; Trapman *et al.*, 2008). One of the most destructive and widespread canker diseases is the European canker caused by *Neonectria ditissima* (Jones and Aldwinckle, 1990). Due to its dangerousness, it is considered as a quarantine pest in some countries (Carstens *et al.*, 2010; Campos *et al.*, 2017). Other canker diseases as the Phomopsis canker (*Diaporthe eres*), the smoky blight canker (*D. seriata*) and the Botryosphaeria canker (*Botryosphaeria dothidea*) are spread in different apple growing regions around the world, where they can cause severe losses (Verma and Sharma, 1999). Infections usually occur through wounds on branches and stems, but the presence of canker agents belonging to the Botryosphaeriaceae family as plant endophytes and latent pathogens has been demonstrated (Slippers and Wingfield, 2007), suggesting that they can rapidly cause disease when the host is under stress (Slippers and Wingfield, 2007).

In addition to canker agents, leaf and fruit pathogens cause damages by defoliating the plant, thus reducing the photosynthetic surface and the productivity, and by directly damaging the fruits, that may become unmarketable (Jones and Aldwinckle, 1990). For instance, powdery mildew (*Podosphaera leucotricha*) is a widespread foliar and fruit pathogen of apples that can cause severe damages on susceptible cultivars (Biggs *et al.*, 2009) and in organic orchards (Holb and Kunz, 2016). In addition, the Alternaria blotch, caused by *Alternaria alternata* apple pathotype, causes leaf spots that may lead to severe defoliation and fruit soft rot (Li *et al.*, 2013). Several fungi belonging to the genus *Phoma* (e.g., *P. pomorum, P. macrostoma, P. glomerata* and *P. exigua*) are the causal agents of the Phoma leaf and fruits spots, which cause defoliation and fruit spotting (Verma and Sharma, 1999). The sooty blotch and flyspeck is a disease caused by a complex of several fungal pathogens (*Dissoconium* sp., *Peltaster* sp., *Ramichloridium* sp., and others) which colonise the surface of apples causing aesthetic damage and downgrading the production from the fresh market (Díaz Arias *et al.*, 2010; Gleason *et al.*, 2011).

Secondary pathogens (e.g., *D. seriata*, sooty blotch and flyspeck disease complex) are potentially controlled with the application of anti-scab treatments (Brown-Rytlewski

and McManus, 2000; Beer *et al.*, 2015; Weber *et al.*, 2016a). Therefore, the reduction of fungicide treatment under organic and low-input disease managements may cause the outbreak of secondary diseases (Ellis et al., 1998; Warner, 1991).

## 1.7 The study of microbial communities with sequencing technologies

### 1.7.1 The study of microbial diversity

The study of microbial diversity relies on microbiological, biochemical and molecular methods (Kirk *et al.*, 2004; Turner *et al.*, 2013). Classic microbiological approaches involve plating and isolating environmental microorganisms using selective media and different growth conditions (Turner *et al.*, 2013). Although these methods are useful for in-depth studies of single microorganisms, they only give information on the culturable fraction of the microbiota, thus they do not give a proper representation of the whole community (Rondon *et al.*, 1999; Turner *et al.*, 2013). Moreover, the choice of selective media and growth conditions only allows the growth of some microorganisms, while suppressing others, favouring the ones with a fast growing rate (Kirk *et al.*, 2004). Since the culturable microorganisms represent less than 1% of the whole microbiota populating an environment (Schloss and Handelsman, 2005), the study of microbial diversity mainly rely on culture-independent DNA-based approaches (Zilber-Rosenberg and Rosenberg, 2008) that skip the laboratory procedures of cultivating and isolating single specimen (Shokralla *et al.*, 2012). The first DNA-based approaches for microbial community analysis were based on DNA hybridization or digestion with restriction enzymes (i.e., Southern blot and restriction fragment length polymorphism, RFLP) (Taberlet *et al.*, 2012). With the PCR-based DNA amplification, genetic fingerprinting techniques such as DGGE (denaturing gradient gel electrophoresis) and TGGE (temperature gradient gel electrophoresis) were developed for the analysis of complex microbial communities (Muyzer and Smalla, 1998). Later on, the development of Sanger sequencing technology allowed the optimization of sequencing-based techniques for species identification (Shokralla *et al.*, 2012; Taberlet *et al.*, 2012). Species identification was carried out amplifying species-specific genomic regions (barcodes) and generating large DNA libraries (Shokralla *et al.*, 2012). DNA libraries were then sequenced and

compared to reference databases assembled with sequences of known species (Hajibabaei *et al.*, 2011). Different barcodes have been standardised for animals (cytochrome oxidase I; COI), bacteria (16S ribosomal DNA; 16S rDNA), fungi (internal transcribed spacer; ITS) and plants (maturase K; *matK* and ribulose-bisphosphate carboxylase; *rbcl*) allowing the identification of species and higher taxonomic levels (Shokralla *et al.*, 2012). However, Sanger technology only allows to sequence 96 specimen at a time, making it an unfeasible approach for environmental samples containing hundreds to thousands of specimen (Hajibabaei *et al.*, 2011). The advent of next-generation sequencing (NGS) technologies facilitated the identification of specimen in environmental samples through high-throughput techniques as DNA metabarcoding and shotgun metagenomics (Taberlet *et al.*, 2012). DNA metabarcoding techniques allow the simultaneous high-throughput identification of multiple species or higher-level taxa from the total DNA extracted from environmental samples (eDNA) (Taberlet *et al.*, 2012) using PCR amplification and sequencing of evolutionarily conserved barcodes, like 16S rDNA, 18S rDNA, COI and ITS (Hajibabaei *et al.*, 2011; Shokralla *et al.*, 2012; Oulas *et al.*, 2015). Metagenomics allows the identification of community structure and genic functions in environmental samples by sequencing the genomes contained in an environmental sample with short random primers (Thomas *et al.*, 2012). Shotgun metagenomics differs than metabarcoding for the absence of a PCR amplification of a specific barcode (Jovel *et al.*, 2016) and not only answers the question ‘who is in the community?’, but also ‘what are they doing?’ and ‘how they interact?’ (Oulas *et al.*, 2015).

### 1.7.2 The Illumina sequencing technology

The first NGS technology developed was the 454 Roche pyrosequencing (Goodwin *et al.*, 2016) that could generate up to 1000 bp-long reads and about 1 million reads per run with a relatively low coverage of up to 700 Mb per run and an error rate of 1% (Oulas *et al.*, 2015; Goodwin *et al.*, 2016). The low resolution of pyrosequencing technology in terms of detection of low-abundant taxa (Huang *et al.*, 2014) and the high costs per Gb (up to 40’000 $ according to Goodwin *et al.* (2016)) were among the major disadvantages of the 454 technology. Although for several years most of the studies involving NGS sequencing of environmental samples employed 454 pyrosequencing instruments thanks to the long sequence obtained (Shokralla *et al.*, 2012), the technology developed by Roche was taken over in the last years by the highly successful Illumina’s set of instruments

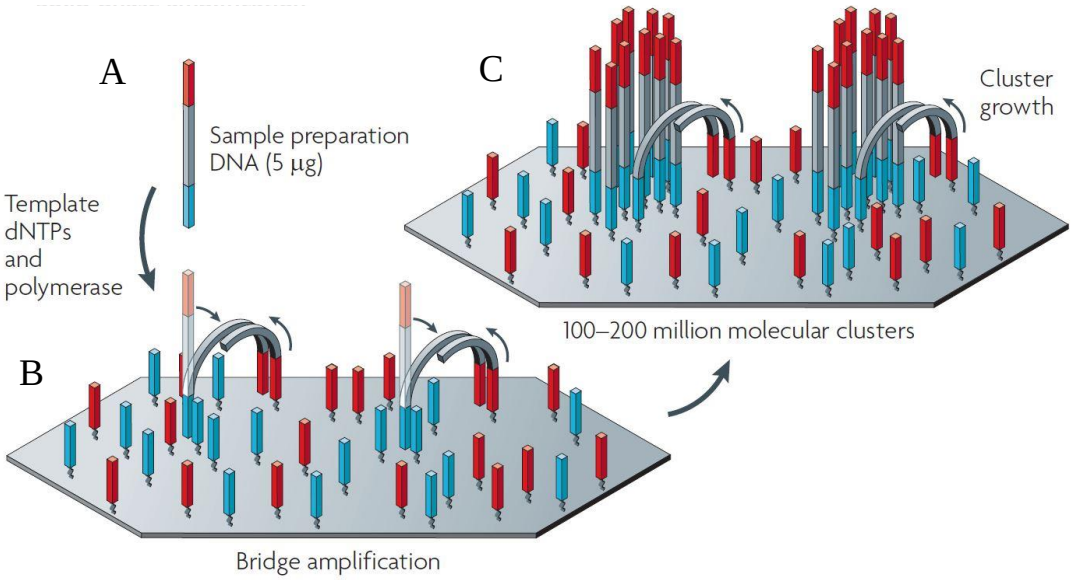



**Figure 8.** Illumina amplification of a DNA fragment (grey). Amplicons are immobilised thanks to the complementarity of the adaptors (red and blue) with the oligos ligated to the flow cell (A). A bridge-PCR amplification (B) generates millions of clusters, each one containing copies of the same amplicon (C). Adapted from Metzker (2010).

(Goodwin *et al.*, 2016). The wide range of Illumina's sequencing platforms is characterised by a high coverage and accuracy (0.1-0.5% error rate), high-throughput (up to 900 Gb of data and three billion reads per run), read length up to 300bp and a considerably low cost per Gb than 454 Roche technology (7-1'000 $ depending on the instrument) (Oulas *et al.*, 2015; Goodwin *et al.*, 2016).

Thanks to the limited costs and to the high coverage provided, nowadays Illumina is the preferred high-throughput NGS technology for metagenomics and metabarcoding analyses (Oulas *et al.*, 2015). Illumina sequencing combines a sequencing-by-synthesis approach with a bridge PCR amplification of a DNA library on the surface of a flow cell (Figure 5) (Mardis, 2008). In metabarcoding experiments of environmental samples, libraries are obtained amplifying a DNA barcode (i.e., 16S rDNA, ITS, etc.) (Shokralla *et al.*, 2012; Schmidt *et al.*, 2013). The amplification is carried out with specific primers containing an adaptor that allows the immobilization on the flow cell (Figure 8A) (Oulas *et al.*, 2015). Sample-specific short barcodes are attached to each amplicon during the amplification to allow samples multiplexing (Oulas *et al.*, 2015). The sequencing reaction happens on a flow cell, which surface has covalently-ligated oligos complementary to the adaptors ligated to the amplicons (Shokralla *et al.*, 2012). During the sequencing reaction, the DNA polymerase generates multiple DNA copies (clusters) of each library DNA fragment through bridge-PRC amplification (Figures 8B and 8C) (Mardis, 2008). The

DNA polymerase incorporates one out of four differentially labelled fluorescent nucleotides attached with a blocking group at the 3'-OH edge, that ensure the incorporation of only one base per flow cycle (Figure 9A) (Shokralla *et al.*, 2012; Oulas *et al.*, 2015). Only the labelled nucleotide complementary to the template DNA is incorporated in the growing strand, while the others are washed away at the end of every flow cycle (Oulas *et al.*, 2015). After the incorporation of every nucleotide, a fluorescence detector identifies the newly incorporated nucleotide in each cluster (Figure 9B) (Oulas *et al.*, 2015) and the 3'-OH blocking group is removed to allow the incorporation of a new labelled nucleotide in the next cycle (Figure 9C) (Mardis, 2008). The sequencing reaction continues until the DNA molecule is sequenced, up to a maximum of 300 bp (Oulas *et al.*, 2015). Sequences are computed for each cluster and a quality filtering algorithm removes low-quality reads (Shokralla *et al.*, 2012). The remarkable amount of data generated by NGS platforms requires downstream computational and bioinformatics analyses to obtain a taxonomic profile of the community, aligning the processed reads with reference databases (Oulas *et al.*, 2015). Some popular reference databases are Greengenes and Ribosomal Database Project (RDP) for 16S rDNA, Silva for 16S and 18S rDNA and Unite for ITS (Oulas *et al.*, 2015).

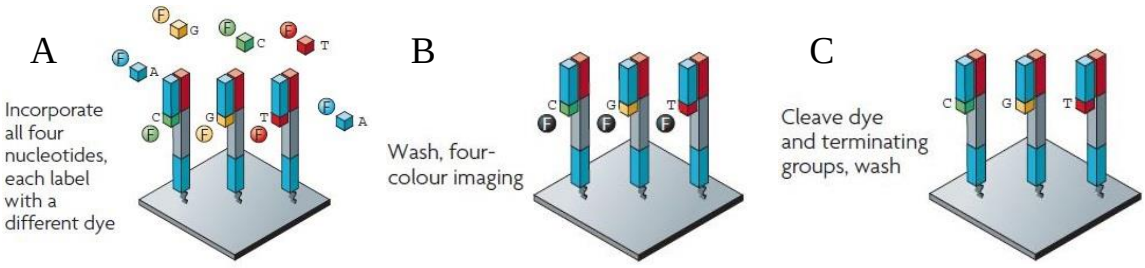



**Figure 9.** Synthesis of the DNA fragment with Illumina's four-dye cyclic reversible termination system. Fluorescent nucleotides containing a 3'-OH blocking group are incorporated in the growing strand, according to the complementarity with the template (A). The free nucleotides are washed away and a laser detects the fluorescence of the incorporated nucleotide (B). The dye and the 3'-OH blocking group are cleaved (C) and the growing DNA strand is ready for another synthesis cycle. Adapted from Metzker (2010).

# Aim of the thesis

The introduction of scab-resistant apple cultivars will allow the reduction of fungicide treatments. Fungicides applied to control apple scab can have side effects against secondary pathogens and the reduction of their applications may cause the outbreak of emerging pathogens. However, scarce information is available on dynamics and physiology of bark-associated microorganisms, such as secondary pathogens and beneficial microorganisms.

The final goal of the present doctoral work is to evaluate the potential changes in the microbiota composition of scab-resistant apple cultivars under low-input disease management i) by the development of metabarcoding-based approaches for the precise study of bark-associated microbial communities, ii) by the evaluation of environmental and agronomic factors affecting population dynamics of plant-associated pathogenic and beneficial microorganisms and iii) by the characterization of fungicide impacts against a bark pathogen under controlled conditions.

For this purpose, the specific aims of this work are:

- to use a microbial ecology approach based on metabarcoding analyses in order i) to estimate proportions and relationships of plant pathogens, beneficial and neutral microorganisms associated with plant bark and ii) to analyse effects of environmental factors (orchard location and climatic conditions), host properties (plant species, cultivar and tissue age) and disease management strategies (integrated and low-input disease management) on the composition of the bark microbiota and on the equilibrium between plant pathogens and beneficial microorganisms (Chapter II and III are referred to this part);
- to use a plant pathology approach on a secondary pathogen (the canker agent *Diplodia seriata*) on apple scab resistant plants (cv. Fujion) in order i) to develop a fast method to assess canker diseases under controlled conditions and ii) to evaluate the impact of conventional fungicides against this secondary pathogen of apple plants (Chapter IV is referred to this part).

# Results chapters

# Chapter II. Tissue age and plant genotype affect the microbiota of apple and pear bark

## 2.1 Summary

Every plant tissue and organ is populated by microbial communities. Despite its importance as reservoir of pathogens and beneficial microorganisms, very little information is available about the bark microbiota. The aim of this work was to determine the composition of the bark microbiota in relation to bark age, host species and cultivar.

This work represented the pilot study to optimize a protocol for the metabarcoding analysis of fungal and bacterial communities of bark that was then applied for the study of population dynamics described in the Chapter III. Bark of young (one year-old) and old (three/four-year-old) shoots was collected from apple (Golden Delicious and Gala cultivars) and pear (*Pyrus communis*; Abate and Williams cultivars) plants at the end of the dormancy stage. Samples were collected in triplicate, ITS and 16S amplicon libraries were prepared after the optimization of DNA extraction, PCR amplification and gel purification. Fungal and bacterial libraries were sequenced with an Illumina MiSeq platform and amplicon reads were further processed and clustered in OTUs at 97% of sequence similarity to obtain the taxonomic composition of the bark microbiota.

Results showed that young and old barks of apple and pear plants share a core microbiota of 159 and 350 common fungal and bacterial OTUs, respectively. Dominant taxa were mainly represented by genera with ubiquitous attitudes (e.g. *Aureobasidium*, *Cryptococcus*, *Deinococcus* and *Hymenobacter* genera), suggesting microbial migration from surrounding environments, such as soil and leaves. Several potential plant pathogenic fungi (e.g. *Alternaria* spp., *Diplodia* spp., *Gibberella* spp., *Peltaster* spp., *Penicillium* spp., *Phoma* spp., *Rosellinia* spp., *Stemphylium* spp. and *Taphrina* spp.) and bacteria (e.g. *Pseudomonas* spp., *Clavibacter* spp., Curtobacterium spp., *Nocardia* spp. and *Pantoea* spp.) were found on apple and pear bark, confirming the role of this plant tissue as a reservoir of phytopathogens. Interestingly, several fungal (e.g. *Arthrinium*, *Aureobasidium*, *Cryptococcus*, *Rhodotorula*, *Saccharomyces* and *Sporobolomyces*) and bacterial genera (e.g. *Bacillus*, *Brevibacillus*, *Burkholderia*, *Deinococcus*, *Lactobacillus*, *Methylobacterium*

and *Sphingomonas*) with potential biocontrol and plant growth promotion properties were found, opening the possibility to extend the research on beneficial microorganisms to the bark environment. The major drivers of community composition were the bark age and the plant species and, to lesser extent, plant cultivar. In particular, the bark age and host genotype affected the equilibrium between pathogenic (e.g. fungal genera: *Alternaria, Penicillium, Rosellinia, Stemphylium* and *Taphrina*; bacterial genera: *Curtobacterium* and *Pseudomonas*) and beneficial microorganisms (e.g. fungal genera: *Arthrinium, Aureobasidium, Rhodotorula* and *Sporobolomyces*; bacterial genera: *Bacillus, Brevibacillus, Methylobacterium, Sphingomonas* and *Stenotrophomonas*).

In conclusion, this work demonstrated that complex fungal and bacterial communities are hosted by pear and apple bark during the dormancy stage, suggesting the possible role of this plant tissue as a microbial overwintering site. Potential pathogenic and beneficial taxa were found and they are possibly in equilibrium in the conditions analysed in this study. In particular, bark is a possible environmental reservoir of plant pathogens that can easily migrate to other host tissues during the plant vegetative season, thanks to wind, rain splashes and agronomical practices.

## 2.2 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 age of the shoot, 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.

**Keywords:** *Malus domestica*, *Pyrus communis*, bark microbiota, metabarcoding, tissue age, plant genotype

## 2.3 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 (Whipps *et al.*, 2008; Vorholt, 2012; Bodenhausen *et al.*, 2014; Leff *et al.*, 2015). 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 (Garbeva *et al.*, 2008; Berg and Smalla, 2009), 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) 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 (Wakelin *et al.*, 2008; Berendsen *et al.*, 2012; Rastogi *et al.*, 2012; Bulgarelli *et al.*, 2013), while only a few of them have studied flower (Junker *et al.*, 2011; Shade *et al.*, 2013a), fruit (Martins *et al.*, 2013; Abdelfattah *et al.*, 2016) 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 bark has a greater diversity and richness of bacterial species than leaves 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).

## 2.4 Materials and methods

### 2.4.1 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 sec. 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 10x magnification.

### 2.4.2 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'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-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 sec, annealing at 59°C for 1 min and extension at 72°C for 45 sec, 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.

### 2.4.3 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 *et al.*, 2010) were used as reference databases to assign the bacterial and fungal taxonomy, respectively. Operational taxonomic unit (OTU)-based analysis was performed in QIIME (Caporaso *et al.*, 2010) to calculate richness (observed OTUs) and diversity (Simpson's index) after random re-*sampling* (999 iterations) and rarefied OTU tables generated at 27,019 and 21,133 read depth for bacteria and fungi, respectively. The Venn diagram of fungal and bacterial OTUs in pear and apple bark was visualised using the online tool JVenn (Bardou *et al.*, 2014). The composition of bacterial and fungal populations was visualised using Krona software (Ondov *et al.*, 2011). Fungal and bacterial genera comprising species with putative plant pathogens (pear and apple pathogens or pathogens of other plants) or beneficial properties (biocontrol agents and plant growth promoters) were identified according to previous literature and consensus sequences were obtained by sequence alignment of OTUs belonging to the same genus using the Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and Ambiguity Consensus Maker tools (https://www.hiv.lanl.gov/content/sequence/CONSENSUS/AmbigCon.html). A neighbour-joining tree was generated on fungal and bacterial consensus sequences using MEGA 7.0 software (Kumar *et al.*, 2016) with the Maximum Likelihood method and 500 replicates of bootstrap testing. Phylogenetic trees were then visualised using iTOL software (Letunic and Bork, 2016).

### 2.4.4 Statistical analysis

The data of observed OTUs, Simpson's index values and CFU counts (normalised by $\log_{10}$ transformation) were analysed using Statistica 13.1 software (Dell, Round Rock, TX, USA). Normal distribution (Kolmogorov-Smirnov test, $p > 0.05$) and variance homogeneity (Cochran's test, $p > 0.05$) were checked, and analysis of variance (ANOVA) was carried out using Tukey's test ($\alpha = 0.05$) to assess significant differences between samples. In order to assess the influence of bark age, plant cultivar and plant species, ANOVA with Chi-squared test was used on observed OTUs and Simpson's index data, and multifactorial ANOVA with the univariate test was used on CFU data.

Bacterial and fungal community structure and diversity were first assessed with unsupervised ordination scaling. For this purpose, rarefied OTU tables were imported in R, Bray-Curtis dissimilarity matrices were calculated and non-metric multidimensional scaling (NMDS) ordination was performed after Wisconsin double standardisation and 999 random starts using the vegan R package (Oksanen *et al.*, 2013). Multivariate analysis of deviance was computed with the mvabund R package (Wang *et al.*, 2012) to determine the effect of bark age (comparison of old and young bark), plant species (comparison of pear and apple bark) and plant cultivars (comparison of Abate, Williams, Golden and Gala bark) on bacterial and fungal data. In more detail, multivariate analysis of deviance was calculated with a likelihood-ratio test after 999 permutations on a generalised linear model, assuming negative binomial distribution (graphically checked) of the data.

In order to assess the influence of bark age and plant species on the relative abundance of fungal and bacterial genera, a permutation pairwise comparison test with 999 permutations was carried out with *p-values* adjusted by false discovery rate (FDR) using the RVAideMemoire R package (https://CRAN.R-project.org/package=RVAideMemoire). In more detail, the test was accomplished with two pairwise comparisons as follows: i) old *vs.* young bark (pooling the data for all plant species for each bark type) and ii) pear *vs.* apple bark (pooling the data for old and young bark for each plant species). Moreover, Bayesian analysis of multivariate count data in metabarcoding and ecology was carried out with the R package, version 1.0.10. (https://CRAN.R-project.org/package=MCMC.OTU) in order to detect significant differences in the relative abundance of fungal and bacterial genera in bark samples, according to the following pairwise comparisons: i) old pear bark (pooling old Abate and Williams bark) *vs.* old apple bark (pooling old Golden and Gala bark); ii) young pear bark (pooling young Abate and Williams bark) *vs.* young apple bark

(pooling young Golden and Gala bark); iii) Abate (pooling old and young bark) *vs.* Williams (pooling old and young bark); iv) Golden (pooling old and young bark) *vs.* Gala (pooling old and young bark); v) old Abate bark *vs.* old Williams bark ; vi) young Abate bark *vs.* young Williams bark ; vii) old Golden bark *vs.* old Gala bark ; viii) young Golden bark *vs.* young Gala bark ; ix) old *vs.* young Abate bark; x) old *vs.* young Williams bark; xi) old *vs.* young Golden bark, and xii) old *vs.* young Gala bark.

## 2.5 Results

### 2.5.1 Structure of the pear and apple bark fungal community

Bark from three/four-year-old shoots (old bark) and one-year-old shoots (youngbark) of Abate and Williams pear cultivars and Golden and Gala apple cultivars was collected and the surface morphology of old bark was found to be rough and rich in cavities (Figs. 1A-1D), while that of young bark was smooth (Fig. 1E-1H). The number of fungal CFUs was higher in old than in young bark for each cultivar (Fig. S1A). In addition to bark age, fungal CFUs differed between pear and apple (plant species) and between Gala and Golden (apple cultivars), according to multifactorial ANOVA. The number of fungal CFUs in old bark was lower in Gala as compared with Golden, William and Abate samples, while the number in young bark was lower in Abate and William as compared with Golden samples.


**Fig. 1.** Bark surface of pear and apple bark. Four-year-old shoots (old bark, A-D) and one-year-old shoots (young bark, E-H) of Abate (A, E) and Williams (B, F) pear cultivars and Golden (C, G) and Gala (D, H) apple cultivars. Images were acquired with a stereoscope (10x magnification).

After quality filtering, 2,050,096 filtered fungal reads were obtained for bark samples (Table S1). The total number of sequences for each replicate ranged from 21,133 to 237,589 and the number of OTUs per replicate ranged from 66 to 183 (Table S1). A total of 430 fungal OTUs were found; 159 of them were shared between old and young bark, while 152 and 119 OTUs were specific for old and young barks, respectively (Table S2). Moreover, pear and apple cultivars shared 75 OTUs in old bark (Fig. 2A) and 58 OTUs in young bark (Fig. 2B). The number of fungal OTUs differed according to the plant cultivar according to ANOVA with Chi-squared test, while no differences were detected in bark age and plant species (Table S3). The number of fungal OTUs was consistent among bark samples, except for greater richness in Williams than in Abate young bark (Fig. S2A). Of the total fungal reads, 97.33% were attributed to a genus (11,823 reads and 55 OTUs) or species (481,834 reads and 228 OTUs), while 0.14% were attributed to a family (734 reads and 32 OTUs; Table S2). The diversity of fungal communities estimated by Simpson's index was influenced by bark age and plant cultivar (Table S3), and was higher in old bark than in young bark of Abate, Golden and Gala (Fig. S2B).

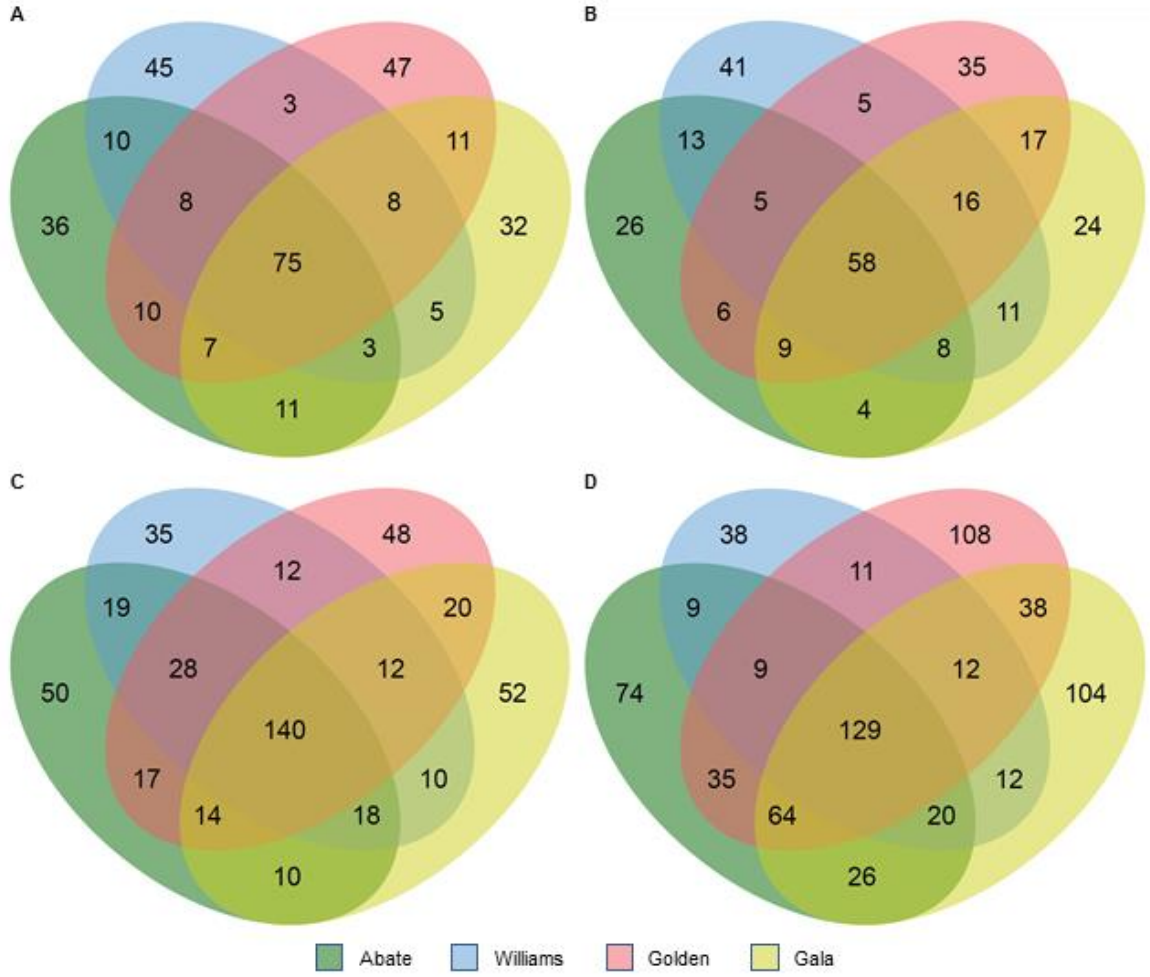



**Fig. 2.** Richness of fungal and bacterial communities of pear and apple bark microbiota. Operational taxonomic units (OTUs) of fungal (A, B) and bacterial (C, D) communities were determined for bark of three/four-year-old shoots (old bark; A, C) and one-year-old shoots (young bark; B, D) collected from Abate (green) and Williams (blue) pear cultivars, and Golden Delicious (red) and Gala (yellow) apple cultivars.

