Microbiological and biochemical investigations on the aetiology of apple replant disease

Author

Lidia Nicola MSc.

Dissertation submitted to the Faculty of Biology of the Leopold-Franzens-Universität Innsbruck in partial fulfilment of the requirements for the degree Doctor of Philosophy (PhD).

Innsbruck, April 2017

Advisors

Univ. Prof. Dr. Heribert Insam
Institute of Microbiology
Universität Innsbruck (Austria)

Univ. Prof. Dr. Ilaria Pertot
Center Agriculture Food Environment,
University of Trento;
Department of Sustainable Agroecosystems and Bioresources,
Edmund Mach Foundation (Italy)

Cover Design & Layout
Lidia Nicola

This work was carried out at the **Edmund Mach Foundation in San Michele all'Adige** (Italy) and the **Institut für Mikrobiologie, Leopold-Franzens-Universität Innsbruck** (Austria).
This dissertation was funded by FIRS>T (FEM International Research School Trentino).

Table of Contents

Abstract

Abstract

Soil health is a key factor for the conservation of biodiverse ecosystems and sustainable agricultural production. Unfortunately, land exploitation due to intense monoculture tends to deplete and exhaust soil resources, giving rise to cultivation problems and harvest reduction. An example is apple replant disease (ARD), one of the major problems of apple production, occurring when apple trees are subsequently planted in the same soil. Despite the efforts in research, the exact aetiology of this disease is still uncertain. The present work investigates the microbial and biochemical complexity of agricultural soils using next generation technologies and especially focusing on the clearing the causes of apple replant disease.

In Chapters II and III, the attention is focused on the microbial communities in ARD-affected soils in field and greenhouse experiments, using next generation sequencing (NGS). In the first situation, soil bacterial and fungal communities were compared in fumigated (dazomet 99%) and untreated soils in an apple orchard where fumigation relived ARD symptoms on apple trees. In Chapter III, the three different hypothesis on the onset of ARD (complex of microbial pathogens – presence of toxins released by old apple trees – nutrient imbalances in soil) were tested by the application of different treatments on ARD-affected soil planted with M9 rootstock in the greenhouse. The results show a change in the microbial balance in ARD-affected soils, with an increased presence in beneficial microorganisms in healthy soils compared to higher concentration of potential pathogens in sick soils. There was also an indication that toxins released by old apple trees might have a role in influencing negatively plant growth.

In Chapter IV, a comprehensive meta-analysis of all the available ARD soil microbial sequencing studies was performed, in order to assemble the vastest data set and analyse it with the same bioinformatics tools to individuate the main drivers in ARD-affected soils. This meta-study confirms the difference in soil microbial communities in ARD affected soils, where a complex of phytopathogenic and nematophagous microorganisms was found, but highlighted also a change in microbial associations, that could be caused by a change in soil chemistry or metabolome. Therefore, more attention should be directed to the measurement of soil parameters, since this would help classify ARD as opportunistic microbial infectious disease, which could be shaped by

a complex combination of environmental parameters affecting microbial communities, ultimately culminating in plant disease.

In Chapter V, the changes in soil phenolic profile after the addition of apple roots were analysed using targeted metabolomics, since autotoxins produced by old apple trees were among the hypothesized causes of ARD. The autotoxicity of old apple roots was also measured on apple seedlings. The addition of apple roots damaged the seedlings, and, at the same time, a high concentration of phlorizin, a phenolic compound known to be phytotoxic, was assessed. The presence of this substance, right after the explanting of apple trees, could contribute to build up the necessary conditions for the onset of ARD.

Finally, in Chapter VI, the attention moves to vineyards, another cultivation that highly exploits the soil, and where new more environmentally compatible ways of managing plants are emerging. In this work, soil microbial communities in vineyards managed with organic, biodynamic and biodynamic with green manure methods were analyzed with NGS. The green manure was the main input of soil microbial biodiversity, with a higher abundance of microorganisms involved in the nitrogen cycle and in the degradation of organic matter.

The results of this work provide insights in the microbial and biochemical complexity of agricultural soils in apple orchards and vineyards, with the ultimate scope of understanding better the multiple mechanisms that rule them, to develop a more environmentally sound management that would improve harvest without long-lasting negative consequences on the ecosystems.

Sommario

La salute del suolo è un fattore di fondamentale importanza per la conservazione di ecosistemi ricchi di biodiversità e di una produzione agricola sostenibile. Purtroppo lo sfruttamento dei terreni da parte di monocolture intensive e ripetute tende a impoverire le risorse del suolo, creando coltivazioni poco vigorose e riduzioni dei raccolti. Un esempio di questi problemi è la "Apple Replant Disease" (ARD), anche detta stanchezza del melo. Essa rappresenta uno dei maggiori problemi nella produzione di mele e avviene quando i meli vengono continuamente reimpiantati nello stesso suolo. Malgrado gli sforzi nella ricerca, l'eziologia esatta della malattia rimane ancora incerta. Questo lavoro di tesi investiga la complessità microbica e biochimica dei suoli agrari usando tecnologie "next generation" e focalizzandosi specialmente sul chiarimento delle cause della "Apple Replant Disease".

Nei Capitoli II e III l'attenzione è incentrata sulle comunità microbiche in suoli affetti da ARD in esperimenti in campo e in serra, usando il "next-generation sequencing" (NGS). Nel primo caso, le comunità batteriche e fungine del suolo sono state comparate in suoli fumigati (dazomet 99%) e non trattati in un meleto dove la fumigazione si era dimostrata efficace nel migliorare i sintomi della ARD. Nel Capitolo III, invece, le tre diverse ipotesi sul'eziologia della ARD (complesso di microorganismi patogeni – presenza di tossine rilasciate dai meli precedenti – squilibrio di nutrienti nel suolo) sono state testate applicando diversi trattamenti ad un suolo affetto da ARD in cui sono poi stati piantati in serra portainnesti M9. I risultati mostrano un cambiamento nell'equilibrio microbico nei suoli affetti da ARD, con una concentrazione maggiore di microorganismi benefici nei suoli sani in confronto ad un'aumentata presenza di potenziali patogeni nei suoli malati. I risultati suggeriscono inoltre che le tossine rilasciate dai meli precedenti potrebbero avere un ruolo nell'influenzare negativamente la crescita delle nuove piante.

Nel Capitolo IV è stata portata a termine una meta-analisi di tutti gli studi che abbiano analizzato le comunità microbiche in suoli affetti da ARD con NGS. Lo scopo era di assemblare un grande set di dati sulla ARD ed analizzarlo con gli stessi strumenti bioinformatici per trovare i fattori chiave della ARD e delle comunità microbiche ad essa associate. Questa meta-analisi conferma la

differenza in composizione nelle comunità microbiche dei suoli affetti da ARD, dove è stato trovato un complesso di microorganismi fitopatogeni e nematofagi. Inoltre, è stato trovato un cambiamento nelle associazioni microbiche, che potrebbe essere causato da una modificazione nel profilo chimico o metabolico del suolo. Di conseguenza, si dovrebbe prestare più attenzione alla misurazione dei parametri del suolo, per capire quale complessa combinazione di parametri ambientali possa provocare cambiamenti nelle comunità microbiche che, a loro volta, possono portare la pianta a sviluppare la ARD, classificando così quest'ultima come malattia infettiva opportunistica.

Nel Capitolo V sono stati analizzati i cambiamenti nel profilo fenolico del suolo dopo l'aggiunta di radici di melo, usando la metabolomica "targeted", per investigare l'ipotesi che le autotossine prodotte dai vecchi meli possano essere tra le cause della ARD. L'autotossicità delle radici dei meli precedenti è stata inoltre misurata sui semenzali di melo. L'aggiunta delle radici ha danneggiato la salute dei semenzali e, allo stesso tempo, è stata anche rilevata un'alta concentrazione di florizina, un composto fenolico conosciuto per essere fitotossico. La presenza di questa sostanza appena dopo l'espianto dei vecchi meli potrebbe contribuire a creare le condizioni necessarie per lo sviluppo della ARD.

Infine, nel Capitolo VI, l'attenzione si sposta sul vigneto, un'altra coltivazione che sfrutta pesantemente il suolo e dove stanno emergendo nuovi modi di gestire le piante più compatibili dal punto di vista ambientale. In questo lavoro, sono state analizzate con NGS le comunità microbiche del suolo in un vigneto gestito con metodo biologico, biodinamico e biodinamico con sovescio. Il sovescio si è rivelato essere il principale input di biodiversità microbica, con una grande abbondanza di microorganismi coinvolti nel ciclo dell'azoto e nella degradazione della materia organica.

I risultati di questo lavoro di tesi forniscono una visione di insieme della complessità microbica e biochimica di suoli agrari in meleti e vigneti, con l'ultimo fine di capire meglio i numerosi meccanismi che li governano per sviluppare una gestione più compatibile con l'ambiente che possa migliorare il raccolto senza conseguenze negative durature sugli ecosistemi.

I. Introduction

Apple replant disease – definition and symptoms

Apple is often grown as a highly specialized monoculture and farmers tend to replant apple trees continually in the same soil for different reasons. Firstly, some environments are particularly suitable for this crop, but not appropriate for other species. Moreover, apple cultivation can be quite profitable, especially in the regions where fruits reach high quality standards. Ultimately, farmers commonly invest money in buying expensive equipment specifically fitting only apple cultivation. These reasons result in management intensification in areas specialized in apple production. In addition, when hail nets are installed in the orchard, farmers replant apple trees on the same row of the previous plantation.

Monoculture can induce negative plant-soil feedbacks (Vukicevich et al. 2016), since low biodiversity can weaken the ecosystem by reducing resilience to disturbances. Specifically, apple replant disease (ARD) or soil sickness is defined as the syndrome occurring when apple plants, repeatedly planted in the same soil, experience stunted growth and decrease in production. Not only do apple trees suffer from replant disease, but also some annual crops (e.g. tomato, wheat, maize, legumes, rice) and other perennial plants like peach and grapevine (Chou 1999; Singh et al. 1999). In grapevine this problem is not as severe as in apple and does not entail substantial production losses. Moreover, in areas renowned for fine wine, a reduced production is actually sought, since it results in a higher quality product.

Apples account more than 40 million tons worldwide (FAO 2014) and are the main fruit crop in Europe, with apple orchards covering 450,000 ha (Eurostat 2016). One of the major apple growing regions, Trentino-Alto Adige, covers almost 70% of the Italian production (Istituto Nazionale di Statistica 2011). In this context, the problem of ARD acquires great importance, since it was estimated that this disease could decrease profitability by 50% throughout the lifespan of the orchard Moreover, the problem can persist even for 20 to 30 years (Klaus 1939) and it cannot be avoided by having a long interval before returning to the same crop.

The most susceptible physiological state of the plant to ARD is shortly after planting (1-3 months) and the main symptom consists in a generally non-lethal growth reduction of above ground and underground parts of apple trees (Hoestra 1968). Additional symptoms can be uneven growth, shortened

internodes, discolored roots, root tip necrosis and general reduction in root biomass, especially with a severe reduction in root hairs. If death of young trees does not occur within the first year, a delay in initial fruit production and reduction in fruit yield and quality are the aspects of ARD with the greatest commercial impact (Mazzola and Manici 2012) The symptomatology usually manifests itself quite evenly on the entire orchard, so the farmer often perceives it as a general decrease or delay in production of the newly replanted orchard when compared to the previous one, not as an acute disease, even if the production losses can be consistent along the years. Hence, it is frequently difficult to have a clear picture of the ARD-affected orchards in an area.

Hypotheses on the aetiology of ARD

Different hypotheses for the causal agents of ARD were proposed starting from the second half of the XX century. The most accredited hypothesis is a biotic cause of the disease, specifically regarding the involvement of soil microorganisms. The first studies focused on finding a complex of microbial pathogens responsible for the onset of ARD. The genera *Cylindrocarpon*, *Rhizoctonia* and the oomycetes *Phytophthora* and *Pythium*, known to be soil-borne apple pathogens, were often found in ARD-affected soils, using classical microbiological investigations (Mazzola 1998; Tewoldemedhin et al. 2011). Moreover, the presence of *Ilyonectria* and *Mortierella*, two other fungal pathogens, was correlated with reduced growth of apple trees (Manici et al. 2013; Mazzola and Manici 2012). However, these fungi failed to be detected regularly in ARD-affected soils. When next-generation sequencing (NGS) became available, this technique was used to get a broad panoramic view on the composition of the entire microbial communities in ARD-affected soils. This technique allowed the study of microbial communities in diseased soils, where no specific pathogen was detected, while a decreased presence of potentially beneficial bacteria (e.g. *Streptomyces* sp., *Bacillus* sp., *Paenibacillus* sp.) when compared to healthy soils was noted (Yim et al. 2015).

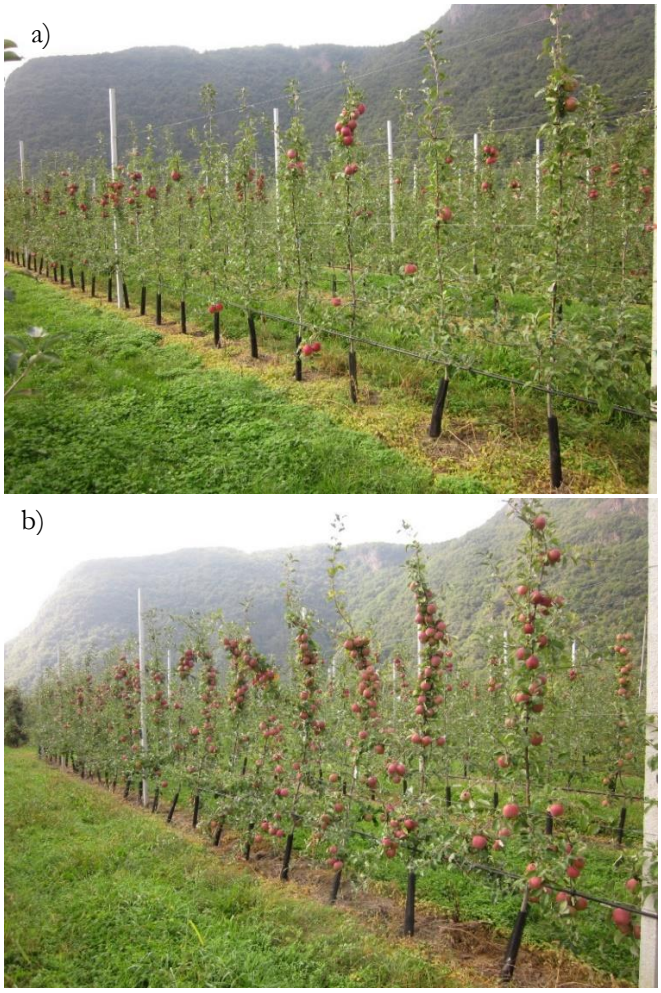


Figure 1. Difference in growth and fruit production in an ARD-affected apple orchard in Trentino-Alto Adige region where some rows (a) were left untreated and others (b) were subjected to pre-plant with dazomet (99%, Basamid® Granulat, Certis Europe, The Netherlands; at a dose rate of 70 mg active substance m^{-2}). The photograph was taken at the end of the second growing season.

The hypothesis of soil microorganisms as causal agents of ARD is further corroborated by the fact that pre-plant fumigation with broad spectrum biocides often manage to reduce symptoms in ARD-affected orchards at the moment of replanting. Fumigation consists in the application of a volatile chemical compound to the soil, which is then covered with plastic film to

favor gas diffusion into it and avoid its dispersion in the environment. The treatment kills most soil-borne pests and pathogens (Eo and Park 2014). However, these products present several drawbacks, including difficulty in application, high cost and potential hazards to human health. For example methyl bromide, one of the most used gas fumigants against soil-borne organisms, became a restricted use pesticide (RUP) for its high acute toxicity to humans and the environment. Its use has been forbidden from 18 March 2009 in the European Union (EU Regulation 2008/753/EC).

Another hypothesis on the biotic origin of ARD is the involvement of root-lesion nematodes. Nematodes belonging to the genus *Pratylenchus*, especially *P. penetrans*, are known to attack various temperate fruit trees and were sometimes detected in ARD-affected soils (Colbran 1979; Mai 1960). However, since the nematodes were not consistently found in orchards affected by replant disease, their role in ARD syndrome seems to be more an aggravating factor in already diseased orchards than the actual causative agent of the disease.

Another hypothesis on ARD is an autotoxic origin of the disease, where old apple trees or their remnants in soil could produce substances that would be toxic to the new, young apple tree, following the principle of allelopathy. Börner (1959) discovered that apple tree roots excrete different phenolic compounds in liquid cultures. Some of these compounds were also found in ARD-affected soils in apple orchards after the explanting of the old trees (Yin et al. 2016). However, root exudation of these substances is quite low during the lifespan of apple plants (Hofmann et al. 2009). Politycka and Adamska (2003) found that remainders of apple roots and leaves from the previous orchard released in soil high amounts of phenolic compounds, which persisted if the soil was incubated at low temperatures.

One further hypothesis on the causes of ARD is nutrient imbalances in soil, due to the strong exploitation by apple monoculture (Forge et. al 2016; von Glisczynski et al 2016). Some attributed it to a decrease in phosphorous availability (Slykhuis and Li 1985), others to a general lack of both nutrients and organic matter (Zhang et al. 2012). Generally, different types of compost were used in numerous trials in orchards affected by ARD to increase soil organic matter and contemporaneously suppress microbial pathogens, but the results were contrasting. Positive growth results were obtained coupling

compost with deep ripping (Braun et al. 2010), or by applying fermented fluid and solid compost (Zhang et al. 2012). More than an immediate positive effect, compost seems to be efficient for maintaining control on replant disease over the years, in orchards that have been highly exploited (Manici et al. 2003). On the other hand, sometimes compost does not have any positive effect, like Yao et al. (2006) demonstrated in his trial on ARD-affected soils.

Use of green manure to improve soil microbial quality and health

Amendment of organic substance is of vital importance for soil quality and health. Specifically, the use of organic fertilizers is increasing, since mineral fertilizers, in the long run, were proven to change the microbial and mycorrhizal colonization of roots (Kleikamp and Joergensen 2006; Schloter et al. 2003) and to reduce the amount of roots (Henry et al. 2005). Moreover, mineral fertilizers contribute heavily to water pollution, since N and P present in these products can leach and impair the water quality in lake and rivers (Potter et al. 2004). On the other hand, organic fertilizers, such as compost, farmyard manure or green manure are recommended, since the release of nutrients in soil is slower and often better synchronized with plant needs. For these reasons, organic fertilizers are the only ones allowed in organic agriculture. Many studies showed that the application of organic amendments increases total soil enzyme activity (Moeskops et al. 2010), improving soil quality and general plant health and nutrition (Baldi et al. 2014; Baldi et al. 2010; Diacono and Montemurro 2010). Moreover, organic fertilizers can also introduce or favor beneficial microorganisms that, either directly or indirectly, can compete or control plant pathogens (Hadar and Papadopoulou 2012) through a property called plant disease suppressiveness (de Bertoldi and Goberna 2010; Noble and Coventry 2005).

Among the different organic fertilizers, green manure is receiving particular interest, especially in viticulture, since it presents several benefits. Green manure in viticulture consists in growing specific crops or plants in the inter-rows of the vineyards and then plowing them under to improve soil quality. Green manure crops can vary and usually include grass mixtures and legume plants, such as vetch, clover, barley and others. From a physical point of view, green manure reduces soil erosion and leaching (Ingels et al. 2005) and increases the soil capacity for retaining water (Reeve et al. 2005). The presence

of cover crops for green manure also provides the necessary environment for the support of predators/parasitoids that could provide pest control (Irvin et al. 2014). Green manure can induce earlier ripening and arrival to full maturity of grape and, above all, it improves the organoleptic characteristics, helping creating a more engaging taste (Rotaru et al. 2011). In soil, green manure increases organic matter available for soil microorganisms and the activity of numerous soil enzymes (Okur et al. 2016). Specifically, it provides large quantities of available nitrogen, because of the low C:N ratio of the legume biomass (Bair et al. 2008). However, little is known on the possible modifications that green manure could induce in soil microbial communities.

PhD thesis objectives and structure

The main objective of the present dissertation was to investigate the microbial and biochemical complexity of agricultural soils using next generation technologies and especially focusing on two important cultivations: apple orchards, with the problem of replant disease, and vineyards, with the effects of green manure in environmentally sustainable managements.

For this purpose, the following specific objectives were addressed in chapters II to VI of this work, in the research fields of agriculture, microbiology and biochemistry:

> 2) To compare soil microbial communities in a fumigated and non-fumigated ARD-affected soil, in an orchard where fumigation alleviated ARD symptoms in order to assess if specific microorganisms could be associated with ARD (**Chapter II**).
>
> 1) To test different hypotheses on the aetiology of ARD using a greenhouse trial where ARD-affected soil underwent different treatments, and to study the effects on apple rootstocks and on the soil microbial community structure (**Chapter III**).
>
> 3) To detect which microorganisms, community mechanisms and environmental parameters can be involved in the onset of ARD by analysing data from all available deep-sequencing studies on microbial communities in ARD-affected soils (**Chapter IV**).

4) To identify and quantify the phenolic compounds released during the decay of apple roots in healthy soil, and also investigating their autotoxicological effect on apple seedlings (**Chapter V**).

5) To investigate the effects of green manure on soil microbial community structure and diversity through NGS techniques in vineyards (organic, biodynamic with or without green manure) (**Chapter VI**).

This thesis is composed of two published scientific articles, two submitted manuscripts and an advanced stage manuscript. The papers are listed below.

Nicola, L., Vrhovsek, U., Soini, E., Insam, H., and Pertot, I. 2016. Phlorizin released by apple root debris is related to apple replant disease. Phytopathologia Mediterranea 55:432-442.

Nicola, L., Turco, E., Albanese, D., Donati, C., Thalheimer, M., Pindo, M., Insam, H., Cavalieri, D., and Pertot., I. 2017. Fumigation with dazomet modifies soil microbiota in apple orchards affected by replant disease. Applied Soil Ecology 113:71-79.

Nicola, L., Insam, H., Pertot, I., Stres, B. 2017. Meta-analysis of microbiomes in soils affected by Apple Replant Disease. Submitted to Plant and Soil

Nicola, L., Turco, E., Thalheimer, M., Pindo, M., Insam, H., Pertot, I. 2017. Apple replant disease may not be caused solely by soil microorganisms. Submitted to Plant Disease.