Global effects on microbiota diversity were analysed using NMDS ordination, which clustered fungal communities according to bark age (Fig. 3A). Multivariate analysis of deviance showed that the composition of fungal communities was affected by bark age, plant species and plant cultivar (Table S4). Fungal communities of old and young bark were dominated by the Ascomycota phylum (70.50 ± 1.31% and 90.71 ± 2.04%, respectively) followed by the Basidiomycota phylum (29.15 ± 1.29% and 9.20 ± 2.04%, respectively) (Fig. 4 and Table S2). Dothioraceae (57.71 ± 1.79% and 82.55 ± 2.39%, respectively) and Pleosporaceae (6.95 ± 1.06% and 7.21 ± 0.54%, respectively) were the most abundant families in old and young bark.

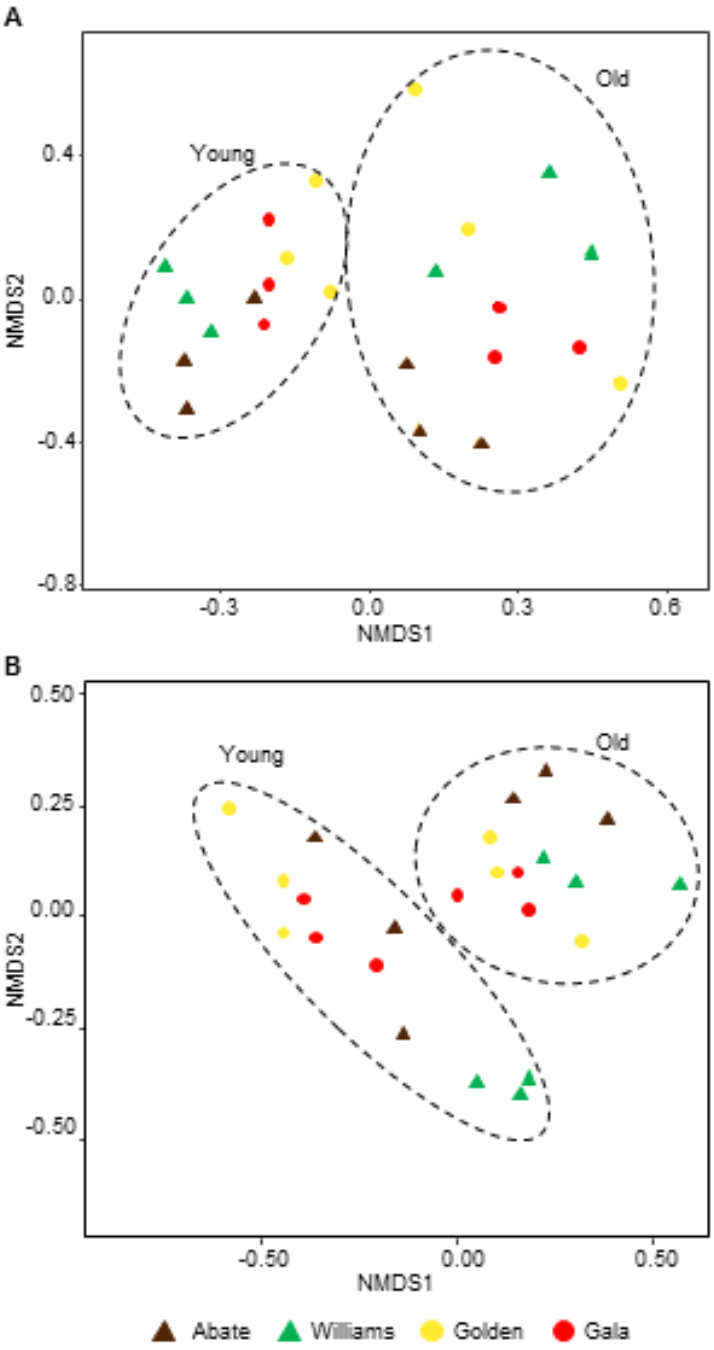



**Fig. 3.** Overview of pear and apple bark microbiota diversity. The diversity of fungal (A) and bacterial (B) bark communities was assessed with non-metric multidimensional scaling (NMDS) ordination of data for three/four-year-old shoots (old bark) and one-year-old shoots (young bark) collected from Abate (brown triangles) and Williams (green triangles) pear cultivars, and Golden Delicious (yellow circles) and Gala (red circles) apple cultivars. The results of multivariate analysis of deviance are reported for fungal and bacterial data in Table S4.

### 2.5.2 Relative abundance and properties of fungal genera in pear and apple bark

Bark microbiota was dominated by seven fungal genera (*Alternaria, Aureobasidium, Cryptococcus, Cystobasidium, Phaeosclera, Rhodotorula* and *Sporobolomyces*). The relative abundance of *Cystobasidium* and *Rhodotorula* was higher in old than in young bark, while that of *Aureobasidium* and S*porobolomyces* was lower (Figs. 4A and 4B; Table S5). In addition, the relative abundance of two (*Rhinocladiella* and *Volucrispora*) and six (*Bensingtonia, Capronia, Chalastospora, Lalaria, Phaeosphaeria* and *Sarcinomyces*) minor genera was higher and lower in old and young bark respectively (Figs. 4C and 4D; Table S5). Conversely, the abundance of three dominant (*Alternaria*, *Cryptococcus* and *Phaosclera*) and 104 minor genera was consistent in old and young bark.

The relative abundance of nine fungal genera differed in pear and apple bark (Figs. S3A and S3B; Table S5). Specifically, the relative abundance of the dominant genera *Alternaria* and *Phaeosclera* was higher in apple than in pear bark. Moreover, two (*Arthrinium* and *Taphrina*) and five minor genera (*Bullera, Leptosphaeria, Protomyces, Setomelanomma* and *Stemphylium*) showed a higher and lower relative abundance in pear than in apple bark, respectively.

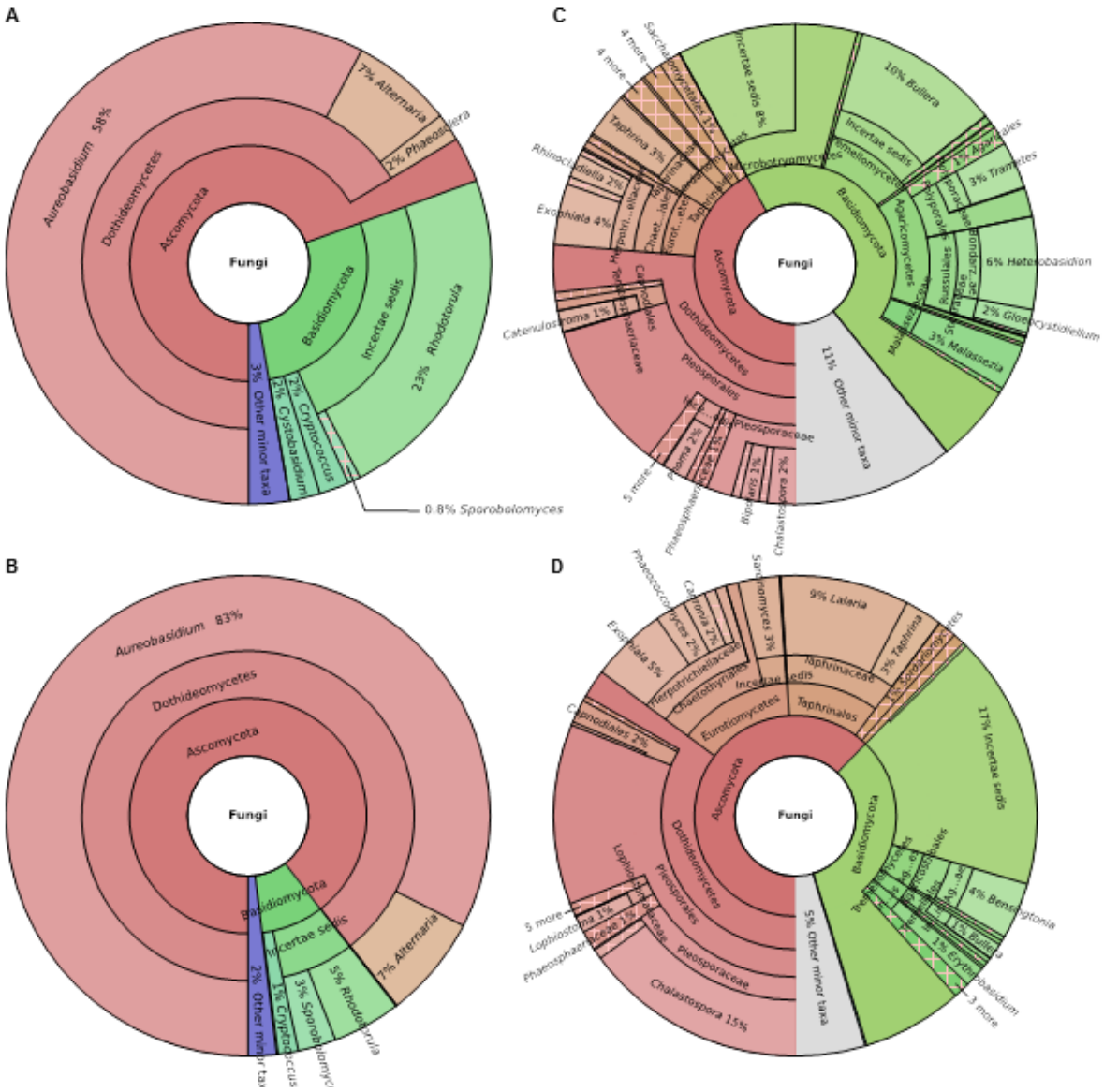



**Fig. 4**. Relative abundance of fungal genera in old and young bark. Dominant (relative abundance > 1.8%; A and B) and rare (C and D) fungal genera were determined for bark of three/four-year-old shoots (old bark, A and C) and one-year-old shoots (young bark, B and D) collected from Abate and Williams pear cultivars, and Golden Delicious and Gala apple cultivars. The mean relative abundance of fungal genera was calculated for old and young bark (pooling data for the four cultivars for each bark type) and permutation pairwise comparison tests were carried out to identify significant changes in relative abundance in old and young bark (Table S5). The total relative abundance of dominant and rare fungal genera was 97.3% (A) and 2.7% (C) in old bark, and 98.1% (B) and 1.9% (D) in young bark, respectively.

When analysing data of old and young bark separately, the relative abundance of *Curvularia* and *Myriangium* was higher in apple than in pear in old and young bark respectively (Table S5). The relative abundance of three genera (*Guehomyces*,

*Leprocaulon* and *Penicillium*) was higher in Abate than in Williams bark and that of one genus (*Hypholoma*) was higher in Golden than in Gala bark. Analysing the old bark of pear cultivars, *Cyphellophora* and *Leptosphaeria* showed higher relative abundance in Williams than in Abate. Moreover, the relative abundance of *Rosellinia* and *Teratosphaeria* was higher and lower in Abate and Williams young bark, respectively. One genus (*Myriangium*) showed higher abundance in Golden than in Gala young bark and all fungal genera showed a consistent abundance in Golden and Gala old bark. In Abate samples, the abundance of two genera (*Coprinellus* and *Phaeophyscia*) and one genus (*Westerdykella*) was higher and lower in old and young bark, respectively. In Williams bark samples, the relative abundance of *Psathyrella* was higher in old than in young bark. The abundance of the genus *Lophium* was higher in young than in old bark of Golden, while no differences in the relative abundance in old and young bark were detected in Gala samples.

Of the bark fungal community, nine genera (*Alternaria, Diplodia, Gibberella, Peltaster, Penicillium, Phoma, Rosellinia, Stemphylium* and *Taphrina*) potentially comprised species with pathogenic activities against the pear and apple, and two of them (*Alternaria* and *Penicillium*) also included species with potential biocontrol activities (Fig. 5A, Table S5). The abundance of two genera (*Alternaria* and *Stemphylium*) and one genus (*Taphrina*) was higher and lower in apple and pear bark respectively, while the abundance of *Diplodia, Gibberella* and *Peltaster* was consistent in bark samples. Nineteen genera potentially containing plant pathogenic species with host targets different from the pear and apple were found. Only the genus *Setomelanomma* showed a higher relative abundance in apple than in pear bark, and the other 18 genera showed a consistent abundance in bark samples (*Bipolaris, Catenulostroma, Deniquelata, Exobasidium, Heterobasidion, Hyphodermella, Hypoxylon, Itersonilia, Monographella, Periconia, Phaeomoniella, Phaeosphaeriopsis, Pyrenochaeta, Pyrenophora, Urocystis, Ustilago, Valsaria* and *Zymoseptoria*). Twelve fungal genera comprising species with potential biocontrol activities were found in total; five of them (*Alternaria, Aureobasidium, Cryptococcus, Rhodotorula* and *Sporobolomyces*) dominated in pear and apple bark (Figs. S3A and S4A) and five (*Coniothyrium, Cryptococcus, Paraconiothyrium, Saccharomyces* and *Saccharomycopsis*) were consistent in bark samples.

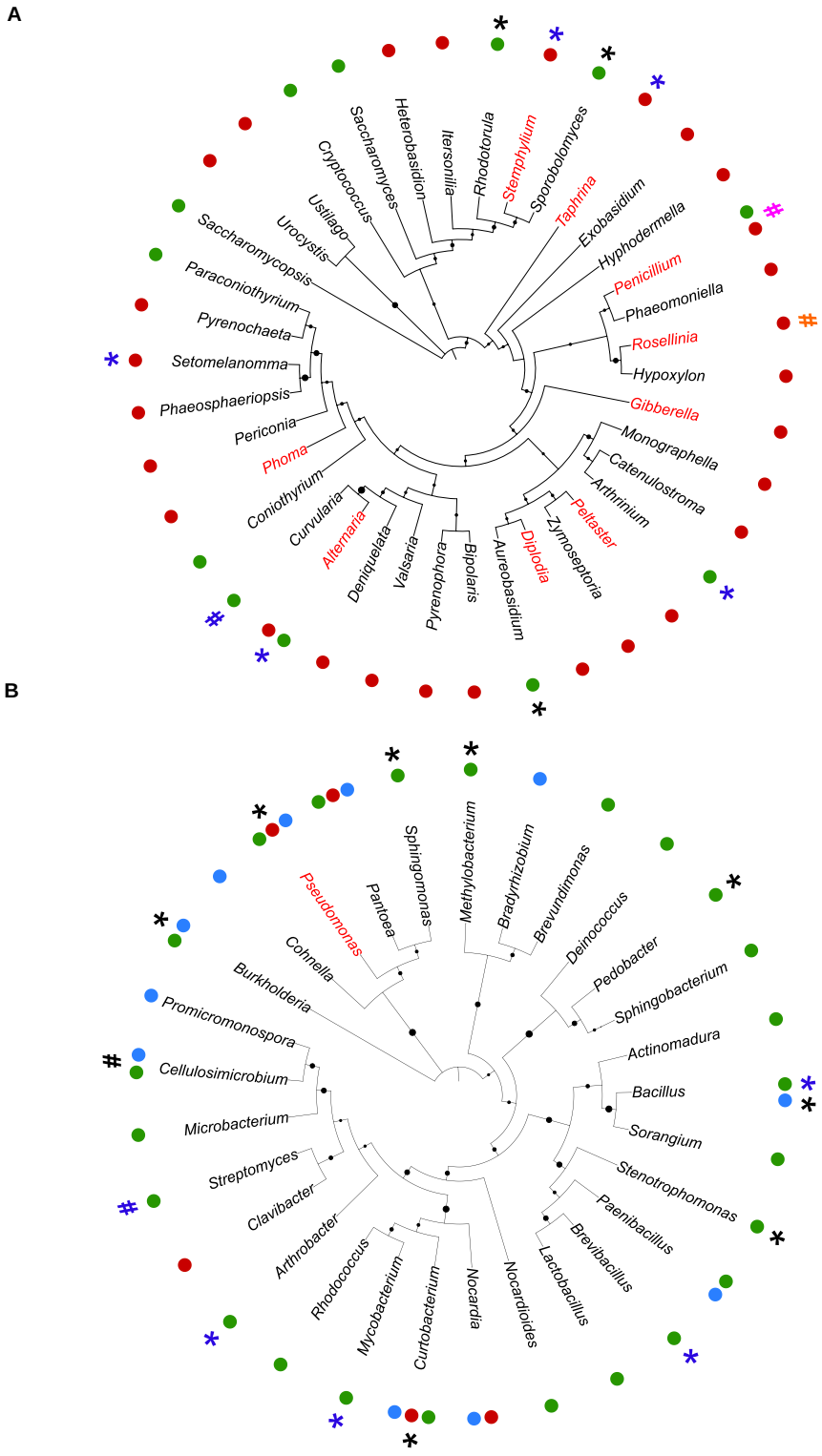



**Fig. 5.** Plant pathogens and beneficial microorganisms in pear and apple bark. Fungal (A) and bacterial (B) genera comprising potential plant pathogens (red circles), biocontrol agents (green circles) and plant growth-promoters (cyan circles) are shown. Pear and apple pathogens are indicated in bold red. Asterisks indicate genera with significant changes in abundance in old and young bark (black) and in pear and apple bark (cyan), according to permutation pairwise comparison tests. Hashtags indicate genera with significant changes in abundance in old pear and apple bark (cyan), young pear and apple bark (black), Abate and Williams bark (pink) and young Abate and Williams bark (orange), according to Bayesian analysis (Table S5 and S7).

### 2.5.3 Structure of the pear and apple bark bacterial community

The number of bacterial CFUs was affected by bark age and apple cultivar, while it was not affected by plant species and pear cultivar according to multifactorial ANOVA (Fig. S1B). In particular, the number of bacterial CFUs was higher in old than in young bark of Abate and William. In young bark, the number of bacterial CFUs was lower in Abate and Williams than in Golden.

A total of 2,757,400 filtered bacterial reads were obtained for bark samples (Table S1). From 27,019 to 378,683 sequences and from 159 to 427 OTUs per sample were obtained for each replicate (Table S1). Of the 824 total bacterial OTUs, 350 were shared between old and young bark, while 135 and 339 were specific for old and young bark, respectively (Table S4). Old (Fig. 2C) and young bark (Fig. 2D) shared 140 and 129 OTUs, respectively. The number of bacterial OTUs differed according to bark age, plant species and plant cultivar (Table S3) and was higher in Golden young bark than in Golden old, Gala old and William young bark (Fig. S2C). Of the total bacterial reads, 95.51% were assigned to a genus (617,292 reads and 402 OTUs) or a bacterial species (2,042 reads and 74 OTUs), while 1.21% were attributed to a family (7,874 reads and 175 OTUs; Table S3). The diversity of bacterial communities estimated by Simpson's index was affected by bark age and was consistent in plant species and cultivars (Fig. S2D and Table S3).

NMDS ordination clustered bacterial communities according to bark age (Fig. 3B). The composition of bacterial communities was affected by bark age, plant species and plant cultivar, according to multivariate analysis of deviance (Table S4). Bacterial communities of old and young bark were dominated by Bacteroidetes (37.23 ± 1.38% and 45.28 ± 2.02%, respectively), Actinobacteria (29.47 ± 1.21% and 12.51 ± 1.58%, respectively), Proteobacteria (22.82 ± 0.87% and 24.15 ± 2%, respectively) and Deinococcus-Thermus (8.87 ± 1.05% and 17.30 ± 3.40%, respectively; Fig. 6 and Table S6). Moreover, Chlamydiae and Chlorobi phyla were found only in old bark, while Nitrospirae, Planctomycetes and Thermotogae were detected only in young bark. The most abundant bacterial families of old and young bark were Cytophagaceae (35.24 ± 1.43% and 44.59 ± 18.47%, respectively), Microbacteriaceae (22.38 ± 1.20% and 11.04 ± 2.04%, respectively), Deinococcaceae (8.87 ± 1.05% and 17.30 ± 12.77%, respectively) and Sphingomonadaceae (13.74 ± 0.85% and 11.20 ± 5.68%, respectively).

### 2.5.4 Relative abundance and properties of bacterial genera in pear and apple bark

The bacterial microbiota was dominated by *Amnibacterium*, *Curtobacterium*, *Deinococcus*, *Frondihabitans*, *Hymenobacter*, *Massilia*, *Methylobacterium*, *Modestobacter*, *Sphingomonas* and *Spirosoma* (Figs. 5A and 5B, Table S7). The relative abundance of five dominant genera (*Amnibacterium*, *Methylobacterium*, *Modestobacter*, *Sphingomonas* and *Spirosoma*) and three dominant genera (*Curtobacterium*, *Hymenobacter* and *Massilia*) was higher and lower in old and young bark respectively, while the abundance of *Deinococcus* and *Frondihabitans* was consistent in old and young bark. The relative abundance of 23 minor genera differed in old and young bark (Figs. 6C and 6D; Table S7).

The relative abundance of 15 minor bacterial genera differed in pear and apple bark, while those of dominant genera did not change in this comparison (Figs. S3C and S3D, Table S7). Specifically, *Bifidobacterium* was more abundant in pear bark, while *Acinetobacter*, *Alloprevotella*, *Aneurinibacillus*, *Arthrobacter*, *Bacillus*, *Brevibacillus*, *Gaiella*, *Haemophilus*, *Mycobacterium*, *Planifilum*, *Porphyromonas*, *Rothia*, *Staphylococcus* and *Symbiobacterium* were more abundant in apple bark. When considering old bark, one bacterial genera (*Streptomyces*) and two bacterial genera (*Ehnydrobacter* and *Segetibacter*) showed lower and higher relative abundance in pear and apple old barks, respectively. Moreover, the relative abundance of one bacterial genus (*Terracoccus*) and three bacterial genera (*Cellulosimicrobium*, *Kingella* and *Rothia*) were higher and lower in pear and apple, respectively.

When analysing data for pear and apple bark separately, four bacterial genera (*Acidocella*, *Criblamydia*, *Dokdonella* and *Saccharomonospora*) showed a higher relative abundance in Abate than in Williams bark. Moreover, *Fonticella* and *Tepidimicrobium* were more abundant in Golden bark, while *Defluviicoccus* and *Kineosporia* were more abundant in Gala bark. On analysing old bark of pear cultivars, two genera (*Agromyces* and *Peredibacter*) and one genus (*Alloprevotella*) genera showed a higher and lower relative abundance in Abate and Williams samples, respectively. In young bark, Abate showed a higher relative abundance of *Demequina* and *Saccharopolyspora* than William. The relative abundance of bacterial genera was consistent in Golden and Gala old bark, while that of *Pseudokinecoccus* was higher in Gala than in Golden young bark. In Abate samples, one genus (*Rhodovastum*) and three genera (*Alloprevotella*, *Geobacillus* and *Skermanella*)

were more abundant in old and young bark, respectively. In William samples, *Dyadobacter* and *Rhizomicrobium* were more abundant in old and young bark, respectively. *Shimazuella* and *Skermanella* showed lower abundance in old than in young bark of Golden and Gala, respectively.

*Pseudomonas* was the only genus that potentially comprised species with pathogenic activities on the apple (i.e. *Pseudomonas syringae* pv *papulans*), as well as

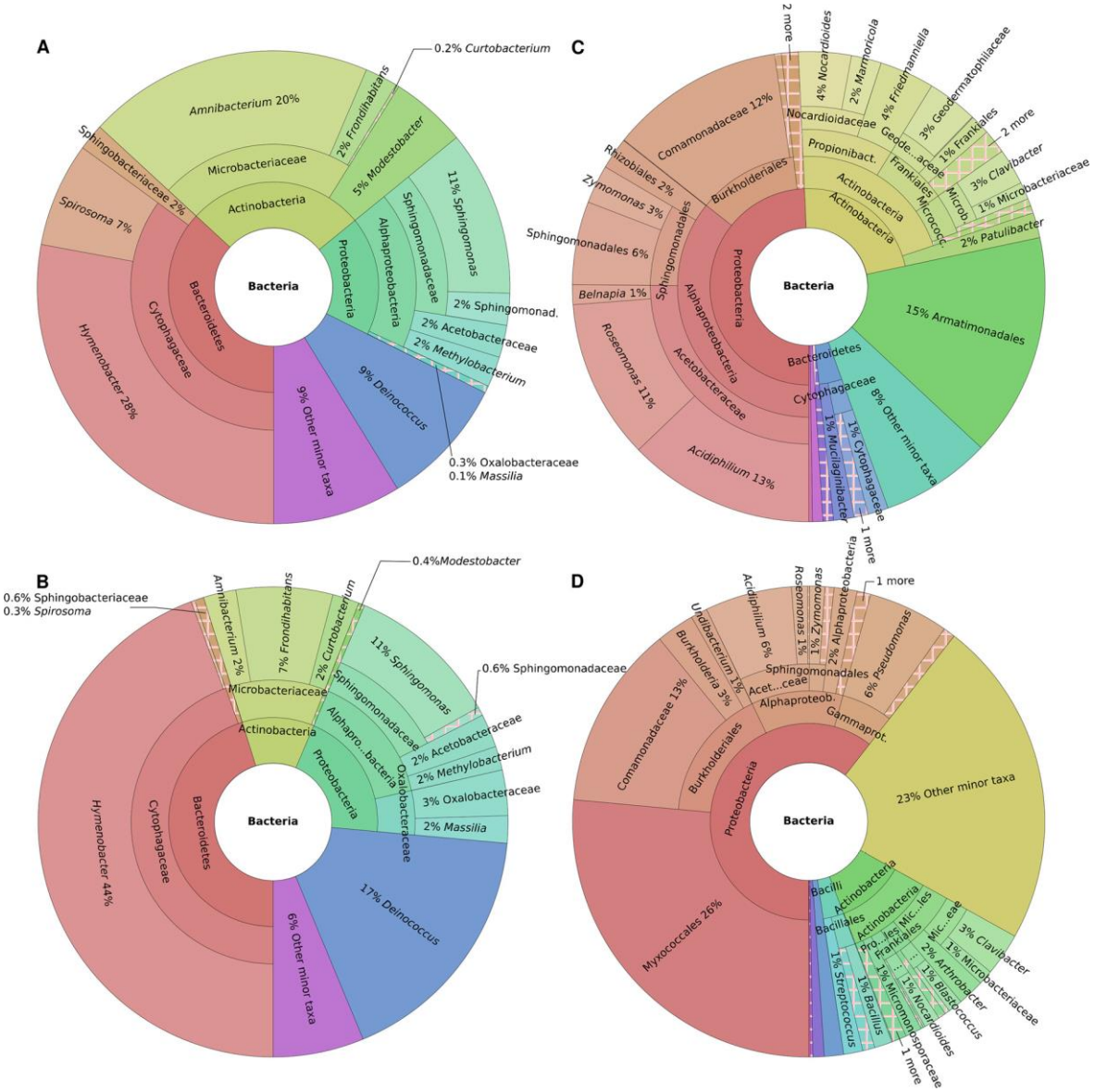



**Fig. 6.** Relative abundance of bacterial genera in old and young bark. Dominant (relative abundance > 1.8%; A and B) and rare (C and D) bacterial genera were determined for the bark of three/four-year-old shoots (old bark, A and C) and one-year-old shoots (young bark, B and D) collected from Abate and Williams pear cultivars, and Golden Delicious and Gala apple cultivars. The mean relative abundance of bacterial genera was calculated for old and young bark, pooling the data for the four cultivars for each bark type, and permutation pairwise comparison tests were carried out to identify significant changes in relative abundance in old and young bark (Table S7). The total relative abundance of dominant and rare bacterial genera was 82.9% (A) and 17.1% (C) in old bark, and 88.6% (B) and 11.4% (D) in young bark, respectively.

biocontrol or plant growth promotion properties (e.g. *Ps. fluorescens* and *Ps. chlororaphis*; Fig. 5B, Table S7). Of the four genera potentially comprising species with pathogenic activities on other plant species, three could also have biocontrol and plant growth promotion activities (*Curtobacterium*, *Nocardia* and *Pantoea*) and three showed a consistent abundance in bark samples (*Clavibacter, Nocardia* and *Pantoea*). Twenty-eight genera putatively comprised species with biocontrol and/or plant growth promotion activities, and four of them (*Curtobacterium*, *Deinococcus*, *Methylobacterium* and *Sphingomonas*) dominated on bark samples (Figs. S3B and S4B).

## 2.6 Discussion

Several above-ground surfaces of plant organs are characterised by scarce nutrient availability and are exposed to various environmental factors (e.g. sunlight, UV radiation, wind and rainfall) that can lead to the diversification of microbial communities (Leff *et al.*, 2015; Zarraonaindia *et al.*, 2015). Moreover, organ age (Vorholt, 2012) and plant genotype (Correa *et al.*, 2007; Whipps *et al.*, 2008; Manter *et al.*, 2010; Brader *et al.*, 2017) can influence the composition of plant-associated microbial communities. While bark can act as a reservoir of beneficial and pathogenic plant microorganisms (Buck *et al.*, 1998; Martins *et al.*, 2013), few studies have analysed the composition of microbial communities residing on this plant tissue (Martins *et al.*, 2013; Lambais *et al.*, 2014; Leff *et al.*, 2015). In this work, we analysed fungal and bacterial communities hosted in pear and apple bark to clarify the influence of bark age (old and young bark) and plant genotype (species and cultivar) on the composition of potential plant pathogens, biocontrol and plant growth promoting agents. The samples of bark analysed in this study hosted populations dominated by *Aureobasidium* and *Alternaria*, which have also been found previously on the bark of other plant species (*Broussonetia papyrifera*, *Celtis occidentalis* and *Ligustrum lucidum*) (De Errasti *et al.*, 2010) and on beech leaves (Cordier *et al.*, 2012). Likewise, the bacterial community in pear and apple bark was dominated by the Actinobacteria class (e.g. *Hymenobacter*) and Microbacteriaceae family (*e.g. Amnibacterium*, *Curtobacterium* and *Frondihabitans*), which have previously been found on the bark of other plant species (e.g. the grapevine, forest plants and *Ginkgo biloba*) (Martins *et al.*, 2013; Lambais *et al.*, 2014; Leff *et al.*, 2015). The analysed bark samples hosted fungal and bacterial taxa (e.g. *Cryptococcus*, *Rhodotorula*, *Sporobolomyces, Bacillus, Deinococcus, Methylobacterium*,

*Paenibacillus* and *Sphingomonas)* that have previously been found on other plant organs, such as plant leaves (Yashiro *et al.*, 2011; Perazzolli *et al.*, 2014; Ortega *et al.*, 2016), fruit (Martins *et al.*, 2013; Gomba *et al.*, 2017; Vepštaitė-Monstavičė *et al.*, 2018) and flowers (Shade *et al.*, 2013a).

Bark age was the major driver of changes in the global structure of fungal and bacterial communities. Specifically, bark age influenced the quantity of culturable fungi and bacteria, fungal and bacterial diversity and bacterial OTU richness. Higher bacterial richness has also been shown previously in old bark (trunk and woody branches) as compared with young bark (green branches) of *G. biloba* (Leff *et al.*, 2015). Coherently, it has been reported that *Vitex lucens* trunks with rough bark host a higher number of epiphytes than smooth-barked trunks of *Knightia excelsa* (Wyse and Burns, 2011) and that bark microsites of peach branches host larger fungal populations than smooth bark surfaces (Buck *et al.*, 1998). More specifically in our study, *Sphingomonas* and *Massilia* abundance was higher and lower in old and young bark respectively, in agreement with Sphingomonadaceae and Oxalobacteraceae abundance on woody branches and green branches of *G. biloba* (Leff *et al.*, 2015). The influence of bark age on fungal and bacterial community structure can possibly be explained by the different surface morphology characterising old and young bark. The surface of old bark is rougher and richer in cavities than that of young bark and this morphology can increase water retention capacity and/or protect microbial cells from adverse environmental conditions (e.g. UV rays, wash-off by rainfall) creating a more favourable habitat for microbial life.

In addition to bark age, the plant genotype (plant species and cultivar) affected the global structure of fungal and bacterial communities of pear and apple bark, and the plant cultivar in particular influenced fungal and bacterial richness and fungal diversity. It has previously been shown that plant genotype can affect the structure of phyllosphere microbial communities (Whipps *et al.*, 2008; Redford *et al.*, 2010), suggesting that each host is able to shape its associated microbiota. In particular, two dominant fungal genera (*Alternaria* and *Phaeosclera*) showed greater abundance in apple than in pear bark. Likewise, the relative abundance of bacterial genera (e.g. *Arthrobacter*, *Bacillus,* G*aiella*, *Mycobacterium* and *Staphylococcus*) differed in pear and apple samples, and it has previously been reported that the read counts of these genera also varied on apple and blackcurrant fruit skins (Vepštaitė-Monstavičė *et al.*, 2018). However, the bark of the closely related plant species studied in this work (*Py. communis* and *M. domestica*) shared

the majority of fungal and bacterial taxa, as previously shown for the phyllosphere of genetically related tropical trees (Kim *et al.*, 2012).

Several genera with potential pathogenic and biocontrol activities have been found among the fungal community of pear and apple bark, demonstrating that bark can act as a reservoir of potential pathogenic and beneficial microorganisms. In particular, nine genera comprising species with potential pathogenic properties for the pear and apple have been identified, such as the dominant genus *Alternaria,* which included potential post-harvest decay agents (*Al. alternata* and *Al. mali*) (Jones and Aldwinckle, 1990). Likewise, minor genera comprising potential pathogens of pear and apple plants have been found, such as agents of shoot diseases (e.g. *Diplodia seriata*) (Jones and Aldwinckle, 1990), root diseases (e.g. *Rosellinia necatrix*) (Jones and Aldwinckle, 1990), leaf diseases (e.g. *Taphrina bullata*) (Cunnington and Mann, 2004) and fruit diseases (e.g. *Gibberella avenacea*, *Penicillium expansum*, *Phoma spp.*, *Stemphylium herbarum* and *S. versicarium*) (Jones and Aldwinckle, 1990; Alberoni *et al.*, 2005; Maxin *et al.*, 2012). Moreover, some genera (e.g. *Alternaria* and *Penicillium*) also comprised species with potential biocontrol activity and they could have multiple roles. For example, *Al. zinniae* and *Al. cassiae* were active against weeds (Charudattan, 2001) and *Pe. oxalicum* was able to control *Fusarium* wilt in the tomato (Larena *et al.*, 2003). In addition to potential pear and apple pathogens, barks hosted 19 genera including potential pathogens of other plant species, such as those affecting fruit plants of agricultural interest [e.g. *Hyphodermella roseae* causing white rot on peach (Sayari *et al.*, 2012)] or causing wood diseases [e.g. *Phaeomoniella chlamydospora* and *Ph. effusa* on grapevine, peach, plum and apricot (Damm *et al.*, 2010) and *Valsaria insitiva* on stone fruits (Žežlina *et al.*, 2016)]. Interestingly, the relative abundance of some plant pathogens differed between plant species (*Alternaria, Setomelanomma, Stemphylium* and *Taphrina*) or cultivars (*Penicillium* and *Rosellinia*). Pear and apple bark also hosted ten potential biocontrol agents, and three of them (*Aureobasidium, Cryptococcus* and *Rhodotorula*) were particularly interesting thanks to their potential biocontrol activities against pear and apple pathogens. Indeed, *Au. pullulans* and *Cr. albidus* were shown to control grey mould and blue mould on post-harvest apple (Fan and Tian, 2001; Mari *et al.*, 2012), while *Rh. mucilaginosa* and *Rh. glutinis* were shown to control blue and grey mould of pears (Zhang *et al.*, 2008) and apples (Li *et al.*, 2011). Moreover, the genera *Saccharomyces* and *Saccharomycopsis* have a wide range of targets, including some post-harvest pathogens such as *Monilinia* spp. and *Botrytis* spp. (Janisiewicz *et al.*, 2010; Nally *et al.*, 2012). The abundance of two (*Aureobasidium* and

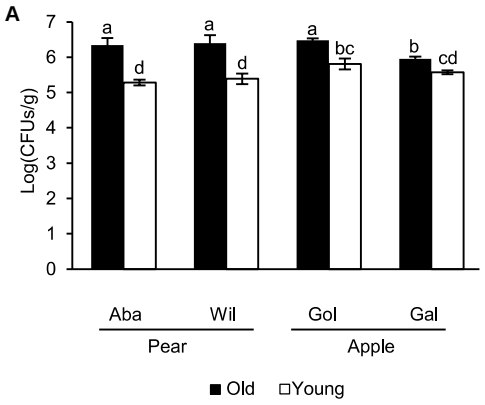
*Sporobolomyces*) and one potential biocontrol genera (*Rhodotorula*) were higher and lower in young and old bark, respectively. Moreover, the abundance of the potential biocontrol genera *Arthrinium* and *Curvularia* were affected by plant species and cultivar, respectively.

Potential pathogenic bacteria have also been found, such as the genus *Pseudomonas,* which comprises the causal agent of blister leaf spot and bark canker on apples (*Ps. syringae* pv. *papulans*) (Horst, 2008). Potential pathogens of other plant species were also found in pear and apple bark (*Clavibacter, Curtobacterium, Nocardia,* and *Pantoea*) and they comprise: *Cl. michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker of tomatoes (Agrios, 2005), the wide host-range phytopathogenic species *Cu. flaccumfaciens* (Locci, 1994; Agrios, 2005), *N. vaccinii,* which causes galls and bud proliferation on the blueberry (Horst, 2008), and *Pa. stewartii* subsp. *Stewartii*, which causes Stewart's wilt of corn (Horst, 2008). However, some of these genera could have multiple properties, such as *Curtobacterium*, *Pantoea* and *Pseudomonas,* which also includes biocontrol and plant growth promoter species (e.g. *Cu. plantarum*, *Pa. agglomerans*, *Ps. fluorescens* and *Ps. chlororaphis*) (Agrios, 2005; Siddiqui, 2005). *Curtobacterium*, *Pantoea* and *Pseudomonas* have previously been found on grapevine bark (Martins *et al.*, 2013), indicating that bark could be a potential source of inoculum for neighbouring plant species. Specifically, in our study *Curtobacterium* and *Pseudomonas* were more abundant in young than in old bark. The bark bacterial community included potential beneficial microorganisms (28 genera in total) that may display beneficial interaction with the plant. For example, the dominant genera *Deinococcus* and *Sphingomonas* contain species with potential biocontrol activities, such as *D. aquaticus* (Yang *et al.*, 2008) and *Sphingomonas* spp. (Innerebner *et al.*, 2011). Likewise, members of the *Lactobacillus* genus (e.g. *L. plantarum*) could have direct biocontrol effects against *Erwinia amylovora* (Roselló *et al.*, 2013), while *Pedobacter spp.* could produce chitinases against *Rhizoctonia solani* and *B. cinerea* (Song *et al.*, 2017), and they could maintain the equilibrium with pathogenic microbial inhabitants. The abundance of six potential biocontrol genera (*Bacillus, Burkholderia, Methylobacterium, Pedobacter, Sphingomonas* and *Stenotrophomonas*) was affected by bark age, while that of six potential biocontrol genera (*Arthrobacter, Bacillus, Brevibacillus, Cellulosimicrobium, Mycobacterium* and *Streptomyces*) was affected by plant species. In addition to biocontrol agents, bacterial genera with potential plant growth-promoting and resistance-inducing traits were found, such as *Bacillus, Bradyrhizobium, Cohnella* and *Paenibacillus* (Siddiqui, 2005; Ratón *et*

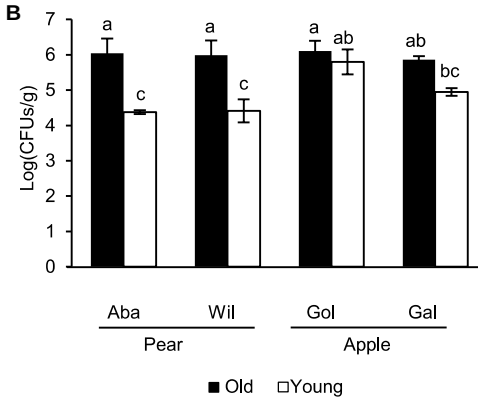
*al.*, 2012), and they would probably need to migrate to the rhizosphere to adequately display their beneficial properties.

In conclusion, complex fungal and bacterial communities were identified in pear and apple bark during the dormancy stage, suggesting the possible role of this plant tissue as a microbial overwintering site. Both potential pathogenic and beneficial taxa were identified, suggesting that plant pathogens and biocontrol agents are possibly in equilibrium in the conditions analysed in this study. In particular, bark is a possible environmental reservoir of plant pathogens that can easily migrate to other host tissues during the plant vegetative season, thanks to wind, rain splashes and agronomical practices. The metabarcoding approach of this study is a powerful tool for investigating the complex composition of bark communities in depth and it demonstrated the role of organ and plant characteristics (bark age and plant genotype) in shaping the associated microbiota. Future steps could include clarification of the influence of environmental conditions and agronomical practices on the composition of bark-associated microbial communities, in order to understand the impact of plant protection management strategies on microbial population dynamics.

## 2.7 Supplementary figures




| Variable | F | *p* |
|---|---|---|
| **Plant species** | 10.44 | **0.0042** |
| **Bark age** | 58.81 | **0** |
| **Pear variety** | 0 | 0.9644 |
| **Apple variety** | 15.51 | **0.0043** |




| Variable | F | *p* |
|---|---|---|
| **Plant species** | 1.46 | 0.2408 |
| **Bark age** | 88.2 | **0** |
| **Pear variety** | 0.62 | 0.4536 |
| **Apple variety** | 52.61 | **0.0001** |

**Figure S1.** Culturable microorganisms of pear and apple bark. Fungal (A) and bacterial (B) colony forming units (CFUs) per gram of bark fresh weight were determined for bark of three/four-year-old shoots (old bark) (old bark, black) and one-year-old shoots (young bark, white) collected from Abate (Aba) and Williams (Wil) pear cultivars, and Golden Delicious (Gol) and Gala (Gal) apple cultivars. Mean $Log_{10}$(CFUs/g) values and standard errors of three replicates are reported. Different letters indicate significant differences, according to Tuckey's test ($p \leq 0.05$). Each table shows factors affecting the number of CFUs, according to a multifactorial analysis of variance with the univariate test ($p \leq 0.05$).

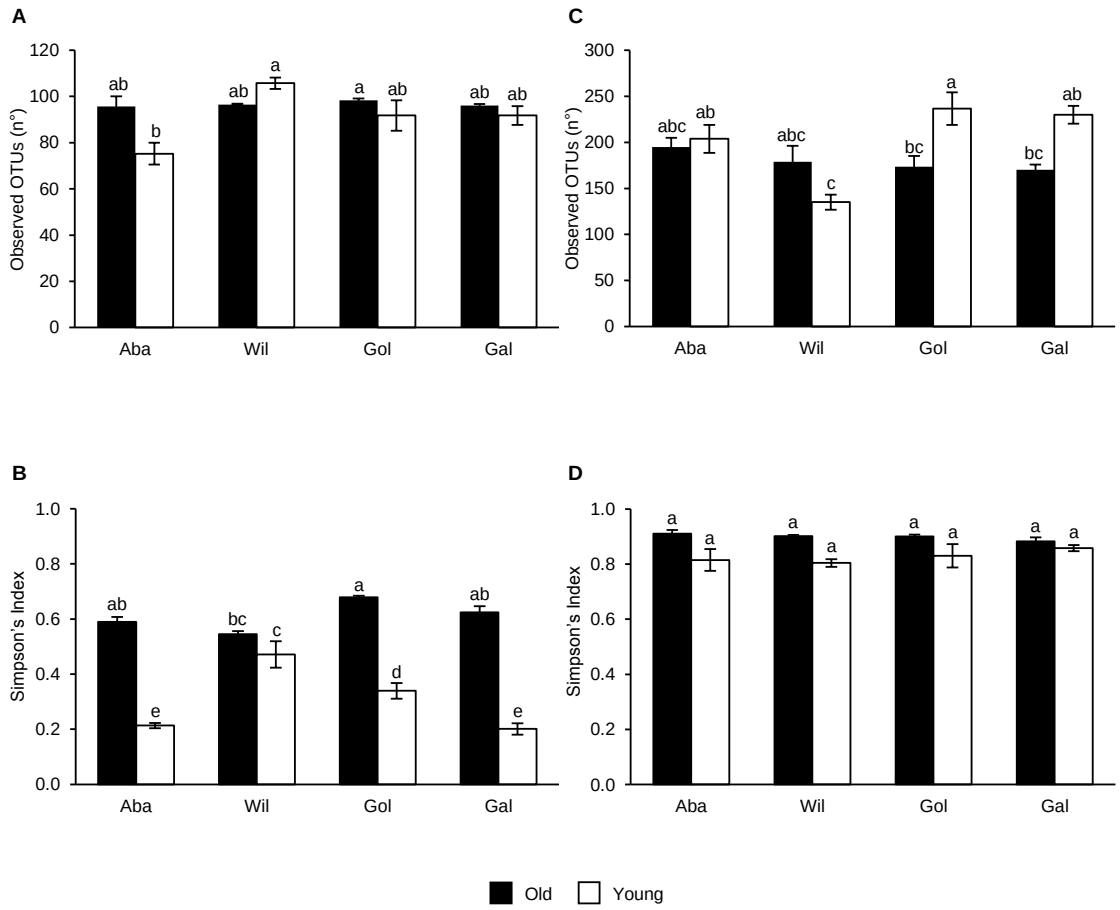



**Figure S2.** Richness and diversity of fungal and bacterial communities of the pear and apple bark microbiota. Richness (A, C), based on number of operational taxonomic units (OTUs) and diversity (B, D), estimated by the Simpson's index, were determined for fungal (A, B) and bacterial (C, D) bark communities of three/four-year-old shoots (old bark, black) and one-year-old shoots (young bark, white) collected from Abate (Aba) and Williams (Wil) pear cultivars, and Golden Delicious (Gol) and Gala (Gal) apple cultivars. Mean and standard error values of three biological replicates are presented for each sample. Different letters indicate significant differences, according to Tuckey's test ($p \leq 0.05$).

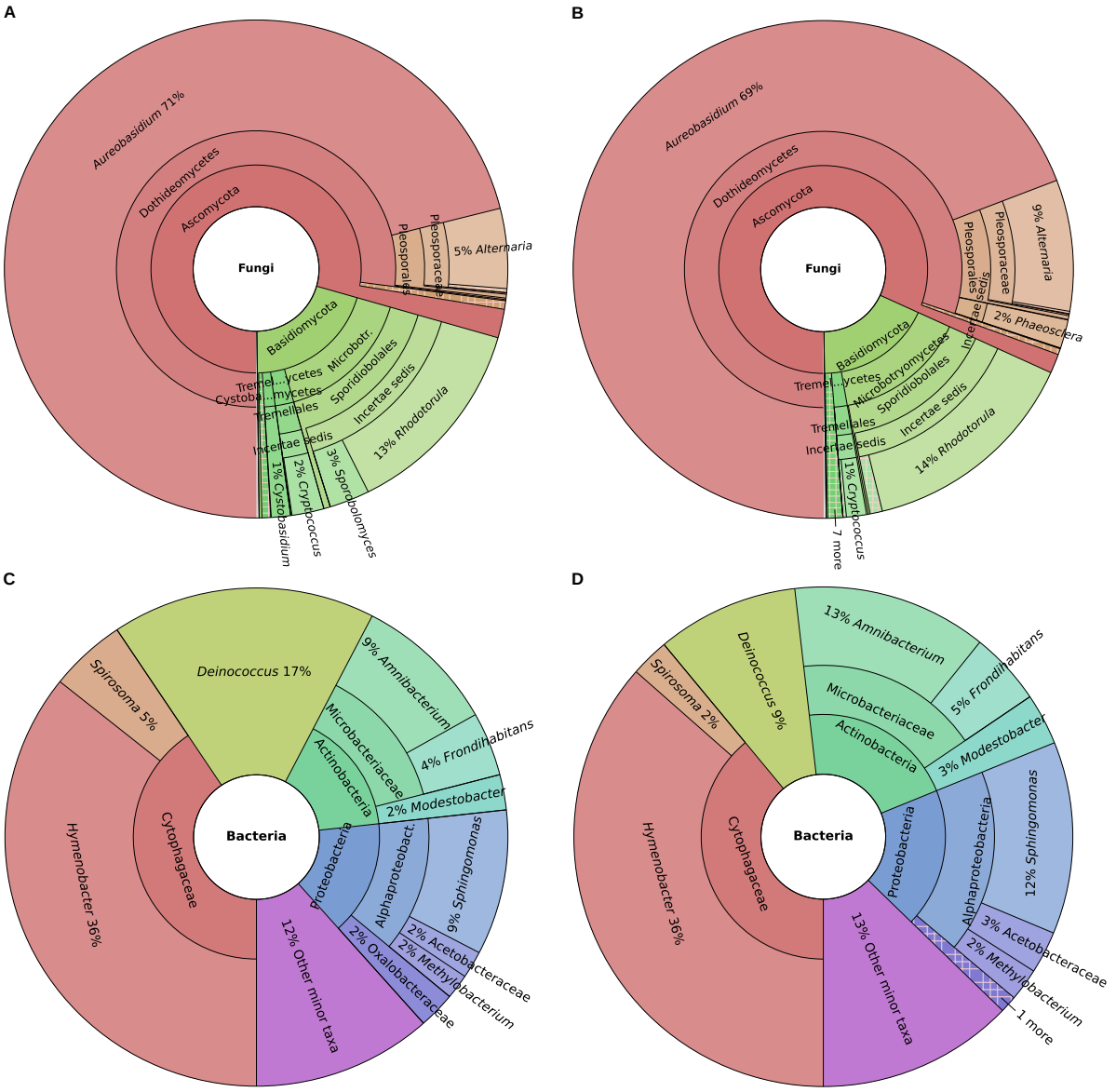



**Figure S3.** Relative abundances of fungal and bacterial genera in pear and apple bark. Dominant (relative abundance >1.8%) fungal (A and B) and bacterial (C and D) genera of pear (A and C) and apple (B and D) bark were determined for three/four-year-old shoots (old bark) and one-year-old shoots (young bark) collected from Abate and Williams pear cultivars, and Golden Delicious and Gala apple cultivars. Mean relative abundances were calculated for pear and apple bark, pooling the data for old and young bark for each plant species (Abate and Williams cultivars of pear and Golden Delicious and Gala cultivars of apple). Permutation pairwise comparison tests were carried out to identify genera with significant changes in relative abundance between pear and apple bark (Tables S5 and S7). The total relative abundance of dominant and fungal and bacterial genera was 96.0% (A) and 88.4% (C) in pear and 97.3% (B) and 87.2% (D) in apple bark, respectively.

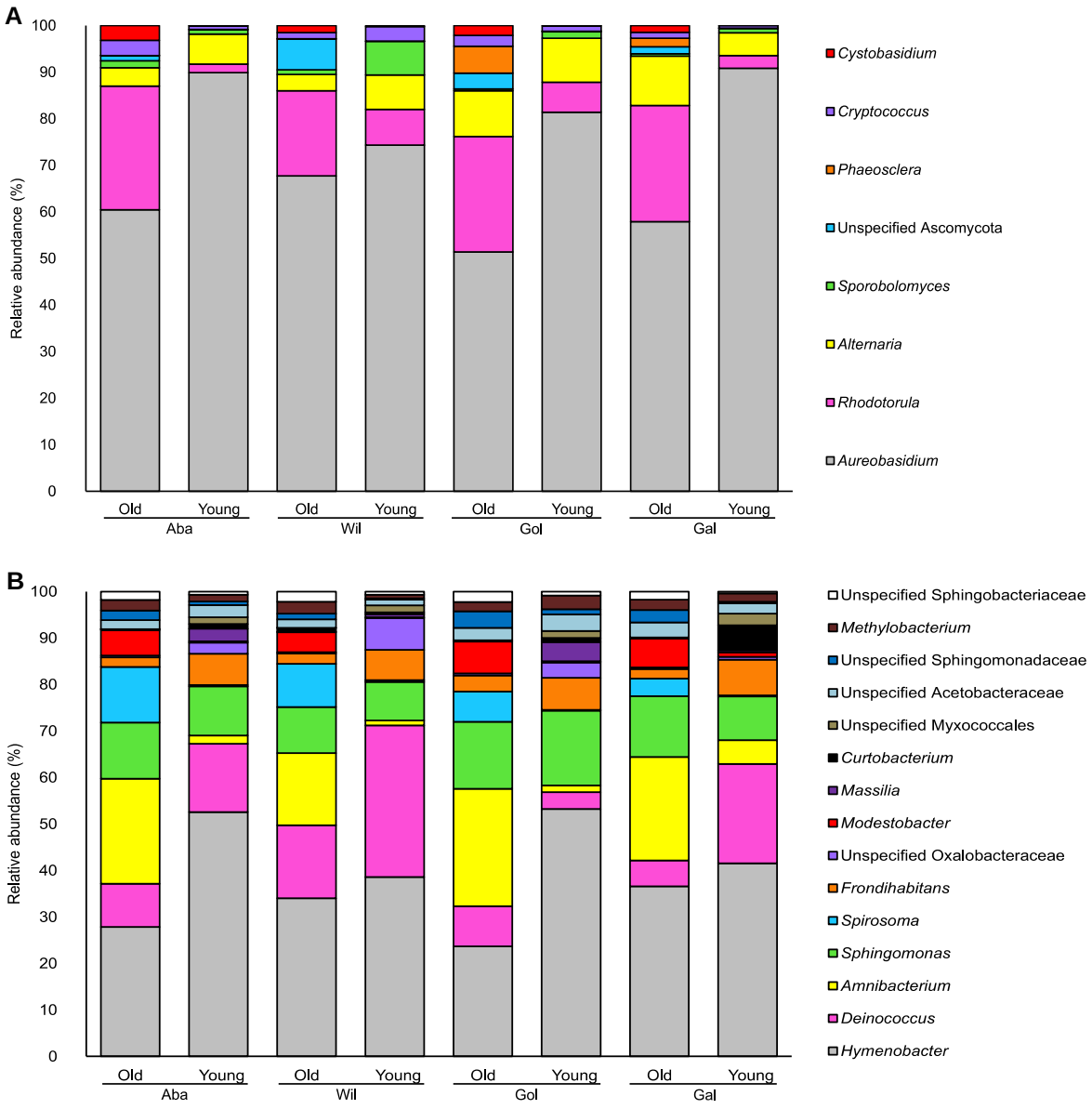



**Figure S4.** Relative abundances of dominant fungal and bacterial genera in pear and apple bark. Relative abundances of dominant (relative abundance > 1.8%) fungal (A) and bacterial (B) genera were determined for bark of three/four-year-old shoots (old bark) and one-year-old shoots (young bark) collected from Abate (Aba) and Williams (Wil) pear cultivars, and Golden Delicious (Gol) and Gala (Gal) apple cultivars. Mean values of three replicates are reported for each sample.