Longa, C. M. O., Nicola, L., Antonielli, L., Mescalchin, E., Zanzotti, R., Turco, E., Pertot, I. Soil microbiota respond to green manure in vineyards managed using environmentally friendly methods. To be submitted to Journal of Applied Microbiology.

References

Bair, K. E., Davenport, J. R., and Stevens, R. G. 2008. Release of available nitrogen after incorporation of a legume cover crop in concord grape. Hortscience 43:875-880.

Baldi, E., Marcolini, G., Quartieri, M., Sorrenti, G., and Toselli, M. 2014. Effect of organic fertilization on nutrient concentration and accumulation in nectarine (Prunus persica var. nucipersica) trees: The effect of rate of application. Sci. Hort. 179:174-179.

Baldi, E., Toselli, M., Marcolini, G., Quartieri, M., Cirillo, E., Innocenti, A., and Marangoni, B. 2010. Compost can successfully replace mineral fertilizers in the nutrient management of commercial peach orchard. Soil Use Manage. 26:346-353.

Börner, H. 1959. The apple replant problem. I. The excretion of phlorizin from apple root residues. Contrib. the Boyce Thompson Inst. 20:39-56.

Braun, P. G., Fuller, K. D., McRae, K., and Fillmore, S. A. E. 2010. Response of 'Honeycrisp (R)' Apple Trees to Combinations of Pre-plant Fumigation, Deep Ripping, and Hog Manure Compost Incorporation in a Soil with Replant Disease. Hortscience 45:1702-1707.

Chou, C. H. 1999. Roles of allelopathy in plant biodiversity and sustainable agriculture. Crit. Rev. Plant Sci. 18:609-636.

Colbran, R. C. 1979. Problems in tree replacement, III. The effects of cultural practices and soil fumigation on root-lesion nematodes and on the growth of apple replants. Aust. J. Agr. Res. 30:113-1123.

de Bertoldi, M., and Goberna, M. 2010. Production and Utilization of Suppressive Compost: Environmental, Food and Health Benefits. in: Microbes at Work. Insam, H., Franke-Whittle, I., Goberna, M. eds. Springer Berlin Heidelberg, pp 153-170.

Diacono, M., and Montemurro, F. 2010. Long-term effects of organic amendments on soil fertility. A review. Agron. Sustainable Dev. 30:401-422.

Eo, J., and Park, K.-C. 2014. Effects of dazomet on soil organisms and recolonisation of fumigated soil. Pedobiologia 57:147-154.

EU Regulation 2008/753/EC. Commission Decision of 18 September 2008 concerning the non-inclusion of methyl bromide in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing that substance (notified under document number C(2008) 5076).

Eurostat. 2016. Agriculture, forestry and fishery statistics. 2016 edition. Luxembourg: Publications Office of the European Union.

FAO. 2014. http://faostat3.fao.org/. (Accessed 8 March 2017)

Forge, T., Neilsen, G., Neilsen, D. 2016. Organically acceptable practices to improve replant succes of temperate tree-fruit crops. Sci. Hort. 200: 205-214.

Hadar, Y., and Papadopoulou, K. K. 2012. Suppressive Composts: Microbial Ecology Links Between Abiotic Environments and Healthy Plants. Pages 133-153 in: Annu.Rev. Phytopathol., Vol 50, vol. 50. N. K. VanAlfen, J. E. Leach and S. Lindow, eds.

Henry, F., Nguyen, C., Paterson, E., Sim, A., and Robin, C. 2005. How does nitrogen availability alter rhizodeposition in Lolium multiflorum Lam. during vegetative growth? Plant Soil 269:181-191.

Hoestra, H. 1968. Replant Disease of apple in the Netherlands. Mededelingen Landbouwhogeschool Wageningen The Netherlands.

Hofmann, A., Wittenmayer, L., Arnold, G., Schicber, A., and Merbach, W. 2009. Root exudation of phloridzin by apple seedlings (Malus x domestica Borkh.) with symptoms of apple replant disease. J. Appl. Bot. Food Qual. 82:193-198.

Ingels, C. A., Scow, K. M., Whisson, D. A., and Drenovsky, R. E. 2005. Effects of cover crops on grapevines, yield, juice composition, soil microbial ecology, and gopher activity. Am. J. Enol. Viticult. 56:19-29.

Irvin, N. A., Pinckard, T. R., Perring, T. M., and Hoddle, M. S. 2014. Evaluating the potential of buckwheat and cahaba vetch as nectar producing cover crops for enhancing biological control of Homalodisca vitripennis in California vineyards. Biol. Control 76:10-18.

Istituto Nazionale di Statistica 2011. http://dati.istat.it/Index.aspx?DataSetCode=DCSP_COLTIVAZ&Lang=it (Accessed 8 March 2017).

Klaus, H. 1939. Das Problem der Bodenmüdigkeit unter Berücksichtigung des Obstbaus. Landwirtschaftliches Jahrbuch 89:413-445.

Kleikamp, B., and Joergensen, R. G. 2006. Evaluation of arbuscular mycorrhiza with symbiotic and nonsymbiotic pea isolines at three sites in the Alentejo, Portugal. J. Plant Nutri. Soil Sc. 169:661-669.

Mai, W. F. 1960. Growth of apple seedlings in relation to soil temperature and inoculation with *Pratylenchus penetrans*. Phytopathol. Notes 50:237-238.

Manici, L. M., Ciavatta, C., Kelderer, M., and Erschbaumer, G. 2003. Replant problems in South Tyrol: role of fungal pathogens and microbial population in conventional and organic apple orchards. Plant Soil 256:315-324.

Manici, L. M., Kelderer, M., Franke-Whittle, I. H., Ruehmer, T., Baab, G., Nicoletti, F., Caputo, F., Topp, A., Insam, H., and Naef, A. 2013. Relationship between root-endophytic microbial communities and replant disease in specialized apple growing areas in Europe. Appl. Soil Ecol. 72:207-214.

Mazzola, M. 1998. Elucidation of the microbial complex having a causal role in the development of apple replant disease in Washington. Phytopathology 88:930-938.

Mazzola, M., and Manici, L. M. 2012. Apple Replant Disease: Role of Microbial Ecology in Cause and Control. Pages 45-65 in: Annu. Rev. Phytopathol., Vol 50, vol. 50. N. K. VanAlfen, J. E. Leach and S. Lindow, eds.

Moeskops, B., Sukristiyonubowo, Buchan, D., Sleutel, S., Herawaty, L., Husen, E., Saraswati, R., Setyorini, D., and De Neve, S. 2010. Soil microbial communities and activities under intensive organic and conventional vegetable farming in West Java, Indonesia. Appl. Soil Ecol. 45:112-120.

Noble, R., and Coventry, E. 2005. Suppression of soil-borne plant diseases with composts: A review. Biocontrol Sci. Techn. 15:3-20.

Okur, N., Kayikcioglu, H. H., Ates, F., and Yagmur, B. 2016. A comparison of soil quality and yield parameters under organic and conventional vineyard systems in Mediterranean conditions (West Turkey). Biol. Agric. Hortic. 32:73-84.

Politycka, B., and Adamska, D. 2003. Release of phenolic compounds from apple residues decomposing in soil and the influence of temperature on their degradation. Pol. J. Environ. Stud. 12:95-98.

Potter, K. M., Cubbage, F. W., Blank, G. B., and Schaberg, R. H. 2004. A watershed-scale model for predicting nonpoint pollution risk in North Carolina. Environ. Manage. 34:62-74.

Reeve, J. R., Carpenter-Boggs, L., Reganold, J. P., York, A. L., McGourty, G., and McCloskey, L. P. 2005. Soil and winegrape quality in biodynamically and organically managed vineyards. Am. J. Enol. Viticult. 56:367-376.

Rotaru, L., Stoleru, V., and Mustea, M. 2011. Fertilization with green manure on Chasselas Dore grape vine as an alternative for sustainable viticulture. J. Food Agric. Environ. 9:236-243.

Schloter, M., Bach, H. J., Metz, S., Sehy, U., and Munch, J. C. 2003. Influence of precision farming on the microbial community structure and functions in nitrogen turnover. Agr. Ecosyst. Environ. 98:295-304.

Singh, H. P., Batish, D. R., and Kohli, R. K. 1999. Autotoxicity: Concept, organisms, and ecological significance. Crit. Rev. Plant Sci. 18:757-772.

Slykhuis, J. T., and Li, T. S. C. 1985. Responses of apple seedlings to biocides and phosphate fertilizers in orchard soils in British-Columbia. Can. J. Plant Pathol. 7:294-301.

Tewoldemedhin, Y. T., Mazzola, M., Botha, W. J., Spies, C. F. J., and McLeod, A. 2011. Characterization of fungi (Fusarium and Rhizoctonia) and oomycetes (Phytophthora and Pythium) associated with apple orchards in South Africa. Eur. J. Plant Pathol. 130:215-229.

von Glisczynski, F., Sandhage-Hofmann, A., Amelung, W, Pude, R. 2016. Biochar-compost substrates do not promote growth and fruit quality of a replanted german apple orchard with fertile haplic luvisol soils. Sci. Hort. 213: 110-114

Vukicevich, E., Lowery, T., Bowen, P., Urbez-Torres, J. R., and Hart, M. 2016. Cover crops to increase soil microbial diversity and mitigate decline in perennial agriculture. A review. Agron. Sustainable Dev. 36.

Yao, S. R., Merwin, I. A., Abawi, G. S., and Thies, J. E. 2006. Soil fumigation and compost amendment alter soil microbial community composition but do not improve tree growth or yield in an apple replant site. Soil Biol. Biochem. 38:587-599.

Yim, B., Winkelmann, T., Ding, G. C., and Smalla, K. 2015. Different bacterial communities in heat and gamma irradiation treated replant disease soils revealed by 16S rRNA gene analysis - contribution to improved aboveground apple plant growth? Front. Microbiol. 6.

Yin, C. M., Xiang, L., Wang, G. S., Wang, Y. F., Shen, X., Chen, X. S., and Mao, Z. Q. 2016. How to Plant Apple Trees to Reduce Replant Disease in Apple Orchard: A Study on the Phenolic Acid of the Replanted Apple Orchard. Plos One 11.

Zhang, Z. B., Chen, Q., Yin, C. M., Shen, X., Chen, X. S., Sun, H. B., Gao, A. N., and Mao, Z. Q. 2012. The effects of organic matter on the physiological features of Malus hupehensis seedlings and soil properties under replant conditions. Sci. Hortic. 146:52-58.

II. Paper 1

Fumigation with dazomet modifies soil microbiota in apple orchards affected by replant disease

Fumigation with dazomet modifies soil microbiota in apple orchards affected by replant disease

Lidia Nicola[a,c*], Elena Turco[a], Davide Albanese[a], Claudio Donati[a], Martin Thalheimer[b], Massimo Pindo[a], Heribert Insam[c], Duccio Cavalieri[a], Ilaria Pertot[a]

[a] Research and Innovation Centre, Fondazione Edmund Mach (FEM), 38010 San Michele all'Adige (TN), Italy
[b] Laimburg Research Centre for Agriculture and Forestry, 39040 Auer (BZ), Italy
[c] Department of Microbiology, University of Innsbruck, 6020 Innsbruck, Austria

* Corresponding author.
Email address: lidia.nicola@fmach.it (L. Nicola).

Applied Soil Ecology January 2017, Volume 113, pp 71–79

DOI: 10.1016/j.apsoil.2017.02.002

Abstract:

Apple replant disease (ARD) is a disorder that affects apple trees when they are replanted in soil where the same species was previously grown. ARD has been known for a long time, but the precise cause is not yet identified. Although ARD is most probably due to a combination of abiotic and biotic factors, the fact that soil fumigation commonly prevents the symptoms, at least temporarily, supports the hypothesis that microorganisms play an important role in it. In order to find possible relations between taxa composition of soil microbial communities and plant growth in ARD-affected orchards, we compared fumigated (dazomet 99%) and untreated soils by using next-generation sequencing (NGS) technologies. Soil sampling was carried out when the difference between fumigated and untreated plots became significant in terms of shoot growth and fruit yield and specifically at the end of the second growing season. Total soil DNA was extracted and two target regions (ITS for fungi and 16S rDNA for bacteria), were pyrosequenced with Roche's 454 Platform. Both bacterial and fungal communities differed significantly in fumigated and untreated soils of our study. Bacillus sp. (ϱ=0.64), Streptomyces sp. (ϱ=0.64), Pseudomonas sp. (ϱ=0.59), and Chaetomium sp. (ϱ=0.85) were some of the taxa positively correlated with asymptomatic apple trees. Although a cause-effect relation with ARD cannot be proven, our results confirm that, fumigation with dazomet reduces ARD symptoms, and also modifies soil microbial communities at length, in particular by increasing the presence of some beneficial microorganisms known for their action against plant pathogens.

Keywords: Apple Replant Disease, Fumigation, Pyrosequencing, Soil microbiota, 16S rDNA, ITS

Highlights:

- Fumigation with dazomet increased growth and yield in ARD-affected apple orchards;
- Fumigation induced medium/long term modification in soil microbial communities;
- A complex of pathogenic fungi was correlated with reduced plant growth
- In fumigated plots, there was an increase in plant growth promoting microorganisms.

1. Introduction

Apples are an important crop, which account for a production of more than 80 million tons worldwide (FAO, 2013). They are commonly grown as a highly specialised monoculture in regions where the climate is particularly favourable for fruit quality. In these areas, characterised by high land value, growers are unlikely to implement crop rotation, so apple orchards are commonly replanted immediately, which quite often results in reduced yield over time. This problem has been named apple replant disease (ARD) (Ross and Crowe, 1973).

The main symptoms of ARD are a general reduction in plant growth, fruit yield and quality; plants have shortened internodes, discoloured roots, root tip necrosis and a reduction in root biomass, which can lead to plant death within the first growing season (Mazzola and Manici 2012). Poor growth and production caused by ARD may decrease profitability by up to 50% throughout the lifespan of the orchard (van Schoor et al. 2009).

The causes of ARD are still unclear, despite the fact that research has been undertaken for decades. The most plausible hypothesis is that ARD is the result of the activity of soil pathogens/parasites, although other factors cannot be excluded (Mazzola and Manici 2012). Fungal species belonging to the *Cylindrocarpon*, *Rhizoctonia*, *Phytophthora* and *Pythium* genera are frequently found in ARD-affected soils, but their presence and frequency can vary from soil to soil (Tewoldemedhin et al. 2011a; Tewoldemedhin et al. 2011b). The role of prokaryotes in ARD has been little investigated and opinions on their

involvement in the disease are contrasting (Hoestra 1968; Mazzola 1998). The severity of ARD symptoms can be influenced by environmental factors, such as water stress and salinity (Redman et al. 2001), general soil fertility (Braun et al. 2010) and the presence of phytotoxic compounds (Tagliavini and Marangoni 1992), hence drawing up a complete picture of the disease aetiology is complex.

ARD has long been studied with classic soil microbiological approaches (i.e. isolation of soil microorganisms on selective agar media and subsequent identification). However, these techniques, well suited for the detection of known pathogens, are inadequate for studying the whole soil microbial community, because only a minimal part of the soil microbial community is cultivable on laboratory media (Guo et al. 2014; van Schoor et al. 2009). The high throughput sequencing technologies allow studying microbial communities in a complex ecosystem (Daniel 2005) and may help in better understanding ARD, by analysing in depth the entire bacterial and fungal community.

Soil disinfestation prior to replanting with pasteurisation or fumigation can partially or temporarily relieve ARD symptoms (Covey et al. 1979; Mai and Abawi 1981), supporting the hypothesis of a microbial role in the syndrome. With fumigation, a volatile chemical compound is applied to the soil, which is then covered with plastic film to favour gas diffusion into it and avoid the dispersion of the active substance during the treatment. The treatment kills most soil-borne pests and pathogens (Eo and Park 2014).

Dazomet (tetrahydro-3,5-dimethyl-2 H-1,3,5-thiadiazine-2-thione) is a granular fumigant that releases methyl isothiocyanate, which is often used to treat soil before apple replanting. It is effective against several pathogenic microorganisms, nematodes and weeds, and this treatment commonly results in enhanced yield in comparison to untreated soils (Otto and Winkler 1993).

The environmental risks of synthetic chemical soil fumigants are frequently debated and no exhaustive information is available on the long term impact of dazomet on soil microbiota. Some studies on the short term effects of dazomet on soil microbial communities in microcosms a few days after application (Eo and Park, 2014; Feld et al., 2015) have shown a decrease in

richness and biodiversity. However, the effects after a longer period of time (e.g. one year or more) have not yet been investigated.

The aim of this study was to compare microbial communities in fumigated and untreated ARD-affected soils in order to verify whether the presence of some groups of soil microorganisms could be associated with ARD. Soil sampling was performed in an apple-growing area in northern Italy and the differences in composition and abundance in the soil microbiome were assessed when ARD symptoms became evident (at the end of the second growing season, 19 months after replanting).

2. Materials and methods

2.1 Study site and composite soil sampling

The study site was located in northern Italy (Trentino-South Tyrol region) in the alluvial plains of the Adige River, an area of intensive apple production (Municipality of Ora, 46.0 N, 11.3 E). The soil at the site originated on recent alluvial deposits and was classified as Typic Fluvaquent, coarse silty, mixed, mesic (Soil Survey Staff, 2010). The area was selected because it is homogeneous in terms of climate and soil type and was continuously cultivated with apple trees for several decades. In this area, trees are commonly planted in rows at a distance of 3.2 m between the rows and 0.8 m long the row. Eight plots of 44 m ×16 m were identified following a randomized block design. On 3 April 2012, just after explanting the previous orchard, half of the plots were fumigated with dazomet (99%, Basamid® Granulat, Certis Europe, Utrecht, The Netherlands; at a dose rate of 70 mg active substance m^{-2}) and half of them were left untreated. The area was subsequently planted with apple trees (cv. Fuji Fubrax grafted onto M9 rootstock) on 26 April 2012. The local guidelines of integrated production were followed (Guidelines for Integrated Pome Cultivation, 2016): mineral fertilisation (ammonium nitrate, 80 kg ha^{-1}) was applied in spring, herbicides were used only along the rows (glyphosate, once every two months during growing season, 1.7 L ha^{-1}); the plant protection program followed the principle of integrated pest management.

Approximately 19 months after fumigation, on 28 October 2013, composite soil samples were collected in each plot (four replicates per treatment). Each

composite soil sample was obtained by mixing five subsamples (20 g each) randomly collected along the row (at a distance of 3 m from each other) at 25 cm from the apple tree and at a depth of 5-20 cm with a soil core sampler. A similar composite sample was also collected in each plot for the chemical analysis of the soil. The soil samples were transferred into sterile plastic containers, sieved with a 0.2 mm mesh size and stored at -80 °C until DNA extraction.

2.2 Soil chemical analysis, cultivable microorganisms enumeration and growth assessment of apple trees

Soil texture was determined manually with the feel method (VDLUFA 1991). Total carbon content of soils was measured according to ISO 10694:1995 protocol (ISO 10694 1995) using a TruSpec CHN (Leco Corporation, St. Joseph, USA); pH was determined in 0.01 mol L^{-1} calcium chloride solution according to DIN EN 15933:2012 (DIN EN 15933 2012) using a digital pH-meter (Schott, Jena, Germany). Soil carbonates were assessed with dilute HCl, assigning effervescence classes on a scale from 0 (no effervescence) to 3 (very strong effervescence). Phosphate and potassium were measured in an extract of calcium-acetate-lactate (CAL), according to ÖNORM L 1087:2012 (ÖNORM L 1087 2012) using ICP-AES (Inductively Coupled Plasma Atomic Emission Spectroscopy). Magnesium, boron, manganese, copper and zinc were determined after extraction with calcium chloride/DTPA (CAT), according to the VDLUFA guidelines (VDLUFA 1991), using ICP-AES.

The count of colony-forming units (CFUs) was carried out following the protocol of Corneo et al. (2013). The colonies of bacteria and fungi were counted after 3 and 6 days of incubation at 27 °C.

ARD severity in the orchard was assessed by measuring shoot growth (current year's shoots with a minimum length of 10 cm; 16 plants per plot) and fruit yield (total amount in kg; 16 plants per plot) (Mazzola and Brown 2010; Yim et al. 2013). Yield assessment was carried out on 22 October 2013, while shoot length was measured on 3 December 2013.

2.3 DNA extraction, PCR amplification and pyrosequencing

Total DNA was extracted from 0.5 g of each composite soil sample using the FastDNA™ SPIN Kit for Soil and the FastPrep® instrument (MP Biomedicals, Santa Ana, USA) according to the manufacturer's instructions.

The extracted DNA was dissolved in 100 µl of DES (DNase/Pyrogen-Free Water) and stored at -20 °C until PCR amplification.

To analyse the soil bacterial communities, the V1-V3 region of 16S rDNA was amplified with PCR using the specific primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (Weisburg et al. 1991) and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al. 1993). A specific forward fusion primer with the sequence combination for identification of individual samples (454 sequence adapter A, key tag, and MID tag) was attached to the 27F, while a common reverse fusion primer with the 454 sequence adapter B and the key tag were attached to the 518R. Soil fungal community was analysed by amplifying the ITS1 fragment of 18S rDNA, using the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns 1993) and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (White et al. 1990), adding the identification sequences as for 16S amplification.

The mixture for bacterial DNA amplification was made up of 1× FastStart High Fidelity Reaction Buffer with 18 mM $MgCl_2$ (Roche, Mannheim, Germany), 0.52 g l^{-1} of Bovine Serum Albumin (BSA) (New England BioLabs Inc., Ipswich, USA), 0.1 mM of dNTPs (Invitrogen, Carlsbad, USA), 0,04 U μl^{-1} of FastStart High Fidelity Enzyme Blend (Roche, Mannheim, Germany), 0.4 µM of each primer and 10 ng of DNA in a final volume of 25 µl. For fungal DNA amplification, the protocol was similar except that 0.2 mM of dNTPs, 0.05 U μl^{-1} of FastStart High Fidelity Enzyme blend, 0.4 µM of each primer, 5 ng of DNA, and no BSA were added. The amplification conditions were as follows: 95 °C for 5 min, 23 cycles at 95 °C for 30 s, 54 °C for 45 s, and 72 °C for 90 s, and a final extension at 72 °C for 10 min for bacterial DNA, and 94 °C for 4 min, 35 cycles at 94 °C for 15 s, 50 °C for 45 s, and 72 °C for 1 min and a final extension at 72 °C for 8 min for fungal DNA. Three independent PCR reactions (technical replicates) were performed for each sample and pooled together. Subsequently, all the PCR products were analysed by gel electrophoresis and cleaned using the AMPure XP beads kit (Beckman Coulter, Brea, USA), following the manufacturer's instructions. The PCR products were then quantified via quantitative PCR using the Library quantification kit Roche 454 titanium (KAPA Biosystems, Boston, USA) and pooled in equimolar proportion in a final amplicon library. The 454 pyrosequencing was carried out on the GS-FLX+ systems using the XL+ chemistry (Roche, Mannheim, Germany), following the manufacturer's instructions.

2.4 Sequence data analysis

Sequence processing was done with the MICCA pipeline (Albanese et al. 2015). The procedure consisted of removing the forward and reverse primers, and then discarding the untrimmed reads. The reads were quality trimmed by removing short sequences (<400 bp for bacteria, <200 bp for fungi) and sequences presenting a low average score (<20 for bacteria, <16 for fungi).

The OTU clustering was performed at 97%, and the UNITE database (Koljalg et al. 2013) and BLAST protocol were used for taxonomic assignment of fungi, while the RDP database and protocol for taxonomy assignment (Cole et al., 2014) were used for bacterial reads, using the "micca-otu-denovo" command of the MICCA pipeline. Multiple alignment with the Greengenes template (DeSantis et al., 2006) was performed with PyNAST for bacteria (Caporaso et al. 2010a), while fungal reads were aligned *denovo* with MUSCLE (Edgar, 2004)

2.5 Statistical analysis

Chemical data, growth parameters and diversity indices were statistically analysed with the PAST software package, version 2.17 (Hammer et al. 2001), using the t test, after checking for normality and homoscedasticity (Shapiro-Wilk test and Levene's test respectively).

Statistical analysis of the sequencing data was performed with the phyloseq R package, ver. 1.6.1 (McMurdie and Holmes 2013). To control biasing effects of sequencing depth, samples were rarefied by subsampling to 90% of the depth of the least abundant sample (15,389 sequences for 16S data, 5,656 sequences for ITS data). Alpha diversity, meaning the microbial diversity within fumigated or untreated soils, was calculated using Observed Species, Simpson, and Shannon indices. Beta diversity, intended as the difference in taxa composition in fumigated and untreated soils, was estimated with multivariate analysis of bacterial and fungal community structure. Specifically, Principal Coordinates Analysis (PCoA) (Gower and Blasius 2005) on unweighted Unifrac distances (Lozupone and Knight 2005) was used. To test whether the fumigated soil microbial community differed significantly from that in untreated soils, PERMANOVA was used, implemented in the vegan R package, ver. 2.0.10 (Oksanen et al. 2013) as the adonis function, and applied to the unweighted Unifrac dissimilarity distance.

Spearman's rank correlation (ϱ) was calculated between the abundance of fungal and bacterial taxa and shoot growth in the same plot to identify which taxa were more abundant in correspondence with greater growth. Taxa present in all samples with an abundance of over 10 in at least one sample and with $\varrho < -0.5$ or $\varrho > 0.5$ correlations were included in the result tables.

3. Results

3.1 Soil properties, soil cultivable microorganisms count and ARD severity at the end of the second growing season

The soil texture was silt-loam in all the plots and no difference in the chemical composition in fumigated and untreated plots was found (Table 1; t test, $p > 0.05$ for each chemical parameter).

Regarding soil cultivable microorganisms, the number of CFUs did not vary significantly according to the soil treatment (t test, $p > 0.05$). In the fumigated plots, we counted $4.3\ 10^6 \pm 2.7\ 10^6$ and $7.5\ 10^4 \pm 5.0\ 10^4$ CFUs g^{-1} dry soil (average $\pm$ standard deviation), for bacteria and fungi, respectively. In the untreated plots there were $4.0\ 10^6 \pm 1.6\ 10^6$ bacterial CFUs g^{-1} dry soil and $5.8\ 10^4 \pm 3.2\ 10^4$ fungal CFUs g^{-1} dry soil. Among cultivable fungi, no root pathogen of apple (*Rosellinia necatrix*, *Armillaria* spp.) was found.

At the end of the second growing season, 19 months after replanting, both shoot growth and fruit yield were lower in plants grown in untreated plots as compared to fumigated ones (t test, $p = 0.003$ and $p = 0.001$ respectively) (Fig. 1), indicating a reduction in ARD symptoms in fumigated soils.

3.2 Soil microbial community composition

Pyrosequencing yielded a total of 191,219 raw pyrotags reads for bacteria and 77,463 reads for fungi. After quality filtering and chimera removal, a total of 150,702 16S sequences and 68,133 ITS sequences remained for community analysis, corresponding to an average $\pm$ standard deviation of 23,902 $\pm$ 1,540 reads and 9,683 $\pm$ 1,478 reads per sample for bacteria and fungi respectively. A total of 4,479 bacterial OTUs and 608 fungal OTUs was detected.

Table 1. Chemical analysis of soil samples taken from fumigated and untreated plots in the selected apple orchards, performed with VDLUFA methods.

Plots	Carbon content	pH	P	K	Mg	B	Mn	Cu	Zn
	%		mg kg-1						
Untreated									
Plot 1	2.6	7.3	96	166	160	0.45	18	14	10
Plot 2	2.2	7.3	91.6	157.7	150	0.4	18	12	10
Plot 3	2.1	7.2	100.4	182.6	150	0.45	19	12	9
Plot 4	1.9	7.3	87.3	182.6	130	0.42	19	12	9
Fumigated									
Plot 1	2	7.3	78.6	207.5	140	0.38	16	10	8
Plot 2	1.8	7.3	91.6	182.6	140	0.4	20	12	10
Plot 3	2.3	7.3	91.6	190.9	140	0.45	19	10	9
Plot 4	1.9	7.3	82.9	182.6	130	0.42	20	13	9

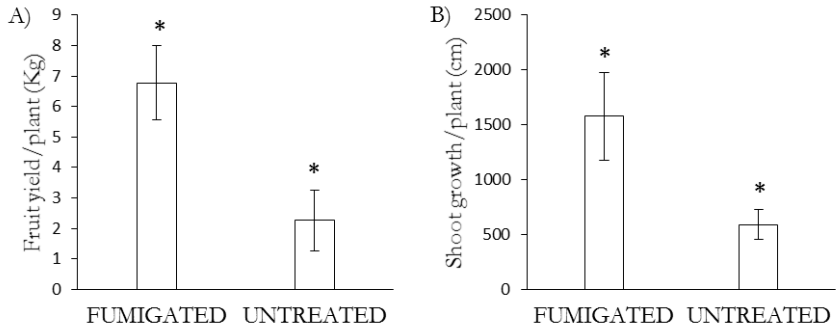


Fig. 1: Fruit yield (A) and shoot growth (B) of apple plants planted in fumigated and untreated plots, from four different sites. Soils were fumigated with dazomet (99%, dose rate of 70 mg active substance m^{-2}) before planting; data were collected 19 months later, at the end of the growing season. 16 plants were assessed for each plot. The bars indicate the standard deviation from the mean. * Significant at 0.05 probability level ($p < 0.05$).

The alpha diversity found in both bacterial and fungal communities in fumigated soils was similar to that in untreated soils. Indeed, the microbial diversity indices, measuring richness and evenness of the microbial communities, did not differ significantly in fumigated and untreated soils (Fig. 2, t test, $p > 0.05$), indicating that, 19 months after fumigation, there was no effect on microbial richness and evenness. The bacterial communities, both in fumigated and untreated soils, were dominated by Proteobacteria, Acidobacteria and Actinobacteria (overall 80% of total reads in both soils), while Ascomycota was by far the predominant fungal phylum (77% in fumigated soil, 82% in untreated soil) in the mycobiota of these soils.

3.3 Soil microbial diversity

When beta diversity was analysed using Principal Coordinate Analysis (PCoA) on unweighted Unifrac distance matrices, in fumigated soils both bacterial and fungal community composition were significantly different from the composition found in untreated soils (Fig. 3, PERMANOVA, $p < 0.05$).

Correlation coefficients between the abundance of microbial taxa and shoot growth were determined. In bactobiota, the phyla TM7, Gemmatimonadetes, Chloroflexi, Firmicutes, and Actinobacteria were positively correlated with

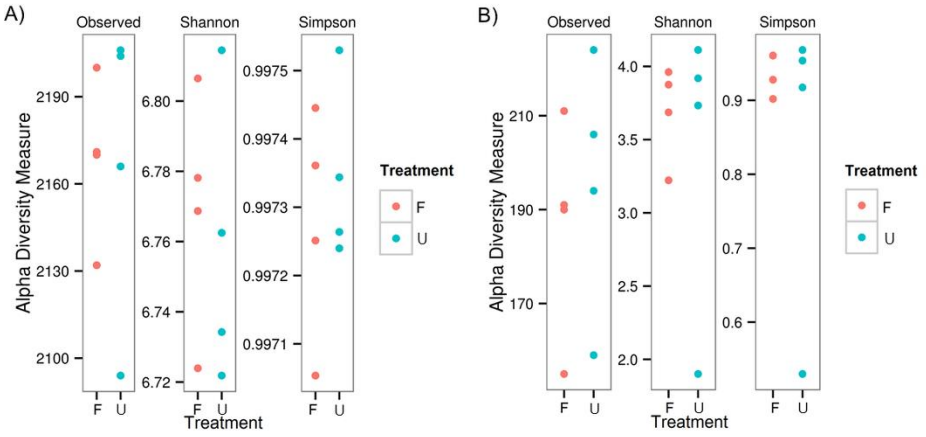


Fig. 2. Richness (Observed Species) and diversity indices (Shannon and Simpson) based on 454 sequencing data for bacteria (A) and fungal (B) communities in fumigated (F) and untreated (U) soil samples. Soils were fumigated with dazomet (99%, dose rate of 70 mg active substance m^{-2}) before planting, and samples were collected from four fumigated and four untreated sites 19 months later, at the end of the growing season.

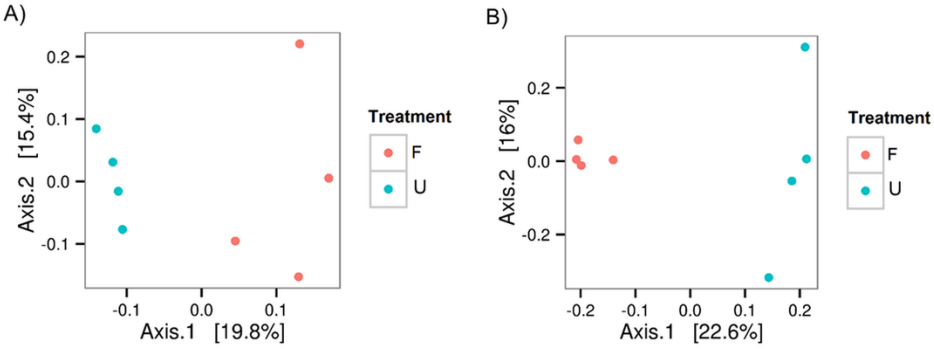


Fig. 3: Principal Coordinate Analysis (PCoA) based on unweighted Unifrac metrics of 454 sequencing microbial data of soil samples taken from the four fumigated (F) and four untreated (U) plots. Soils were fumigated with dazomet (99%, dose rate of 70 mg active substance m^{-2}) before planting and samples were collected from four fumigated and four untreated sites 19 months later, at the end of the growing season. Each point represents the composition of the soil microbiota of one sample. A: Bactobiota, B: Mycobiota.

growth, while Planctomycetes, Acidobacteria and Nitrospira had a negative correlation (Table 2). At genus level, genera associated with plant growth promotion, such as *Bacillus* sp. (ϱ = 0.64), *Streptomyces* sp. (ϱ = 0.64), and *Pseudomonas* sp. (ϱ = 0.59), were found to be positively correlated with shoot growth (Table 3). The bacterial genera most negatively correlated with shoot growth were *Nakamurella* sp. (ϱ = -0.89), *Geobacter* sp. (ϱ = -0.88) and *Nitrospira* sp. (ϱ = -0.86).

Table 2. Spearman's rank correlation coefficients (ϱ) between the abundance of soil-borne bacterial and fungal phyla, obtained by 454 sequencing, and shoot growth in apple trees. The relative abundance of the phyla in fumigated (F) and untreated (U) soils and the percent change in abundance in F soils compared to U soils are also presented. Soil samples were collected in apple orchards in the Trentino-South Tyrol region (Italy) in four plots fumigated with dazomet (99%, dose rate of 70 mg active substance m^{-2}), and in four untreated plots.

Phylum	Spearman's correlation	Relative abundance in U	Relative abundance in F	Change in F compared to U
	ϱ	----------%----------		
Bacterial Phyla				
TM7	0.76	0.07	0.19	+ 164.79
Gemmatimonadetes	0.69	1.45	1.76	+ 21.93
Chloroflexi	0.65	1.1	1.23	+ 11.67
Firmicutes	0.64	1.54	2.31	+ 50.29
Actinobacteria	0.57	6.83	8.01	+ 17.33
Planctomycetes	-0.52	0.94	0.74	- 21.23
Acidobacteria	-0.67	15.9	14.58	- 8.30
Nitrospira	-0.88	1.18	0.65	- 45.07
Fungal Phyla				
Zygomycota	-0.52	0.67	0.5	- 34.54
Ascomycota	-0.55	42.08	37.85	- 10.06

In fungal phyla, no pronounced correlation with growth was found (Table 2). At genus level, *Chaetomium* sp. (ϱ = 0.86), *Pseudallescheria* sp. (ϱ = 0.79), and *Myrmecridium* sp. (ϱ = 0.76) were among those positively correlated with shoot

growth, while the pathogenic genera *Ilyonectria* sp. (ϱ = -0.67), growth, while the pathogenic genera *Ilyonectria* sp. (ϱ = -0.67), *Pyrenochaeta* sp. (ϱ = -0.73) and *Mortierella* sp. (ϱ = -0.50) were among those negatively correlated with shoot growth (Table 4).

Table 3. Spearman's rank correlation coefficients (ϱ) between the abundance of soil-borne bacterial genera, obtained by 454 sequencing, and shoot growth in apple trees. The relative abundance of the genera in fumigated (F) and untreated (U) soils and the percent change in abundance in F soils compared to U soils are also presented. Soil samples were collected in apple orchards in the Trentino-South Tyrol region (Italy) in four plots fumigated with dazomet (99%, dose rate of 70 mg active substance m^{-2}), and in four untreated plots.

Genus	Spearman's correlation	Relative abundance in U	Relative abundance in F	Change in F compared to U
	ϱ	----------%----------		
Adhaeribacter	0.96	0.03	0.09	+ 194.44
Gp16	0.9	1.17	1.56	+ 33.14
Microbacterium	0.83	0.03	0.09	+ 168.42
Saccharothrix	0.82	0.02	0.06	+ 169.23
Nitrosospira	0.8	0.01	0.09	+ 960.00
Clostridium III	0.8	0.01	0.04	+ 228.57
Phenylobacterium	0.79	0.04	0.09	+ 145.45
Micromonospora	0.76	0.12	0.32	+ 80.77
TM7 genera incertae sedis	0.76	0.12	0.32	+ 164.79
Methylobacillus	0.76	0.01	0.21	+ 3966.67
Gp1	0.75	0.08	0.14	+ 71.43
Armatimonadetes gp4	0.73	0.36	0.44	+ 24.53

Continues on next page >

> Continues from previous page

Genus	Spearman's correlation	Relative abundance in U	Relative abundance in F	Change in F compared to U
	ϱ	----------%----------		
Catelliglobosispora	0.73	0.03	0.07	+ 175.00
Acidovorax	0.71	0.01	0.29	+ 2371.43
Sporocytophaga	0.7	0	0.04	+ 2100.00
Gemmatimonas	0.69	2.47	3.01	+ 21.93
Ammoniphilus	0.69	0.04	0.11	+ 214.29
Massilia	0.68	0.22	0.38	+ 73.08
Gp7	0.67	1.58	2.12	+ 34.36
Steroidobacter	0.66	0.47	0.65	+ 36.88
Legionella	0.66	0.02	0.05	+ 128.57
Streptomyces	0.64	0.17	0.38	+ 127.00
Bacillus	0.64	0.76	1.36	+ 77.70
Caldilinea	0.64	0.45	0.62	+ 36.57
Nocardia	0.64	0.03	0.08	+ 135.00
Solirubrobacter	0.61	0.28	0.41	+ 46.06
Patulibacter	0.6	0.03	0.04	+ 52.94
Pseudomonas	0.6	0.26	0.81	+ 210.32
Clostridium sensu stricto	0.5	0.06	0.13	+ 111.11
Hyphomicrobium	0.5	0.39	0.45	+ 16.38
Gp17	-0.51	1.37	0.92	- 32.68
Marmoricola	-0.55	0.14	0.1	- 26.19

Continues on next page >

> Continues from previous page

Genus	Spearman's correlation	Relative abundance in U	Relative abundance in F	Change in F compared to U
	ϱ	----------%----------		
Terrimonas	-0.67	0.39	0.19	- 49.78
Gp6	-0.69	13.66	12.11	- 11.38
Dongia	-0.71	0.48	0.23	- 52.65
Gp5	-0.74	0.84	0.5	- 40.56
Gp9	-0.75	0.33	0.2	- 39.49
Pedobacter	-0.76	0.19	0.12	- 38.26
Methylotenera	-0.76	0.12	0.04	- 66.67
Angustibacter	-0.77	0.04	0.01	- 69.57
Gp11	-0.79	0.52	0.24	- 53.38
Nitrospira	-0.88	2.01	1.11	- 45.07
Geobacter	-0.88	0.26	0.12	- 52.63
Nakamurella	-0.9	0.05	0.03	- 34.48

Table 4. Spearman's rank correlation coefficients (ϱ) between the abundance of soil-borne fungal genera, obtained by 454 sequencing, and shoot growth in apple trees. The relative abundance of the genera in fumigated (F) and untreated (U) soils and the percent change in abundance in F soils compared to U soils are also presented. Soil samples were collected in apple orchards in the Trentino-South Tyrol region (Italy) in four plots fumigated with dazomet (99%, dose rate of 70 mg active substance m^{-2}), and in four untreated plots.

Genus	Spearman's correlation	Relative abundance in U	Relative abundance in F	Change in F compared to U
	ϱ	----------%----------		
Chaetomium	0.86	0.08	1.49	+ 1830.00
Pseudallescheria	0.79	0.01	2.26	+ 29133.33
Myrmecridium	0.76	0.19	0.74	+ 289.19
Pseudeurotium	0.7	0.01	0.04	+ 700.00
Preussia	0.66	0.03	0.19	+ 554.55
Lindtneria	0.61	0.1	0.07	+ 866.67
Geopyxis	0.58	0.03	0.16	+ 376.92
Lectera	0.53	0	0.14	+ 5500.00
Mortierella	-0.5	0.61	0.34	+ 79.55
Didymosphaeria	-0.52	0.17	0.01	- 92.19
Clonostachys	-0.55	1.23	0.17	- 86.16
Cryptococcus	-0.61	0.79	0.05	- 93.79
Ochroconis	-0.64	0.09	0.02	- 46.67
Ilyonectria	-0.67	1.35	0.72	- 97.92
Pyrenochaeta	-0.73	0.12	0	- 100.00
Tricharina	-0.75	0.04	0	- 78.79

4. Discussion

4.1 Overall differences in microbial communities after 19 months from fumigation.

As expected, microbiological techniques did not allow identifying significant differences in bacterial or fungal CFUs between fumigated and untreated soils. Despite the risk of PCR biases, NGS technology is, to date, one of the

best approaches to have a comprehensive view of the microbial community, because cultivation techniques can capture less than 1% of the microbial biodiversity of soil and they were already found insufficient to fully describe the microbial complexity in several previous studies of ARD (Mazzola and Brown, 2010; Tewoldemedhin et al., 2011b). Soil fumigation increased the shoot growth and yield of apple trees almost three-fold in ARD-affected soils in the second growing season, confirming dazomet as an effective measure against ARD. This experimental set up allowed having both healthy and ARD-affected plants in the same orchards (similar agronomic practices and climatic conditions), which is not easy to find in intensive apple-growing areas, and we could compare the microbial communities in healthy and ARD-affected soils with very similar characteristics. Several studies have dealt with short term effect of fumigation on soil microorganisms and on microbial recolonization in artificial conditions (Dominguez-Mendoza et al., 2014; Eo and Park, 2014) and in field (Bonanomi et al. 2008), but this is the first metagenomic comparison of fumigated and untreated soil in presence of ARD at the end of the second growing season. In our study site there were no significant differences in alpha diversity in fumigated and untreated soils (Fig. 2), indicating that the richness and the evenness of the microbial community in fumigated soils were comparable to those in untreated soils. On the contrary, when measurements were taken immediately after treatment with dazomet, Bonanomi et al. (2008) found a decreased bacterial richness in fumigated soils. In our case, there was sufficient time (almost 19 months) for the microorganisms to recolonise the fumigated soil, and their community had similar evenness and richness to the ones of the untreated soil, confirming the high resilience of soil (Mocali et al. 2015).

As regards beta diversity, using phospholipid fatty acid (PFLA) analysis, Eo and Park (2014) found that the composition of microbial communities in soils treated with dazomet was different from the composition in untreated soil seven days after fumigation. This was also confirmed with NGS techniques by Dominguez-Mendoza et al. (2014), who analysed soil fumigated with ethanol-free chloroform ten days after fumigation. Our results provide strong evidence that this difference in beta diversity persists over time; indeed, we found that both fungal and bacterial communities in fumigated soils were significantly different from those in untreated soils 19 months after fumigation, at the end of the second growing season. This suggests that when recolonization of fumigated soils took place, the populations of

microorganisms in fumigated soils reached a different equilibrium in the community, as compared to untreated soil, even if the plots were neighbouring.