## 2.8 Supplementary tables

Supplementary tables associated with this chapter can be found in the online version of the article at https://doi.org/10.1016/j.micres.2018.04.002 and in the DropBox folder:
https://www.dropbox.com/home/PhD%20Thesis%20Elena%20Arrigoni/Supplementary%20material

| Sample | Replicate | Fungi | | | Bacteria | | |
|---|---|---|---|---|---|---|---|
| | | Total reads | Identified OTUs | Simpson Index | Total reads | Identified OTUs | Simpson Index |
| **Aba Old** | 1 | 100831 | 122 | 0.60 | 141212 | 275 | 0.88 |
| | 2 | 56494 | 98 | 0.62 | 95874 | 224 | 0.93 |
| | 3 | 48113 | 109 | 0.56 | 147791 | 264 | 0.92 |
| **Aba Young** | 1 | 21133 | 66 | 0.23 | 33365 | 226 | 0.74 |
| | 2 | 52588 | 97 | 0.22 | 38968 | 192 | 0.83 |
| | 3 | 100251 | 114 | 0.20 | 94890 | 297 | 0.88 |
| **Wil Old** | 1 | 133350 | 110 | 0.53 | 112907 | 190 | 0.89 |
| | 2 | 86008 | 122 | 0.57 | 31213 | 203 | 0.90 |
| | 3 | 110000 | 109 | 0.54 | 32673 | 206 | 0.91 |
| **Wil Young** | 1 | 79215 | 117 | 0.43 | 99393 | 191 | 0.83 |
| | 2 | 56567 | 125 | 0.42 | 83345 | 172 | 0.80 |
| | 3 | 112879 | 126 | 0.57 | 126755 | 159 | 0.78 |
| **Gol Old** | 1 | 99481 | 124 | 0.69 | 222646 | 250 | 0.89 |
| | 2 | 112966 | 111 | 0.67 | 66728 | 185 | 0.91 |
| | 3 | 100845 | 124 | 0.68 | 251464 | 295 | 0.90 |
| **Gol Young** | 1 | 65140 | 117 | 0.34 | 167111 | 327 | 0.75 |
| | 2 | 58815 | 108 | 0.29 | 124243 | 304 | 0.84 |
| | 3 | 237589 | 183 | 0.39 | 378683 | 427 | 0.90 |
| **Gal Old** | 1 | 100916 | 128 | 0.63 | 174412 | 245 | 0.85 |
| | 2 | 62859 | 108 | 0.66 | 78904 | 221 | 0.89 |
| | 3 | 73751 | 104 | 0.58 | 70218 | 188 | 0.90 |
| **Gal Young** | 1 | 71707 | 132 | 0.24 | 27019 | 211 | 0.86 |
| | 2 | 55689 | 123 | 0.16 | 42584 | 267 | 0.88 |
| | 3 | 52909 | 105 | 0.20 | 115002 | 304 | 0.84 |

**Table S1.** Sequencing results of fungal and bacterial communities of pear and apple bark. Number of total quality filtered reads, identified operational taxonomic units (OTUs) and Simpson index values obtained for fungi and bacteria of pear and apple bark. The fungal ITS and bacterial 16S rRNA fragments were amplified from DNA extracted from bark of three/four-year-old shoots (old bark) and one-year-old shoots (young bark) collected from Abate (Aba) and Williams (Wil) pear cultivars, and Golden Delicious (Gol) and Gala (Gal) apple cultivars. Data of three replicates (named from 1 to 3) are shown for each sample.

**Table S2.** Fungal operational taxonomic units (OTUs) of pear and apple bark. Read counts are reported for each fungal OTU identified using the UNITE database for each replicate (named from 1 to 3) of bark samples collected from three/four-year-old shoots (old bark) and one-year-old shoots (young bark) of Abate (Aba) and Williams (Wil) pear cultivars, and Golden Delicious (Gol) and Gala (Gal) apple cultivars. Taxonomy indicates kingdom (k), phylum (p), class (c), order (o), family (f), genus (g) and species (s) of identified OTUs.

## Fungi

| Variable | OTUs | | | Simpson | | |
|---|---|---|---|---|---|---|
| | Resid. Dev. | *p* value | Significance | Resid. Dev. | *p* value | Significance |
| Bark age | 0.30231 | 0.082808 | NS | 1.7664 | 2.20E-16 | *** |
| Plant cultivar | 0.21708 | 0.004737 | ** | 1.2711 | 1.70E-05 | *** |
| Plant species | 0.30188 | 0.814186 | NS | 1.7655 | 0.7823 | NS |
| Bark age:Plant Species | 0.30183 | 0.937511 | NS | 1.5412 | 1.95E-05 | *** |
| Bark age:Plant Species:Plant cultivar | 0.12787 | 0.003575 | ** | 0.1965 | 2.20E-16 | *** |

## Bacteria

| Variable | OTUs | | | Simpson | | |
|---|---|---|---|---|---|---|
| | Resid. Dev. | *p* value | Significance | Resid. Dev. | *p* value | Significance |
| Bark age | 0.80539 | 0.012659 | * | 0.045191 | 1.57E-05 | *** |
| Plant cultivar | 0.3052 | 0.001194 | ** | 0.038672 | 0.8969 | NS |
| Plant species | 0.7069 | 0.005616 | ** | 0.04434 | 0.5389 | NS |
| Bark age:Plant Species | 0.47805 | 2.43E-05 | *** | 0.039163 | 0.1296 | NS |
| Bark age:Plant Species:Plant cultivar | 0.21264 | 0.027221 | * | 0.03653 | 0.6218 | NS |

## Legend

| Significance | |
|---|---|
| ***p* value** | **Code** |
| > 0.05 | not significative (NS) |
| < 0.05 | * |
| < 0.01 | ** |
| < 0.001 | *** |

**Table S3.** Factors affecting bacterial operational taxonomic units (OTUs) and Simpson's indexes. Differences in the number of OTUs and in the Simpson's indexes were calculated using an analysis of variance with Chi-squared test on fungal and bacterial data of three/four-year-old shoots (old bark) and one-year-old shoots (young bark) collected from Abate and Williams pear cultivars, Golden Delicious and Gala apple cultivars. Results of pairwise comparisons between old and young bark (bark age), pear and apple bark (plant species), Abate, Williams, and Golden Delicious and Gala (plant cultivar) and their interactions are reported.

## Fungi

| Variable | Deviance | *p* value | Significance |
|---|---|---|---|
| Bark age | 448.6 | 0.001 | *** |
| Plant species | 295.5 | 0.001 | *** |
| Plant cultivar | 323.7 | 0.044 | * |
| Bark age:Plant Species | 129.4 | 0.007 | ** |
| Bark age:Plant Species:Plant cultivar | 213.5 | 0.002 | ** |

## Bacteria

| Variable | Deviance | *p* value | Significance |
|---|---|---|---|
| Bark age | 931.7 | 0.001 | *** |
| Plant species | 407.7 | 0.017 | * |
| Plant cultivar | 871.8 | 0.001 | *** |
| Bark age:Plant Species | 309.4 | 0.007 | ** |
| Bark age:Plant Species:Plant cultivar | 402.2 | 0.002 | ** |

## Legend

| Significance | |
|---|---|
| ***p* value** | **Code** |
| > 0.05 | not significative (NS) |
| < 0.05 | * |
| < 0.01 | ** |
| < 0.001 | *** |

**Table S4.** Multivariate analysis of deviances of fungal and bacterial communities. The multivariate analysis of deviances with a likelihood-ratio test and 999 permutations was obtained on fungal and bacterial bark communities of three/four-year-old shoots (young bark) and one-year-old shoots (young bark) collected from Abate and Williams pear cultivars, Golden Delicious and Gala apple cultivars. Results of comparisons between old and young bark (Bark age), pear and apple (Plant species), Abate, Williams, and Golden Delicious and Gala (plant cultivar) and their interactions are reported.

**Table S5.** Relative abundances of fungal genera. Relative abundances of fungal genera were obtained for bark of three/four-year-old shoots (old bark) and of one-year-old shoots (young bark) collected from Abate (Aba) and Williams (Wil) pear cultivars and from Golden Delicious (Gol) and Gala (Gal) apple cultivars. Dominant genera (relative abundance > 1.8%) are reported in red. Fungal genera (Column A) that comprise species with putative plant pathogenic or beneficial properties (Column C) were identified according to previous literature (Column D) and each genera was classified as: pear and apple pathogen, plant pathogen, biocontrol agent (BCA) or neutral (column B). Genera with significant differences in the relative abundance were identified with permutation pairwise comparison tests in the comparisons of Columns E-K: old vs. young bark (pooling the data for all plant species for each bark type) and Columns L-R: pear vs. apple bark (pooling the data for old and young bark for each plant species); or with a Bayesian analysis in the comparisons of Columns S-Y: old pear bark (pooling Abate and Williams old bark) vs. old apple bark (pooling Golden and Gala old bark); Columns Z-AF: young pear bark (pooling Abate and Williams young bark) vs. young apple bark (pooling Golden and Gala young bark); Columns AG-AM: Abate (pooling old and young bark) vs. Williams (pooling old and young bark); Columns AN-AT: Golden (pooling old and young bark) vs. Gala (pooling old and young bark); Columns AU-BA: old Abate bark vs. old Williams bark; Columns BB-BH: young Abate bark vs. young Williams bark; Columns BI-BO: old Golden bark vs. old Gala bark; Columns BP-BV: young Golden bark vs. young Gala bark; Columns BW-CC: old vs. young Abate bark; Columns CD-CJ: old vs. young Williams bark; Columns CK-CQ: old vs. young Golden bark and Columns CR-CX: old vs. young Gala bark. Bold cells are referred to the genera with significant differences in relative abundances for each pairwise comparison. The total number of genera with significant changes in abundance for each comparison is reported in square brackets.

**Table S6.** Bacterial operational taxonomic units (OTUs) of pear and apple bark. Read counts are reported for each bacterial OTU identified using the SILVA database for each replicate (named from 1 to 3) of bark samples collected from three/four-year-old shoots (old bark) and one-year-old shoots (young bark) of Abate (Aba) and Williams (Wil) pear cultivars, and Golden Delicious (Gol) and Gala (Gal) apple cultivars. Taxonomy indicates kingdom (k), phylum (p), class (c), order (o), family (f), genus (g) and species (s) of identified OTUs.

**Table S7.** Relative abundances of bacterial genera. Relative abundances of bacterial genera were obtained for bark of three/four-year-old shoots (old bark) and of one-year-old shoots (young bark) collected from Abate (Aba) and Williams (Wil) pear cultivars and from Golden Delicious (Gol) and Gala (Gal) apple cultivars. Dominant genera (relative abundance > 1.8%) are reported in red. Bacterial genera (Column A) that comprise species with putative plant pathogenic or beneficial properties (Column C) were identified according to previous literature (Column D) and each genera was classified as: pear and apple pathogen, plant pathogen, biocontrol agent (BCA), plant growth promoter (PGP) or neutral (column B). Genera with significant differences in the relative abundance were identified with permutation pairwise comparison tests in the comparisons of Columns E-K: old vs. young bark (pooling the data for all plant species for each bark type) and Columns L-R: pear vs. apple bark (pooling the data for old and young bark for each plant species); or with a Bayesian analysis in the comparisons of Columns S-Y: old pear bark (pooling Abate and Williams old bark) vs. old apple bark (pooling Golden and Gala old bark); Columns Z-AF: young pear bark (pooling Abate and Williams young bark) vs. young apple bark (pooling Golden and Gala young bark); Columns AG-AM: Abate (pooling old and young bark) vs. Williams (pooling old and young bark); Columns AN-AT: Golden (pooling old and young bark) vs. Gala (pooling old and young bark); Columns AU-BA: old Abate bark vs. old Williams bark; Columns BB-BH: young Abate bark vs. young Williams bark; Columns BI-BO: old Golden bark vs. old Gala bark; Columns BP-BV: young Golden bark vs. young Gala bark; Columns BW-CC: old vs. young Abate bark; Columns CD-CJ: old vs. young Williams bark; Columns CK-CQ: old vs. young Golden bark and Columns CR-CX: old vs. young Gala bark. Bold cells are referred to the genera with significant differences in relative abundances for each pairwise comparison. The total number of genera with significant changes in abundance for each comparison is reported in square brackets.

# Chapter III. Environmental conditions and disease management strategies influence the composition of fungal and bacterial communities associated to apple barks

## 3.1 Summary

Plant disease management strategies can influence the composition of the plant-associated microbiota. Scab-resistant apple cultivars are compatible with low-input disease management and organic agriculture. However, the reduction of fungicide treatments for the apple scab control may cause outbreaks of emerging pathogens. Although the risk of emerging secondary pathogens is real under low-input disease management, no information is available on the effect of low-input disease management on population composition and dynamics of plant-associated microbial communities. The aim of this work was to assess the impact of disease management strategies and environmental conditions on bark microbial communities, particularly on potential plant pathogens and biocontrol agents, in order to predict the risk of secondary pathogen outbreaks under low-input disease management applied on scab resistant cultivars.

Plants of the scab-resistant cultivar Fujion were analysed in two experimental orchards located in northern Italy (orchard 1 and orchard 2) for two consecutive seasons (2016 and 2017) and samples were collected at three time points per season. Integrated and a low-input disease management for apple scab control were applied to each orchard in two separated field plots. The protocol for the metabarcoding analysis of bark microbial communities discussed in Chapter II was adopted to investigate the fungal and bacterial communities of young (one year-old shoots) and old (three/four-year-old shoots) barks. A clustering approach based on sequence variants was used to recover microbial sub-operationa taxonomic units (sOTU). Bark microbial communities were dominated by sOTU belonging to the fungal genera *Aureobasidium*, *Leptosphaeria*, *Tumularia* and *Davidiella*; and the bacterial genera *Frondihabitans*, *Deinococcus*, *Amnibacterium* and *Hymenobacter*, in agreement with the findings described in Chapter II. In addition, the potential plant pathogens *Davidiella* spp. (anamorph *Cladosporium* spp.), *Leptosphaeria* spp. and *Curtobacterium* spp. were detected among the dominant fungal and bacterial taxa. Potential fungal canker agents (*Botryosphaeria* spp., *Diaporthe* spp., *Diplodia* spp. and *Neonectria* spp.), beneficial fungi (*Cryptococcus* spp., *Rhodotorula* spp., *Sporobolomyces*

spp. and *Trichoderma* spp.) as well as pathogenic (*Erwinia* spp., *Pseudomonas* spp., and *Rathayibacter* spp.) and beneficial bacteria (*Methylobacterium* spp. and *Nocardioides* spp.) were identified, confirming the role of bark as reservoir of potential phytopathogenic and beneficial microorganisms. Results showed that the bark age and orchard location strongly affected fungal and bacterial richness and diversity. Generalised linear models showed that old bark harboured a higher fungal and bacterial richness (observed sOTUs) and diversity (Shannon index) than young bark at the majority of the analysed time points. Within the same fungal (e.g., *Aureobasidium, Sporobolomyces* and *Davidiella*) and bacterial genera (*Hymenobacter, Sphingomonas, Massilia* and others), some sOTUs were more abundant in old bark, while others were more abundant in the young one, suggesting a habitat specialization of microbial species and subspecies. Similarly, the orchard location affected the abundances of sOTU belonging to some potential plant pathogenic genera as *Alternaria* spp., *Diplodia* spp., *Phoma* spp. and *Curtobacterium* spp.

Disease management strategies affected the abundance of sOTU assigned to potential apple and other plant pathogens and biocontrol agents for each bark age, such as *Alternaria* spp., *Cryptococcus* spp., *Curtobacterium* spp., *Erwinia* spp., *Pseudomonas* spp. and *Sphingomonas* spp. In particular, the low-input disease management strategy positively affected the relative abundances of sOTUs assigned to potential genera with pathogenic properties for apple (*Alternaria*) and for other plants (*Leptosphaeria, Curtobacterium, Rathayibacter* and *Rhizobacter*). The low-input strategy positively affected the relative abundances of sOTUs assigned to potential beneficial genera (*Cryptococcus, Rhodotorula, Methylobacterium* and *Nocardioides*). Conversely, the integrated disease management strategy positively affected the relative abundances of two potential bacterial pathogens of apple (*Erwinia* and *Pseudomonas*).

In conclusion, this work demonstrated that the bark age and the orchard location are major drivers of microbial diversity and composition at different phenological stages. The effect of disease management strategies on the composition of bark microbiota was demonstrated for each location and bark age, suggesting that bark microbiota can respond differently to disease management depending on the environmental conditions and organ characteristics. In particular, the disease management can affect potential phytopathogens and beneficial microorganisms, possibly altering their equilibrium and eventually leading to pathogen outbreaks. Additional analyses are in progress in order to better u understand the influence of disease management strategies and climatic conditions on phytopathogenic and beneficial microorganisms.

## 3.2 Abstract

Plants host a large variety of microbial communities that can be affected by environmental conditions and agronomic practices. Despite the role of bark as a potential reservoir of plant pathogens, no information is available on the effect of low-input disease management on population composition and dynamics of bark microbiota. The aim of this work was to assess the impact of disease management strategies and environmental conditions on bark microbial communities, in order to estimate the risk of secondary pathogen outbreaks under low-input disease management applied on scab resistant cultivars. Integrated and a low-input disease management strategies were applied in two experimental orchards for two consecutive seasons and fungal and bacterial sub-operational taxonomic units (sOTUs) were identified in young (one year-old shoots) and old (three/four-year-old shoots) barks by a metabarcoding approach. The bark age and orchard location were the major drivers of fungal and bacterial diversity. In particular, higher fungal and bacterial richness and diversity were found in old bark as compared with young bark. Microbial communities were dominated by *Aureobasidium*, *Cryptococcus, Rhodotorula, Leptosphaeria, Tumularia*, *Hymenobacter, Curtobacterium* and *Frondihabitans* genus. The relative abundance of several sOTUs was affected by the disease management strategy. In particular, the low-input disease management positively affected the relative abundances of genera comprising beneficial species (e.g. *Cryptococcus*, *Rhodotorula, Methylobacterium* and *Nocardioides*) and plant pathogens (e.g. *Alternaria, Leptosphaeria*, *Curtobacterium*, *Rathayibacter* and *Rhizobacter*).

**Keywords**: Malus domestica, bark microbiota, metabarcoding, integrated disease management, scab-resistant cultivar, secondary pathogens

## 3.3 Introduction

Plants are colonised by a wide range of microorganisms that can establish commensal, symbiotic and pathogenic interactions with their host (Hassani *et al.*, 2018). Some plant-associated microorganisms exert beneficial effects on host growth and health producing phytohormones (Tsavkelova *et al.*, 2006), enhancing nutrient acquisition (Pii *et al.*, 2015), protecting against biotic (Berendsen *et al.*, 2012) or abiotic stresses (Rolli *et al.*, 2015) and contributing to environmental adaptation (Bulgarelli *et al.*, 2013). As a consequence of its multiple functions, the plant microbiota impacts plant fitness and productivity (Hassani *et al.*, 2018) and an increasing attention was given to factors that influence the composition of crop-associated microbial communities (Busby *et al.*, 2017). Environmental conditions (Leff *et al.*, 2015; Zarraonaindia *et al.*, 2015; Shen *et al.*, 2018), tissue age (Vorholt, 2012; Arrigoni *et al.*, 2018) and plant genotype (Correa *et al.*, 2007; Whipps *et al.*, 2008; Manter *et al.*, 2010; Brader *et al.*, 2017) have been reported as key determinants of the microbial community composition. Moreover, agronomic practices can influence the composition of plant-associated microbial communities and the effect of plant disease managements have been reported for some crops (Ottesen *et al.*, 2009; Schmid *et al.*, 2011; Leff and Fierer, 2013; Glenn *et al.*, 2015; McGarvey *et al.*, 2015; Abdelfattah *et al.*, 2016; Karlsson *et al.*, 2017). In particular, the phyllosphere community composition differed under organic and conventional disease management on apple plants (Ottesen *et al.*, 2009; Glenn *et al.*, 2015; Abdelfattah *et al.*, 2016).

Apple (*Malus domestica*) is one the most important fruit crops worldwide and it represents the dominant fruit sector in Europe in terms of economic relevance (Forti and Henrard, 2016). However, the majority of commercially relevant cultivars are susceptible to several destructive diseases, such as apple scab, powdery mildew and fire blight (Jones and Aldwinckle, 1990). Apple scab, caused by *Venturia inaequalis*, is the most important disease under temperate climates (Bowen *et al.*, 2011) and it attacks fruits and leaves, causing severe losses in terms of fruit quality and yield (Belete and Boyraz, 2017). Frequent fungicide treatments are commonly applied in the integrated disease management against apple scab (Belete and Boyraz, 2017) and up to 30 treatments per season are required for a satisfactory disease control on susceptible cultivars (Soriano *et al.*, 2009). Thus, anti-scab fungicides represent the largest fraction of treatments of the apple disease management (MacHardy, 2000; Didelot *et al.*, 2016).

As a result of frequent applications, fungicide residues can be found on fresh apples (Baker *et al.*, 2002; Li *et al.*, 2005), as well as in soils (Li *et al.*, 2005) and ambient air (Coscollà *et al.*, 2010) of apple growing areas. Fungicides have been demonstrated to negatively affect human health (Costa *et al.*, 2008; Fantke *et al.*, 2012) and to develop fungicide resistance in *V. inaequalis* (Chapman *et al.*, 2011). The increasing concern about fungicides residues (Simon *et al.*, 2011) and their impact on human health and the environment (Fantke *et al.*, 2012) led several countries to direct regulations aiming at reducing fungicide applications (Hillocks, 2012; Skevas *et al.*, 2013). In particular, the introduction of scab-resistant apple cultivars can reduce fungicide applications as compared with susceptible cultivars (Ellis *et al.*, 1998; Simon *et al.*, 2011; Didelot *et al.*, 2016). However, fungicides applied to control apple scab can have side effects and potentially act against secondary pathogens, such as *Diplodia seriata* (Brown-Rytlewski and McManus, 2000; Beer *et al.*, 2015), sooty blotch and flyspeck disease complex (Weber *et al.*, 2016a). Therefore, the introduction of scab-resistant apple cultivars, with a consequent reduction in fungicide sprays under low-input disease management, may cause the outbreak of secondary diseases (Warner, 1991; Ellis *et al.*, 1998). Although the risk of pathogen outbreaks (Ellis *et al.*, 1998), scarce information is available on precise effects of low-input disease managements on population composition and dynamics of plant-associated microorganisms. Most of the studies on disease management impacts have been focused on the soil (Hartmann *et al.*, 2015), leaf (Ottesen *et al.*, 2009; Schmid *et al.*, 2011; Leff and Fierer, 2013; Perazzolli *et al.*, 2014; Glenn *et al.*, 2015; Karlsson *et al.*, 2017) and fruit microbiota (Ottesen *et al.*, 2009; Jensen *et al.*, 2013; Leff and Fierer, 2013; McGarvey *et al.*, 2015; Abdelfattah *et al.*, 2016). However, no studies analysed disease management impacts on bark microbial communities, despite the key role of this tissue as reservoir of plant pathogens (Buck *et al.*, 1998; Martins *et al.*, 2013; Arrigoni *et al.*, 2018). The aim of this study was to understand the effect of environmental conditions and disease management strategies on the structure of fungal and bacterial communities associated to apple bark according to the tissue age. The final goal was to estimate the influence of integrated and low-input disease management on potential plant pathogens and biocontrol agents residing on the apple bark in two different orchards and two consecutive seasons, in order to predict risks of pathogen outbreaks under low-input strategies on scab resistant cultivars.

## 3.4 Results

### 3.4.1 Structure, composition and properties of fungal communities

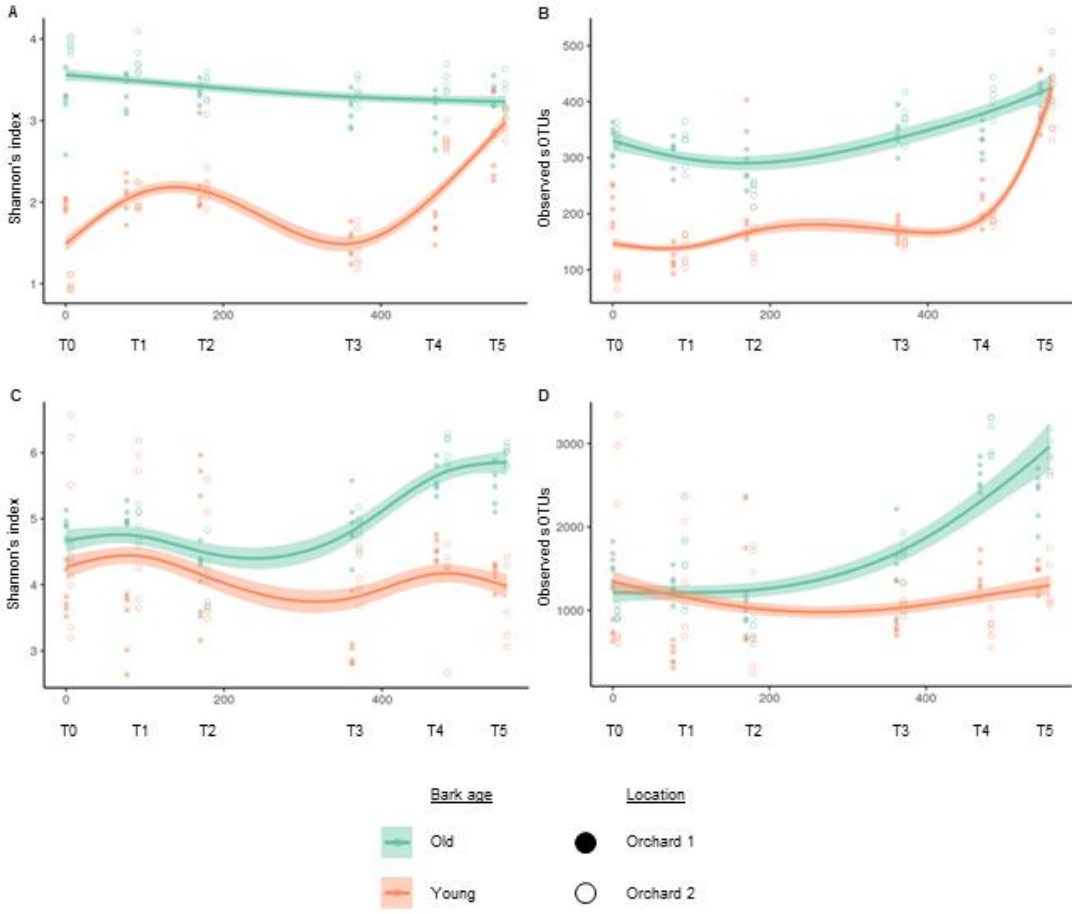



**Figure 1.** Fungal (A, B) and bacterial (C, D) diversity, estimated with Shannon's index (A, C), and richness, expressed as observed sOTUs (B,D), for old (green) and young (orange) bark in orchard 1 (full circle) and orchard 2 (empty circle) at five time points (from T0 to T5). Generalised addictive models were used to generate temporal series of diversity (ITS: $p < 2 \times 10^{-16}$, 16S: $p = 4.78 \times 10^{-16}$) and richness values for each bark age and location. Data from integrated and low-input disease management strategies were pooled.

After quality filtering, a total of 9,843,605 filtered fungal reads and 2423 sub-operational taxonomic units (sOTUs) were obtained from bark samples according to a sequence variant analysis (Table S1). The total number of sequences for each replicate ranged from 15,364 to 93,754 and the number of sOTUs per replicate ranged from 64 to 526 (Table S2). Fungal diversity, estimated by the Shannon index, was higher in old as compared with young bark at all the time points, according to a generalised additive model (GAM; $p < 2 \times 10^{-16}$; Fig.

1A) and to a pairwise test with a generalized linear model (GLM) at second (T2, $p = 8.3 \times 10^{-7}$) and last time point (T5, $p = 5.5 \times 10^{-4}$). The fungal richness, assessed as number of observed sOTUs, was higher in old than young bark according to a GAM ($p < 2 \times 10^{-16}$) and to pairwise GLM at T2 ($p = 1.8 \times 10^{-3}$), but not at T5 ( $p = 0.1$; Fig. 1B).