In our study most of the bacterial phyla that positively correlated with plant growth and that were more abundant in the fumigated soils, were Gram positive. This result is in line with what found a few days after fumigation by Eo and Park (2014) and Bonanomi et al. (2008), confirming that Gram positive bacteria are more resistant to fumigation than Gram negative ones and that this advantage persists in medium-term also in the field, 19 months after fumigation. More specifically, Dominguez-Mendoza et al. (2014) found an increased abundance of the bacterial phyla Actinobacteria and Firmicutes in soil ten days after fumigation with ethanol-free chloroform, and these phyla have also been found in composts associated with increased disease suppression (Franke-Whittle et al., unpublished results). Similarly, in our experiment, Actinobacteria and Firmicutes were the most abundant phyla positively correlated with growth, hence more abundant in fumigated soils (Table 2). Both phyla are considered copiotrophs in soil, meaning that they thrive in conditions of high nutrient availability, exhibiting high growth rates (Eilers et al. 2010; Fierer et al. 2007; Lienhard et al. 2014). These bacteria may be able to exploit decomposable organic material from the microorganisms killed by fumigation and start soil recolonisation. Contrary to Dominguez-Mendoza et al. (2014), we found Chloroflexi and Gemmatimonadetes more abundant in fumigated soils than untreated ones. This difference between the two studies could be explained by the different sampling time and the growth rate of the above-mentioned taxa. In fact, ten days after fumigation (Dominguez-Mendoza et al. 2014), the fastest bacterial colonizers (e.g. Actinobacteria and Firmicutes) are commonly more represented, while the slow-growing ones could emerge only after a certain time. The most abundant phylum negatively correlated with plant growth was Acidobacteria, which comprises oligotrophic soil bacteria (Fierer et al. 2007; Schimel and Schaeffer 2012), perfectly adapted to environments with low nutrient availability, such as our untreated soils. Our results suggest that this imbalance between bacterial phyla, due to initial repopulation of fumigated soils, also remains 19 months after fumigation in the field.

4.2 Differences in microbial community composition in fumigated and ARD-affected soils

Among the microbial genera negatively correlated with shoot growth, we found a complex of three potentially pathogenic fungi: *Ilyonectria* sp., *Pyrenochaeta* sp. and *Mortierella* sp. (Table 4). *Ilyonectria* sp., previously part of the genus *Cylindrocarpon*, is commonly associated with root rot in many woody plants, including the apple (Cabral et al. 2012). This genus was detected among the *Cylindrocarpon*-like fungi reducing plant growth through root infection in ARD-affected orchards in Italy, Austria and Germany (Manici et al. 2013). The genus *Pyrenochaeta* includes many plant pathogenic species and it was isolated in apple and pear orchards affected by die-back symptoms in South Africa (Cloete et al. 2011). *Mortierella* sp. is a Zygomycota fungus that live in soil, usually colonising roots, and it is associated with the onset of ARD (Mazzola and Manici 2012; Utkhede and Li 1989).

In this study we did not find other microorganisms that are often linked to ARD, such as *Phytophthora, Pythium,* and *Rhizoctonia* (Tewoldemedhin et al. 2011a). *Phytophthora* and *Pythium* were not detected because the primers used for amplification and sequencing do not target oomycetes (Sapkota and Nicolaisen 2015). In any case, although these fungal pathogens have often been detected in ARD-affected soils, they were never unequivocally linked to the causes of ARD, which seems to be the result of multiple biotic interactions and not only the effect of roots pathogens.

As regards the microbial genera positively correlated with shoot growth, a group of microorganisms with biocontrol or plant growth promoting potential was found: the fungus *Chaetomium* sp. (Table 4), and the bacteria *Microbacterium* sp., *Micromonospora* sp., *Streptomyces* sp., *Bacillus* sp., and *Pseudomonas* sp. (Table 3).

Chaetomium sp. is an ubiquitous fungal genus and it is widespread in soil and decaying plant material (Samson et al. 1984). Strains belonging to this genus are widely used as biocontrol agents and biofertilisers in many cultivations (Soytong et al. 2001; Vasanthakumari and Shivanna 2014), for example *Chaetomium globosum* ND35, used as fungal fertiliser in ARD-affected soils, significantly increased root mass and improved soil enzyme activity (Song et al. 2015). *Chaetomium* sp. was also found to be significantly more abundant in

soils treated with fumigation and Brassicaceae seed meal than in the ARD-affected untreated soil using metagenomic analysis (Mazzola et al. 2015).

Microbacterium sp. is considered a plant growth promoting bacterium (Esitken et al. 2009) and successfully increases the yield, growth, and nutrition of apple trees, together with *Bacillus* sp. (Karlidag et al. 2007). *Micromonospora* sp. is a metabolically versatile Actinobacterium and thus early coloniser of fumigated soils (Dominguez-Mendoza et al. 2014), and it is used as biocontrol agent for various crops (Hirsch and Valdes 2010). A high presence of *Streptomyces* sp. is known to have the potential to control soil-borne pathogens (Kinkel et al. 2012), for example, this bacterium was identified as the main player acting against *Rhizoctonia* root rot in the rhizosphere of apple trees planted in disease suppressive soils (Mazzola 2007).

Bacillus sp. is an important microbial antagonist of pathogens and it enhances plant growth and hold off fungal pathogens in ARD-affected apple orchards (Utkhede and Li 1989; Van Schoor and Bezuidenhout 2014). Like *Bacillus* sp., *Pseudomonas* sp. produces plenty of antifungal substances to suppress soil-borne pathogens, for example, *Pseudomonas putida* NRRL B-30041 is able to decrease the effects of replant disease in fruit trees by controlling pathogenic fungi (Mazzola 1999).

Other microbial genera that have been found to be associated with ARD in other metagenomics-based studies were *Dongia* sp., *Nitrospira* sp. (Franke-Whittle et al. 2015), Gp6, Gp7, and *Geobacter* sp. (Yim et al. 2015), while Gp16, *Solirubrobacter* sp. (Franke-Whittle et al. 2015), *Phenylobacterium* sp. and *Gemmatimonas* sp. (Yim et al. 2015) were found in healthy soils, but the role of these microorganisms in ARD has still to be thoroughly investigated.

4.3 Conclusion

In this study soil fumigation led to a reduction in ARD symptoms (enhancement of shoot growth and fruit yield) at the end of the second growing season, as compared to untreated soil and the high-throughput sequencing revealed differences in the soil microbial community composition, which may be involved in the ARD. However, the modification was quite limited and consisted of a slight imbalance between plant beneficial and pathogenic microorganisms in fumigated and untreated soils. In particular, a complex of potentially pathogenic fungi (*Ilyonectria* sp., *Pyrenochaeta* sp. and *Mortierella* sp.) was found to be negatively correlated with apple tree growth,

while a strong positive correlation was found with plant growth promoting microorganisms (*Chaetomium* sp. *Microbacterium* sp., *Micromonospora* sp., *Streptomyces* sp., *Bacillus* sp., and *Pseudomonas* sp.), suggesting that ARD might be the result not only of an increase in soil-borne pathogens, but also of a reduction in plant beneficial microorganisms. Further advanced approaches, as for example full metabolomic analysis of soil or transcriptomic analysis of plant root tissues, may add additional information to help identifying the complex of causal agents of ARD.

References

Albanese, D., Fontana, P., De Filippo, C., Cavalieri, D., Donati, C., 2015. MICCA: a complete and accurate software for taxonomic profiling of metagenomic data. Scientific Reports 5.

Bonanomi, G., Chiurazzi, M., Caporaso, S., Del Sorbo, G., Moschetti, G., Felice, S., 2008. Soil solarization with biodegradable materials and its impact on soil microbial communities. Soil Biol. Biochem. 40, 1989-1998.

Braun, P.G., Fuller, K.D., McRae, K., Fillmore, S.A.E., 2010. Response of 'Honeycrisp (R)' Apple Trees to Combinations of Pre-plant Fumigation, Deep Ripping, and Hog Manure Compost Incorporation in a Soil with Replant Disease. HortScience 45, 1702-1707.

Cabral, A., Groenewald, J.Z., Rego, C., Oliveira, H., Crous, P.W., 2012. Cylindrocarpon root rot: multi-gene analysis reveals novel species within the *Ilyonectria radicicola* species complex. Mycol. Prog. 11, 655-688.

Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., Knight, R., 2010. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26, 266-267.

Cloete, M., Fourie, P.H., Damm, U., Crous, P.W., Mostert, L., 2011. Fungi associated with die-back symptoms of apple and pear trees, a possible inoculum source of grapevine trunk disease pathogens. Phytopathol. Mediterr. 50, 176-190.

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Brown, C. T., Porras-Alfaro, A., Kuske, C. R., Tiedje, J. M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis Nucl. Acids Res. 42, D633-D642

Corneo, P.E., Pellegrini, A., Cappellin, L., Roncador, M., Chierici, M., Gessler, C., Pertot, I., 2013. Microbial community structure in vineyard soils across altitudinal gradients and in different seasons. FEMS Microbiol. Ecol. 84, 588-602.

Covey, R.P., Jr., Benson, N.R., Haglund, W.A., 1979. Effect of soil fumigation on the apple replant disease in Washington. Phytopathology 69, 684-686.

Daniel, R., 2005. The metagenomics of soil. Nature Reviews Microbiology 3, 470-478.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G. L., 2006. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl Environ Microbiol 72, 5069-72.

DIN EN 15933, 2012. Sludge, treated biowaste and soil - Determination of pH.

Dominguez-Mendoza, C.A., Bello-Lopez, J.M., Navarro-Noya, Y.E., de Leon-Lorenzana, A.S., Delgado-Balbuena, L., Gomez-Acata, S., Ruiz-Valdiviezo, V.M., Ramirez-Villanueva, D.A., Luna-Guido, M., Dendooven, L., 2014. Bacterial community structure in fumigated soil. Soil Biol. Biochem. 73, 122-129.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32(5), 1792-97.

Eilers, K.G., Lauber, C.L., Knight, R., Fierer, N., 2010. Shifts in bacterial community structure associated with inputs of low molecular weight carbon compounds to soil. Soil Biol. Biochem. 42, 896-903.

Eo, J., Park, K.-C., 2014. Effects of dazomet on soil organisms and recolonisation of fumigated soil. Pedobiologia 57, 147-154.

Esitken, A., Pirlak, L., Ipek, M., Donmez, M.F., Cakmakci, R., Sahin, F., 2009. Fruit Bio-thinning by Plant Growth Promoting Bacteria (PGPB) in Apple cvs. Golden Delicious and Braeburn. Biol. Agric. Hortic. 26, 379-390.

FAO, 2013. http://faostat3.fao.org/ (accessed 25/02/2016).

Feld, L., Hjelmsø M.H., Nielsen M.S., Jacobsen A.D., Rønn R., Ekelund F., Krogh P.H, Strobel B.W., Jacobsen C.S., 2015. Pesticide Side Effects in an Agricultural Soil Ecosystem as Measured by amoA Expression Quantification and Bacterial Diversity Changes. Plos One 10.

Fierer, N., Bradford, M.A., Jackson, R.B., 2007. Toward an ecological classification of soil bacteria. Ecology 88, 1354-1364.

Franke-Whittle, I.H., Manici, L.M., Insam, H., Stres, B., 2015. Rhizosphere bacteria and fungi associated with plant growth in soils of three replanted apple orchards. Plant Soil 395, 317-333.

Franke-Whittle, I.H., Fernandez-Delgado Juarez, M., Insam, H., Schweizer, S., Kelderer, M., Rühmer, T., Baab, G., Henfrey, J., Topp, A., Naef, A., Manici, L, unpublished results. Microbial composition of composts originating from 4 different countries, and their effects on apple replant disease.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113-118.

Gower, J.C., Blasius, J., 2005. Multivariate prediction with nonlinear principal components analysis: Theory. Qual. Quant. 39, 359-372.

Guidelines for Integrated Pome Cultivation, 2016. Agrios. http://www.agrios.it/guidelines.html (last accesed 03/03/2016).

Guo, H., Mao, Z., Jiang, H., Liu, P., Zhou, B., Bao, Z., Sui, J., Zhou, X., Liu, X., 2014. Community analysis of plant growth promoting rhizobacteria for apple trees. Crop Protect. 62, 1-9.

Hammer, O., Harper, D.A.T., Ryan, P.D., 2001. PAST: paleontological statistics software package for education and data analysis. Palaentol. Electron. 4.

Hirsch, A.M., Valdes, M., 2010. *Micromonospora*: An important microbe for biomedicine and potentially for biocontrol and biofuels. Soil Biol. Biochem. 42, 536-542.

Hoestra, H., 1968. Replant Disease of apple in the Netherlands. Mededelingen Landbouwhogeschool Wageningen The Netherlands.

ISO 10694, 1995. Soil quality -- Determination of organic and total carbon after dry combustion (elementary analysis).

Karlidag, H., Esitken, A., Turan, M., Sahin, F., 2007. Effects of root inoculation of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient element contents of leaves of apple. Sci. Hortic-Amsterdam 114, 16-20.

Kinkel, L.L., Schlatter, D.C., Bakker, M.G., Arenz, B.E., 2012. *Streptomyces* competition and co-evolution in relation to plant disease suppression. Res. Microbiol. 163, 490-499.

Koljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F.S., Bahram, M., Bates, S.T., Bruns, T.D., Bengtsson-Palme, J., Callaghan, T.M., Douglas, B., Drenkhan, T., Eberhardt, U., Duenas, M., Grebenc, T., Griffith, G.W.,

Hartmann, M., Kirk, P.M., Kohout, P., Larsson, E., Lindahl, B.D., Luecking, R., Martin, M.P., Matheny, P.B., Nguyen, N.H., Niskanen, T., Oja, J., Peay, K.G., Peintner, U., Peterson, M., Poldmaa, K., Saag, L., Saar, I., Schuessler, A., Scott, J.A., Senes, C., Smith, M.E., Suija, A., Taylor, D.L., Telleria, M.T., Weiss, M., Larsson, K.-H., 2013. Towards a unified paradigm for sequence-based identification of fungi. Mol. Ecol. 22, 5271-5277.

Lienhard, P., Terrat, S., Prevost-Boure, N.C., Nowak, V., Regnier, T., Sayphoummie, S., Panyasiri, K., Tivet, F., Mathieu, O., Leveque, J., Maron, P.-A., Ranjard, L., 2014. Pyrosequencing evidences the impact of cropping on soil bacterial and fungal diversity in Laos tropical grassland. Agron. Sustain. Dev. 34, 525-533.

Lozupone, C., Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71, 8228-8235.

Mai, W.F., Abawi, G.S., 1981. Controlling Replant Diseases of Pome and Stone Fruits in Northeastern United-States by Preplant Fumigation. Plant Dis. 65, 859-864.

Manici, L.M., Kelderer, M., Franke-Whittle, I.H., Ruehmer, T., Baab, G., Nicoletti, F., Caputo, F., Topp, A., Insam, H., Naef, A., 2013. Relationship between root-endophytic microbial communities and replant disease in specialized apple growing areas in Europe. Appl. Soil Ecol. 72, 207-214.

Mazzola, M., 1998. Elucidation of the microbial complex having a causal role in the development of apple replant disease in Washington. Phytopathology 88, 930-938.

Mazzola, M., 1999. Control of replant disease of tree fruits with Pseudomonas putida. Google Patents.

Mazzola, M., 2007. Manipulation of rhizosphere bacterial communities to induce suppressive soils. J. Nematol. 39, 213-220.

Mazzola, M., Brown, J., 2010. Efficacy of Brassicaceous Seed Meal Formulations for the Control of Apple Replant Disease in Conventional and Organic Production Systems. Plant Dis. 94, 835-842.

Mazzola, M., Hewavitharana, S.S., Strauss, S.L., 2015. Brassica Seed Meal Soil Amendments Transform the Rhizosphere Microbiome and Improve Apple Production Through Resistance to Pathogen Reinfestation. Phytopathology 105, 460-469.

Mazzola, M., Manici, L.M., 2012. Apple Replant Disease: Role of Microbial Ecology in Cause and Control, in: VanAlfen, N.K., Leach, J.E., Lindow, S. (Eds.), Annual Review of Phytopathology, Vol 50, pp. 45-65.

McMurdie, P.J., Holmes, S., 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. Plos One 8.

Mocali, S., Landi, S., Curto, G., Dallavalle, E., Infantino, A., Colzi, C., d'Errico, G., Roversi, P.F., D'Avino, L., Lazzeri, L., 2015. Resilience of soil microbial and nematode communities after biofumigant treatment with defatted seed meals. Ind. Crop. Prod. 75, 79-90.

Muyzer, G., Dewaal, E.C., Uitterlinden, A.G., 1993. Profiling of Complex Microbial-Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S Ribosomal-RNA. Appl. Environ. Microbiol. 59, 695-700.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2013. Vegan: Community Ecology Package. R package version 2.0-10. http://CRAN.R-project.org/package=vegan.

ÖNORM L 1087, 2012. Chemical analysis of soils — Determination of "plant-available" phosphorus and potassium by the calcium-acetate-lactate (CAL)-method.

Otto, G., Winkler, H., 1993. Soil fumigation for controlling replant problems in apple orchards. Acta Hortic., 91-95.

Redman, R.S., Dunigan, D.D., Rodriguez, R.J., 2001. Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? New Phytol. 151, 705-716.

Ross, R.G., Crowe, A.D., 1973. Replant disease in apple orchard soil. Can. Plant Dis. Surv. 53, 144-146.

Samson, R., Hoekstra, E., Lund, F., Filtenborg, O., Frisvad, J., 1984. Introduction of Food- and Airborne fungi, 6th edition. Centraalbureau voor Schimmelcultures, Utrecht.

Sapkota, R., Nicolaisen, M., 2015. An improved high throughput sequencing method for studying oomycete communities. J. Microbiol. Methods 110, 33-39.

Schimel, J.P., Schaeffer, S.M., 2012. Microbial control over carbon cycling in soil. Front. Microbiol. 3.

Soil Survey Staff, 2010. Keys to Soil Taxonomy, 11th ed. USDA-Natural Resources Conservation Service, Washington DC.

Song, F., Wang, S., Zhang, X., Gao, K., Yin, C., Chen, X., Mao, Z., 2015. Effects of Chaetomium globosum ND35 Fungal Fertilizer on Continuous Cropping Soil

Microorganism and Malus hupehensis Seedling Biomass. Acta Hortic. Sin. 42, 205-213.

Soytong, K., Kanokmedhakul, S., Kukongviriyapa, V., Isobe, M., 2001. Application of *Chaetomium* species (Ketomium((R))) as a new broad spectrum biological fungicide for plant disease control: A review article. Fung. Divers. 7, 1-15.

Tagliavini, M., Marangoni, B., 1992. Growth of peach as affected by decomposition of own root residues in soil. Plant Soil 145, 253-260.

Tewoldemedhin, Y.T., Mazzola, M., Botha, W.J., Spies, C.F.J., McLeod, A., 2011a. Characterization of fungi (*Fusarium* and *Rhizoctonia*) and oomycetes (*Phytophthora* and *Pythium*) associated with apple orchards in South Africa. Eur. J. Plant Pathol. 130, 215-229.

Tewoldemedhin, Y.T., Mazzola, M., Labuschagne, I., McLeod, A., 2011b. A multi-phasic approach reveals that apple replant disease is caused by multiple biological agents, with some agents acting synergistically. Soil Biol. Biochem. 43, 1917-1927.

Utkhede, R.S., Li, T.S.C., 1989. Evaluation of *Bacillus subtilis* for potential control of apple replant disease. J. Phytopathol. 126, 305-312.

Van Schoor, L., Bezuidenhout, K., 2014. Potential use of compost extract and *Bacillus* inoculants in combination with compost in managing apple replant disease. SA Fruit Journal June/July, 62-67.

van Schoor, L., Denman, S., Cook, N.C., 2009. Characterisation of apple replant disease under South African conditions and potential biological management strategies. Sci. Hortic-Amsterdam 119, 153-162.

Vasanthakumari, M.M., Shivanna, M.B., 2014. Biological control of sorghum anthracnose with rhizosphere and rhizoplane fungal isolates from perennial grasses of the Western Ghats of India. Eur. J. Plant Pathol. 139, 721-733.

VDLUFA, 1991. Band I Die Untersuchung von Böden, volume I, chapter D2.1 4. Aufl, ISBN 978-3-941273-13-9.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S Ribosomal DNA Amplification for Phylogenetic Study. J. Bacteriol. 173, 697-703.

White, T., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: a guide to methods and applications. Academic Press, New York, USA, pp. 315–322.

Yim, B., Smalla, K., Winkelmann, T., 2013. Evaluation of apple replant problems based on different soil disinfection treatments-links to soil microbial community structure? Plant Soil 366, 617-631.

Yim, B., Winkelmann, T., Ding, G.-C., Smalla, K., 2015. Different bacterial communities in heat and gamma irradiation treated replant disease soils revealed by 16S rRNA gene analysis - contribution to improved aboveground apple plant growth? Front. Microbiol. 6.

III. Paper 2

Apple replant disease may not be caused solely by soil microorganisms

Apple replant disease may not be caused solely by soil microorganisms

Lidia Nicola[a,b], Elena Turco[a], Martin Thalheimer[c], Massimo Pindo[a], Heribert Insam[b], Ilaria Pertot[ad*].

[a] Research and Innovation Centre, Fondazione Edmund Mach (FEM), 38010 San Michele all'Adige (TN), Italy
[b] Department of Microbiology, University of Innsbruck, 6020 Innsbruck, Austria
[c] Laimburg Research Centre for Agriculture and Forestry , 39040 Auer (BZ), Italy
[d] Center Agriculture Food Environment, University of Trento

* Corresponding author.

E-mail address: ilaria.pertot@fmach.it (I. Pertot)

Manuscript submitted to Plant Disease

Abstract:

Apple Replant Disease (ARD) is a key problem in apple production, occurring when apple trees are replanted in the same soil. Three different hypotheses are currently proposed regarding the aetiology: microbial origin, the presence of toxins, and nutrient imbalances in soil. The aim of this work was to test these hypotheses using specific soil treatments (fumigation, addition of a fungal biocontrol agent, soil washing and compost addition) to determine the response of M9 rooted cuttings and soil microbial communities in bulk soil and the rhizosphere using metabarcoding. Addition of compost or a biocontrol agent (*Trichoderma atroviride* SC1) did not reduce ARD symptoms in either year, but changed the microbial communities in bulk soil. Intensive irrigation and fumigation restored plant growth, however inconsistently in the two years. The microbial communities in fumigated soils were significantly different from those in the untreated control in both years, while soil washing did not modify them. A number of OTUs known to promote plant growth were found to be positively correlated with shoot growth. These results indicate that although microorganisms have a role in ARD development, there could be other contributing factors, such as the presence of autotoxins in soil, especially when close to replanting.