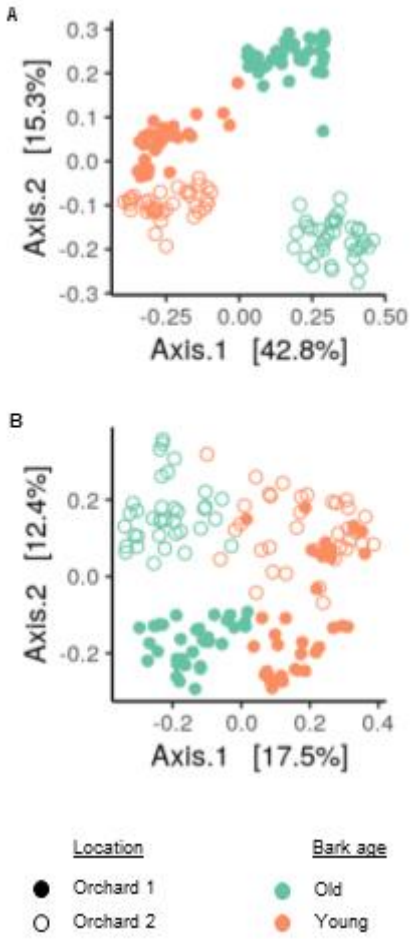



**Figure 2.** Fungal (A) and bacterial (B) diversity of old (green) and young (orange) barks in orchard 1 (full circle) and orchard 2 (empty circle). Distances between samples were measured with a permutational multivariate analysis of variance (PERMANOVA; nested model, 9999 permutations) using Bray-Curtis dissimilarities (Table S3) and visualised with a principal component analysis (PCoA). Data from integrated and low-input disease management strategies were pooled**.**

Global effects on fungal diversity were calculated with a permutational multivariate analysis of variance (PERMANOVA) on the Bray-Curtis dissimilarity, which clustered fungal communities according to bark age and location (Fig. 2A and Table S3A). Similar results were obtained when the fungal diversity was calculated with a PERMANOVA using UniFrac distances (Table S3B). The divergence of Bray-Curtis dissimilarity (Fig. 3A) and UniFrac distance values (Fig. 3B) was higher in old bark as compared with young bark for the majority time points (T0 used as reference) in both orchards.

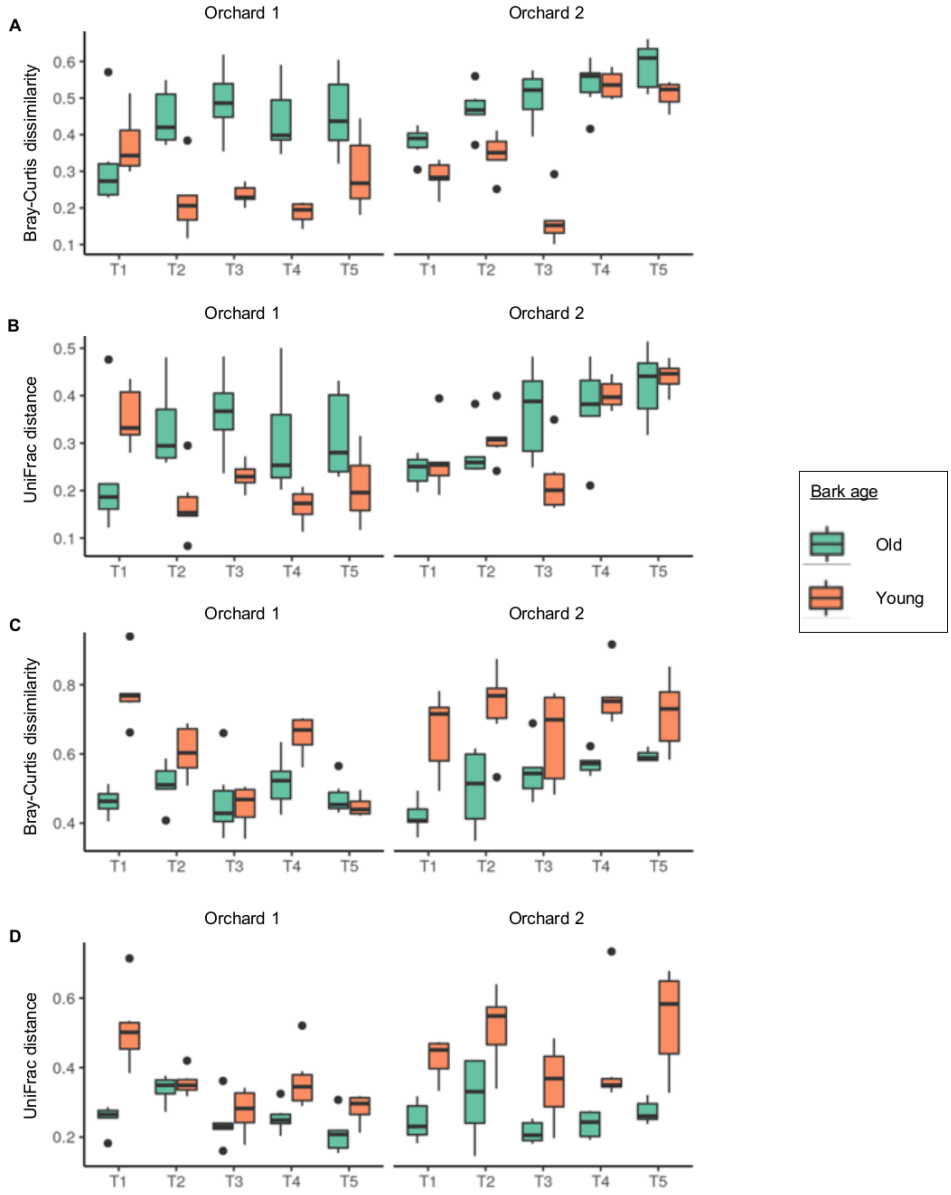



**Figure 3.** Differences in the diversity of fungal (A, B) and bacterial (C, D) communities were calculated as pairwise comparison between each time point (from T1 to T5) and T0 as differences in Bray-Curtis dissimilarities (A, C) and in weighted UniFrac distances (B, D) for old (green) and young (orange) bark. Data from the two orchards and disease management strategies were pooled.

Bark fungal community was dominated by Ascomycota phylum (78.2 ± 0.02%, 1171 sOTUs), followed by Basidiomycota (13.6 ± 0.08%, 455 sOTUs), Chytridiomycota (0.01 ± $8.01 \cdot 10^{-4}$%, 6 sOTUs) and Zygomycota (0.002 ± $6.25 \cdot 10^{-4}$%, 2 sOTUs). Dominant sOTUs (mean relative abundance higher than 1.1%) belonged to *Aureobasidium*, *Leptosphaeria*, *Tumularia*, *Davidiella* (two sOTUs), *Alternaria*, *Cryptococcus*, *Rhodotorula* (two sOTUs) and *Sporobolomyces* genus (Fig. 4A). The relative abundance of all the dominant sOTUs was influenced by the time point and orchard location, while that of 12 dominant sOTUs was affected by the bark age (Table S4A). Interestingly, the disease management affected the relative abundance of the dominant sOTUs belonging to *Aureobasidium*, *Cryptococcus*, *Davidiella* (sOTU 9) and *Rhodotorula* (sOTUs 13 and 17) genera (Table S4A). In more details, a total of 139 and 372 fungal sOTUs were affected by the orchard location at T2 and T5 respectively, with 77 fungal sOTUs affected by the orchard location at both T2 and T5 time points (Table S5). The sOTUs were classified as apple pathogens or plant pathogens if the genus to which they were assigned potentially included pathogenic species towards apple and other plant species, respectively, and as biocontrol agents if it potentially included species with biocontrol activity. Among the significant sOTUs at T2, three, five and six genera comprising potential apple pathogens (*Alternaria*, *Diaporthe* and *Phoma*), plant pathogens (*Cadophora*, *Devriesia*, *Entyloma*, *Heterobasidion*, *Leptosphaeria*) and biocontrol agents (*Aureobasidium*, *Cryptococcus*, *Filobasidium*, *Paraconiothyrium Rhodotorula* and *Sporobolomyces*) were found, respectively (Table S5). At T5, four, six and five sOTUs belonging to potential apple pathogens (*Alternaria*, *Diaporthe*, *Diplodia* and *Phoma*), plant pathogens (*Cadophora*, *Devriesia*, *Entyloma*, *Leptosphaeria*, *Setomelanomma* and *Taphrina*) and biocontrol genera (*Aureobasidium*, *Cryptococcus*, *Paraconiothyrium*, *Rhodotorula* and *Sporobolomyces*) were affected by orchard location, respectively (Table S5). In summary, the relative abundances of sOTUs belonging to three, four and five potential apple pathogenic (*Alternaria*, *Diaporthe* and *Phoma*), plant pathogenic (*Cadophora*, *Devriesia*, *Entyloma* and *Leptosphaeria*) and biocontrol genera (*Aureobasidium*, *Cryptococcus*,*Paraconiothyrium*, *Rhodotorula* and *Sporobolomyces*) were affected by the orchard location at both the T2 and T5 time points (Table S5).

The disease management affected the relative abundance of nine and 16 sOTUs on young and 11 and 14 sOTUs on old barks at T2 and T5, respectively (Table S5). Among them, the relative abundance of sOTUs assigned to potential apple pathogens (*Alternaria*),plant pathogens (*Leptosphaeria*) and biocontrol agents (*Cryptococcus* and

*Rhodotorula*) was lower in integrated as compared with in low-input disease management (Table S6A). The relative abundance of *Filobasidium* was higher in integrated as compared with low-input disease management. In addition, four out of five significant sOTUs assigned to *Aureobasidium* genus were higher in integrated as compared with low-input disease management.

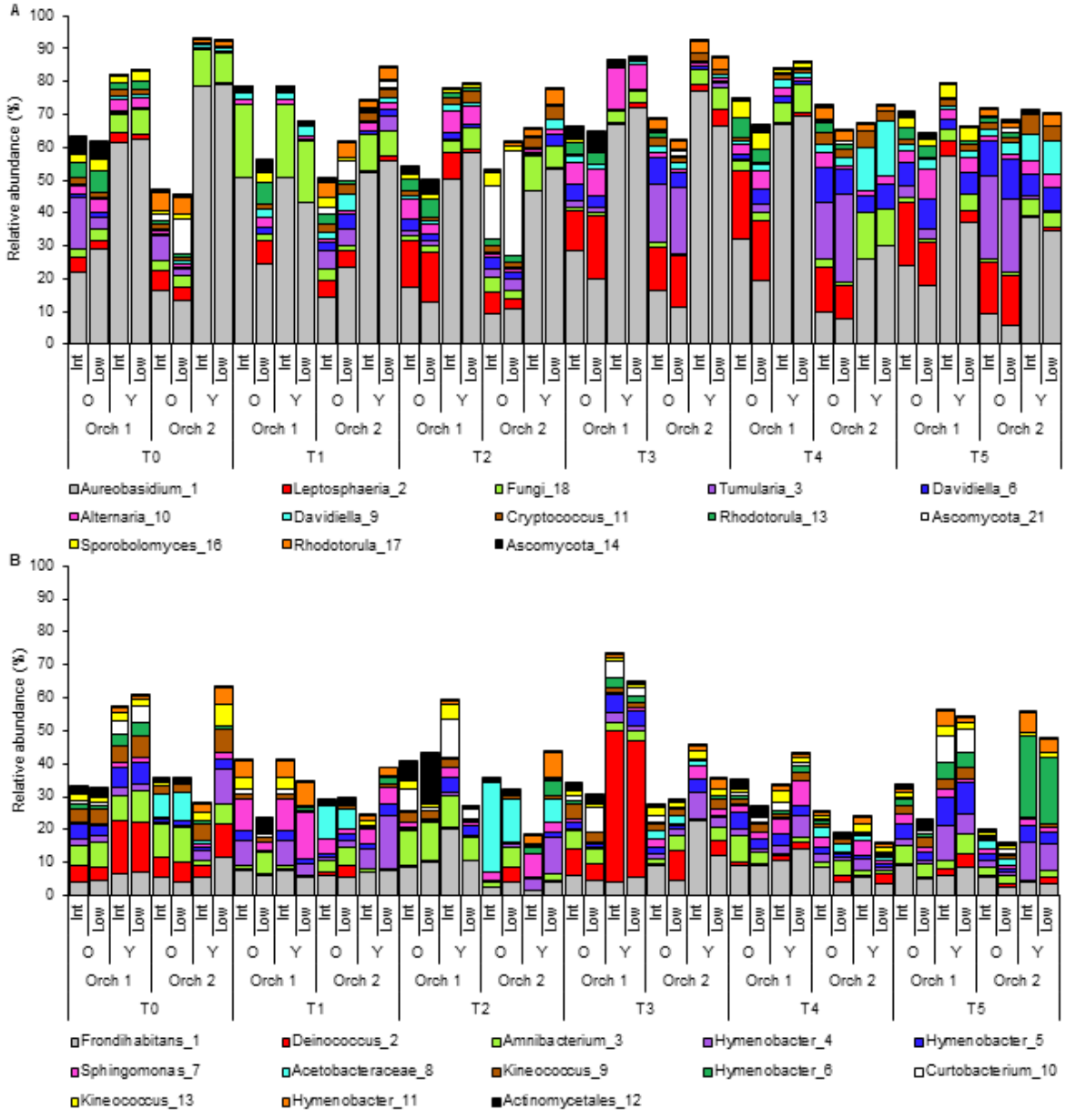



**Figure 4.** Fungal (A) and bacterial (B) dominant sub-operational taxonomic units (sOTUs; relative abundance > 1.1%) are reported for old (O) and young (Y) bark as average of three biological replicates collected at five time points (from T0 to T5) from two orchards (Orch 1 and Orch 2) under integrated (Int) and low-input (Low) disease management strategies.

### 3.4.2 Structure and composition of bacterial communities

A total of 8,746,945 filtered bacterial reads was obtained after quality filtering and 7856 sOTUs were detected in bark samples (Table S7). The total number of sequences for each replicate ranged from 30,623 to 97,654 and the number of sOTUs per replicate ranged from 242 to 3343 (Table S5). Bacterial diversity, estimated by the Shannon index, was higher in old as compared with the young bark at T3, T4 and T5 according to a GAM ($p = 4.78 \times 10^{-16}$) (Fig. 1C) and at T5 ($p = 5.4 \times 10^{-7}$) and not at T2 ($p = 0.52$) according to a pairwise test with a GLM. The bacterial richness, expressed as observed sOTUs, differed in young and old bark according to a GAM ($p = 5.13 \times 10^{-16}$) and to a pairwise GLM at T5 ($p = 5.01 \times 10^{-7}$), but not at T2 ( $p = 0.437$; Fig. 1D).

The PERMANOVA on the Bray-Curtis dissimilarity highlighted significant effects of location and bark age on bacterial diversity ($p < 1 \times 10^{-4}$; Fig. 2B and Table S3C) as confirmed by UniFrac distances (Table S3D). The divergence of Bray-Curtis dissimilarity (Fig. 3C) and UniFrac distance values (Fig. 3D) was higher in old bark as compared with young bark for the majority time points (T0 used as reference) in both orchards, except for T2, T3 and T5 of orchard 1.

Bark bacterial community was dominated by the Actinobacteria (35.4 ± 0.003%, 2623 sOTUs), Proteobacteria (31 ± 0.002%, 2670 sOTUs), Bacteroidetes (23.3 ± 0.004%, 1336 sOTUs) and Deinococcus-Thermus phylum (6.9 ± 0.03%, 166 sOTUs). The dominant bacterial sOTUs (mean relative abundance higher than 1.1%) were assigned to the genera *Frondihabitans, Deinococcus, Amnibacterium, Hymenobacter* (four sOTUs), *Sphingomonas, Kineococcus* (two sOTUs) and *Curtobacterium* (Fig. 4B). The relative abundance of all the dominant bacterial sOTUs was affected by time point and bark age, while the orchard location and the disease management strategy affected nine and eight of dominant sOTUs, respectively (Table S4B). In more details, the orchard location affected the relative abundance of 575 and 1074 sOTUs at T2 and T5 respectively, and 219 of them were affected by the orchard location at both the time points (Table S8). The sOTUs were classified as apple pathogens or plant pathogens if they were assigned to a genus which potentially included pathogenic species towards apple and other plant species, respectively, and as biocontrol agents if it potentially included species with biocontrol and plant growth promotion activity. Among the significantly affected sOTUs at T2, two (*Curtobacterium* and *Rhizobacter*) and five genera (*Actinoplanes*, *Methylobacterium*, *Nocardioides*, *Rhizobium Rhodococcus* and *Sphingomonas*) comprising bacterial species with potential

plant pathogenic and biocontrol properties were found, respectively (Table S8). At T5, sOTUs assigned to one (*Pseudomonas*) and four (*Curtobacterium, Pantoea, Ralstonia* and *Rhizobacter*) genera with potential pathogenic properties for apple and other plants were found, respectively (Table S8). In addition, six genera (*Burkholderia, Methylobacterium, Mycobacterium, Nocardioides, Pedobacter* and *Sphingomonas*) with potential biocontrol and plant promotion activities were detected (Table S8). In complex, the orchard location affected the relative abundance of sOTUs belonging to one genus (*Curtobacterium*) that included species with potential pathogenic activities and three genera (*Methylobacterium, Nocardioides* and *Sphingomonas*) with potential biocontrol and plant growth promotion activities at both time points T2 and T5 (Table S8).

The disease management strategy affected the relative abundances of 38 and 165 sOTUs on young barks and of 69 and 249 sOTUs on old barks at T2 and T5, respectively (Table S8). More precisely, 67 sOTUs assigned to 12 genera were less abundant in integrated as compared with low-input disease management, considering young and old barks (Table S6B). Among them, sOTUs assigned to three genera (*Curtobacterium, Rathayibacter* and *Rhizobacter*) with potential plant pathogenic properties and two (*Methylobacterium* and *Nocardioides*) included species with potential biocontrol and plant growth promotion activities (Table S6B). Likewise, the relative abundance of sOTUs belonging to two genera (*Erwinia* and *Pseudomonas*) including species with pathogenic activity for apple was higher in integrated as compared with in low-input disease management (Table S6B).

## 3.5 Discussion

Plant organs are colonised by complex microbial communities which can exert beneficial effects on the plant, increasing its fitness and productivity (Hassani *et al.*, 2018) and contributing to its adaptation to the environment (Bulgarelli *et al.*, 2013). External perturbation factors can influence the composition of plant-associated microbial communities, such as environmental conditions (Shen *et al.*, 2018), organ age (Arrigoni *et al.*, 2018), plant genotype (Brader *et al.*, 2017) and disease management strategies (Karlsson *et al.*, 2017). In particular, the reduction of fungicide applications on scab-resistant apple cultivars may cause outbreaks of secondary pathogens (Warner, 1991; Ellis *et al.*, 1998) but no precise information is available on its impact on the microbiota

associated to reservoir organs. In this study, the influence environmental conditions and disease management strategies was investigated on the composition and dynamics of fungal and bacterial communities associated with apple bark. Bark was dominated by the fungal genera *Aureobasidium, Cryptococcus, Rhodotorula,* and *Sporobolomyces, which* have been previously found on apple and blackcurrant fruits (Arrigoni *et al.*, 2018; Shen *et al.*, 2018; Vepštaitė-Monstavičė *et al.*, 2018). In particular, the genus *Aureobasidium* has been previously demonstrated to be a ubiquitous resident of apple plants tissues (He *et al.*, 2012; Glenn *et al.*, 2015; Arrigoni *et al.*, 2018). In addition, the dominant fungal genera *Leptosphaeria* and *Tumularia* were found on apples (Vepštaitė-Monstavičė *et al.*, 2018) and in the roots of *Aralia elata* and *A. continentalis* (Paul *et al.*, 2007). Bacterial communities were dominated by the genera *Hymenobacter, Curtobacterium* and *Frondihabitans,* which have previously been found on bark of apple and pear trees (Arrigoni *et al.*, 2018), grapevine (Martins *et al.*, 2013) and forest plants (Lambais *et al.*, 2014) as well as on apple and blackcurrant fruits (Vepštaitė-Monstavičė *et al.*, 2018), on apple flowers (Shade *et al.*, 2013b) and leaves (Glenn *et al.*, 2015).

Bark age and the orchard location were the major drivers of microbial richness and diversity. In particular, higher fungal and bacterial richness was recorded in old bark, confirming previous findings on apple, pear and *Ginkgo biloba* (Leff *et al.*, 2015; Arrigoni *et al.*, 2018). The orchard location is another major driver for the fungal and bacterial diversity and composition of bark microbial communities. Differences in the composition of plant-associated microbial communities were detected among urban and peri-urban apple orchards (Shen *et al.*, 2018) and among different grapevine (Bokulich *et al.*, 2014; Pinto *et al.*, 2015) and blackcurrant growing regions (Vepštaitė-Monstavičė *et al.*, 2018). The influence of orchard location in the composition of fungal and bacterial communities can be explained by the different environmental conditions that can lead a differentiation of plant-associated microbial communities in space and in time (Bokulich *et al.*, 2014). Indeed, the time points during two growing season revealed differences in the relative abundance of dominant fungal and bacterial genera. A high intra-seasonal variability has been previously demonstrated for soil (Bell *et al.*, 2009), airborne (Bowers *et al.*, 2012) and phyllosphere (Pinto *et al.*, 2014; Copeland *et al.*, 2015; Fort *et al.*, 2016) microbial communities, which can be explained with variations in rainfalls and climatic conditions (Bell *et al.*, 2009; Bowers *et al.*, 2012) and soil influences (Copeland *et al.*, 2015). Moreover, the plant developmental stage has been demonstrated to influence the bacterial catabolic activity in soils (Papatheodorou *et al.*, 2008), composition of rhizosphere

(Houlden *et al.*, 2008; Chaparro *et al.*, 2014) and phyllosphere microbial communities (Pinto *et al.*, 2014; Forti and Henrard, 2016). Similarly, the phenological stage of apple plants and environmental conditions in the two locations and seasons could play a key role in shaping the composition of fungal and bacterial communities of apple bark.

In addition, the disease management strategy affected the relative abundances of several fungal and bacterial sOTUs, and some of them increased in relative abundance in the low-input disease management, such as those belonging to the genera *Alternaria, Cryptococcus, Leptosphaeria, Rhodotorula, Curtobacterium, Methylobacterium, Nocardioides, Rathayibacter* and *Rhizobacter*. Previous studies demonstrated that the reduction of fungicide application in organic farming allowed to recover larger microbial communities and unique taxa from cider vinegar (Štornik *et al.*, 2016), apple phyllosphere (Glenn *et al.*, 2015), almond drupes (McGarvey *et al.*, 2015), grapevine (Pinto *et al.*, 2014) and soybean (da Costa Stuart *et al.*, 2018). In this study, the low-input disease management positively affected the relative abundance of fungal and bacterial genera with potential biocontrol and plant growth promotion activities, such as *Cryptococcus, Rhodotorula, Methylobacterium* and *Nocardioides*, confirming that fungicides applied on integrated strategy may have an impact on non-target organisms (Abdelfattah *et al.*, 2016). Likewise, the relative abundance of sOTUs belonging to potential apple and other plant pathogens such as *Alternaria, Leptosphaeria, Curtobacterium*, *Rathayibacter* and *Rhizobacter* were lower in the integrated disease management than in the low-input strategy. In general, the disease management strategies influenced the balance between potential pathogens and beneficial microorganisms, as previously shown for grapevine (Pinto *et al.*, 2014). However, the influence of disease management strategies on bark microbial communities depends on the organ age, suggesting that resilience of the bark microbiota may be influenced by the bark surface that changes with the organ age (Arrigoni *et al.*, 2018). Several fungal and bacterial sOTUs assigned to genera with pathogenic, biocontrol and growth promotion activities were found on apple bark. For example, the genera *Alternaria, Botryosphaeria, Diplodia, Diaporthe* and *Neonectria* comprise species with pathogenic activities for apple plants and some canker agents, as well as potential post-harvest decay agents as *Penicillium* (Jones and Aldwinckle, 1990). Likewise, potential bacterial sOTUs assigned to genera with potential pathogenic activities towards apple plants were detected, such as *Erwinia* and *Pseudomonas*. The abundance of sOTUs belonging to apple (*Alternaria*) and other plant (*Leptosphaeria*, *Curtobacterium*, *Rathayibacter* and *Rhizobacter*) increased low-input disease management, suggesting a potential outbreak

those pathogens as a consequence of the reduced fungicide treatments under low-input disease management of scab-resistant apple plants and an infection risk for other plant species.

## 3.6 Conclusions

The complex fungal and bacterial communities residing on apple bark were influenced by bark age, orchard location and collection time point of the growing season. Moreover, the disease management strategy influenced the composition of bark microbiota in young and old barks. Several potential plant pathogens and biocontrol agents were detected and their abundance varied according to the disease management strategy. In particular, the higher abundance of apple pathogens (*Alternaria*) and other plant pathogens (*Leptosphaeria*, *Curtobacterium*, *Rathayibacter* and *Rhizobacter*) in the low-input strategy suggested the possible outbreaks of these phytopathogenic genera as a consequence of the reduction of fungicide applications and the possibility of inoculum source of other plant species. The metabarcoding approach used in this study has the potential for detailed characterisation of plant-associated microbial sOTUs and for the investigation of the effect of external impacts on their composition. Future steps may include the application of this approach in order to estimate impacts of innovative agronomic practices (e.g. training systems and rain covers) on plant-associated microbial populations on a longer time span.

## 3.7 Experimental procedures

### 3.7.1 Experimental design

Apple plants of the scab-resistant cultivar ‘Fujion’ were analysed in two experimental orchards, namely orchard 1 (‘Ceriano’, Monza-Brianza; latitude, 45.630217 N; longitude, 9.095419 E; altitude, 226 m a.s.l.) and orchard 2 (‘Maiano’, Trento; latitude, 46.360794 N; longitude, 11.040881 E; altitude, 648 m a.s.l.) in northern Italy (Figure S1). Orchards differed in topography, slope, exposure, training system, planting pattern, plant age (Table S9) and phenological phases showed an average delay of two weeks in the orchard 2 as compared with the orchard 1 (Tables S10 and S11). Integrated and a low-input

disease management for apple scab control was applied to each orchard in two separated field plots located 100 m apart from each other, starting from T0 (orchard 1, 24 March 2016; orchard 2, 30 March 2016; Figure S1, Tables S10 and S11). The number of treatments per season ranged from 12 to 24 and from five to seven for integrated and low-input disease management depending on the orchard location and season, respectively. Integrated and low-input disease management corresponded to practices commonly applied to scab-susceptible (Belete and Boyraz, 2017) and scab-resistant cultivars (Simon *et al.*, 2011; Didelot *et al.*, 2016) in commercial orchards, respectively. Samples were collected at three time points in two consecutive seasons (2016 and 2017) from each location (orchard 1 and 2) and disease management strategy (integrated and low-input), for a total of six time points (T0-T5), corresponding to the phenological phases (Chapman and Catlin, 1976): pink cluster (T0 and T3 in 2016 and 2017, respectively), 30 mm of fruit diameter (T1 and T4 in 2016 and 2017, respectively) and two weeks before harvest (T2 and T5 in 2016 and 2017, respectively). Daily maximum and minimum temperature and rainfalls were recorded in both orchards by meteorological stations located nearby (Figure S2; Tables S10 and S11).

### 3.7.2 Sample collection, apple bark microbiota isolation and sequencing

From each location, disease management strategy and time point, bark samples 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 five randomly selected plants according a split-plot sampling design, as reported by Arrigoni *et al.* (2018). Briefly, bark curls (20 mm long, 5 mm wide and 1 mm thick) were collected using a fire-sterilised scalpel and each sample (0.5 g of a pool of 30 bark curls) was ground into sterile stainless steel jars (2.5 ml of cold sterile 0.85% NaCl solution) with a mixer-mill disruptor (MM 400, Retsch, Germany).

DNA extraction, amplification of the fungal internal transcribed spacer 2 (ITS2; primer ITS3 forward 5′-CATCGATGAAGAACGCAG-3′ and ITS4 reverse 5′-TCCTSSSCTTATTGATATGC-3′) and bacterial V5-V7 region of 16S rDNA (primer 799 forward 5′-AACMGGATTAGATACCCKG-3′ and 1175 reverse 5′-ACGTCRTCCCCDCCTTCCT-3′), purification, indexing, quantification, and library preparation for the Illumina MiSeq sequencing (PE300) were carried out as described by

Arrigoni *et al.* (2018). Sequences were deposited at the Sequence Read Archive of NCBI (http://www.ncbi.nlm.nih.gov/sra) under the BioProject number PRJNA495750.