1. Introduction

Plants in the Rosaceae family suffer when replanted in the same soil. This phenomenonis particularly severe in the apple, where it is called apple replant disease (ARD). The symptoms of ARD are a reduction in plant growth, shortened internodes, discoloured roots, root tip necrosis and a general decrease in root biomass (Mazzola and Manici 2012). These symptoms result in a significant decrease in yield quantity and quality.

Several hypotheses have been proposed in relation to the causal agents of ARD. The first regards the release of toxic substances into the soil from the roots of the preceding apple trees, or as result of the degradation of root residues in the soil. Indeed, the roots of apple trees excrete different phenolic compounds in liquid cultures (Börner 1959) and some of these compounds, in particular phlorizin, were detected in soils amended with ground apple roots and were significantly correlated with impaired growth of apple seedlings (Nicola et al. 2016). Phlorizin can inhibit the respiratory rate and

enzyme activities of the tricarboxylic acid cycle in apple roots (Wang et al. 2012; Yin et al. 2016a), and was also detected in the soil of ARD-affected orchards (Yin et al. 2016b).

The second hypothesis is that ARD results from the activity of a complex of microbial pathogens. Indeed, classic microbiological investigations have often detected the *Cylindrocarpon*, *Rhizoctonia* and *Pythium* genera, which host several phytopathogenic species, in ARD-affected orchards (Braun 1991; Jaffee et al. 1982; Mazzola 1998). In addition, other analyses have associated the presence of fungal pathogens belonging to the *Ilyonectria* and *Mortierella* genera in the soil to reduced plant growth in ARD-affected orchards (Manici et al. 2013; Mazzola and Manici 2012; Nicola et al. 2017). Furthermore, metabarcoding analysis identified a decrease in species belonging to beneficial microorganisms (e.g. *Streptomyces* sp., *Bacillus* sp., *Paenibacillus* sp., *Chaetomium* sp. and *Microbacterium* sp.) in ARD-affected soil, as compared to healthy soil (Nicola et al. 2017; Yim et al. 2015).

Soil nutrient imbalances, due to prolonged apple monoculture and limited addition of organic supplements, are also associated with ARD (Forge et. al. 2016; von Glisczynski et al. 2016), so different strategies to increase organic matter in soil have been adopted in ARD-affected soil, with variable results. The addition of fresh organic waste can increase the presence of soil-borne pathogens like *Pythium* spp. and *Rhizoctonia* spp. (Manici et al. 2004). In contrast, the use of products from organic waste, such as compost, can suppress microbial pathogens and contemporaneously increase soil organic matter (Mandelbaum and Hadar 1990). The application of compost to reduce ARD symptoms has also had varied results. For example, Braun et al. (2010) found increased plant growth following compost treatment coupled with deep ripping, and both solid compost and digestate significantly increased the growth of *Malus hupehensis* Rehd. (Zhang et al. 2012). Moreover, long-term application of organic matter in orchards was proven to be efficient in maintaining control of replant disease over the years (Manici et al. 2003). In contrast, other trials did not find any positive effect linked to the addition of compost to ARD-affected soils (Yao et al. 2006).

The aim of this study was to test these different hypotheses under controlled conditions, in which ARD-affected soil underwent different treatments: soil washing, fumigation or addition of a biocontrol agent and addition of compost, to test the hypotheses regarding the presence of toxins, microbial

origin and nutrient imbalance respectively. The effects of these treatments on the growth of rootstock and soil microbial composition were monitored usinghigh throughput sequences.

2. Materials and methods

2.1 Experimental design and soil sampling

The experiment was repeated with the same methodology in two successive years (2013 and 2014). The experimental site was an apple orchard in northern Italy (46.0 N, 11.3 E), which had been uninterruptedly cultivated with apple trees for several decades and was affected by ARD. In this orchard, the last replanting was on 26 April 2012. The classic symptoms of ARD were present in the entire orchard. The soil at the site was classified as Typic Fluvaquent, coarse silty, mixed, mesic (Soil Survey Staff, 2010), with a pH of 7.3 and silt loam as soil texture. The trees were planted with a distance of 3.2 m between the rows and 0.8 m along the row. On 6 June 2013 and 3 June 2014, approximately 200 litres of soil were collected along the rows, by sampling at a distance of 25 cm from each apple tree, at a depth of 5-20 cm. The collected soil was sieved through 4 mm mesh and divided into six equal lots, then treated as follows. The first lot was fumigated by adding 0.4 g litres^{-1} of dazomet (99%, Basamid® Granulat, Certis Europe, The Netherlands) and incubated in a sealed plastic bag for 14 days, then left to ventilate for seven days (F treatment). The second lot was amended with 1:5 (v:v) of organic compost (C treatment), made from mowed material and residues from wine and fruit production. The compost (Ecorott s.r.l., Italy) contained 20.3% organic carbon and had a C/N-ratio of 13.5. The third lot was amended with 3.8 $\cdot 10^9$ conidia litres^{-1} of *Trichoderma atroviride* SC1 (10^{10} viable spores g^{-1}; Vintec; Belchim Crop Protection), irrigated and incubated at 22 °C for 14 days (TR treatment). The fourth lot was divided into 20 fabric-lined 2-litre pots with expanded clay at the bottom and irrigated with half of its volume in water every day for ten days (washing), then mixed and left to dry on a bench (W treatment); the remaining lots (fifth and sixth) were left untreated.

The treated soil lots were placed in 1.6-litre pots with a 1 cm layer of expanded clay at the bottom. A rooted cutting of M9 rootstock (35 cm tall) was planted in each pot (replicate) and six replicates per soil treatment were arranged (n=36). The fifth and sixth lot represented the untreated controls for the

experiment: one was planted as described above (U treatment) and the other was not planted (UU treatment). The pots were kept at 20 °C in a greenhouse and irrigated by drop irrigation (approximately 300 ml per week). Sampling of the bulk soil was carried out when the soil was collected in the field (T0), taking three samples from the mixed soil, and three months after planting (T3), sampling three pots for each treatment (n=18). The rhizosphere of the corresponding M9 plants was also collected (n=15), according to the method described by Bulgarelli et al. (2012). At T3, ARD severity was assessed in each of the six replicates by measuring the dry mass of roots and aerial shoots,. Counting of colony-forming units (CFUs) in the soil samples was carried out at T3, following the protocol of Corneo et al. (2013). The colonies of bacteria and fungi were counted after 3 and 6 days of incubation at 27 °C.

2.2 DNA extraction, PCR amplification and pyrosequencing

Total DNA was extracted from 0.5 g of each soil sample using the FastDNA™ SPIN Kit for Soil and the FastPrep® instrument (MP Biomedicals, Santa Ana, USA), according to the manufacturer's instructions. Extracted DNA was dissolved in 100 µl of DES (DNase/Pyrogen-Free Water) and stored at -20 °C until PCR amplification. To analyse the soil microbial communities, specific primers for pyrosequencing were used: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (Weisburg et al. 1991) and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al. 1993) for bacteria, ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns 1993) and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (White et al. 1990) for fungi.

The PCRs were performed according to the protocols described by Nicola et al (2017). The PCR products were then quantified via quantitative PCR using the Roche 454 titanium library quantification kit (KAPA Biosystems, Boston, USA) and pooled in equimolar proportion in a final amplicon library. 454 pyrosequencing was carried out on GS-FLX+ systems, using the XL+ chemistry (Roche, Mannheim, Germany), following the manufacturer's instructions.

2.3 Sequence data analysis

Sequence processing was done with the MICCA pipeline (Albanese et al. 2015). The procedure consisted of removing the forward and reverse primers, and then discarding untrimmed reads. The reads were quality trimmed by removing short sequences (<400 bp for bacteria, <200 bp for fungi) and

sequences presenting a high error rate (>0.75 both for bacteria and fungi). De novo greedy OTU clustering was performed, and the UNITE database (Koljalg et al. 2013) was used for taxonomic assignment of fungi, while the RDP database and protocol for taxonomy assignment (Cole et al. 2014) were used for bacterial reads. Multiple alignment with the Greengenes template (DeSantis et al. 2006) was performed with PyNAST for bacteria (Caporaso et al. 2010). Sequence data were made available at the NCBI SRA database under the BioProject number PRJNA381040

2.4 Statistical analysis

Mass measurements, diversity indices and CFU counts were statistically analysed with the PAST software package, version 3.14 (Hammer et al. 2001), using Pearson correlation, ANOVA and Tukey's test, after checking for normality and homoscedasticity (Shapiro-Wilk test and Levene's test respectively). Statistical analysis of the sequencing data was performed with the phyloseq R package, ver. 3.4 (McMurdie and Holmes 2013). To control biasing effects of sequencing depth, samples were rarefied by subsampling to 90% of the depth of the least abundant sample.

Alpha diversity (microbial diversity within the differently treated soils), was calculated using the Observed Species, Simpson, and Shannon indices. Beta diversity (differences in taxa composition among the differently treated soils) was estimated with multivariate analysis of bacterial and fungal community structure. Specifically, principal coordinates analysis (PCoA) (Gower and Blasius 2005) and canonical correspondence analysis (CCA), both on Bray-Curtis distances (Lozupone and Knight 2005), were used. To test whether soil microbial communities differed significantly among the treatments, PERMANOVA was used, implemented in the vegan R package, ver. 2.4.1 (Oksanen et al. 2013), and applied to the Bray-Curtis dissimilarity distance matrices; it was then corrected for multiple comparisons with the False Discovery Rate (FDR). A Procrustes test was applied to the CCA ordinations to correlate the bacterial and fungal beta-diversity response to different soil treatments. Wilcoxon tests were carried out on OTU abundance to detect differentially abundant OTUs between untreated ARD-affected soils (U) and other treatments for each year; the p values were corrected for multiple comparisons with the FDR. Spearman's rank correlation (ϱ) was calculated between the abundance of microbial taxa and total aerial mass, to identify which taxa were more abundant in correspondence with reduced ARD

symptoms. Taxa present in all samples with an abundance of over 10 in at least one sample and with $\varrho < -0.5$ or $\varrho > 0.5$ correlations were included in the result tables.

3. Results

As regards soil cultivable microorganisms, the number of bacterial CFUs in fumigated samples was significantly higher than in most of the other treatments, both in 2013 and 2014 (ANOVA, $p = 0.006$ and $p = 0.007$, respectively; Table 1). For fungi, on the other hand, no difference among treatments was detected (ANOVA, $p = 0.394$ in 2013 and $p = 0.189$ in 2014; Table 1). No colonies of *Rosellinia necatrix* and *Armillaria* spp., which are root pathogens of the apple, were found among the cultivable fungi.

Table 1. Average counts of colony-forming units in soil samples collected from ARD-affected soils undergoing different treatments. The measurements were taken three months after transplanting the plants (M9 cuttings). ANOVA and Tukey's test were applied separately each year. Different letters indicate significant differences ($\alpha = 0.05$). W = washing treatment; F = fumigation with dazomet; TR = addition of *Trichoderma atroviride* SC1 conidia; C = addition of compost; U = untreated control; UU = unplanted untreated control.

	Bacteria		**Fungi**	
	2013	**2014**	**2013**	**2014**
W	$9.34\ 10^6$ *ab*	$1.85\ 10^6$ *b*	$1.63\ 10^5$ *a*	$6{,}67\ 10^4$ *a*
F	$1.48\ 10^7$ *a*	$4.02\ 10^6$ *a*	$2.13\ 10^5$ *a*	$1.75\ 10^5$ *a*
TR	$8.73\ 10^6$ *ab*	$2.19\ 10^6$ *b*	$3.63\ 10^5$ *a*	$6.25\ 10^4$ *a*
C	$6.94\ 10^6$ *b*	$2.36\ 10^6$ *ab*	$1.92\ 10^5$ *a*	$1.67\ 10^4$ *a*
U	$5.28\ 10^6$ *b*	$2.44\ 10^6$ *ab*	$3.50\ 10^5$ *a*	$4.17\ 10^4$ *a*
UU	$4.69\ 10^6$ *b*	$1.75\ 10^6$ *b*	$2.63\ 10^5$ *a*	$2.50\ 10^4$ *a*

Pyrosequencing yielded a total of 1,630,788 raw pyrotag reads for bacteria; of these, 739,926 reads were from bulk soil and 890,862 from the rhizosphere. Fungi yielded a total of raw pyrotag reads of 948,435, of which 522,573 from bulk soil and 425,862 from the rhizosphere. After quality filtering and chimera removal, a total of 1,363,322 16S sequences and 716,813 ITS sequences remained for community analysis, corresponding to an average ± standard

deviation of 18,935 ± 6,195 reads and 9,956 ± 4,368 reads per sample for bacteria and fungi respectively. A total of 16,083 bacterial OTUs and 2,839 fungal OTUs was counted. Proteobacteria, Actinobacteria and Bacteroidetes were the dominant bacterial phyla in soil (overall 86% of total reads), while fungal communities were dominated by Ascomycota (90% of total reads).

As regards ARD severity, statistical analysis was done separately for each year, because the effect of the treatments was different in the two years. Soil treatments significantly affected the mass of both roots (Fig. 1; ANOVA, p=0.026, p=0.025 in 2013 and 2014 respectively) and the aerial part of plants (ANOVA p=0.031, p=0.000 in 2013 and 2014 respectively). Since the mass of the aerial parts of plants was comparable for the treatments and highly correlated with the effect on the mass of roots (Pearson correlation, R=0.98 and R=0.94 in 2013 and 2014 respectively), only the mass of aerial parts is shown (Fig. 2). In both years, no effect resulting from the addition of *T. atroviride* SC1 to TR soils was detected on the growth of plants as compared to the untreated control. The aerial mass of plants grown in C and U soils was consistently lower in both years. Contrasting results for the two years were noticed with F and W treatments. In the first year, M9 plants grown in W soil had higher growth (ANOVA, p = 0.000), while in the second year they did not differ significantly from the plants grown in untreated soil. On the other hand, in 2014 the plants with significantly higher growth were those grown in F soils (ANOVA, p = 0.031), which on the contrary did not grow well in 2013.

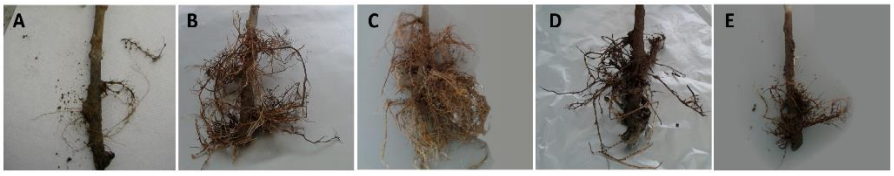


Fig. 1. Roots of apple plants (rooted cutting of M9 rootstock) three months after transplanting in ARD-affected soil undergoing different treatments (pictures refer to the trial carried out in 2014). A: untreated control; B: washing treatment; C: fumigation with dazomet; D: addition of *Trichoderma atroviride* SC1 conidia; E: addition of compost.

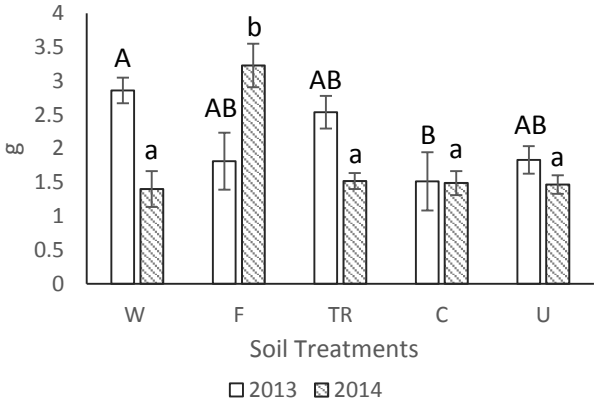


Fig. 2. Measurement of the total aerial mass of M9 plants in ARD-affected soil undergoing different treatments. The measurements were taken three months after transplanting the plants (M9 rootstock). Bars indicate the standard error of the mean. ANOVA and Tukey's test were applied separately each year. Different letters (uppercase for 2013 and lowercase for 2014) indicate significant differences (α = 0.05).W = washing treatment; F = fumigation with dazomet; TR = addition of *Trichoderma atroviride* SC1 conidia; C = addition of compost; U = untreated control.

As regards the alpha (within-sample) diversity of soil microbial communities, bacteria in F soils had a significantly lower Shannon diversity index, both in bulk soil and in the rhizosphere (Fig. 3 a, b). Fungi, on the other hand, were affected by fumigation only in bulk soil and not in the rhizosphere (Fig. 3 c, d). The addition of *T. atroviride* SC1 to TR soils decreased fungal diversity in bulk soil, because of its massive dominance against other taxa.

As regards beta (between-samples) diversity, the origin of the sample (bulk soil or rhizosphere) had a considerable influence on the taxonomic composition of the microbial samples, while the soil treatments had a lower impact (Fig. 4 and 5). Likewise, in PCoA graphs showing microbial beta diversity (Fig. 6), the clusters of bulk soil and rhizosphere samples were very distant from each other, especially for bacteria. This difference between bulk soil and the rhizosphere was confirmed by PERMANOVA ($p = 0.001$ for bacteria and fungi), which also detected a statistically significant effect of the soil treatments on the communities for both bacteria and fungi. With more restricted examination of bulk soil and the rhizosphere separately (Fig. 7), the samples clustered more tightly in bulk soil than in the rhizosphere, according to the treatments, especially in fungi, meaning that rhizosphere microbial

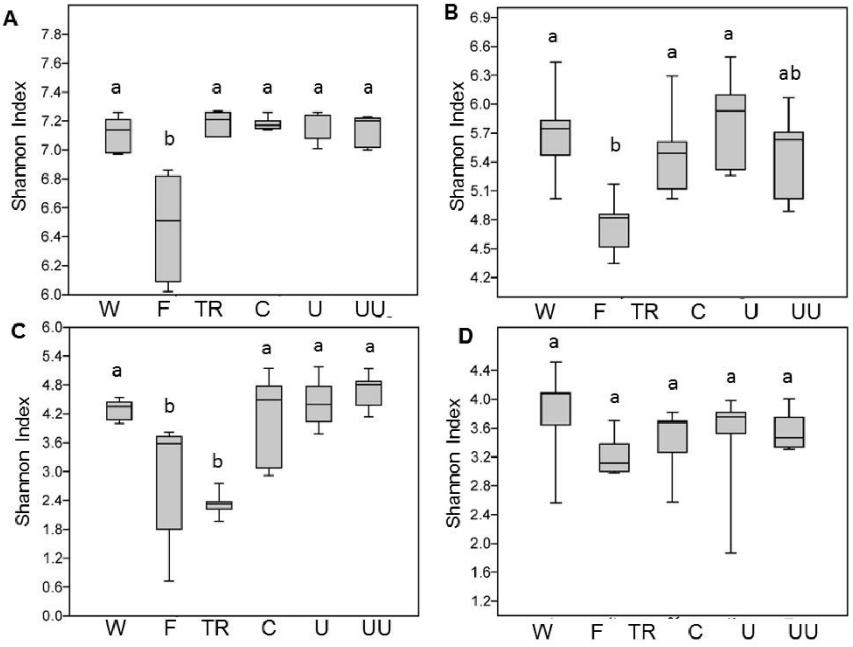


Fig. 3. Box plot representing Shannon diversity indices for bacterial (A, B) and fungal (C, D) communities in ARD-affected soil exposed to the different treatments. The bulk soil (A, C) and rhizosphere (B, D) samples were collected three months after transplanting the plants (M9 rootstock). ANOVA and Tukey's tests were applied and different letters indicate significant differences ($\alpha = 0.05$). W = washing treatment; F = fumigation with dazomet; TR = addition of *Trichoderma atroviride* SC1 conidia; C = addition of compost; U = untreated control; UU = unplanted untreated control.

Fig. 4. Bar plot of the relative abundance of bacterial communities at order level in bulk soil and the rhizosphere affected by ARD and exposed to different treatments. The samples were collected three months after transplanting the plants (M9 rootstock). R = samples from the rhizosphere; S = samples from bulk soil; W = washing treatment; F = fumigation with dazomet; TR = addition of *Trichoderma atroviride* SC1 conidia; C = addition of compost; U = untreated control; UU = unplanted untreated control.

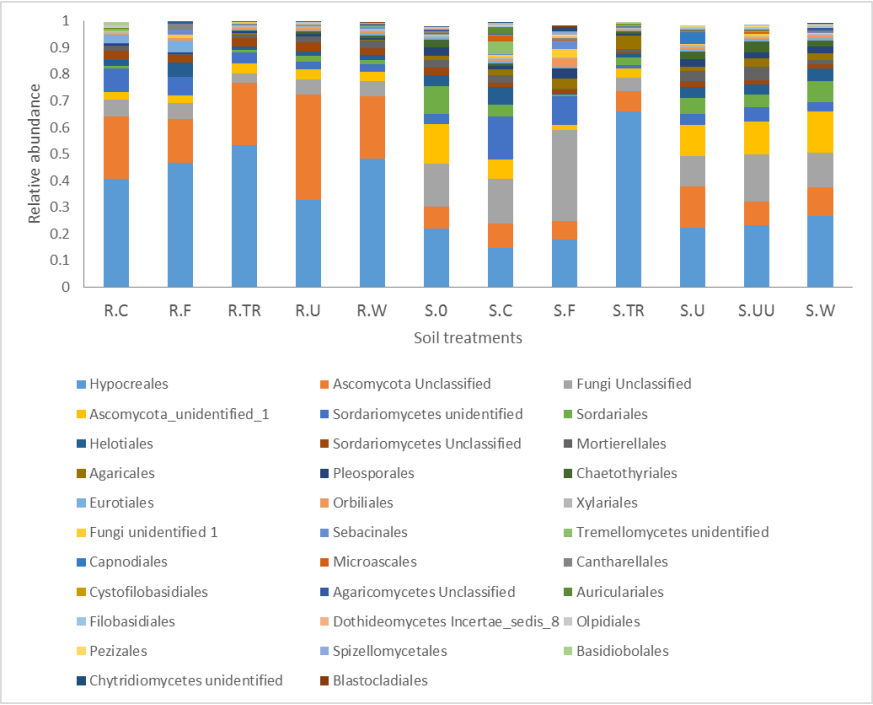


Fig. 5. Bar plot of the relative abundance of fungal communities at order level in bulk soil and the rhizosphere affected by ARD and exposed to different treatments. The samples were collected three months after transplanting the plants (M9 rootstock). R = samples from the rhizosphere; S = samples from bulk soil; W = washing treatment; F = fumigation with dazomet; TR = addition of *Trichoderma atroviride* SC1 conidia; C = addition of compost; U = untreated control; UU = unplanted untreated control.

communities were less influenced by the different treatments. In any case, the treatments significantly shaped the bacterial and fungal communities in bulk soil and the rhizosphere (PERMANOVA, p= 0.001 for bacteria both in bulk soil and the rhizosphere; p = 0.001 and p = 0.002 for fungi in bulk soil and the rhizosphere respectively). Specifically, F soil hosted significantly different fungal and bacterial soil communities, both in the rhizosphere and bulk soil. Compost addition significantly influenced fungal and bacterial communities in the bulk soil of C samples, while in the rhizosphere the effect was not present. A significant change between the bacterial community at time 0 (0) and almost all other treatments was also observed. The addition of *T. atroviride* SC1 significantly changed the fungal communities in the TR rhizosphere and bulk soil compared to U soils. On the other hand, W soils did not show any significant modification in terms of the microbial communities compared to the untreated control (U).

Bacteria and fungi reacted in a very similar way to the treatments: indeed, the correlation between the response of fungi and bacteria was very high (Procrustes test, 0.94, m12=0.123), with a very strong significance (p = 0.0001).

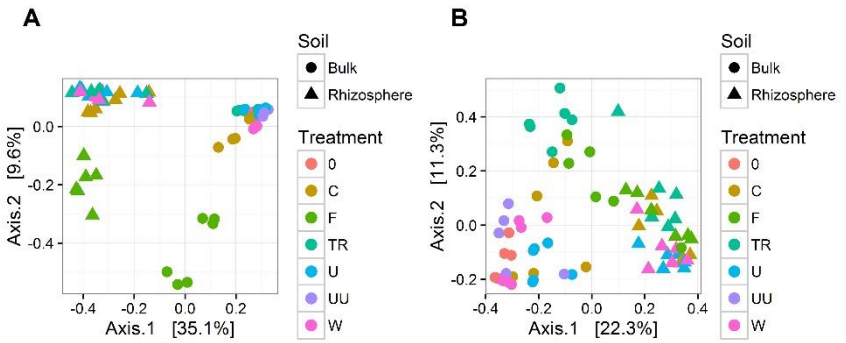


Fig. 6. Principal Coordinate Analysis based on Bray-Curtis metrics of 454 microbial sequencing data for bulk soil and rhizosphere samples taken from ARD-affected soil exposed to different treatments. Each point represents the composition of the soil microbiota of one sample. A: bactobiota, B: mycobiota. W = washing treatment; F = fumigation with dazomet; TR = addition of *Trichoderma atroviride* SC1 conidia; C = addition of compost; U = untreated control; UU = unplanted untreated control. The analysis was carried out three months after transplanting the plants (M9 rootstock).

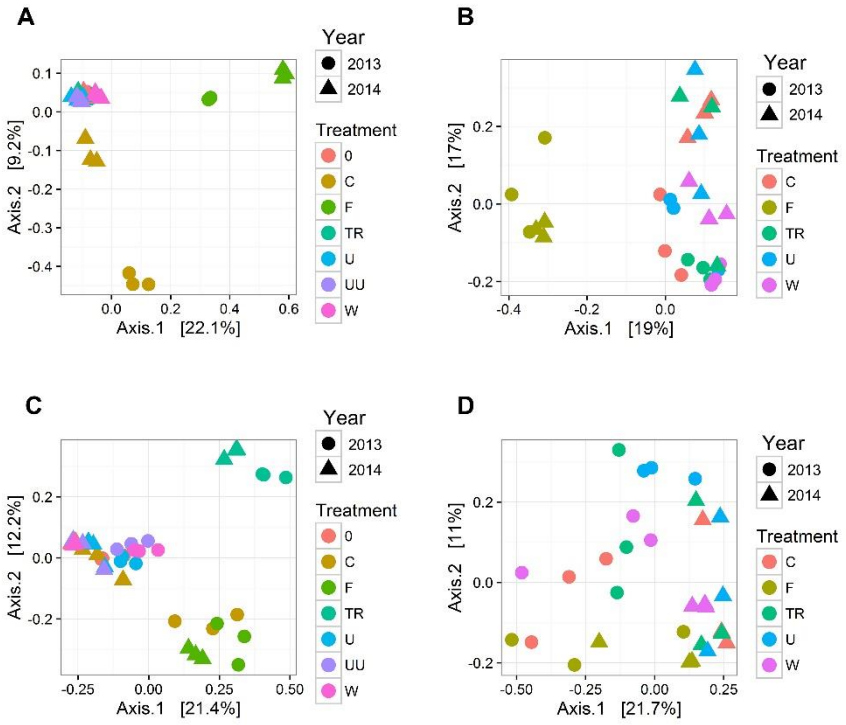


Fig. 7. Principal Coordinate Analysis based on Bray-Curtis metrics of 454 microbial sequencing data for bulk soil (A, C) and rhizosphere samples (B, D) taken from ARD-affected soil exposed to different treatments. Each point represents the composition of the soil microbiota of one sample. A: bactobiota in bulk soil, B: bactobiota in the rhizosphere; C: mycobiota in bulk soil; D: mycobiota in the rhizosphere; W = washing treatment; F = fumigation with dazomet; TR = addition of *Trichoderma atroviride* SC1 conidia; C = addition of compost; U = untreated control; UU = unplanted untreated control. The analysis was carried out three months after transplanting the plants (M9 rootstock).

Because of differences in trends for the two years (the highest growth was in W soils and F soils, in 2013 and 2014 respectively), Wilcoxon tests were applied separately each year to the taxonomical composition of bacteria and fungi in F and W samples as compared to those in U samples, to see if there were any changes in the two years, but no OTU with a significantly different abundance was detected. Moreover, the correlation between the total aerial mass of rooted cuttings and OTU abundance was analysed. A group of

bacteria known for their biocontrol activity or their part in mineral soil cycles was found to be positively correlated in bulk soil with aerial mass (Table 2), while in the rhizosphere the biocontrol agent *Nocardioides* sp. was detected. In the rhizosphere, only one bacterium was negatively correlated with aerial mass (*Salinibacterium* sp., ϱ = -0.55), while no negatively correlated bacteria were found in bulk soil. As for fungi, just one OTU at genus or lower level was positively correlated with aerial mass, *Ilyonectria macrodydima* (ϱ = +0.51).

Table 2: Spearman's rank correlation coefficients (ϱ) between the abundance of soil-borne bacterial genera, obtained by 454 sequencing, and the total aerial mass of M9 cuttings. The samples of bulk soil were collected three months after transplanting the plants (M9 cuttings) in ARD-affected soil undergoing different soil treatments.

OTUs	**Spearman's correlation (ϱ)**
Pseudoxanthomonas sp.	0.65
Bosea sp.	0.64
Methylobacillus sp.	0.60
Sphingopyxis sp.	0.57
Dyadobacter sp.	0.55
Sphingomonas sp.	0.54
Niastella sp.	0.54
Asanoa sp.	0.52
Sphingobium sp.	0.51
Chitinophaga sp.	0.51
Rhizobacter sp.	0.50

4. Discussion

The spatial origin of the samples (bulk soil *vs.* rhizosphere) was the main factor shaping the composition of the microbial communities in the samples. Similarly to several other plants, the rhizosphere microbial community of apple plants differs from that of bulk soil. In plants, the "rhizosphere effect" on microorganisms (Hiltner 1904) is the result of root architecture (Hunter et al. 2014), changes in pH and redox gradients and the presence of exudates (Schmidt et al. 2011). In return for creating a suitable niche for

microorganisms, the plant obtains specific nutrients, phytohormones and protection against phytopathogens (Peiffer et al. 2013). As expected, in our experiments the rhizosphere acted as a 'buffer', cushioning the effect of the soil treatments: indeed, the addition of compost, for example, changed the microbial communities in bulk soil, but did not significantly modify rhizosphere composition. Fumigation was the only soil treatment with a significant effect on both bacterial and fungal rhizosphere communities.

In terms of the effects on plants, the lowest growth was observed in U and C soils in both years. As U soil was the untreated control in our experimental design, stunted plant growth was expected. Compost addition was ineffective in relieving ARD symptoms, since in both years plants in C soils had a level of root and shoot growth comparable with the untreated control. Although in some studies the addition of compost has given positive results (Zhang et al. 2012), others did not find any appreciable difference in plant growth (Yao et al. 2006). In our trials, the addition of compost did not relieve ARD symptoms, but its addition significantly changed the microbial communities in bulk soil. This change may be irrelevant for expression of the ARD syndrome or insufficient to prevent it, because being confined to bulk soil, it could not reach the rhizosphere communities.

Although *T. atroviride* SC1 is a good biocontrol agent, which has been successfully used in soil to reduce the incidence of *Armillaria* spp. (Pellegrini et al. 2014) and *R. necatrix* (Pasini et al. 2016), it did not restore plant growth in ARD-affected soil samples in our trials. The addition of *T. atroviride* SC1 indeed modified fungal community composition in the treated soils, with a substantial increase in Hypocreales (Fig. 3b), which indicates that *T. atroviride* SC1 filled in the available ecological niches in the soil during the timeframe of the experiment. However, the biocontrol treatment did not reduce ARD symptoms, indicating that fungal pathogens were not the main cause of ARD or that the biocontrol agent was not able efficiently antagonise possible microbial agents of ARD.

The washing treatment was designed to test the hypothesis that hydrosoluble autotoxic compounds are a possible cause of ARD. In 2013, this was the treatment that reduced ARD symptoms most, but not in 2014. On the other hand, fumigation, which was used in our trials to test whether soil microorganisms were responsible for ARD, was the most successful treatment in 2014. Fumigation had a highly significant effect on bulk soil

microbial alpha and beta diversity in both years. In addition, CFU counts detected a significant increase in cultivable bacteria in the soil communities of F soils after soil recolonisation by the microorganisms surviving fumigation or introduced afterwards. Both rhizosphere and bulk soil microbial communities changed in F soils in a similar way in 2013 and 2014. The positive effect of fumigation against ARD symptoms observed in 2014 has been confirmed by many previous studies (Braun et al. 2010; Mai and Abawi 1981; Nicola et al. 2017). However, this effect is often inconsistent (Yao et al. 2006), as we noticed in the experiment in 2013, when fumigation did not improve apple plant growth in ARD-affected soil. The fact that fumigation was not effective during 2013, while the washing treatment was, could suggest that microorganisms may not be the direct causal agents of ARD. The presence of autotoxic compounds could be involved in ARD. For example phlorizin is one of the main phenolic compounds released both by living and dead apple roots in soil (Hofmann et al. 2009; Nicola et al. 2016). This water-soluble compound was found, together with other polyphenols, in the soil from which apple trees were explanted (Yin et al. 2016b) and has an autotoxic effect against new apple plants (Nicola et al. 2016). Further research should clarify whether the presence of toxins in soil after explanting can trigger the onset of ARD in the orchard. Indeed, in 2013, the success of the washing treatment could indicate the involvement of autotoxins or other damaging substances present in the soil after the removal of trees in the previous year. Over time, the soil microbial communities in explanted soils may also start to change, due to modifications in the metabolic environment, and their negative influence on plants may grow stronger, hence the success of fumigation in 2014. Future studies should focus on the metabolic impact of old apple trees on the soil and also on the function changes in soil microbial communities and not only on the taxonomy.

In bulk soil, a number of bacteria found in the literature to be involved in soil mineral cycles or to be associated with biocontrol or plant growth promotion were found to be highly correlated with total aerial mass. For example, the *Pseudoxanthomonas* genus is known for its nitrogen-fixing activity, and some strains can produce siderophores and have ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity, all indications of its potential plant growth promotion ability (Castellano-Hinojosa et al. 2016). Three genera belonging to Sphingomonadaceae were also found to be associated with enhanced plant growth. The first, *Sphingomonas* sp., was also found in healthy soil in another

study on ARD (Yim et al 2015) and several strains belonging to this genus showed biocontrol activity against the pathogen *Pseudomonas syringae* (Innerebner et al. 2011). *Sphingobium* sp. can inhibit the pathogen *Pythium in vitro* (Burgos-Garay et al. 2014), and was found to have antagonistic potential against *Fusarium oxysporum* (Fu et al. 2017)together with *Dyadobacter*, another positively correlated genus found in this work, known as PGPR (Debode et al. 2016). The *Sphingopyxis* genus, which can produce high concentrations of auxins (Dias et al. 2009), can act as a biocontrol agent against *F. oxysporum,* together with *Bosea* sp. (Fujiwara et al. 2016), a bacterium with nitrogen-fixing potential (Duque Jaramillo et al. 2013) The sole bacterium in the rhizosphere found to be positively correlated with growth was *Nocardioides* sp., acting as a biocontrol agent against the pathogen *Gaeumannomyces graminis* (Coombs et al. 2004). Surprisingly, *I. macrodydima*, usually associated with black foot disease in the grapevine (Agusti-Brisach et al. 2014), was the only fungal OTU that was positively correlated with growth in our experiment, suggesting that this particular strain had no pathogenic effect on apple plants in the tested conditions. The only OTU found to be negatively correlated with growth was *Salinibacterium* sp., an actinobacterium whose relationship with plants is still unknown. However, this study also underlined the limitations of this technique, and further research using techniques like shot-gun sequencing or transcriptomics for example, could clarify the role of OTUs correlating with increased growth of plants in metabarcoding studies on ARD.

In conclusion, our work confirmed the resilience of soil microbial communities to perturbing factors; indeed, apart from pre-plant fumigation, which modified both bacteria and fungi in bulk soil and the rhizosphere, the other soil treatments did not cause major changes. Variability was noticed between the two years in terms of the effect on plant growth, but this could not be associated with any significant changes in the microbial communities. This result suggests that although microorganisms may have a role in ARD development, there could be other contributing factors, for example the presence of autotoxic compounds in soil, especially close to the time of explanting, which could in turn influence the metabolism of microbial populations. To better clarify this hypothesis, further research should focus on continuous monitoring of both the metabolic and microbiological profile of ARD-affected soil from explanting onwards, to understand the dynamics of interaction between the biotic and abiotic soil components that could lead to ARD symptoms.

References

Agusti-Brisach, C., Mostert, L., and Armengol, J. 2014. Detection and quantification of Ilyonectria spp. associated with black- foot disease of grapevine in nursery soils using multiplex nested PCR and quantitative PCR. Plant Pathol. 63:316-322.

Albanese, D., Fontana, P., De Filippo, C., Cavalieri, D., and Donati, C. 2015. MICCA: a complete and accurate software for taxonomic profiling of metagenomic data. Scientific Reports 5.

Börner, H. 1959. The apple replant problem. I. The excretion of phlorizin from apple root residues. Contrib. of the Boyce Thompson Inst. 20:39-56.

Braun, P. G. 1991. The combination of *Cylindrocarpon lucidum* and *Pythium irregulare* as possible cause of apple replant disease in Nova Scotia. Can. J. Plant Pathol. 13:291-297.

Braun, P. G., Fuller, K. D., McRae, K., and Fillmore, S. A. E. 2010. Response of 'Honeycrisp (R)' Apple Trees to Combinations of Pre-plant Fumigation, Deep Ripping, and Hog Manure Compost Incorporation in a Soil with Replant Disease. Hortscience 45:1702-1707.

Bulgarelli, D., Rott, M., Schlaeppi, K., van Themaat, E. V. L., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F. O., Amann, R., Eickhorst, T., and Schulze-Lefert, P. 2012. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. Nature 488:91-95.

Burgos-Garay, M. L., Hong, C., and Moorman, G. W. 2014. Interactions of Heterotrophic Bacteria from Recycled Greenhouse Irrigation Water with Plant Pathogenic Pythium. Hortscience 49:961-967.

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. 2010. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26:266-267.

Castellano-Hinojosa, A., Correa-Galeote, D., Palau, J., and Bedmar, E. J. 2016. Isolation of N-2-fixing rhizobacteria from *Lolium perenne* and evaluating their plant growth promoting traits. J. Basic Microb. 56:85-91.

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Brown, C. T., Porras-Alfaro, A., Kuske, C. R., and Tiedje, J. M. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42:D633-D642.

Coombs, J. T., Michelsen, P. P., and Franco, C. M. M. 2004. Evaluation of endophytic actinobacteria as antagonists of *Gaeumannomyces graminis* var. tritici in wheat. Biol. Control 29:359-366.

Corneo, P. E., Pellegrini, A., Cappellin, L., Roncador, M., Chierici, M., Gessler, C., and Pertot, I. 2013. Microbial community structure in vineyard soils across altitudinal gradients and in different seasons. Fems Microbiol. Ecol. 84:588-602.

Debode, J., De Tender, C., Soltaninejad, S., Van Malderghem, C., Haegeman, A., Van der Linden, I., Cottyn, B., Heyndrickx, M., and Maes, M. 2016. Chitin Mixed in Potting Soil Alters Lettuce Growth, the Survival of Zoonotic Bacteria on the Leaves and Associated Rhizosphere Microbiology. Front. Microbiol. 7.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., and Andersen, G. L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microb. 72:5069-5072.

Dias, A. C. F., Costa, F. E. C., Andreote, F. D., Lacava, P. T., Teixeira, M. A., Assumpcao, L. C., Araujo, W. L., Azevedo, J. L., and Melo, I. S. 2009. Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. World J. Microb. Biot. 25:189-195.

Duque Jaramillo, P. M., Guimaraes, A. A., Florentino, L. A., Silva, K. B., Abrahao Nobrega, R. S., and de Souza Moreira, F. M. 2013. Symbiotic nitrogen-fixing bacterial populations trapped from soils under agroforestry systems in the Western Amazon. Sci. Agric. 70:397-404.

Forge, T., Neilsen, G., Neilsen, D. 2016. Organically acceptable practices to improve replant succes of temperate tree-fruit crops. Sci. Hort. 200: 205-214.

Fu, L., Penton, C. R., Ruan, Y., Shen, Z., Xue, C., Li, R., and Shen, Q. 2017. Inducing the rhizosphere microbiome by biofertilizer application to suppress banana *Fusarium* wilt disease. Soil Biol. Biochem. 104:39-48.

Fujiwara, K., Iida, Y., Someya, N., Takano, M., Ohnishi, J., Terami, F., and Shinohara, M. 2016. Emergence of Antagonism Against the Pathogenic Fungus *Fusarium oxysporum* by Interplay Among Non-Antagonistic Bacteria in a Hydroponics Using Multiple Parallel Mineralization. J. Phytopathol. 164:853-862.

Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113-118.

Gower, J. C., and Blasius, J. 2005. Multivariate prediction with nonlinear principal components analysis: Theory. Qual. Quant. 39:359-372.

Hammer, O., Harper, D. A. T., and Ryan, P. D. 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4.

Hiltner, L. 1904. Über neuere Erfahrungen und Probleme auf dem Gebieteder Bodenbakteriologie unter besonderer Berücksichtigung der Gründüngungund Brache. ArbDLG 98:59-78.

Hofmann, A., Wittenmayer, L., Arnold, G., Schicber, A., and Merbach, W. 2009. Root exudation of phloridzin by apple seedlings (Malus x domestica Borkh.) with symptoms of apple replant disease. J. Appl. Bot. Food Qual. 82:193-198.

Hunter, P. J., Teakle, G. R., and Bending, G. D. 2014. Root traits and microbial community interactions in relation to phosphorus availability and acquisition, with particular reference to *Brassica*. Front. Plant Sci. 5.

Innerebner, G., Knief, C., and Vorholt, J. A. 2011. Protection of Arabidopsis thaliana against Leaf-Pathogenic *Pseudomonas syringae* by *Sphingomonas* Strains in a Controlled Model System. Appl. Environ. Microb. 77:3202-3210.

Jaffee, B. A., Abawi, G. S., and Mai, W. F. 1982. Role of soil microflora and Pratylenchus penetrans in an Apple Replant Disease. Phytopathol. 72:247-251.

Koljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., Bates, S. T., Bruns, T. D., Bengtsson-Palme, J., Callaghan, T. M., Douglas, B., Drenkhan, T., Eberhardt, U., Duenas, M., Grebenc, T., Griffith, G. W., Hartmann, M., Kirk, P. M., Kohout, P., Larsson, E., Lindahl, B. D., Luecking, R., et al. 2013. Towards a unified paradigm for sequence-based identification of fungi. Mol. Ecol. 22:5271-5277.

Lozupone, C., and Knight, R. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microb. 71:8228-8235.

Mai, W. F., and Abawi, G. S. 1981. Controlling Replant Diseases of Pome and Stone Fruits in Northeastern United-States by Preplant Fumigation. Plant Dis. 65:859-864.

Mandelbaum, R., and Hadar, Y. 1990. Effects of available carbon source on microbial activity and Suppression of *Pythium aphanidermatum* in compost and peat container media Phytopathol. 80:794-804.

Manici, L. M., Caputo, F., and Babini, V. 2004. Effect of green manure of *Pythium* spp. population and microbial communities in intensive cropping systems. Plant Soil 263:133-142.

Manici, L. M., Ciavatta, C., Kelderer, M., and Erschbaumer, G. 2003. Replant problems in South Tyrol: role of fungal pathogens and microbial population in conventional and organic apple orchards. Plant Soil 256:315-324.

Manici, L. M., Kelderer, M., Franke-Whittle, I. H., Ruehmer, T., Baab, G., Nicoletti, F., Caputo, F., Topp, A., Insam, H., and Naef, A. 2013. Relationship between root-endophytic microbial communities and replant disease in specialized apple growing areas in Europe. Appl. Soil Ecol. 72:207-214.

Mazzola, M. 1998. Elucidation of the microbial complex having a causal role in the development of apple replant disease in Washington. Phytopathol. 88:930-938.

Mazzola, M., and Manici, L. M. 2012. Apple Replant Disease: Role of Microbial Ecology in Cause and Control. Pages 45-65 in: Annu. Rev. . Phytopathol., Vol 50. N. K. VanAlfen, J. E. Leach and S. Lindow, eds.

McMurdie, P. J., and Holmes, S. 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. Plos One 8.

Muyzer, G., Dewaal, E. C., and Uitterlinden, A. G. 1993. Profiling of Complex Microbial-Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S Ribosomal-RNA. Appl. Environ. Microb. 59:695-700.

Nicola, L., Vrhovsek, U., Soini, E., Insam, H., and Pertot., I. 2016. Phlorizin released by apple root debris is related to apple replant disease. Phytopathol. Mediterr. 55:432-442.