### 3.7.3 Bioinformatic analysis

Sequences were processed using the open-source MICCA (v1.7.0) software (Albanese *et al.*, 2015). Raw forward and reverse 16S reads were truncated at 250 bp, after which they were merged (assembled) using the procedure described in (Edgar and Flyvbjerg, 2015). Overlapping paired-end reads with an overlap length smaller than 60 and with more than 15 mismatches were discarded. After forward and reverse primers trimming, merged reads shorter than 360 bp and with an expected error rate higher than 0.75% were removed. Filtered sequences were denoised using the UNOISE (Edgar, 2016) algorithm. Denoising methods (Callahan *et al.*, 2016; Amir *et al.*, 2017; Nearing *et al.*, 2018) allow to correct sequencing errors and determine real biological sequences at single nucleotide resolution by generating amplicon sequence variants, also called sOTUs according to Amato *et al.* (2018). sOTUs were taxonomically classified using the Ribosomal Database Project (RDP) Classifier v2.11 (Wang *et al.*, 2007). Multiple sequence alignment (MSA) was performed on the denoised reads applying the Nearest Alignment Space Termination (NAST) algorithm (DeSantis *et al.*, 2006) and the phylogenetic tree was inferred using FastTree v2.1.8 (DeSantis *et al.*, 2006; Price *et al.*, 2010).

Raw forward and reverse ITS sequences were truncated and merged as for 16S reads. After forward and reverse primers trimming, merged reads shorter than 275 bp and with an expected error rate higher than 0.50% were removed. Filtered sequences were denoised using the UNOISE algorithm and denoised reads (sOTUs) were taxonomically classified using the RDP Classifier v2.11 and the UNITE (Kõljalg *et al.*, 2005) database. Multiple sequence alignment was performed on the denoised reads using MUSCLE v3.8.31 (Edgar, 2004) and phylogenetic tree was inferred using FastTree. Finally, sOTUs with less than 75% similarity to the sequences present in the UNITE database (clustered at 85%, release 2017/12/01) were discarded using VSEARCH (Rognes *et al.*, 2016) v2.3.4.

After taxonomic classification, sOTUs corresponding to chloroplasts and mitochondrial sequences were discarded and downstream analyses were performed using R v3.4.2 and phyloseq v1.25.3 (McMurdie and Holmes, 2013) and vegan v2.5-2 packages. Samples were rarefied (without replacement) at 30,000 (16S) and 15,000 (ITS) reads. In

this step, five sOTUs were removed from ITS samples. Fungal and bacterial genera comprising species with potential plant pathogens or beneficial properties (biocontrol agents and plant growth promoters) were identified according to previous literature.

### 3.7.4 Statistical analysis

In order to assess the influence of orchard location on the relative abundance of fungal and bacterial sOTUs, a two-tailed t-test was carried out using its specific function in Microsoft ExCel (Microsoft, Redmond, Washington, USA) at T2 and T5 time points. In more detail, the test was accomplished pooling the data for bark age and disease management strategies for each of the time point analysed (orchard 1 *vs.* orchard 2). Likewise, the same procedure was adopted to assess the influence of the disease management strategies on the relative abundances of fungal and bacterial sOTUs for each bark age at time point T2 and T5, pooling the samples as follows: i) integrated management *vs.* low-input management on young bark and ii) integrated management *vs.* low-input management on old bark. The influence of bark age, orchard location and disease management strategy on the relative abundances of dominant fungal and bacterial sOTUs was assessed with a factorial ANOVA and t-test, using the Statistica 13.1 software (Dell, Round Rock, TX, USA).

## 3.8 Supplementary figures


**Figure S1**. Locations of the orchard 1 (‘Ceriano’) and orchard 2 (‘Maiano’) in Northern Italy studied in this work.

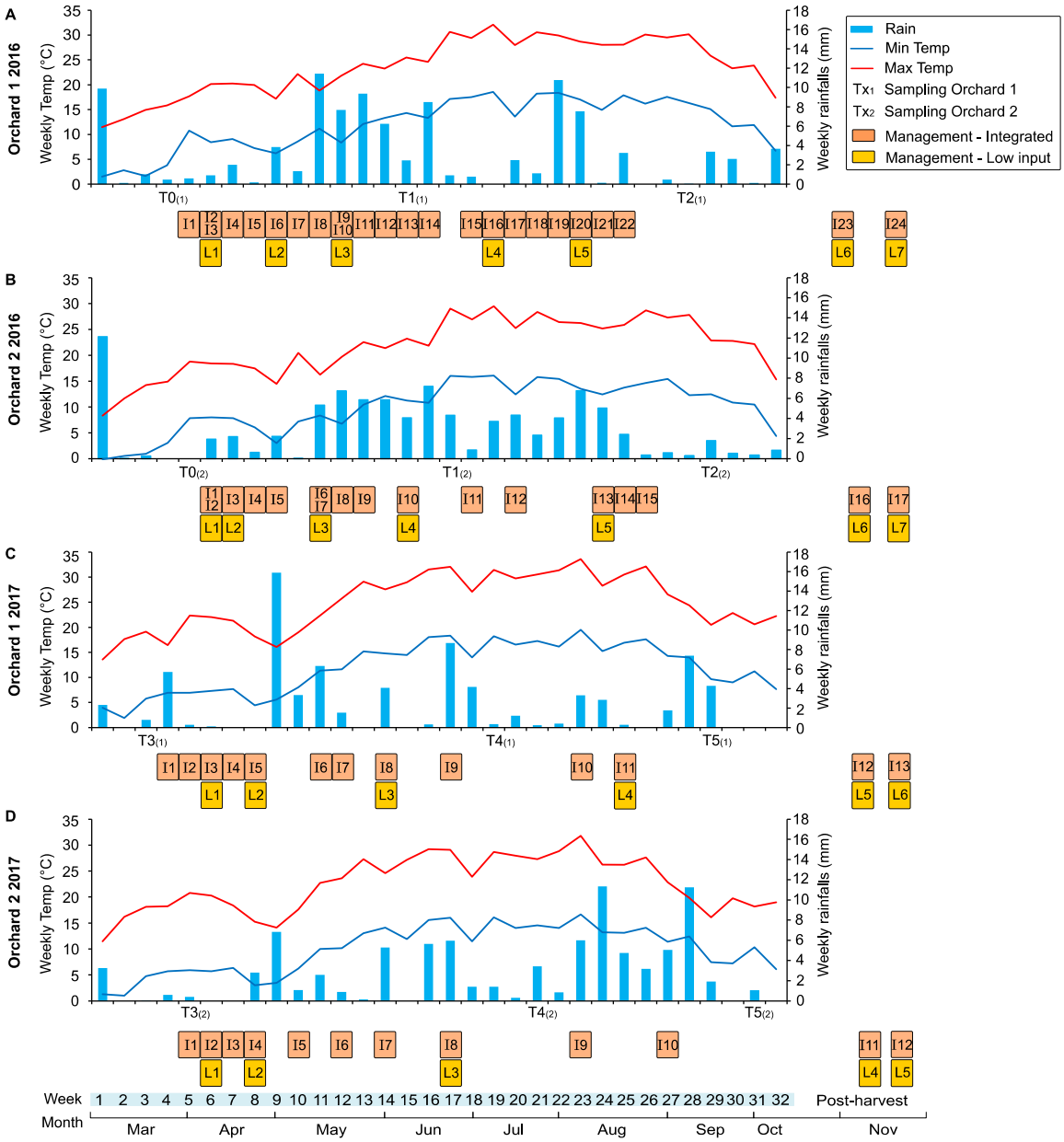



**Figure S2**. Climatic conditions of orchard 1 (A, C) and orchard 2 (B, D) in 2016 and 2017 season. Weekly climatic data are calculated as the average of daily minimum and maximum temperatures and as sum of daily rainfalls (Tables S10 and S11). Consequential numbers of orchard treatments are shown for integrated (I, orange boxes) and low-input (L, yellow boxes) disease management, as better detailed in Tables S10 and S11. The phenological phases showed an average delay of two weeks in the orchard 2 as compared with the orchard 1 and time points of sample collection (from T0 to T5) are indicated for each orchard.

## 3.9 Supplementary tables

Supplementary tables associated with this chapter can be found in the DropBox Folder:
https://www.dropbox.com/home/PhD%20Thesis%20Elena%20Arrigoni/Supplementary%20material

**Table S1.** Fungal sub-operational taxonomic units (sOTUs) of apple bark. Rarefied read counts are reported for each fungal sOTU identified using the UNITE database for each replicate (named from 1 to 3) of bark samples collected from one-year-old shoots (young bark; Y) and three/four-year-old shoots (old bark;O) collected for two consecutive years at three time points per year (2016: from T0 to T2, 2017: from T3 to T5) in two orchards (Orch 1 and Orch 2) under integrated (Int) and low-input (Low) disease management strategies.

**Table S2.** Sequencing results. Number of total quality filtered reads and identified sub-OTUs (s-OTUs) obtained for fungi and bacteria of bark. Fungal ITS and bacterial 16S rDNA fragments were amplified from DNA extracted from bark of three/four-year-old shoots (old bark) and one-year-old shoots (young bark) collected in three replicates for two consecutive years at three time points per year in two orchards under integrated and low-input disease management strategies. Data of three replicates (named from 1 to 3) are shown for each sample.

**Table S3.** Influence of bark age and orchard location on the diversity of fungal (A, B) and bacterial (C, D) communities of apple bark. Microbial diversity was estimated with a permutational multivariate analysis of variance (PERMANOVA) using Bray-Curtis dissimilarities (A, C) and weighted UniFrac distances (B, D) (nested models, 9999 permutations) from bark of three/four-year-old shoots (old bark) and one-year-old shoots (young bark) collected in three replicates for two consecutive years at three time points per year in two orchards under integrated and low-input disease management strategies. Results about the comparison between locations (orchard 1 and orchard 2) and bark age (young and old bark, nested in location) are shown.

**Table S4.** The influence of time point, orchard location, disease management strategy and bark age on the relative abundances of dominant (> 1.1% mean relative abundance) fungal (A) and bacterial (B) sub-operational taxonomic units (sOTUs) was calculated with a factorial analysis of variance (ANOVA). Significant interactions are marked with asterisk.

**Table S5.** Influence of orchard location and bark age on fungal sub-operational taxonomic units (sOTUs). Fungal sOTUs (column A) were obtained from bark of three/four-year-old shoots (old bark) and one-year-old shoots (young bark) collected in three replicates for two consecutive years at three time points per year in two orchards under integrated and low-input disease management strategies and identified using the UNITE database (columns B-G). Mean relative abundances were calculated at T2 (columns H and I) and T5 (columns M and N) for each location pooling data of bark age and disease management. A fold change was calculated as the ratio between the mean relative abundances of orchard 1 and orchard 2 for each sOTU (columns J and O). Significant differences in the relative abundances between orchard 1 and orchard 2 were identified with a t-test for T2 (column K) and T5 (column P). Significant sOTUs ($p < 0.05$) are indicated with an asterisk (columns L and Q). Likewise, mean relative abundances were calculated for each disease management strategy on young (columns R-V and AB-AF) and old barks (columns W-AA and AG-AK) at T2 (columns R-AA) and T5 (AB-AK) pooling the data of the orchard locations. A fold change was calculated as the ratio between the mean relative abundances of integrated and low-input disease managements for young (columns T and AD) and old barks (columns Y and AI) for each sOTU. Significant differences in the relative abundances between integrated and low-input disease managements were identified with t-test on young (columns U and AE) and old barks (columns Z and AJ) for T2 and T5. Significant sOTUs ($p < 0.05$) are indicated with an asterisk for young (columns V and AF) and old barks (columns AA and AK). The sOTUs discussed in the manuscript are reported in red.

**Table S6.** Fungal (A) and bacterial (B) sOTUs with significant difference in the relative abundance between integrated and low-input disease management strategies at T2 and T5 time points and in young and old barks. sOTUs with significant changes (t-test $p \leq 0.05$) in the relative abundance between integrated and low-input disease management strategies were selected from Table S5 merging the data of the two orchards. A putative functional activity towards the hosts was assigned to each genus according to the literature and each

genus was classified as apple pathogen, plant pathogen (pathogen of other plant species), biocontrol agent (BCA) or neutral.

**Table S7.** Bacterial sub-operational taxonomic units (sOTUs) of apple bark. Rarefied read counts are reported for each bacterial sOTU identified using the SILVA database for each replicate (named from 1 to 3) of bark samples collected from one-year-old shoots (young bark; Y) and three/four-year-old shoots (old bark;O) collected in three replicates for two consecutive years at three time points per year (2016: from T0 to T2, 2017: from T3 to T5) in two orchards (Orch 1 and Orch 2) under integrated (Int) and low-input (Low) disease management strategies.

**Table S8.** Influence of orchard location and bark age on bacterial sub-operational taxonomic units (sOTUs). Bacterial sOTUs (column A) were obtained from bark of three/four-year-old shoots (old bark) and one-year-old shoots (young bark) collected in three replicates for two consecutive years at three time points per year in two orchards under integrated and low-input disease management strategies and identified using the UNITE database (columns B-G). Mean relative abundances were calculated at T2 (columns H and I) and T5 (columns M and N) for each location pooling data from bark age and disease management. A fold change was calculated as the ratio between the mean relative abundances of orchard 1 and orchard 2 for each sOTU (columns J and O). Significant differences in the relative abundances between orchard 1 and orchard 2 were identified with a t-test for T2 (column K) and T5 (column P). Significant sOTUs ($p < 0.05$) are indicated with an asterisk (columns L and Q). Likewise, mean relative abundances were calculated for each disease management strategy on young (columns R-V and AB-AF) and old barks (columns W-AA and AG-AK) at T2 (columns R-AA) and T5 (AB-AK) pooling the data of the orchard locations. A fold change was calculated as the ratio between the mean relative abundances of integrated and low-input disease managements for young (columns T and AD) and old barks (columns Y and AI) for each sOTU. Significant differences in the relative abundances between integrated and low-input disease managements were identified with a t-test on young (columns U and AE) and old barks (columns Z and AJ) for T2 and T5. Significant sOTUs ($p < 0.05$) are indicated with an asterisk for young (columns V and AF) and old barks (columns AA and AK). The sOTUs discussed in the manuscript are reported in red.

**Table S9.** Geographical location and agronomics characteristics of the orchard 1 (‘Ceriano’) orchard 2 (‘Maiano’).

**Table S10.** Disease management and climatic data of 2016 season. Disease management and climatic data are reported for the orchard 1 (columns C-P) and orchard 2 (columns Q-AD) orchards for 32 weeks (columns A-B) of 2016 season. Phenological phases (columns C-D and Q-R, respectively), according to the descriptions of (Chapman and Catlin, 1976), and sample collection time points (columns E-F and S-T, respectively) are reported for the orchard 1 and 2. Dates (columns G and U) and treatments of integrated (columns H and V) and low-input (columns I and W) disease management are listed for each orchard, including the product name, active substance, applied dose and target pathogen or pest (columns J-M and X-AA). Anti-scab products are reported in red. Consequential numbers were assigned to disease management treatments of integrated (abbreviated as I) and low-input (abbreviated as L) disease management. Weekly climatic data are calculated as the average of daily minimum and maximum temperatures and as sum of daily rainfalls for the orchard 1 (columns N-P) and orchard 2 (columns AB-AD).

**Table S11.** Disease management and climatic data of 2017 season. Disease management and climatic data are reported for the orchard 1 (columns C-P) and orchard 2 (columns Q-AD) orchards for 32 weeks (columns A-B) of 2017 season. Phenological phases (columns C-D and Q-R, respectively), according to the descriptions of (Chapman and Catlin, 1976), and sample collection time points (columns E-F and S-T, respectively) are reported for the orchard 1 and 2. Dates (columns G and U) and treatments of integrated (columns H and V) and low-input (columns I and W) disease management are listed for each orchard, including the product name, active substance, applied dose and target pathogen or pest (columns J-M and X-AA). Anti-scab products are reported in red. Consequential numbers were assigned to disease management treatments of integrated (abbreviated as I) and low-input (abbreviated as L) disease management. Weekly climatic data are calculated as the average of daily minimum and maximum temperatures and as sum of daily rainfalls for the orchard 1 (columns N-P) and orchard 2 (columns AB-AD).

# Chapter IV. A fast and reliable method for *Diplodia seriata* inoculation of trunks and fungicide efficacy assessment on potted apple plants under greenhouse conditions

## 4.1 Summary

Fungicides applied in integrated pest management programmes against apple scab can also act against secondary pathogens. Thus, the introduction of scab-resistant apple cultivars, and the consequent reduction in fungicide applications, may increase the incidence of secondary pathogens. For instance, the canker, leaf-spot and fruit rot agent *Diplodia seriata* is a secondary pathogen and represents an emerging disease in organic orchards, where the application of fungicides is limited. Despite potential outbreaks of this pathogen, no fast and reproducible protocols for artificial inoculation of *D. seriata* and fungicide efficacy tests are available for apple plants. The aim of this study was to optimise a protocol to inoculate and assess *D. seriata* on potted apple plants under controlled conditions and to evaluate the fungicide efficacy against trunk canker using conventional spray applications on the scab-resistant cultivar Fujion. This work enriches the present doctoral thesis with a practical study on the efficacy of common fungicides on an emerging secondary pathogen, complementing the ecological studies of Chapters II and III.

For this purpose, a method for *D. seriata* growth, inoculation, disease assessment and fungicide efficacy tests was developed. Fungal growth and pycnidia production were assessed in different growth media, temperatures and photoperiods and a fast protocol for mycelial growth and spore production was optimised. Five common anti-scab fungicides were tested *in vitro* for their activity against mycelial and conidial growth and dithianon was selected for further efficacy tests under greenhouse conditions. Different methods for the inoculation of apple trunks and shoots with *D. seriata* mycelium and conidia were tested. The best protocol was selected to inoculate apple plants and they were sprayed at four different time points before and after *D. seriata* inoculation. Canker symptoms were assessed and the *D. seriata* DNA was quantified with quantitative real-time PCR (qPCR).

Results showed that *D. seriata* mycelium and pycnidia production were maximized on respectively potato dextrose agar (PDA) in complete darkness and water agar (WA) supplemented with pine needles with a 16:8 hour light:dark photoperiod for 28 days at

27°C. Moreover, best protocol for *D. seriata* inoculation was obtained with mycelium plugs on wounded apple trunks and shoots. The best inoculation protocol was obtained with mycelium plugs on wounded apple trunks and shoots. Efficacy tests of commercial fungicides revealed that captan, dithianon and fluazinam inhibited *D. seriata* mycelial and conidial growth *in vitro*, while penconazole and ziram did not. However, dithianon spray applications did not reduce trunk canker severity and *D. seriata* DNA amount in plants inoculated under greenhouse conditions. This optimised protocol for fast and precise assessment of fungicide efficacy is suitable for further investigating the effects of other fungicides against *D. seriata* under greenhouse conditions and to estimate the impact of fungicide spray reduction in organic orchards.

## 4.2 Abstract

*Diplodia seriata* is a polyphagous and widespread pathogen that infects trunks, shoots, fruits and leaves of apple plants. Fungicides applied under integrated pest management programmes can act against *D. seriata*. However, the introduction of scab-resistant apple cultivars and the consequent reduction in fungicide applications under low-input disease managements may increase the incidence of *D. seriata*. Despite potential outbreaks of trunk canker, no fast and reproducible protocols for artificial inoculation of *D. seriata* and fungicide efficacy tests are available for apple plants. In this work, protocols for mycelial plug- and conidial suspension-inoculation of trunks and shoots were optimised; canker disease assessment was carried out on potted apple plants under greenhouse conditions and coupled with *D. seriata* DNA quantification with quantitative PCR (qPCR). Efficacy tests of commercial fungicides revealed that captan, dithianon and fluazinam inhibited *D. seriata* mycelial growth and conidial viability in vitro, while penconazole and ziram did not. However, dithianon spray applications did not reduce trunk canker severity and *D. seriata* DNA amount in artificially inoculated plants under greenhouse conditions. This optimised protocol for fast and precise assessment of fungicide efficacy is suitable for further investigating the effects of other fungicides against *D. seriata* under greenhouse conditions.

**Keywords:** *Botryosphaeria obtusa*; *Malus domestica*; apple stem canker; *Diplodia* infection protocol; qPCR quantification

## 4.3 Introduction

*Diplodia seriata* (synonym *Botryosphaeria obtusa*) is a plant pathogen belonging to the Botryosphaeriaceae family (phylum Ascomycota) that has been isolated from several different hosts worldwide (Phillips *et al.*, 2007; Úrbez-Torres and Gubler, 2009; Mondello *et al.*, 2017; Pouzoulet *et al.*, 2017; Spagnolo *et al.*, 2017). In particular, *D. seriata* causes trunk canker and shoot dieback, fruit black rot and leaf frog-eye spot on apple plants (Phillips *et al.*, 2007). Trunk canker is characterised by reddish brown lesions that turn smoky and develop series of alternate rings, rapidly becoming elliptical in shape (Naqvi, 2007). The bark surface becomes rough and cracked with possible callus depositions around the wounds and reddish brown stains on the wood underneath (Naqvi, 2007). *Diplodia seriata* fruit infection (namely black rot) initially appears as small raised purplish lesions on young fruits and develops into large, brown, firm lesions on ripening apples that may finally rot the entire fruit (Venkatasubbaiah *et al.*, 1991). Leaf infections (namely frog-eye leaf spot) initially appear as reddish-brown flecks that develop into circular brown lesions, often surrounded by a purple halo, followed by leaf chlorosis and abscission (Venkatasubbaiah *et al.*, 1991). In addition, *D. seriata* is a polyphagous pathogen and causes pear (Choudhury *et al.*, 2014), grapevine (Úrbez-Torres *et al.*, 2008), olive (Kaliterna *et al.*, 2012) and mulberry trunk canker (Arzanlou and Dokhanchi, 2013), olive (Moral *et al.*, 2008) and loquat rot (Palou *et al.*, 2013), and canker of ornamental plants (e.g. *Cotoneaster salicifolius*) (Bobev *et al.*, 2008) and forest plants (e.g. *Castanea sativa*) (Dar and Rai, 2017). Trunk cankers and infected fruits left in the orchard are the main source of pathogen inoculum (Beer *et al.*, 2015), as well as infected tissues of other plant species in close proximity (Cloete *et al.*, 2011). Thus, the removal of diseased shoots and fruits (Brown-Rytlewski and McManus, 2000; Beer *et al.*, 2015) and the use of resistant cultivars (Biggs and Miller, 2004) are recommended agronomic practices to limit the spread of *D. seriata*.

Conidia spread from inoculum sources to healthy trunks, shoots, fruits or leaves during rain and penetrate through natural openings, such as wounds, lenticels and stomata (Naqvi, 2007). In particular, trunk and shoot infections can occur mainly through pruning wounds in winter or in summer, when trees are more vulnerable to canker development due to possible drought stress (Brown-Rytlewski and McManus, 2000). However, *Diplodia* spp. have consistently been found at low levels in asymptomatic apple and pear bark, acting as a potential source of new infection (Arrigoni *et al.*, 2018). Likewise, fungi

belonging to Botryosphaeriaceae have been found as endophytes and latent pathogens in woody plants (Slippers and Wingfield, 2007) and endophytic Botryosphaeriaceae can rapidly cause disease when their hosts are under stress (Slippers and Wingfield, 2007).

*Diplodia seriata* black rot causes significant losses in organic orchards (up to 10% estimated in northern Germany), but damage is less frequent under integrated pest management (IPM) (Beer *et al.*, 2015). Thus, it has been hypothesised that fungicides applied to control apple scab, flyspeck or sooty blotch can also act against *D. seriata* (Brown-Rytlewski and McManus, 2000; Beer *et al.*, 2015). Indeed, fungicides (benomyl, kresoxim-methyl and trifloxystrobin) directly applied by topical wound treatment reduced the incidence of *D. seriata* trunk canker in apple trees (Brown-Rytlewski and McManus, 2000) and fungicide sprays against apple scab can control sooty blotch and flyspeck disease (Weber *et al.*, 2016b). This is most probably related to the side effect of fungicides applied against the main pathogen (i.e. *Venturia inaequalis*), which can reduce the inoculum of some secondary ones. However, the introduction of scab-resistant apple cultivars can reduce fungicide applications as compared with susceptible cultivars (Simon *et al.*, 2011; Didelot *et al.*, 2016) and the consequent reduction in fungicide applications under low-input disease managements of scab-resistant apple cultivars may cause outbreaks of several newly emerging pathogens, *D. seriata* included (Ellis *et al.*, 1998). An increasing risk of canker outbreaks was also hypothesised due to climate change, which exposes plants to the risk of abiotic stress (Slippers and Wingfield, 2007), indicating that specific fungicide applications will most probably be required to control canker agents in the future (Ellis *et al.*, 1998). Although the risk of emerging secondary pathogens is real, no fast and reproducible protocols are available to assess the efficacy of conventional spray applications against *D. seriata* canker under greenhouse conditions. Most of the studies on *D. seriata* infection and management have focused on apple black rot (Biggs and Miller, 2004; Beer *et al.*, 2015) and grapevine trunk disease (Úrbez-Torres and Gubler, 2009; Pitt *et al.*, 2012; Mondello *et al.*, 2017). Trunk canker diseases are rarely studied due to the long incubation time and the difficulties in reproducing infection under controlled conditions. The aim of this study was to optimise a fast and reliable protocol to inoculate and assess *D. seriata* on potted apple plants and to evaluate fungicide efficacy against trunk canker using conventional spray applications under greenhouse conditions.

## 4.4 Materials and methods

### 4.4.1 Fungal isolates

Two strains of *D. seriata* (namely S and VT) were isolated as single spore cultures from trunks of *Malus domestica* plants with canker symptoms in the 'Scurelle' (latitude, N46.0647636; longitude, E11.510519599999952; altitude, 482 m) and 'Vigo di Ton' orchards (latitude, N46.2655852; longitude, E11.085680799999977; altitude, 375 m) in the province of Trento (northern Italy). The strains were stored in glycerol at -80°C in the fungal collection of the Fondazione Edmund Mach and they are freely available upon request. The strains were identified by morphology and molecular methods. For the latter, *D. seriata* was grown on potato dextrose agar (PDA, Oxoid, Basingstoke, United Kingdom) for 14 days at 27°C and DNA was extracted form 0.2 g of mycelium using the FastDNA spin kit for soil (MP Bimedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. DNA was quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with a Synergy2 microplate reader (BioTek, Winooski, VT, USA) and the internal transcribed spacer (ITS) region was amplified using the forward primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990; Halwachs *et al.*, 2017). PCR amplification was carried out using the FastStart HighFidelity PCR system (Roche, Branford, CT, USA) with 1 µl of extracted DNA (10 ng), 0.25 mM deoxynucleoside triphosphates, 1% (w:v) bovine serum albumin, 4% (v:v) dimethyl sulphoxide, 0.3 µM of each primer and 2.5 U of FastStart High-Fidelity DNA polymerase (Roche) in 50 µL of reaction and the following programme: denaturation at 95°C for 5 min, 32 cycles of amplification at 95°C for 30 s, annealing at 60°C for 1 min, extension at 72°C and final extension at 72°C for 10 min. PCR product was purified with the Illustra ExoProStar reaction (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, United Kingdom) and single strand sequences were obtained with an ABI PRISM 3730xl DNA analyser (Applied Biosystems, Thermo Fisher Scientific) using the forward primer ITS5, according to the manufacturer's instructions (Sequencing Service of the Fondazione Edmund Mach). Sequences were aligned against the National Center for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov) to confirm *D. seriata* identity and they were deposited at the Genbank database of NCBI

(https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers MH174673 (*D. seriata* S) and MH174674 (*D. seriata* VT).