Nicola, L., Turco, E., Albanese, D., Donati, C., Thalheimer, M., Pindo, M., Insam, H., Cavalieri, D., and Pertot., I. 2017. Fumigation with dazomet modifies soil microbiota in apple orchards affected by replant disease. Appl. Soil Ecol. 113: 71-79.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., and Wagner, H. 2013. Vegan: Community Ecology Package. R package version 2.0-10. http://CRAN.R-project.org/package=vegan.

Pasini, L., Prodorutti, D., Pastorelli, S., and Pertot, I. 2016. Genetic Diversity and Biocontrol of *Rosellinia necatrix* Infecting Apple in Northern Italy. Plant Dis. 100:444-452.

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., Buckler, E. S., and Ley, R. E. 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. P. Natl Acad Sci USA110:6548-6553.

Pellegrini, A., Prodorutti, D., and Pertot, I. 2014. Use of bark mulch pre-inoculated with Trichoderma atroviride to control *Armillaria* root rot. Crop Prot. 64:104-109.

Schmidt, H., Eickhorst, T., and Tippkoetter, R. 2011. Monitoring of root growth and redox conditions in paddy soil rhizotrons by redox electrodes and image analysis. Plant Soil 341:221-232.

Survey Staff, 2010. Keys to Soil Taxonomy, 11th ed. USDA-Natural Resources Conservation Service, Washington DC.

von Glisczynski, F., Sandhage-Hofmann, A., Amelung, W, Pude, R. 2016. Biochar-compost substrates do not promote growth and fruit quality of a replanted german apple orchard with fertile haplic luvisol soils. Sci. Hort. 213: 110-114

Wang, Q., Hu, Y., Zhou, H., Zhan, X., Mao, Z., and Zhu, S. 2012. Effects of phloridzin on the tricarboxylic acid cycle enzymes of roots of Malus hupehensis Rehd. Sci. Agric. Sin. 45:3108-3114.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. 1991. 16S Ribosomal DNA Amplification for Phylogenetic Study. J. Bacteriol. 173:697-703.

White, T., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315–322 in: PCR Protocols: a guide to methods and applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, eds. Academic Press, New York, USA.

Yao, S. R., Merwin, I. A., Abawi, G. S., and Thies, J. E. 2006. Soil fumigation and compost amendment alter soil microbial community composition but do not improve tree growth or yield in an apple replant site. Soil Biol. Biochem. 38:587-599.

Yim, B., Winkelmann, T., Ding, G. C., and Smalla, K. 2015. Different bacterial communities in heat and gamma irradiation treated replant disease soils revealed by 16S rRNA gene analysis - contribution to improved aboveground apple plant growth? Front. Microb. 6.

Yin, C. M., Xiang, L., Wang, G. S., Wang, Y. F., Shen, X., Chen, X. S., and Mao, Z. Q. 2016a. How to Plant Apple Trees to Reduce Replant Disease in Apple Orchard: A Study on the Phenolic Acid of the Replanted Apple Orchard. Plos One 11.

Yin, C. M., Hu, Y. L., Wang, G. S., Zhang, X. F., Zhou, H., Shen, X., Chen, X. S., and Mao, Z. Q. 2016b. Effect of main phenolic acids of the apple replanted soil on the roots of *Malus hupehensis* Rehd. seedlings. Sci. Agric. Sin. 49:961-969.

Zhang, Z. B., Chen, Q., Yin, C. M., Shen, X., Chen, X. S., Sun, H. B., Gao, A. N., and Mao, Z. Q. 2012. The effects of organic matter on the physiological features of *Malus hupehensis* seedlings and soil properties under replant conditions. Scientia Horticulturae 146:52-58.

IV. Paper 3

Meta-analysis of microbiomes in soils affected by Apple Replant Disease

Meta-analysis of microbiomes in soils affected by Apple Replant Disease

Lidia Nicola[a,b]*, Heribert Insam[b], Ilaria Pertot[a], Blaz Stres[c]

[a]Research and Innovation Centre, Fondazione Edmund Mach (FEM), 38010 San Michele all'Adige (TN), Italy

[b] Institute of Microbiology, University of Innsbruck, Innsbruck, Austria

[c] Group for Microbiology and Microbial Biotechnology, Department of Animal Science, University of Ljubljana, Ljubljana, Slovenia

* Corresponding author.
Email address: lidia.nicola@fmach.it (L. Nicola).

Manuscript submitted to Plant and Soil

Abstract:

Aims. The aim of this study was to identify the presumed microbiological drivers in soils affected by apple replant disease (ARD) using a meta-analytical approach.

Methods. Based on a literature search on the available deep-sequencing studies on ARD-affected soil microbiomes, data on environmental variables and molecular techniques were extracted, together with the raw sequencing data from public databases. The sequencing datasets of bacteria and fungi were analyzed using a taxonomic approach in mothur, using SILVA and UNITE databases, respectively.

Results. Both bacterial and fungal communities in ARD-affected soils had a significantly different structure and were genetically different from those in healthy soils. A pool of co-occurring microbial OTUs was also identified in ARD-affected soils. For bacteria, most of the genetic variability was explained with the available meta-data; however, the different molecular methods accounted for 25% of the variability. For fungi, instead, most of the variability remained unexplained and was not influenced by the differences in molecular methods among the studies. The variables that affected most the microbial communities were the presence of ARD, the soil treatments and the plant rootstock.

Conclusions. This meta-analysis indicated that ARD might be defined as an opportunistic microbial infectious disease, created by certain prevailing environmental conditions affecting microbial metabolism.

Keywords: apple replant disease, soil, microbial communities, Next Generation Sequencing.

1. Introduction

One gram of soil can contain from 10^3 to 10^7 bacterial species (Schloss and Handelsman 2006) and soil is one of the most complex and diverse habitats. Uncovering soil microbial diversity has always been a challenge, due to the sheer abundance of species, the fact that just a small percentage of them is cultivable, and to the intrinsic physicochemical properties of the soil matrix, all interacting with environmental parameters. To provide insight into complex microbial networks in these systems, studies over the past ten years have routinely adopted Next-Generation Sequencing (NGS).

This has led to the accumulation of studies presenting data produced with different platforms (e.g. Ion torrent, Roche 454 and Illumina), using various DNA extraction procedures and experimental conditions (Bonilla-Rosso et al. 2012; Claesson et al. 2010; Inceoglu et al. 2010). In addition, distinct bioinformatic approaches (e.g. MG-RAST, QIIME) with their inherent biases could lead to contrasting results and interpretations (D'Argenio et al. 2014). These limitations can hamper a wide comparison and generalization of the results of available studies. The study of the soil microbiome in replanted apple orchards and accompanying apple replant disease (ARD) can be an example of this set of problems.

Apple replant disease is a complex syndrome that causes reduced growth and production in apple trees that are replanted in the same soil. Moreover, plants show shortened internodes, discoloured roots, root tip necrosis and a reduction in root biomass, which can lead to plant death within the first growing season (Mazzola and Manici 2012). The reduction in growth and production caused by ARD may decrease profitability up to 50% throughout the life cycle of the orchard (van Schoor et al. 2009). The disease aetiology is not yet clear, but the most endorsed hypothesis is a change in soil microbial communities (Mazzola and Manici 2012). In general, there are four approaches to the role of the microbiota present in orchard soils and their effect on the development of ARD. The first approach is the "specific ARD hypothesis". It states that only a small part of the microbiota found in the soil is involved in diseases. However, problems of this hypothesis are diseases which also arise in the absence of these pathogenic microorganisms or that no diseases occur despite their presence. For example, fungal species belonging to the *Cylindrocarpon*, *Rhizoctonia*, *Phytophthora* and *Pythium* genera are frequently found in ARD-affected soils, but their presence and frequency can

vary from soil to soil (Tewoldemedhin et al. 2011a; Tewoldemedhin et al. 2011b). In analogy the dental plaques hypotheses (Marsh, 1994), the "non-specific ARD hypothesis" states that many different micoorganisms in soil are responsible for the development of ARD. These diseases in turn are the result of the interaction between the microorganisms in the soil and the host. The "ecological ARD hypothesis" supposes an equilibrium shift of certain key factors for the development of the disease; that microbial pathogens are only a small part of the resident microbiota supports this hypothesis. The recent "keystone-pathogen-hypothesis" (Rosier et al. 2014) proposes an interaction of one key player with the host, triggering a host response that makes it more susceptible to a number of other pathogens.

The advent of NGS helped understanding better the microbiome of soils in ARD apple orchards and this technology was used in several studies, targeting fungi and bacteria and, mainly, investigating the effect of different soil treatments on ARD severity and microbial communities (Mazzola et al. 2015; Nicola et al. 2017; Yim et al. 2015). However, comparing the end-point results of these studies is difficult, since different approaches and pipelines were used in sequence generation and analysis making it hard to spot a definite trend that could help defining the aetiology of ARD.

In this meta-analysis, we collected and integrated all the studies on ARD that used NGS and analyzed their raw sequences together and this inclusive procedure allowed to elucidate the main microbiological drivers in ARD-affected soils. In addition, we also inferred on the role of different environmental conditions, locations or molecular methods on the variability of microbial communities in the soil.

2. Materials and methods

2.1 Search and selection of relevant literature and data extraction

A literature search was performed on Web of Knowledge database, looking for all the studies on apple replant disease that analyzed the soil microbiome through metagenomics approaches. The combinations of key words used for the search were: "apple replant disease" AND "soil" AND "microbiome", "apple replant disease" AND "soil" AND "454", "apple replant disease" AND "soil" AND "Illumina". On May 3, 2016 the literature search, using these keywords, produced a total of six hits (Online Resource 1). After

omitting abstracts from conferences, studies on plants other than apple and studies that did not report on amplicon-metagenomic datasets, four studies investigating bacterial or fungal communities in ARD-affected soils were retained. Two additional studies produced by the authors of this meta-analysis, but not yet published at the time, were included, since they met the inclusion criteria. The following information was extracted from these six studies: soil niche (bulk soil or rhizosphere), study type, rootstock, soil treatments, number of orchards sampled, number of samples, duration of the experiment, health of the plant, year and season of sampling, location, global positioning system (GPS) coordinates , altitude, soil texture, pH, organic matter, DNA extraction method, amplified region, primers used, sequencing technique, sequencing depth, software used for data analysis and number of reads after denoising. The samples were also categorized as healthy or diseased based on the growth of the apple tree: samples taken from ARD-affected soils whose apple trees experienced stunted growth were considered diseased (reduction of growth: RG), while if there was a statistically significant increase in growth compared these soils, those samples were considered healthy (H).

2.2 Sequence analysis

Sequence files from past publications were obtained from the NCBI Sequence Read Archive database (SRA). The 64-bit version of the source code SRA Toolkit for Ubuntu Linux version 2.6.0 were downloaded from NCBI and used to programmatically access data housed within SRA and convert it from the SRA format to fastq or sff formats. The SRA Toolkit contains a series of independent data-"dump" utilities that allow for conversion of SRA data into different file formats. Fastq-dump was used to convert data to fastq and fasta format.

Some of the datasets resulted in an interleaved distribution of sequences. In order to be able to parse the mixed datasets into sample specific data files described in published papers (Franke-Whittle et al. 2015; Mazzola et al. 2015; Yim et al. 2015), authors were asked to submit a design file of their experiment. The lack of design file prevented the use of interleaved sequence mix, therefore the study by Sun et al. (2014) had to be omitted from downstream analyses.

Data sets containing bacterial and fungal sequences were grouped and separated. A tab-delimited mapping file describing experimental conditions of each study was created from the published literature.

Sequences were analyzed using mothur (Schloss et al., 2009) with the SILVA (Quast et al. 2013) and UNITE (Koljalg et al. 2013) databases. Phylotype approach was used in order to minimize the bias in alignment or binning steps of bacterial and fungal sequences due to different length or gene section. Sample analysis scripts were generalized between studies in order to minimize differences in bioinformatics approaches. The resulting *.taxonomy and *.count_table files were merged and served as basis for generation of *.shared files specific for bacteria and fungi. Bacterial and fungal samples were subsampled to 2500 sequences, omitting the samples with fewer sequences (n_B=132, n_F=68). To cover the developed approach a novel utility was suggested to the developers of the program and was just recently integrated (mothur v.1.39.1 - the February 2017 release of mothur).

2.3 Statistical analysis

Bacterial and fungal datasets were analyzed separately in mothur. Initially, alpha diversity estimates (rarefaction curves, various parametric and non-parametric diversity indices) were calculated in order to compare microbial diversity within samples and to check whether the sequencing depth was sufficient.

The beta (between-sample) diversity analyses were used to test for differences in structure of microbial communities in terms of phylogenetic composition parsimony, (unweighted UNIFRAC), abundance of particular clades (weighted UNIFRAC), analysis of molecular variance (AMOVA), homogeneity of molecular variance (HOMOVA). Lefse, random forest and other approaches were used to test for the congruency in sample assignment, i.e. classification efficiency of samples to healthy-diseased classes. Metastats, instead, was used to identify the taxa that were differentially abundant between different H-RG classes ($p<0.05$, after False Discovery Rate correction).

OTU association networks (nonlinear Spearman association, $p<0.005$, and $R^2>0.8$) was used to identify taxa that were positively or negatively associated with each other across groups of samples.

This resulted in four tables of tightly associated microbial taxa (Bacteria-H, Bacteria-RG; Fungi-H, Fungi-RG) that were further analyzed in Cytoscape (V3.2.0) and the characteristics of their co-occurrence networks were recorded to disentangle the difference between healthy-diseased classes.

In order to provide the overall insight into the extent of explained variance of bacterial or fungal microbial community structure, information pertaining experimental setup, soil characteristics, sample distribution, geographic location were all agglomerated from published literature. The assembled datasets were analyzed using variation partitioning as implemented in R (vegan, version 2.4-1).

3. Results

3.1 Literature search results and characteristics of the eligible studies

The list of studies taken into consideration and their characteristics are outlined in the Supplementary Materials (Online Resource 2 and 3, respectively). The studies were conducted between 2011 and 2014 in the state of Washington (USA), in Germany, Austria and Italy, where soil samples from replanted apple orchards were studied. Three were field studies, while the other three took soil from orchards to set up bioassays in the greenhouse. All of the included studies focused on soil bacterial communities (n=140 samples in total), while three studies provided also data on fungi (n=73 samples in total).

3.2 Sequence analysis

3.2.1 Alpha (within sample) diversity

The metagenomic analysis of the sequences with taxonomic binning resulted in a total of 1,747,545 reads for bacteria, with samples from 42 to 49,928 reads, and 578,450 reads for fungi, with samples from 10 to 45,799 reads. Since the great diversity in number of reads in the samples after filtering and in order to compare them, we subsampled at 2500 reads both bacterial and fungal samples, entailing a loss of 8 bacterial and 5 fungal samples [in both cases from Mazzola et al. (2015)]. The rarefaction curves of bacterial and fungal samples showed that, subsampling at 2500 reads, the number of OTUs was getting close to the plateau (Fig. 1 A and B). The richness and

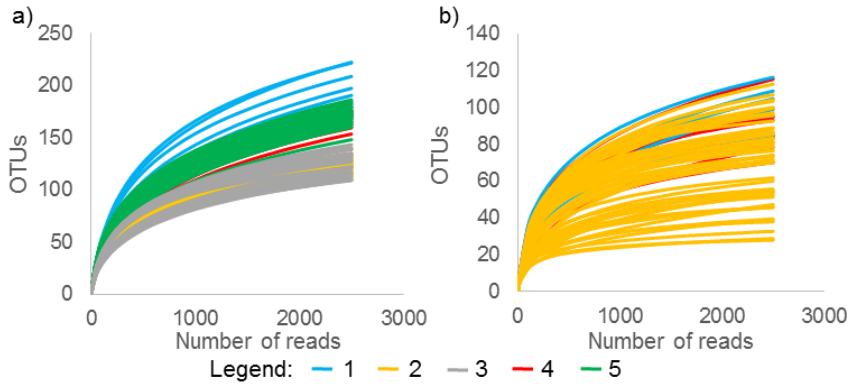


Fig. 1 Bacterial (a) and fungal (b) rarefaction curves at the subsampling threshold (2500 reads). Abbreviations: 1= Franke-Whittle et al (2015); 2= Mazzola et al. (2015); 3= Yim et al. (2015); 4= Nicola et al. (2017); 5= Peruzzi et al. (2017).

evenness of the samples were strongly related to the study they came from: the studies by Franke-Whittle et al. (2015) and Peruzzi et al. (2017) had the highest bacterial richness, while those of Mazzola et al. (2015) and Yim et al. (2015) produced the lowest. In fungal samples, the richness and evenness varied also within the studies. For bacteria, samples with a reduction of growth (RG) had a significantly lower Inverse Simpson Index, meaning a less diverse community, while no differences were noticed for fungi. The most abundant bacterial phyla in soil were Proteobacteria (35%), Actinobacteria (16%), Bacteroidetes (14%) and Acidobacteria (7%), while the fungal communities were dominated by Ascomycota (64%) and Basidiomycota (9%).

3.2.2 Beta (between sample) diversity

As regards beta diversity, the data presented some structure, as shown in Online Resource 4, clustering according to the study where they came from. The same data were subjected to several statistical tests in order to understand if the microbial communities differed according to the soil health state. For both bacteria and fungi, RG samples had a community structure that was significantly different from that in healthy samples (Parsimony: $p<0.05$) and the communities in the two groups were genetically different (AMOVA: $p<0.05$). Bacterial communities in RG soils had also a significantly higher

variation compared to communities in healthy samples (HOMOVA: $p<0.05$), but this trend was not detectable in fungal communities. Using the Random Forest algorithm, 106 bacterial samples out of 140 and 53 fungal samples out of 73 were correctly assigned according to the RG-H design, with an error rate of 0.20 and 0.22, respectively, but it appears more likely to assign H samples correctly rather than RG samples, in both bacteria and fungi (Table 1).

Table 1: Classification of the microbial samples following the H-RG design, according to the Random-Forest algorithm. The assignment error rate for bacteria was 0.20, for fungi was 0.22. H = healthy samples; RG = ARD-affected samples with reduction of growth.

	Bacteria		**Fungi**	
	H	**RG**	**H**	**RG**
H	53	6	38	4
RG	20	53	11	15

3.3.3 Differentially abundant taxa between healthy and diseased

Thanks to the metastats algorithm in mothur, a group of bacteria involved in the nitrogen cycle (*Opitutus* sp., *Bradyrhizobium* sp., and *Rhodanobacter* sp.) was significantly more abundant in healthy soils (Table 2), while a high number of other microbial genera was significantly more abundant in RG soils, among them some plant pathogens (*Chitinophaga* sp., *Acidovorax* sp., *Podosphaera* sp., *Volutella* sp., *Neonectria* sp., *Lecythophora* sp.; Table 3 and 5). In those soils, also many nematophagous microorganisms thrived (*Microbacterium* sp., *Purpureocillium lilacinum*, *Pochonia chlamydosporia*, *Malassezia restricta*, *Malassezia globosa*; Table 3 and 5).

Table 2: Bacterial OTUs found significantly more abundant in H soils (t statistics). P-values underwent a Benjamini-Yekutieli correction for multiple comparisons. Only OTUs with an abundance greater than 0.05 and identified at genus level are included in this list.

OTUs	**Relative abundance (%)**	**p-value**
Solirubrobacter sp.	0.17	0.03
Opitutus sp.	0.13	0.03
Bradyrhizobium sp.	0.12	0.03
Rhodanobacter sp.	0.07	0.03
Caulobacter sp.	0.07	0.03

Table 3: Bacterial OTUs found significantly more abundant in RG soils (t statistics). P-values underwent a Benjamini-Yekutieli correction for multiple comparisons. Only OTUs with an abundance greater than 0.05 and identified at genus level are included in this list.

OTUs	**Relative abundance (%)**	**p-value**
Flavobacterium sp.	3.55	0.03
Nitrospira sp.	2.72	0.03
Candidatus Chloroacidobacterium sp.	1.77	0.03
Flexibacter sp.	0.84	0.03
Arthrobacter sp.	0.70	0.03
Methylibium sp.	0.54	0.03
Humicoccus sp.	0.48	0.03
Mycobacterium sp.	0.46	0.03
Microlunatus sp.	0.45	0.03
Pedobacter sp.	0.45	0.03
Variovorax sp.	0.43	0.03
Steroidobacter sp.	0.42	0.03
Microbacterium sp.	0.41	0.03
Planctomyces sp.	0.40	0.03

Continues on next page >

> Continues from previous page

OTUs	Relative abundance (%)	p-value
Duganella sp.	0.34	0.05
Pirellula sp.	0.34	0.03
Massilia sp.	0.32	0.03
Marmoricola sp.	0.31	0.03
Polaromonas sp.	0.29	0.03
Iamia sp.	0.26	0.03
Nocardioides sp.	0.23	0.03
Chryseobacterium sp.	0.19	0.03
Pseudonocardia sp.	0.14	0.03
Agromyces sp.	0.13	0.03
Chitinophaga sp.	0.12	0.03
Blastococcus sp.	0.10	0.03
Friedmanniella sp.	0.10	0.03
Fluviicola sp.	0.07	0.03
Aeromicrobium sp.	0.07	0.03
Aquicella sp.	0.07	0.03
Arenimonas sp.	0.06	0.03
Ideonella sp.	0.06	0.03
Ilumatobacter sp.	0.06	0.03
Herminiimonas sp.	0.06	0.00
Acidovorax sp.	0.06	0.03
Solitalea sp.	0.06	0.03
Clostridium sp.	0.06	0.03
Sediminibacterium sp.	0.06	0.01

Table 4: Fungal OTUs found significantly more abundant in H soils (t statistics). P-values underwent a Benjamini-Yekutieli correction for multiple comparisons. Only OTUs identified at least at genus level are included in this list.

OTUs	**Relative abundance (%)**	**p-value**
Thermomyces lanuginosus	0.09	0.00
Mastigobasidium sp.	0.03	0.00
Lectera longa	0.02	0.00
Articulospora sp.	0.02	0.01
Auricularia sp.	0.01	0.00
Leucoagaricus nympharum	0.01	0.00
Cryptococcus victoriae	0.01	0.01
Phaeosphaeria sp.	0.01	0.01
Rhizoctonia oryzae	0.003	0.01

Table 5: Fungal OTUs found significantly more abundant in RG soils (t statistics). P-values underwent a Benjamini-Yekutieli correction for multiple comparisons. Only OTUs identified at least at genus level are included in this list.