### 4.4.2 Growth conditions of *Diplodia seriata* strain S and VT

Mycelial growth of *D. seriata* S and *D. seriata* VT was assessed on four growth media: i) 15 g $L^{-1}$ technical agar (Sigma-Aldrich, Saint Louis, MO, USA) in distilled water (water agar, WA), ii) 39 g $L^{-1}$ PDA (Oxoid), iii) 2.4 g $L^{-1}$ potato dextrose broth (Fluka, Sigma-Aldrich) supplemented with 15 g $L^{-1}$ technical agar (PDB+A, Oxoid) and iv) 2.4 g $L^{-1}$ potato dextrose broth (Fluka, Sigma-Aldrich) supplemented with 15 g $L^{-1}$ technical agar (Oxoid) and 10 g $L^{-1}$ of carboxymethyl-cellulose (PDB+A+C, Sigma-Aldrich). Plates were incubated at 27°C for 28 days under two photoperiod conditions: complete darkness and 16:8 hour light:dark photoperiod. Three double-sterilised pine needles (5 cm long) were added to each WA plate, in order to stimulate the production of pycnidia, as previously reported for the Botryosphaeriaceae species (Slippers and Wingfield, 2007; Amponsah *et al.*, 2008). A mycelial plug (5 mm in diameter) of a seven day-old culture of *D. seriata* S or *D. seriata* VT grown on PDA was placed in each Petri dish (90 mm in diameter). The mycelial diameter was measured after every three days of incubation at 27°C and the daily growth rate was then calculated by dividing the maximum diameter (measure of the diameter registered before complete plate coverage) by the number of days of incubation. Pycnidia production on PDA, PDB+A and PDB+A+C was assessed visually as percentage of dish surface covered by *D. seriata* pycnidia (pycnidia density) 28 days after incubation at 27°C. Pycnidia production on WA supplemented with pine needles was expressed as the number of pycnidia produced per cm of needle length by counting under a stereoscope (SMZ800, Nikon, Tokyo, Japan) 28 days after incubation at 27°C. Four replicates (plates) were assessed for each medium, photoperiod condition and fungal strain and the experiment was carried out twice.

### 4.4.3 *Diplodia seriata* inoculation of apple plants

Two protocols for inoculation (mycelial plug and conidial suspension) and two plant tissues (trunks and shoots) were compared. To prepare the mycelial plug, *D. seriata* S was grown on PDA for 28 days at 27°C in complete darkness, and plugs (5 mm in

diameter) were obtained with a flame-sterilised cork borer under sterile conditions. To prepare the conidial suspension, *D. seriata* S was grown on WA supplemented with three double-sterilised pine needles for 28 days at 27°C with a 16:8 hour light:dark photoperiod. Thirty sporulated pine needles were transferred to 30 mL of distilled sterile water, ten sterile stainless steel spheres were added and the vial was shaken by vortexing at the maximum speed for 2 min, in order to break the pycnidia and allow the release of conidia. The conidial suspension was vacuum-filtered using a membrane with a 160 μm porosity (160 μm NY6H, Millipore, Burlington, VT, USA) in order to remove intact pycnidia and needle fragments. The conidial concentration was adjusted to $1\times10^5$ conidia $mL^{-1}$ using a Thoma cell counting chamber under a light microscope (Eclipse 80i, Nikon) and the suspension was applied with a compressed air hand sprayer.

Three-year-old apple plants belonging to the apple scab-resistant cv. Fujion were planted in six-L pots and grown under greenhouse conditions at 25 ± 1°C with a 16:8 hour light:dark photoperiod and relative humidity (RH) of 60 ± 10% for three months, until each plant reached the petal fall phenological stage (Chapman and Catlin, 1976). Longitudinal wounds (20 mm long, 8 mm wide and 2 mm deep) were made on three-year-old apple trunks and one-year-old shoots with a fire-sterilised scalpel without totally removing the outer bark. Each wound on the *D. seriata*-inoculated plants was inoculated with a mycelial plug (5 mm in diameter) or sprayed with 300 μL of conidial suspension ($1\times10^5$ conidia $mL^{-1}$). On each plant, eight wounds were made alternately on diametrically opposite sides on the trunk, in order to have a distance of more than 30 cm between wounds longitudinally along the trunk and to avoid canker lesion overlapping. Four wounds were inoculated with the mycelial plug and four sprayed with the conidial suspension. Likewise, eight wounds were made on four separate shoots, four of them inoculated with the mycelial plug and four sprayed with the conidial suspension. As a control, a second group of plants was prepared and each wound was mock inoculated with a sterile PDA plug (5 mm in diameter) or with 300 μL of sterile distilled water. Five replicates (plants) were obtained for *D. seriata* S-inoculated and control plants.

Each wound was covered with the outer bark and wrapped with laboratory plastic film (Parafilm, Bemis, Neenah, WI, USA) for seven days to maintain high humidity and reduce the risk of contamination, as reported for *D. seriata* inoculation on the grapevine (Spagnolo *et al.*, 2017). Plants were incubated at 95 ± 5% RH and 25 ± 1°C for 48 h and then maintained under greenhouse conditions in a randomised complete block design. Sixty days after inoculation, trunks and shoots were cut into sections (15 cm long that was

sufficient to include the whole canker) containing the inoculation site and surface-sterilised by incubating in 1% v:v sodium hypochlorite for 45 sec, then washed twice in sterile distilled water for 60 sec. Trunk sections were peeled to remove 2-3 mm of superficial tissue layers and a calliper was used to measure the longitudinal canker length. Disease severity and incidence were assessed for each wound of mycelial plug- and conidial suspension-inoculated trunks and shoots. For each replicate (plant), disease severity was assessed as the mean of canker length (mm) of four inoculated wounds and disease incidence was expressed as the percentage (%) of infected wounds. In order to remove the effect of the wound scar, the canker length of mock-inoculated plants with sterile PDA plugs and sterile distilled water was subtracted from the disease severity scores of mycelial plug and conidial suspension *D. seriata*-inoculated plants, respectively.

### 4.4.4 Fungicide efficacy tests against *Diplodia seriata in vitro*

Five commercial fungicides commonly used in apple protection programmes (Table 1) (Longo *et al.*, 2017) were tested against *D. seriata* mycelial growth and conidial viability *in vitro*. To assess effectiveness against mycelial growth, a small mycelial portion (2 mm long and 1 mm wide) was collected with a flame-sterilised bacteriological needle from a 28-day-old *D. seriata* S culture on PDA in complete darkness and transferred to a 2 mL sterile plastic tube containing 1 mL of sterile distilled water (control) or a fungicide solution at the concentration recommended on the label (Table 1). To assess activity against conidial viability, a conidial suspension was prepared as described above for apple plant inoculation and 500 µL of conidial suspension ($1\times10^5$ conidia $mL^{-1}$) was added to 500 µL of sterile distilled water (control) or a 2-fold concentrated fungicide solution in a 2 mL collection tube, reaching the recommended concentration of each fungicide.

Samples were incubated at room temperature for 5 h under orbital shaking at 80 rpm and centrifuged at 8000 × *g* for 3 min. The supernatant was discarded and the pellet was washed three times by suspending in 1 mL of sterile distilled water and centrifuging at 8000 × *g* for 3 min, in order to remove fungicide residues. Each mycelial portion was plated on PDA and diameter of mycelial growth was measured three days after incubation at 27°C. Pelleted conidia were resuspended in 200 µL of sterile distilled water, 100 µL of each suspension was plated on PDA and conidial viability was assessed as colony forming units per unit of suspension volume (CFU $ml^{-1}$) two days after incubation at 27°C. Six

replicates (tubes) were prepared for each fungicide and inoculation procedure, and the experiment was carried out twice.

**Table 1.** List of fungicides tested against *Diplodia seriata*.

| Active ingredient | Main target apple pathogen | Commercial name | Manufacturer | Dosage (g $L^{-1}$) |
|---|---|---|---|---|
| Captan | *Venturia inaequalis* | Merpan 80 WDG | Adama | 1.5 |
| Dithianon | *V. inaequalis* | Delan 70 WG | Basf Crop Protection | 0.4 |
| Fluazinam | *V. inaequalis* | Banjo | Adama | 0.7 |
| Ziram | *V. inaequalis* | Diziram 76 WG | FMC Foret S.A. | 2.0 |
| Penconazole | *Podosphaera leucotricha*, *V. inaequalis* | Topas 10 EC | Syngenta | 0.3 |

### 4.4.5 Fungicide treatments on apple plants inoculated with *Diplodia seriata* under greenhouse conditions

Three-year-old apple plants grown under greenhouse conditions (cv. Fujion) were inoculated on the trunk with the *D. seriata* S mycelial plug (5 mm in diameter) and conidial suspension (300 µL of $1\times10^5$ conidia $mL^{-1}$) as described above. Eight wounds were obtained alternately on diametrically opposite sides of each plant trunk: four wounds were inoculated with the mycelial plug and four wounds were sprayed with the conidial suspension. Plants were treated with dithianon seven days before inoculation, one day before inoculation, one day after inoculation or seven days after inoculation, at the dosage recommended in the manufacturer's instructions (Table 1) using a compressed air hand sprayer, with an application rate comparable to conventional fungicide treatments under field conditions (0.1 kg $ha^{-1}$ for young plants). As a control, plants were sprayed with water one day before *D. seriata* S inoculation. The plastic film was removed from each wound before fungicide or water treatments and a new plastic film was then applied once the plant tissues had dried completely. A group of mock-inoculated plants with a sterile PDA plug (5 mm in diameter) or with 300 µL of sterile distilled water was prepared to assess the effect of the wound scar, as described above. Plastic films were removed seven days after inoculation and plants were kept for 60 days under greenhouse conditions. Trunks were cut into sections (15 cm long), surface-sterilised and peeled to assess disease severity and incidence of mycelial plug-inoculated (four trunk sections per plant) and conidial suspension-inoculated plants (four trunk sections per plant), as described above. Four

wood pieces (20 mm long, 5 mm wide and 1 mm thick) were collected with a fire-sterilised scalpel from each trunk section. Two wood samples were obtained from each plant, one from mycelial plug-inoculated trunks (16 wood pieces pooled in equal amount) and one form conidial suspension-inoculated trunks (16 wood pieces pooled in equal amount), and they were stored at -20°C for DNA extraction and *D. seriata* S quantification. Five replicates (plants) were analysed for each treatment in a randomised complete block design and the experiment was carried out twice.

### 4.4.6 DNA extraction and *Diplodia seriata* quantification with real-time quantitative PCR

Each wood sample (one from mycelial plug-inoculated trunks and one form conidial suspension-inoculated trunks of each plant) was 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 as described by Arrigoni *et al.* (2018). DNA was extracted from 500 µl of each ground wood sample using the FastDNA spin kit for soil (MP Bimedicals) according to the manufacturer's instructions and quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher Scientific) with a Synergy2 microplate reader (BioTek). *D. seriata* was quantified with real-time quantitative PCR (qPCR) using the primer pair DseCQF (5'-CTCTGCAATCGCTGACCCTTG-3') and DseCQR (5'-ACGTGTTTGTCTAACTAGTAGAGAGTACC-3') that previously showed high sensitivity and positive correlation between the qPCR-quantified *D. seriata* DNA amount and *D. seriata* incidence (Pouzoulet *et al.*, 2017). qPCR assay was carried out using the KAPA SYBR FAST qPCR Master Mix at the concentration recommended in the manufacturer's instructions (1×; Kapa Biosystems, Cape Town, South Africa) with 0.5 µM of each primer and DNA template ranging in concentration from 0.5 to 4 ng/µL in 25 µL of reaction. Amplification was performed using a Roche Light Cycler 480 (Roche Diagnostics GmbH, Germany) with the following programme: 95°C for 3 min; 40 cycles of 95°C for 3 sec, 62°C for 40 sec and an additional melting analysis from 60°C to 95°C according to Pouzoulet *et al.* (2017). Negative and standard controls containing nuclease-free water and 0.01 ng of pure *D. seriata* DNA respectively were included in every run. A standard curve for absolute quantification of *D. seriata* in apple samples was obtained in triplicate by mixing serial dilutions of *D. seriata* S DNA (1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1

pg) with 10 ng of apple DNA extracted from axenic apple plants grown *in vitro*. Wood samples of five replicates (plants) were analysed for each treatment and inoculation procedure, the experiment was carried out twice.

### 4.4.7 Statistical analysis

Data were analysed with Statistica 13.1 software (Dell, Round Rock, TX, USA). Normal distribution (Kolmogorov-Smirnov test, $p \leq 0.05$) and variance homogeneity of the data (Levene's or Chochran's test, $p \leq 0.05$) were checked and non-parametric tests were used when these parametric assumptions were not respected. Each experimental repetition was analysed individually and the Kruskal–Wallis test was used to demonstrate equivalent results in the two repetitions of each experiment ($p > 0.05$, non-significant differences between the two experimental repetitions). For each experiment, data from the two experimental repetitions were pooled and the Kruskal–Wallis test ($p \leq 0.05$) was used to detect significant differences among treatments. Interaction among factors affecting the *D. seriata* growth, pycnidia production, severity and incidence were assessed according to the Kruskal–Wallis test ($p \leq 0.05$), merging data for growth media, fungal strains, inoculated plant organ (trunks and shoots) or inoculation procedures (mycelial plug and conidial suspension).

## 4.5 Results

### 4.5.1 *Diplodia seriata* propagation and inoculation under greenhouse conditions

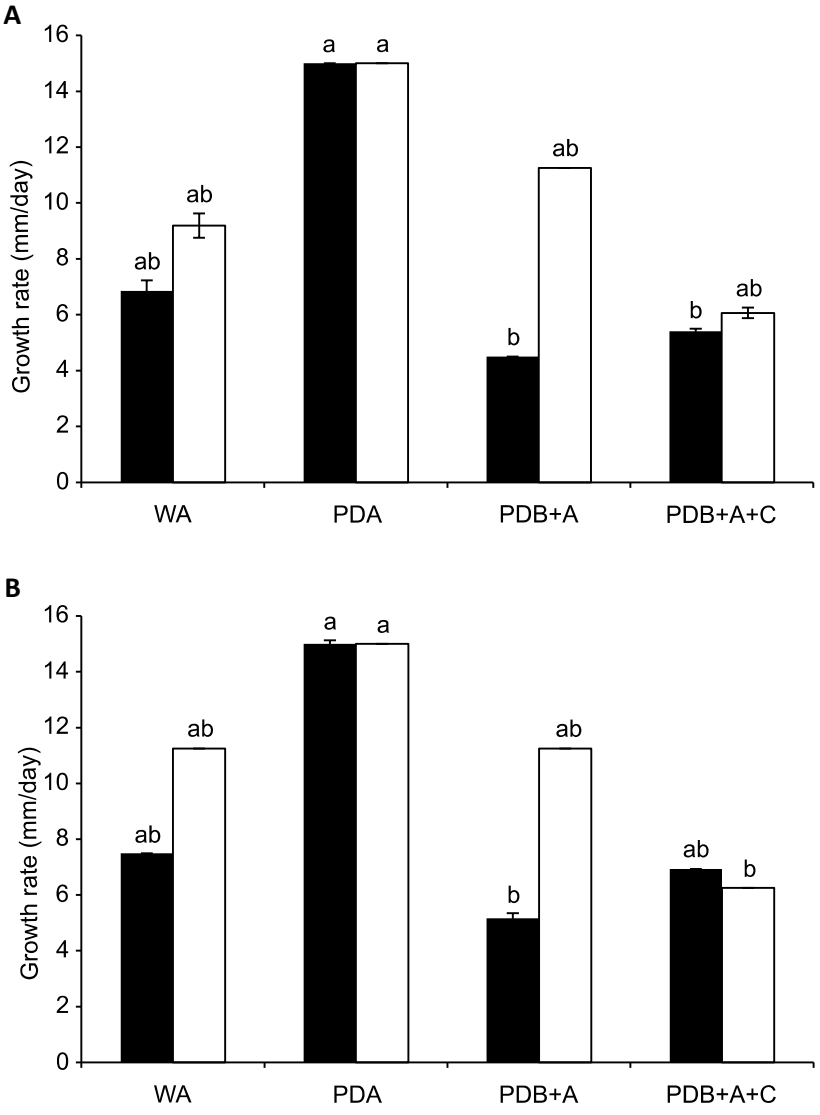



**Figure 1.** Mycelial growth rate of the *Diplodia seriata* strain S (black) and *D. seriata* strain VT (white) grown a) in complete darkness and b) with a 16:8 hour light:dark photoperiod on water agar (WA), potato dextrose agar (PDA), potato dextrose broth supplemented with agar (PDB+A) and PDB+A supplemented with cellulose (PDB+A+C) for 28 days at 27°C. The Kruskal-Wallis test showed no significant differences between the two experimental repetitions ($p > 0.05$, n = 4 replicates per experiment) and data from the two experiments were pooled. Mean and standard error values for eight replicates (dishes) from the two experiments are shown for each fungal strain and growth medium. Different letters indicate significant differences according to the Kruskal-Wallis test ($p \leq 0.05$).

The highest growth rate was found for *D. seriata* S and *D. seriata* VT on PDA (Figure 1) and no differences were found between the two photoperiod conditions on merging data for growth media and fungal strains (Kruskal-Wallist test, $p$ = 0.264). Pycnidia production, expressed as pycnidia density and pycnidia number, was comparable among growth media and fungal strains (Figure 2), while it was higher with a 16:8 hour light:dark photoperiod as compared with the complete darkness on merging data for growth media and fungal strains (Kruskal-Wallis test, $p = 3.9 \times 10^{-8}$ and $9.4 \times \times 10^{-4}$, respectively).

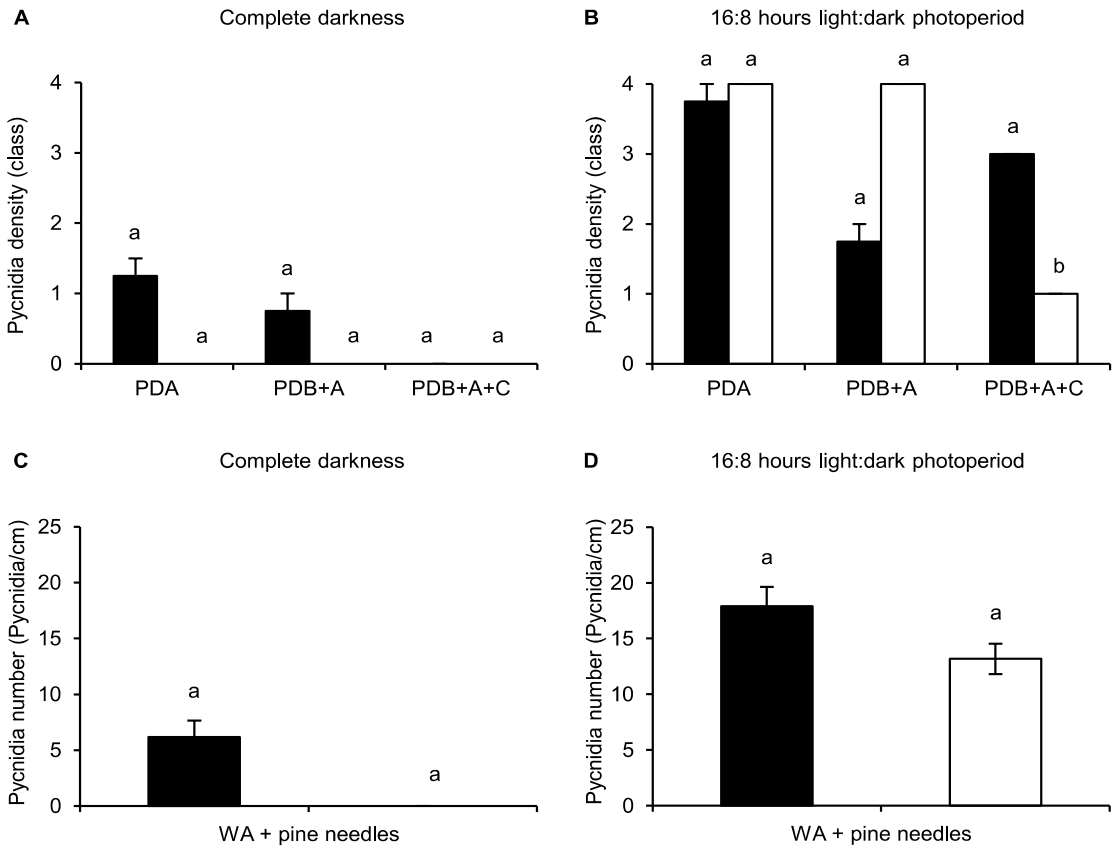



**Figure 2.** Pycnidia production from *Diplodia seriata* S (black) and *D. seriata* VT (white) grown on potato dextrose agar (PDA), potato dextrose broth supplemented with agar (PDB+A), PDB+A supplemented with cellulose (PDB+A+C) and water agar supplemented with pine needles (WA + pine needles). Pycnidia production was assessed a, b) as the percentage of dish surface covered by *D. seriata* pycnidia and c, d) as the number of pycnidia per centimetre of pine needles 28 days after growth in complete darkness and with a 16:8 hour light:dark photoperiod at 27°C. The Kruskal-Wallis test showed no significant differences between the two e experimental repetitions ($p > 0.05$, n = 4 replicates per experiment) and data from the two experiments were pooled. Mean and standard error values of eight replicates (dishes) from the two experiments are shown for each fungal strain and growth medium. Different letters indicate significant differences according to the Kruskal-Wallis test ($p \leq 0.05$).

*Diplodia seriata* severity was higher in mycelial plug-inoculated than conidial suspension-inoculated trunks (Figure 3), while no differences were found between the two inoculation procedures on shoots (Figure 4a).


**Figure 3.** Inoculation procedure of a) three-year-old apple trunk b) cut to obtain a longitudinal wound. Each wound was c) inoculated with a mycelial plug or sprayed with the conidial suspension (example picture as in the panel b) and d) covered with laboratory plastic film. The disease severity was assessed on e) mycelial plug-inoculated and f) conidial suspension-inoculated trunks 60 days after incubation under greenhouse conditions. Length (cm) is specified by the ruler on the right.

Significant effects of the inoculation procedure (Kruskal–Wallis test, $p = 0.002$) and not plant organ (Kruskal–Wallis test, $p = 0.449$) on *D. seriata* severity were found, merging data for trunks and shoots and data for conidial suspension and mycelial plug inoculation respectively. Likewise, the inoculation procedure (Kruskal–Wallis test, $p = 0.002$) and not plant organ (Kruskal–Wallis test, $p = 0.307$) affected *D. seriata* incidence.

In particular, mycelium plug inoculation on shoots resulted in a higher disease incidence as compared with conidial suspension inoculation on trunks (Figure 4b).

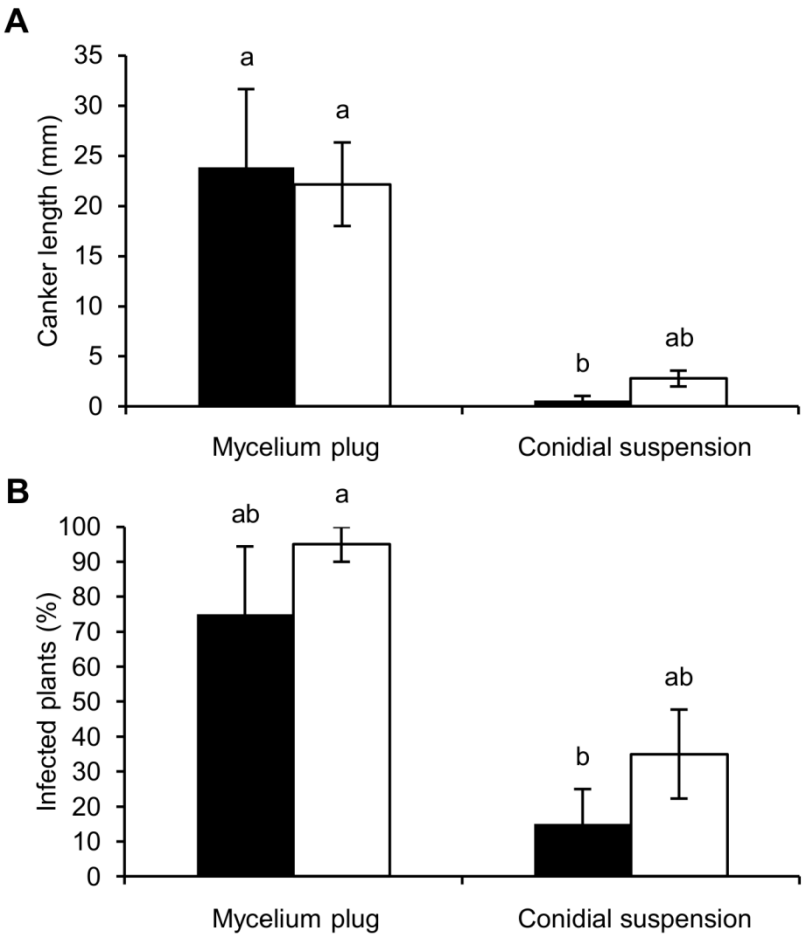



**Figure 4.** *Diplodia seriata* S a) disease severity and b) incidence assessed as canker length and as percentage of infected plants 60 days after incubation under greenhouse conditions, respectively. Three-year-old trunks (black) and one-year-old shoots (white) of apple plants were inoculated with a *Diplodia seriata* S mycelial plug or conidial suspension. Mean and standard error values are shown for five replicates (plants) for each plant organ and inoculation procedure. Different letters indicate significant differences according to the Kruskal-Wallis test ($p \leq 0.05$).

### 4.5.2 Effect of commercial fungicides against *Diplodia seriata*

Incubation of *D. seriata* S with dithianon, captan and fluazinam reduced mycelial growth (Figure 5a) and conidial viability *in vitro* (Figure 5b). On the other hand, penconazole and ziram did not affect *D. seriata* S mycelial growth and conidial viability *in vitro*.

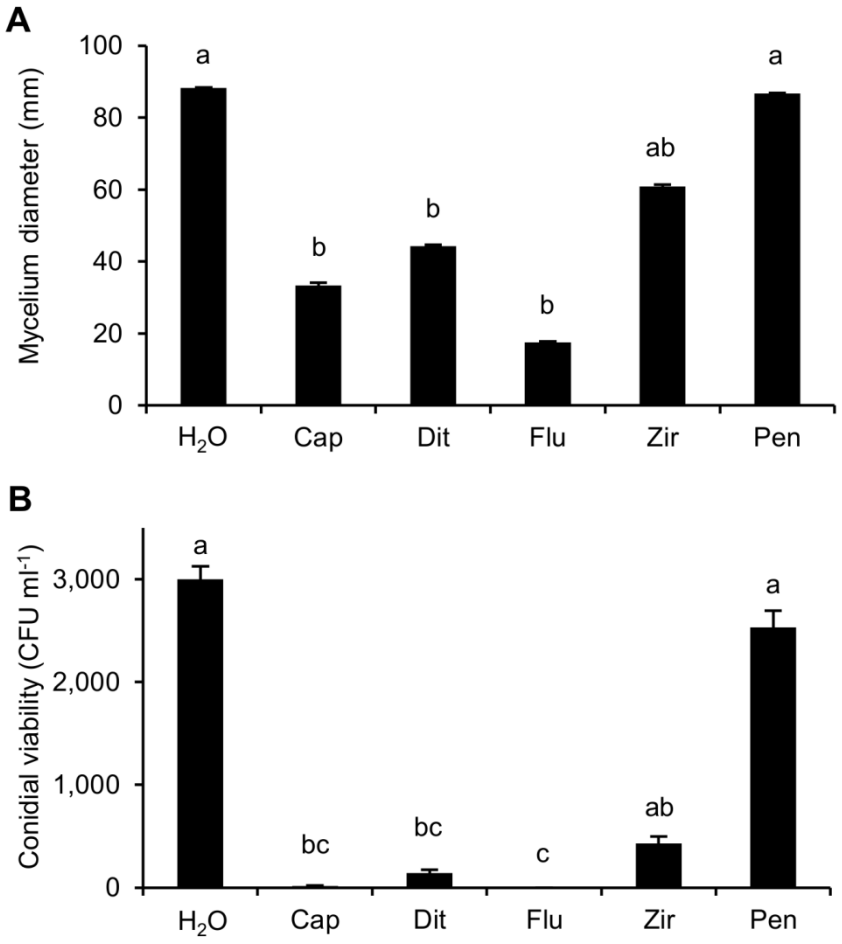



**Figure 5.** *Diplodia seriata* S a) mycelial growth and b) conidial viability after incubation in water ($H_2O$) or in presence of a commercial fungicide: captan (Cap), dithianon (Dit), fluazinam (Flu), ziram (Zir) or penconazole (Pen). Mycelia and conidia were incubated for 5 h with the different treatments and plated on potato dextrose agar. Mycelial growth and conidial viability were assessed respectively as the mycelial diameter and as colony forming units per unit of volume (CFU $ml^{-1}$) three and two days after incubation at 27°C. The Kruskal-Wallis test showed no significant differences between the two experimental repetitions ($p > 0.05$, n = 6 replicates per experiment) and data from the two experiments were pooled. Mean and standard error values for 12 replicates (dishes) from the two experiments are shown for each treatment. Different letters indicate significant differences according to the Kruskal-Wallis test ($p \leq 0.05$).

Dithianon was applied before or after *D. seriata* S inoculation of greenhouse-grown plants and it did not reduce disease severity (Figure 6a), disease incidence (Figure S1) and *D. seriata* DNA amount (Figure 6b) on mycelial plug- and conidial suspension-inoculated trunks. Disease severity, disease incidence and the *D. seriata* DNA amount were affected by the inoculation procedure (Kruskal–Wallis test, $p = 4.0 \cdot \times \cdot 10^{-7}$, $p = 7.1 \cdot \times \cdot 10^{-7}$ and $p = 3.5 \cdot \times \cdot 10^{-5}$, respectively) and they higher in mycelial plug-inoculated than in conidial suspension-inoculated trunks, merging data for water- and dithianon-treated plants.

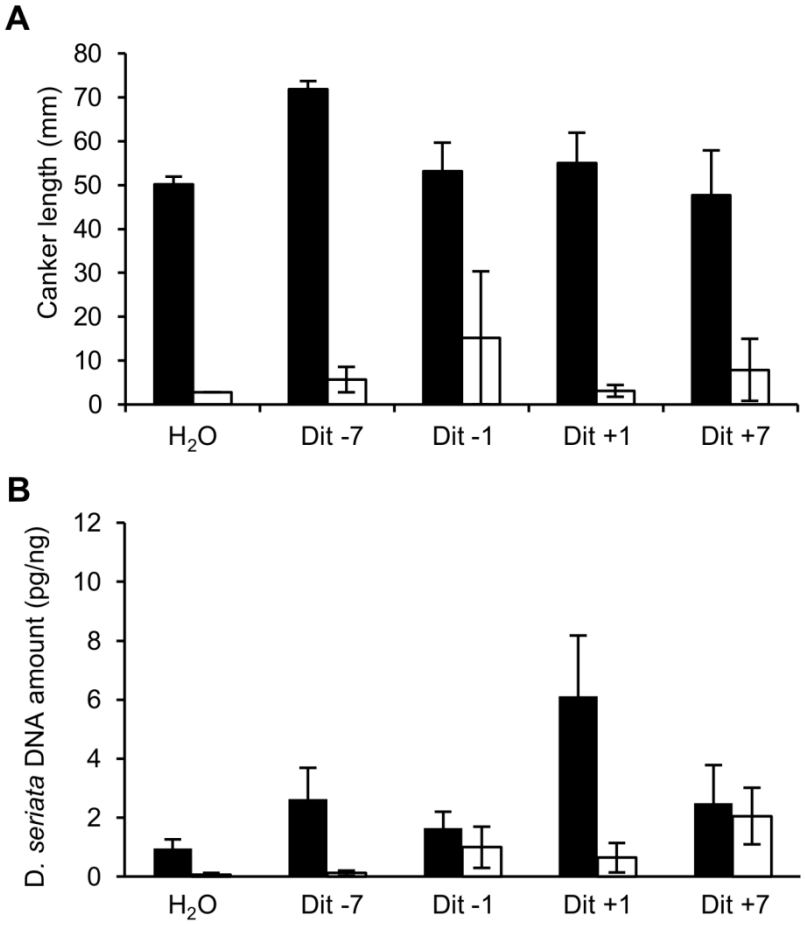



**Figure 6.** *Diplodia seriata* S a) disease severity and b) DNA amount for three-year-old trunks of apple plants inoculated with a mycelium plug (black) or conidial suspension (white) 60 days after incubation under greenhouse conditions. Plants were treated with water one day before inoculation ($H_2O$) or with dithianon seven days (Dit -7) or one day (Dit -1) before inoculation, and one day (Dit +1) or seven days (Dit +7) after inoculation. The Kruskal-Wallis test showed no significant differences between the two experiments ($p > 0.05$, n = 5 replicates per experiment), and data from the two experiments were pooled. Mean and standard error values for ten replicates (plants) from the two experiments are shown for each plant organ and inoculum. No significant differences among treatments of mycelium- and conidia-inoculated plants were found according to the Kruskal-Wallis test ($p \leq 0.05$).

## 4.6 Discussion

*Diplodia* spp. have been found in asymptomatic apple bark (Arrigoni *et al.*, 2018) and Botryosphaeriaceae genera can persist as latent pathogens of trees (Slippers and Wingfield, 2007), suggesting that their presence on the plant could be linked to possible plant infection and disease. In apple orchards, *Diplodia* spp. inoculum is plausibly controlled by fungicide applications in plant protection programmes (Ellis *et al.*, 1998). Therefore, the reduction in chemical fungicides under low-input disease management of scab-resistant cultivars (Simon *et al.*, 2011; Didelot *et al.*, 2016) may lead to canker outbreaks (Ellis *et al.*, 1998). In order to investigate fungicide efficacy against apple trunk canker, a fast and reproducible method for *D. seriata* inoculation, disease assessment and fungicide efficacy test *in vitro* and on apple plants was developed. *Diplodia seriata* S mycelia and pycnidia were obtained on PDA in complete darkness and WA supplemented with pine needles with a 16:8 hour light:dark photoperiod respectively, in agreement with previous studies on Botryosphaeriaceae species (Kim *et al.*, 2005; Slippers and Wingfield, 2007; Amponsah *et al.*, 2008; Phillips *et al.*, 2013; Dheepa *et al.*, 2018). Pycnidia production of *D. seriata* was higher with a 16:8 hour light:dark photoperiod than in complete darkness, confirming the role of photoperiod in spore production of the Botryosphaeriaceae species (Kim *et al.*, 2005; Dheepa *et al.*, 2018), particularly with pine needles that maximised pycnidia production in Botryosphaeriaceae species (Slippers and Wingfield, 2007; Amponsah *et al.*, 2008; Phillips *et al.*, 2013).

In a previous study, a method for the inoculation of apple plants with *Botryosphaeria dothidea* and *B. obtusa* mycelial plugs was developed under field conditions, resulting in high variability between experiments (Brown-Rytlewski and McManus, 2000). The lack of reproducibility was attributed to the fluctuating environmental conditions in the field (Brown-Rytlewski and McManus, 2000) and to overcome this possible source of variability we developed a method for the artificial inoculation of *D. seriata* and fungicide efficacy test on apple trunks under greenhouse conditions. Mycelial plug inoculation produced severe canker symptoms on the trunks and shoots of potted apple plants, and disease severity was higher in mycelial plug-inoculated as compared with conidial suspension-inoculated plants, in agreement with Botryosphaeria dieback (e.g. *D. mutila* and *Neofusicoccum* spp.) in the grapevine (Amponsah *et al.*, 2011). *Diplodia seriata* severity on apple trunks and shoots was comparable, indicating that tissue age did not affect disease development under controlled conditions, as shown for grapevine

shoots and trunks inoculated with *Neofusicoccum luteum* (Amponsah *et al.*, 2012b). In the efficacy tests *in vitro*, three commercial fungicides (fluazinam, dithianon and captan) reduced *D. seriata* viability and growth, while penconazole and ziram did not. Dithianon was reported to have little effect against *D. mutila* on grapevine plants (Amponsah *et al.*, 2012a) and demethylation-inhibiting fungicides showed different efficacy levels against *D. seriata* and *D. mutila in vitro* (Torres *et al.*, 2013), indicating their species-specific susceptibility. Penconazole has previously been reported as an inhibitor of *D. seriata* mycelial growth *in vitro* (Pitt *et al.*, 2012; Mondello *et al.*, 2017) and it showed lower efficacy than fluazinam (Pitt *et al.*, 2012). Differences in penconazole efficacy could be related to the specific *D. seriata* strains used in this study and/or to the efficacy test protocols used. In particular, mycelia and conidia were incubated in the fungicide solution for 5 h and then plated on PDA in our assays, while mycelial plugs were plated on PDA supplemented with the fungicide in the protocol of Pitt *et al.* (2012), possibly leading to longer exposure to penconazole. Due to its frequent applications in IPM apple orchards (Blommers, 1994), dithianon was selected for efficacy tests under greenhouse conditions and it showed no reduction in *D. seriata* severity, incidence and DNA amount in mycelial plug- and conidial suspension-inoculated plants. Discrepancies between *in vitro* and *in planta* efficacy tests have also been described for Botryosphaeria dieback agents (Mondello *et al.*, 2017), indicating that fungicide deposition, adhesion and penetration in host tissues can influence the efficacy (Zabkiewicz, 2007). Moreover, topical wound treatments of benomyl, kresoxim-methyl and trifloxystrobin were able to reduce the incidence of *D. seriata* trunk canker under field conditions (Brown-Rytlewski and McManus, 2000), indicating that the mode of application and the active ingredient affected fungicide efficacy.

In this work, methods for *D. seriata* inoculation and quantification were optimised on a scab-resistant apple cultivar (Fujion) and efficacy tests of commercial fungicides were carried out *in vitro* and with conventional spray application under greenhouse conditions. Although *D. seriata* mycelial growth and conidial viability were inhibited by three fungicides *in vitro* (dithianon, captan and fluazinam), trunk canker symptoms were not affected by spray applications of dithianon under greenhouse conditions, suggesting that its application does not significantly contribute to indirect *D. seriata* control in IPM. These optimised methods can be used to accurately assess fungicide spray efficacy against *D. seriata* and to precisely estimate the impact of fungicide spray reduction of low-input disease managements on scab-resistant apple cultivars.

## 4.7 Supplementary figures

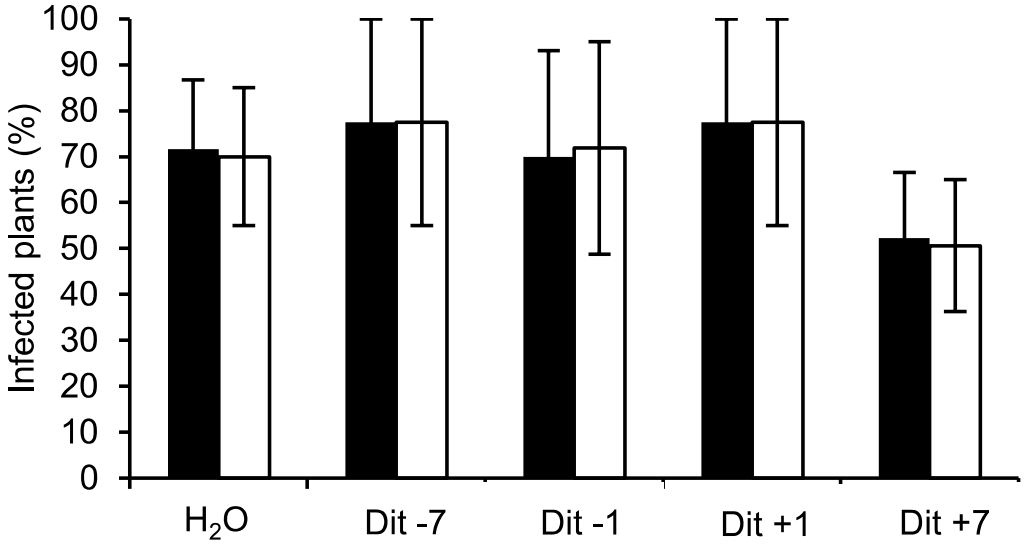



**Figure S1.** *Diplodia seriata* S disease incidence of three-year-old trunks of apple plants inoculated with a mycelial plug (black) or conidial suspension (white) 60 days after incubation under greenhouse conditions. Plants were treated with water one day before inoculation ($H_2O$) or with dithianon seven days before inoculation (Dit -7), one day before inoculation (Dit -1), one day after inoculation (Dit +1) or seven days after inoculation (Dit +7). The Kruskal-Wallis test showed no significant differences between the two experiments ($p > 0.05$, $n = 5$ replicates per experiment), and data from the two experiments were pooled. Mean and standard error values for ten replicates (plants) from the two experiments are shown for each plant organ and inoculation procedure. No significant differences among treatments of mycelial plug- and conidial suspension-inoculated plants were found according to the Kruskal-Wallis test ($p > 0.05$).

# Chapter V. Discussion

Plant microbiota plays crucial roles in plant fitness and in the adaptation to the environment, establishing beneficial and detrimental interactions with the plant host (Bulgarelli *et al.*, 2013; Mendes *et al.*, 2013; Berg *et al.*, 2015). Microbial communities colonise every plant tissue and organ (Turner *et al.*, 2013) and their composition is deeply influenced by the nutritional and environmental characteristics of the plant compartment (Kowalchuk *et al.*, 2010; Berg *et al.*, 2015). The composition of plant-associated microbial communities is influenced by plant genotype (Berg *et al.*, 2015), environmental conditions (Perazzolli *et al.*, 2014; Hamonts *et al.*, 2017), organ age (Vorholt, 2012) and agronomical practices (Leff and Fierer, 2013; Abdelfattah *et al.*, 2016). In particular, previous studies on soil (Hartmann *et al.*, 2015), leaf (Ottesen *et al.*, 2009; Schmid *et al.*, 2011; Perazzolli *et al.*, 2014; Glenn *et al.*, 2015) and fruits (Jensen *et al.*, 2013; McGarvey *et al.*, 2015; Abdelfattah *et al.*, 2016) demonstrated that disease management strategies can influence the composition of plant-associated microbiota. With the aim to reduce the use of fungicides in the management of apple diseases, several scab-resistant cultivars have been introduced in the market and they are compatible with a low-input disease management strategy (Ellis *et al.*, 1998; Simon *et al.*, 2011; Didelot *et al.*, 2016). The reduction of fungicide treatments may affect the proportions and the equilibrium between pathogenic and beneficial microorganisms populating the microbiota and eventually cause the outbreak of secondary pathogens, which are indirectly controlled under conventional and integrated disease management (Warner, 1991; Ellis *et al.*, 1998; Beer *et al.*, 2015). However, most of the studies about plant-associated microbial communities are focused on phylloplane, rhizosphere (Berg *et al.*, 2014) and endosphere (Berg *et al.*, 2015), while other plant compartments such as bark, flowers and fruits remain poorly studied. Despite the potential role of bark as a reservoir of pathogenic and beneficial microorganisms (Buck *et al.*, 1998; Martins *et al.*, 2013), few studies have been focused on the composition of its associated microbial communities and no studies about the influence of disease management strategies on the composition of bark microbial communities are available. In this doctoral work, the composition of bark- associated microbial communities in relation to bark age, plant species and cultivar was investigated (Chapter II). A protocol for the metabarcoding analysis of fungal and bacterial communities residing on pear (Abate and Williams cultivars) and apple (Golden Delicious and Gala) bark was optimised. Bark hosts

complex microbial communities, despite the harsh conditions that can hinder microbial growth and survival (Buck *et al.*, 1998), such as scarce nutrient and water availability, presence of complex polymers as lignin and hemicellulose and of compounds with antimicrobial activity (Pearce, 1996; Valentín *et al.*, 2010). The major drivers of the structure and composition of fungal and bacterial communities of bark were the bark age and the plant species, with minor effects attributed to plant cultivar. Such complex microbial life is probably harboured in lenticels and surface cracks that may retain more water and nutrients, favouring microbial growth and proliferation (Buck *et al.*, 1998). The presence of several cracks on the surface of old barks may cause a higher retention of water and nutrients, favouring microbial growth, while the smooth surface of young bark may offer less protection from UV radiation and wash-off. The plant species influenced the global structure of fungal and bacterial communities as well as the relative abundance of some fungal and bacterial genera. However, the bark of the closely related pear and apple plants studied in this work shared the majority of fungal and bacterial taxa, as previously reported for other genetically related plant species (Kim *et al.*, 2012). Several genera with potential pathogenic and beneficial properties were found on apple and pear barks including some potential pathogens for pear and apples like *Alternaria, Diplodia, Rosellinia, Penicillium, Phoma* and *Pseudomonas* and for other plant species. These findings demonstrate that bark represents a microbial overwintering site and a reservoir of plant pathogens that can probably migrate on other plant organs or on other plants, initiating the pathogenic process.

The second part of this doctoral work (Chapter III) used the methodology optimised during the first part to assess the impact of disease management (integrated and low-input strategies) and environmental conditions (two orchards and two consecutive seasons) on the composition and dynamics bark microbial communities of a scab-resistant apple variety (cv Fujion). Young and old bark was studied in two experimental orchards where integrated and low-input disease management strategies were applied in two separated field plots. The role of bark age as a major driver of microbial diversity, richness and composition was confirmed. In agreement with the findings of the Chapter II, several potential plant pathogens (fungal genera: *Alternaria, Botryosphaeria, Cadophora, Diaporthe, Diplodia, Phoma, Taphrina* and bacterial genera: *Curtobacterium, Erwinia, Pantoea, Pseudomonas, Ralstonia, Rathayibacter*) and beneficial microorganisms (fungal genera: *Aureobasidium, Cryptococcus, Rhodotorula, Sporobolomyces* and bacterial genera: *Burkholderia, Methylobacterium, Pseudomonas, Sphingomonas*) were found on apple

bark, confirming its role as reservoir of plant pathogens and beneficial microorganisms. The other major driver of microbial richness and diversity was the orchard location that also influenced the abundances of dominant sOTUs. The two experimental orchards were located in a flat (province of Monza-Brianza, Italy) and in a mountainous region (province of Trento, Italy) and the different environmental conditions could influence the structure and composition to microbial communities, in agreement with previous studies on grapevine (Bokulich *et al.*, 2014; Pinto *et al.*, 2014). Previous studies demonstrated the influence of the location in the composition of microbial communities in urban and peri-urban apple orchards (Shen *et al.*, 2018) and in different grapevine (Bokulich *et al.*, 2014; Pinto *et al.*, 2015) and blackcurrant growing regions (Vepštaitė-Monstavičė *et al.*, 2018). In addition, the influence of the sampling time and disease management strategy on the relative abundance of fungal and bacterial sOTUs was investigated. The relative abundance of several fungal and bacterial sOTUs significantly differed according to the time point, indicating that specific microorganisms are subjected to seasonal fluctuations. Similar fluctuations were observed in other environments (Bell *et al.*, 2009; Gilbert *et al.*, 2009; Bowers *et al.*, 2012; Copeland *et al.*, 2015) and they can be attributed to the variations in rainfalls and climatic conditions that influence microbial growth (Bell *et al.*, 2009; Bowers *et al.*, 2012). The disease management strategy influenced the composition of fungal and bacterial communities of the old and young barks and it affected the relative abundances of some taxa with potential plant pathogenic and biocontrol functions. In particular, the low-input disease management increased the relative abundances of fungal and bacterial genera with potential biocontrol and plant growth promotion activities (e.g. *Cryptococcus, Rhodotorula, Methylobacterium* and *Nocardioides*), suggesting that fungicides applied on integrated strategy may have an impact on non-target organisms (Abdelfattah *et al.*, 2016). At the same time, the low-input strategy increased the abundance of some potential apple and other plant pathogens such as *Alternaria, Leptosphaeria, Curtobacterium* and *Rathayibacter*. These findings suggest that the disease management strategies modify the equilibrium between pathogens and beneficial microorganisms residing on bark, possibly leading to the outbreak of secondary diseases under low-input protection strategies. On the other hand, the potential threat represented by an increase of phytopathogens could be balanced by the increase of beneficial microorganisms observed in this work and in other studies (Glenn *et al.*, 2015; McGarvey *et al.*, 2015; Štornik *et al.*, 2016; da Costa Stuart *et al.*, 2018). The influence of disease management strategies on bark microbial communities

depends on the organ age, suggesting that the organ structure could partially protect the microbial community from external perturbations.

The first two works (Chapter II and III) demonstrated the presence of several plant pathogens on bark, among which some canker agents were detected, such as *Botryosphaeria, Diaporthe* and *Diplodia.* The trunk canker agent *D. seriata* represents an emerging threat in apple production when the application of fungicides is limited (Trapman *et al.*, 2008) and it has been isolated from more than 34 hosts worldwide (Phillips *et al.*, 2007). The aim of the third part of this doctoral work (Chapter IV) was to evaluate the impact of commercial fungicides against this widespread emerging pathogen. A fast and reproducible method for the growth, inoculation and disease assessment of *D. seriata* was optimised on scab-resistant apple plants under greenhouse conditions. Three commercial fungicides commonly used against apple scab (fluazinam, dithianon and captan) inhibited the growth of mycelium and conidia *in vitro*. Due to its frequent applications in the integrated management of apple orchards (Blommers, 1994) dithianon was selected for the fungicide efficacy tests on apple plants and it did not reduce canker symptoms and *D. seriata* DNA amount in plant tissues under greenhouse conditions. Deposition, adhesion, penetration in host tissues and the mode of application can influence the fungicide efficacy, possibly explaining the discrepancies between *in vitro* and *in vivo* assays (Brown-Rytlewski and McManus, 2000; Zabkiewicz, 2007; Mondello *et al.*, 2017). Thus, additional tests with other fungicides and secondary pathogens are required to provide a more complete overview of fungicide side effects against poorly-studied diseases, in order to precisely estimate risk of a low-input disease management strategy on the emergence of secondary diseases.

# Chapter VI. Conclusions and future perspectives

During the present doctoral work, the influence of a low-input disease management on the composition and dynamics of plant-associated microbiota was investigated by a metabarcoding analysis of bark-associated fungal and bacterial communities and the characterization of a canker agent. Bark age, orchard location, plant species, plant cultivar and disease management strategies significantly affected the structure and dynamics of bark associated fungi and bacteria. In particular, the low-input disease management strategies influenced the abundances of plant pathogens on the scab-resistant apple cultivar studied in this work, influencing their equilibrium with the beneficial microorganisms and possibly leading to the outbreak of secondary pathogens. The impact of commercial fungicides was assessed against the bark pathogen *D. seriata in vitro* and under greenhouse controlled conditions, demonstrating its inhibition by three fungicides *in vitro*, but not by an anti-scab fungicide under greenhouse conditions.

This study demonstrated the potential of use of the metabarcoding approach as a tool to monitor the variation of the composition and dynamics of plant microbiota. In particular, this approach allows to precisely study the effect of environmental, host and agronomical changes on the composition of plant-associated pathogenic, beneficial and neutral microorganisms. This approach can be further used to investigate the influence of innovative farming practices on the composition of bark microbiota and to monitor the dynamics of pathogens and beneficial microorganisms. Moreover, this approach could also be applied, with appropriate modifications in the PCR amplification approach, to investigate the composition and dynamics of plant-associated algae and protozoa, in order to give a more complete understanding of the plant holobiont. Likewise, the methodology optimised for the disease assessment of *D. seriata* can be further used for the assessment of canker symptoms under field conditions and to investigate the susceptibility of other apple cultivars to the pathogen.

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# List of publications and training activities

Peer reviewed publications with impact factor

- Arrigoni E, Antonielli L, Pindo M, Pertot I and Perazzolli M, 2018. Tissue age and plant genotype affect the microbiota of apple and pear bark. Microbiological Research; 211:57-68.

- Arrigoni E, Albanese D, Oliveira Longa C M, Angeli D, Donati C, Ioriatti C, Pertot I and Perazzolli M (2018). Environmental conditions and disease management strategies influence the composition of fungal and bacterial communities associated to apple barks. In preparation for ISME Journal.

- Arrigoni E, Oliveira Longa C M, Angeli D, Soini M, Pertot I and Perazzolli M, 2018. A fast and reliable method for *Diplodia seriata* inoculation of trunks and fungicide efficacy assessment on potted apple plants under greenhouse conditions. Phytopathologia Mediterranea, Accepted.

Peer reviewed publications without impact factor

- Arrigoni E, Antonielli L, Pindo M, Pertot I and Perazzolli M, 2018. The composition of apple and pear bark microbiota suggest microbial migrations from soil. IOBC-WPRS Bulletin; 135:149-151

- Arrigoni E, Antonielli L, Pindo M, Pertot I and Perazzolli M, 2017. Unravelling the composition of apple and pear bark microbiota through a metabarcoding approach. IOBC-WPRS Bulletin, Accepted.

Oral communications and potsers

- Arrigoni E, Antonielli L, Pindo M, Pertot I and Perazzolli M Unravelling the composition of apple and pear bark microbiota through a metabarcoding approach. Oral presentation at the 11$^{th}$ International IOBC-WPRS Workshop. 26-30 June 2017 Jurmala, Latvia

- Arrigoni E, Antonielli L, Pindo M, Pertot I and Perazzolli M. The composition of apple and pear bark microbiota suggests microbial migrations from soil. Poster presentation and flash talk and winner of the best poster award at the Future IPM 3.0. 15-20 October 2017 Riva del Garda (TN), Italy

- Arrigoni E, Angeli D, Oliveira Longa C M, Antonielli L, Pindo M, Pertot I and Perazzolli M. Insights into apple and pear bark microbiota: characterizing

composition and population dynamics of beneficial and pathogenic microorganisms. Poster presentation at the 17th International Symposium on Microbial Ecology (ISME17). 12-17 August 2018 Leipzig, Germany

- Arrigoni E. Unravelling dynamics and composition of apple and pear bark microbiota under different chemical inputs by metabarcoding approaches. Oral presentation for the annual workshop of the PhD School in Agricultural Sciences and Biotechnology of the University of Udine – 22 September 2016 Udine, Italy

- Arrigoni E. Unravelling dynamics and composition of apple and pear bark microbiota under different chemical inputs by metabarcoding approaches. Oral presentation for the annual workshop of the PhD School in Agricultural Sciences and Biotechnology of the University of Udine – 24 May 2017 Udine, Italy

- Arrigoni E, Pertot I, and Perazzolli M. Unravelling dynamics and composition of apple bark microbiota under different plant protection strategies. Poster presentation at the Ph.D. Expo at the University of Udine - Udine 24 May 2018

- Arrigoni E. Biotecnologie Vegetali e Microbiche: cosa farò da grande. Oral presentation (in Italian) of personal career to bachelor and master students during the open days at the Department of Agriculture, Food and Environment of the University of Pisa. 19 April 2018 Pisa, Italy

Courses and summer schools

- Summer School "Data analysis in applied biology" organised by the PhD School in Agricultural Sciences and Biotechnology of the University of Udine. 6-10 September 2017 Paluzza (UD), Italy

- Summer School “System Biology” organised by the PhD School in Agricultural Sciences and Biotechnology of the University of Udine. 3-7 September 2018 Udine, Italy

- Course “Understanding statistic tests: from experimental design to data analysis” organised by the Department of Sustainable Agroecosystems and Bioresources of the Edmund Mach Foundation. November 2017 San Michele all’Adige (TN), Italy

- Course "Molecular phylogenetics and evolution" organised by the Department of Sustainable Agroecosystems and Bioresources of the Edmund Mach Foundation. December 2017 San Michele all’Adige (TN), Italy

- Workshop **“**IPM for a sustainable agriculture: future trends**”** organised by Edmund Mach Foundation, Laimburg Research Centre and Free University of Bozen. 11 December 2015 Bolzano, Italy

Seminars

- **Zenoni S. "**The identification of putative master regulators of the transition from immature to mature organ phase in grapevine by an integrated network analysis**".** 5 November 2015, Edmund Mach Foundation. San Michele all'Adige (TN), Italy

- **D'Incà E. "**Functional analysis of four NAC trancription factors in grapevine by transient and stable grapevine transformation**".** 5 November 2015, Edmund Mach Foundation

- **Gramazio T. "**Zero Risk Goal". 20 June 2016, Edmund Mach Foundation. San Michele all'Adige (TN), Italy

- **Marchesini A. "**Relationships among the three levels of biodiversity - genes, species and ecosystems: an empirical study with alpine amphibians from Trentino**".** 8 March 2017, Edmund Mach Foundation. San Michele all'Adige (TN), Italy

- **Gobbi A. "**The hunt for the wine terroir". 20 November 2017, Edmund Mach Foundation. San Michele all'Adige (TN), Italy

- **Anderluh G. "**Structural basis for Nep1-like protein – plant membrane interactions". 14 February 2018, Edmund Mach Foundation. San Michele all'Adige (TN), Italy

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