OTUs	**Relative abundance (%)**	**p-value**
Scutellinia torrentis	0.04	0.01
Purpureocillium lilacinum	0.03	0.00
Pochonia chlamydosporia	0.02	0.01
Cystofilobasidium capitatum	0.02	0.00
Didymosphaeria sp.	0.02	0.00
Malassezia restricta	0.01	0.02
Volutella sp.	0.01	0.00
Podosphaera sp.	0.01	0.00
Coprinellus sp.	0.01	0.02
Scutellinia sp.	0.01	0.01
Acremonium polychromum	0.01	0.00
Entoloma graphitipes	0.01	0.00
Neonectria sp	0.01	0.00
Lecythophora sp.	0.01	0.01

Continues on next page >

> Continues from previous page

OTUs	Relative abundance (%)	p-value
Cryptococcus sp.	0.005	0.02
Malassezia globosa	0.004	0.02
Hyphoderma sp.	0.004	0.00

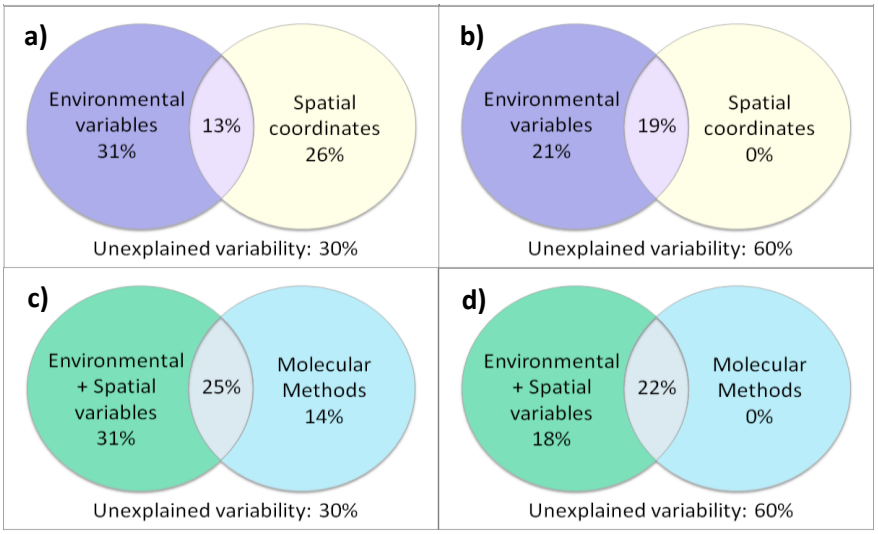


Fig. 2 Variation partitioning for bacterial (a and c) and fungal (b and d) communities, with the standard environmental vs. spatial approach (a and b) and the molecular methods vs. environmental-spatial approach (c and d). The percentages indicate the amount of variability explained by the variables.

3.3.4 Parameters associated with structure of microbial communities

Variation partitioning uncovered that the whole set of environmental parameters collected from every study and the spatial coordinates of the sampling locations explained 70% of the variation in bacterial communities and 40% of that in fungal communities (Fig. 2a and b). The environmental variables alone explained 31% and 21% of variation in bacterial and fungal communities, respectively, while the spatial variables explained 26% and 0%, respectively. The overlaps between environmental and spatial variables were quite consistent (Fig. 2a and b), indicating an inter-dependence of the variables. When analyzing the involvement of molecular methods as

generators of variation against the spatial and environmental variables, the molecular methods explained 25% of variability in bacterial communities, while they did not appear to explain any variability on their own in fungal communities (22% of overlap with spatial and environmental variables; Fig 2c and d). The amount of total explained variability did not vary from the first variation partitioning analysis (70% for bacteria and 40% for fungi) (Fig. 2c and d). The variables that turned out to be non-redundant, shaping the bacterial and fungal communities, were "health" (if the soil was ARD-affected or not), "rootstock" (the type of apple rootstock planted in the soil), and "soil treatment" (the different treatments the soils underwent in order to be relieved by ARD).

3.3.5 Microbial networks

Bacterial soil association network (SAN) in RG soils was more clustered than healthy SAN (clustering coefficient 0.697 *vs.* 0.545), had lower number of connected components (5 *vs.* 8), had lower diameter (network diameter 8 *vs* 11), was 2.5 times more centralized (network centralization 0.285 *vs.* 0.099), contained 39% shorter characteristic path length (2.22 *vs.* 3.61) and a six-fold larger average number of neighbors (42.23 *vs.* 7.29). In addition, the RG network was almost four-fold more dense (network density 0.181 *vs.* 0.049), but less heterogeneous than healthy SAN (network heterogeneity 0.778 *vs.* 0.90). Overall, 33 OTUs were shared between H and RG networks (49% and 24% of the total number of bacterial OTUs in the healthy and diseased network, respectively). The shared OTUs were rearranged in different clusters with different partners, according to the soil health state. For example one cluster in the H network was composed mainly by bacteria involved in the nitrogen cycle and attributed to be plant growth promoting rhizobacteria PGPR (*Nitrosococcus* sp, *Sphingobacterium* sp. *Mesorhizobium* sp.), while in another cluster there was a complex of nitrogen fixing, phosphate solubilizing and plant degrading bacteria (*Derxia* sp., *Cupriavidus* sp., *Agromyces* sp.). In the RG network, the nematophagous bacterium *Microbacterium* sp. and the phytopatogenic *Chitinophaga* sp. were found in the same cluster.

The fungal soil association network (SAN) in RG soils was slightly less clustered than healthy SAN (clustering coefficient 0.407 *vs.* 0.433), had equal number of connected components (7) and network diameter (5), was slightly less centralized than healthy (network centralization 0.261 *vs* 0.235), contained

larger characteristic path length (2.269 *vs.* 1.788) and a slightly lower average number of neighbors (5.073 *vs.* 5.3) than healthy SAN. In addition, the RG network was slightly less dense (0.127 *vs.* 0.136), but more heterogeneous than healthy SAN (network heterogeneity 0.963 *vs.* 0.847). Six OTUs were shared between the clusters n. 1 of H and RG networks (43 % and 46 % of the total number of fungal OTUs in the network, respectively). Among them there was *Cylindrocarpon* sp. a fungal pathogen considered involved in ARD: it was present in both soils, but in RG one it was linked with two other pathogens of apple (*Mortierella* sp. and *Armillaria* sp.).

4. Discussion

4.1 Study characteristics and sequence analysis

In our meta-analysis of deep-sequencing data sets, we analyzed the largest cohort of ARD samples than any study before within a wide range of study locations. The number of data sets on fungi was lower than those on bacteria, even if, historically, fungi were indicated more often as possible causal agents of ARD (Tewoldemedhin et al. 2011a; Tewoldemedhin et al. 2011b). This fact calls for a revision of established experimental protocols guiding studies in the field of ARD to place more emphasis on fungi as well and increase the number of relevant studies including fungal aspects as well.

To assemble the most comprehensive data set, individual data sets with different characteristics were assembled from the three studies focusing on rhizosphere microbial community (Franke-Whittle et al. 2015; Mazzola et al. 2015; Yim et al. 2015) and also from the other two analyzing bulk soil (Nicola et al. 2017, Peruzzi et al 2017). Usually, rhizosphere and bulk soil host different microbial communities (Uroz et al. 2016; van Bruggen et al. 2016), but in our work, given all the different variables taken into consideration, the origin of the samples did not act as a main non-redundant variable, shaping the communities, according to the variation partitioning analysis.

Nowadays, the fast technological development gives more precise measurements each time, but, unfortunately, it is very difficult to compare the results produced by different technologies, which tend to be abandoned too fast to produce a big enough set of data to analyze through a meta-analysis, especially in certain research fields. Thanks to the separate taxonomic binning approach developed in this work, we were able to analyze together sequences

produced by different technologies (Roche 454 and Illumina Miseq). In addition, the developed modular approach enables us to continually add novel datasets to the existing data collection as they become published. This way, the geographical coverage may be extended and the same analytical approach may be re-run allowing consistency.

Applying the same parameters for quality trimming to all data sets produced samples of a wide size range and it forced us to eliminate the smaller samples. This result enlightens the need for a stringent standardization of read quality parameters and bioinformatic analysis pipelines in soil microbial deep-sequencing studies to ensure reproducibility of results across a range of samples, otherwise inclusion and analysis of (too) low quality sequences might pose serious risks, leading to inaccurate interpretations.

4.2 Alpha (within sample) diversity: healthy soils contain more diverse communities

The richness and evenness of the samples depended a lot on the study they came from, a fact that was probably caused by the adoption of different sequencing techniques: for example, Peruzzi et al. (2017), using Miseq Illumina Sequencing, had the highest Inverse Simpson Index values. The Inverse Simpson Index of Bacteria was significantly lower in RG soils than in H soils, suggesting the existence of a less diverse microbial community in RG than H soils. This could mean that a rich and diverse soil microbial community could be a key factor in growing healthy apple trees, contradicting the results of Mazzola et al. (2015). On the other hand, it could be the healthy apple trees that promote a richer and more diverse soil microbial community.

4.3 Beta (between sample) diversity: identification of previously missed microbial pathogens

The fact that both fungal and bacterial communities were significantly different in H and RG soils is a strong indication of a change in the microbial balance. This discovery led to the identification of differentially abundant genera. The identified taxa only partially correspond to the ones found correlated with H or RG soils in their original papers, because widening the perspective onto different types of soil and having a larger sample size allowed us to detect novel trends, too subtle to be detected in any single study.

Among the microorganisms significantly more abundant in H soils, we found bacteria involved in the nitrogen cycle. *Opitutus* sp. can reduce nitrate to nitrite

(Chin et al. 2001) and it was more abundant in soils that underwent reductive disinfestation (RSD), an environmentally friendly and broad-spectrum method to eradicate soil pathogens and favor positive microorganisms (Liu et al. 2016), compared to untreated infested soils. *Bradyrhizobium* sp. is a nitrogen fixing cyanobacterium often found in plant rhizosphere (Hayat et al. 2010) and it is considered to be a PGPR since it can produce phytohormones like auxins and cytokinins (Nizampatnam et al. 2015). Strains of *Rhodanobacter* sp., on the other hand, can perform complete denitrification in soil (Prakash et al. 2012), can act as antagonists to fungal pathogens (De Clercq et al. 2006) and were found as dominant characters in microbial communities in RSD soils (Huang et al. 2016). Since the nitrogen-cycle related bacteria were found to be of particular importance in healthy soils, the lack of these bacteria in RG soils indicates that the excess carbon available from decaying roots might be responsible for a change in nitrogen metabolism of replanted soils.

In RG soils, instead, a group of microbial pathogens was significantly more abundant (*Podosphaera* sp., *Volutella* sp., *Neonectria* sp., *Lecythophora* sp., *Acidovorax* sp., *Chitinophaga* sp.). The fungus *Podosphaera* sp. can cause powdery mildew in apple plants (Baumgartner et al. 2015), while *Volutella* sp., found in soils where *Rosaceae* are grown (Postma et al. 2010), can be a pathogen for several plants, among them boxwood and legumes. *Neonectria* sp. is a ubiquitous soil-borne pathogen with a wide host range, including apple, and it was thought to be a potential causal agent of ARD (Braun 1991; 1995). *Lecythophora* sp. grows commonly in soil and can be a pathogenic agent of soft-rot wood (Hale and Eaton 1985), causing also wood necrosis on *Prunus* (Damm et al. 2010). The bacterium *Acidovorax* sp. can be a pathogen of cucurbits (Zimerman-Lax et al. 2016) and rice (Cui et al. 2016), while *Chitinophaga* sp. was detected in the rhizosphere of diseased wheat plants (Yin et al. 2013) and it was also found associated with the nematode *Acrobeloides maximus* (Baquiran et al. 2013).

In regards to nematophagous microorganisms, RG soils hosted also more abundantly some *Microbacterium* sp., which can be a pathogen of *Caenorhabditis elegans* (Meisel and Kim 2014), *Malassezia globosa* and *Malassezia restricta,* which are associated with the nematode genus *Malenchus* (Renker et al. 2003), and *Purpureumcillium lilacinum* and *Pochonia chlamidospora*, which are considered biological control agents against plant parasitic nematodes (Manzanilla-Lopez et al. 2013; Timper and Parajuli 2012). The presence of these nematophagous microorganisms in RG soils could indicate the increased presence of

nematodes in these soils, a parameter that was beforehand linked with ARD, especially the nematode *Pratylenchus penetrans* (Jaffee et al. 1982), but that was not measured in any of the studies taken into consideration. Future amplicon studies should include also these taxa to capture all microbial kingdoms in one analysis.

4.4 Existing and novel parameters associated with structure of microbial communities

The extent of unexplained variability, especially for fungi (60%), shows that there could well exist additional environmental, chemical and other (e.g. also microbial) parameters that have not been included into analyses or were not effectively measured at appropriate scales but are nevertheless associated with microbial communities, in addition to stochastic events.

This is in line with our observation that many of the studies in the field of ARD did not report on the same set of soil physico-chemical characteristics, neither in the same nor convertible units. This effectively opens a new venue for future consolidation of research, adopting a common practice in future publications. Specifically, the fact that 0% of the variability of fungal communities was explained by either spatial variables or molecular methods alone induced the idea that also the scarcity of fungal samples and the taxonomic fungal databases, still not as rich as the ones for bacteria, had a role in this result. There is also the need to standardize the molecular methods adopted to get the sequencing reads, as quite a large part of variability in bacterial communities (25%) was caused by this factor.

Among the measured variables, "health" (if the soil was ARD-affected or not), "rootstock" (the type of apple rootstock planted in the soil), and "soil treatment" (the different treatments the soils underwent in order to be relieved by ARD) were the most important parameters correlated to the distribution of microbial communities. Although "soil treatment" was quite expected, since treatments like fumigation and heat can easily change the microbial communities (Eo and Park 2014; Yim et al. 2013), the fact that the variable "health" is non redundant is a further confirmation of the effective change in soil microbial communities in ARD-affected soils. The variable "rootstock", quite often not considered much in the studies, can be of renewed importance in future studies, to see if certain rootstocks can create a microbial community that can improve growth in replanted soils.

4.5 Microbial networks: is soil chemistry responsible for different behavior of existing taxa?

Two-way contrasting changes in microbial association networks of bacteria and fungi distinguish RG from H state, where bacterial trends are opposite to those in fungal networks. It seems improbable that the same pathogens would cause ARD through its own acute infection of plants in the field, especially because an identical complex of microbial pathogens was not detected in every ARD-affected soil. Therefore, this could be an indication that ARD is instead caused by a change in association of microbes and a modification of their metabolism, due to changes in extracellular environmental chemistry of soil, caused by decomposition of remaining roots left in place during replant activities, supporting the "ecological ARD hypothesis", where a change in key soil factors alters the equilibrium in soil ecosystem, leading to the disease (Fig. 3). An example that the presence of a certain pathogen does not necessarily lead to effective ARD outburst, is the fungal pathogen *Cylindrocarpon* sp. that was present in both RG and H networks. However, it was associated to two other fungal pathogens of apple in RG soils, *Mortierella* sp. and *Armillaria* sp.. This calls for concerted efforts, linking multifaceted aspects of ARD: microorganisms, soil chemistry, soil physics, nutrients, gene expression, metabolites, etc. Theoretical studies based on mathematical modeling suggested that spatial isolation and carbon resource heterogeneity could limit competition in soils, thereby supporting the high diversity and a more uniform community structure (Carson et al. 2010; Zhou et al. 2002). Understanding additive effects of mechanisms that may control community structure, such as spatial isolation, has important implications for preservation of biodiversity, management of microbial communities for bioremediation, biocontrol of root diseases, and improved soil fertility. Although the parameter of soil connectivity was not reported in any of the ARD studies, it might be of central importance in ARD as low pore connectivity is commonly experienced by soil bacteria under field conditions in soils, whereas the decaying root systems in RG soils represents venues for continuous substrate resource flush, moisture buildup and hence increasing physical connectivity

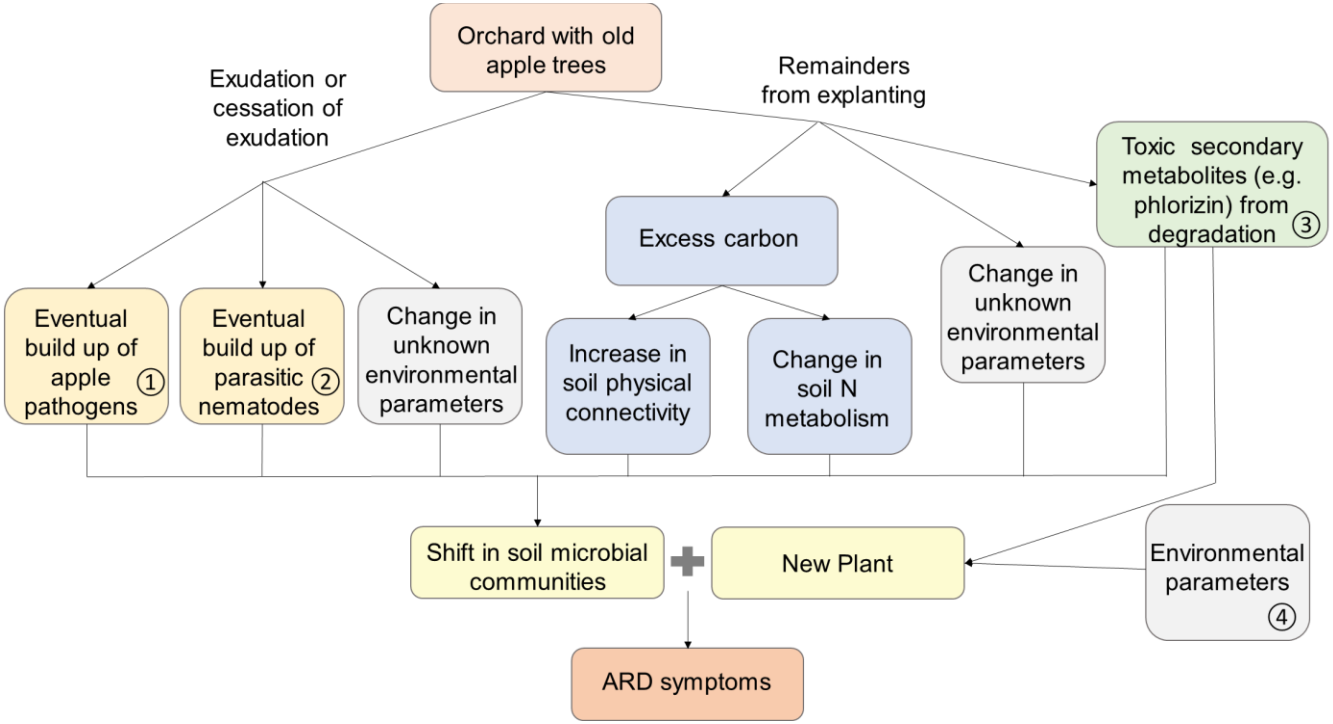


Fig. 3 Suggested cycle leading to apple replant disease (ARD), supported by the ecological ARD hypothesis. ① Tewoldemedhin et al. 2011a, Tewoldemedhin et al. 2011b; ② Jaffee et al. 1982; ③ Nicola et al. 2016, Yin et al. 2016 ④ Hoestra 1968, Huang et al 2013.

4.6 Conclusion

This meta-analysis, thanks to the taxonomic binning approach, managed to analyze together all the deep-sequencing data sets available on microbial communities in soils with a reduction in growth due to ARD, confirming that they host different microbial communities than healthy soils. In addition to a higher presence of phytopathogenic and nematophagous microoganisms in ARD-affected soils, it is interesting to notice also a change in the microbial associations, that can be due to a change in the soil environmental chemistry and metabolome, a parameter deserving renewed interest in future studies, especially now that other powerful tools like metabolomics (liquid state NMR, GC-MS) and metaproteomics are becoming available.

Our results also point to the importance of precise descriptions of soil environments that could provide ground for identification of key soil parameters or metabolites that steer soil microbial communities towards infectious phenotypes. In essence, this endeavour would also help to classify ARD as an opportunistic microbial infectious disease, which is mostly shaped by a complex constellation of appropriate environmental parameters affecting microbial physiology and their mutual interplay, ultimately culminating in plant disease.

Future concerted studies linking information from microbial metagenomes, soil metabolites and soil physico-chemical parameters will thus have the potential to disentangle the causative and associative network of parameters leading to the development of systemic effects that reflect at plant level and are known as ARD.

Acknowledgments

BS was in part supported through visiting professorship awarded by University of Innsbruck, Institute of Microbiology.

LN was supported by a scholarship from Fem International Research School of Trentino (FIRS>T, Edmund Mach Foundation).

Michael Fink and Martin Pöll are acknowledged for their help during the setup of mothur on HPCC mach@uibk.ac.at. The computational results presented have been achieved (in part) using the HPC infrastructure of the University of Innsbruck.

Contributions

Experimental outline: BS; Data assembly: LN; Analyzed the data: BS; Statistical analyses: LN, BS; Drafted the manuscript: LN, BS; Provided ideas, support, intellectual content and resources: LN, BS, HI, IP. All authors contributed with their intellectual input, provided and wrote parts of the manuscript, and agreed on the final version of the manuscript.

References

Baquiran, J. P., Thater, B., Sedky, S., De Ley, P., Crowley, D., Orwin, P. M. 2013. Culture-Independent Investigation of the Microbiome Associated with the Nematode *Acrobeloides maximus*. PLoS ONE 8

Baumgartner, I. O., Patocchi, A., Frey, J. E., Peil, A., Kellerhals, M. 2015. Breeding Elite Lines of Apple Carrying Pyramided Homozygous Resistance Genes Against Apple Scab and Resistance Against Powdery Mildew and Fire Blight. Plant. Mol. Biol. Rep. 33:1573-1583

Bonilla-Rosso, G., Eguiarte, L. E., Romero, D., Travisano, M., Souza, V. 2012. Understanding microbial community diversity metrics derived from metagenomes: performance evaluation using simulated data sets. FEMS Microbiol. Ecol. 82:37-49

Braun, P. G. 1991. The combination of *Cylindrocarpon lucidum* and *Pythium irregulare* as possible cause of apple replant disease in Nova Scotia. Can. J. Plant. Pathol. 13:291-297

Braun, P. G. 1995. Effects of *Cylindrocarpon* and *Pythium* species on apple seedlings and potential role in apple replant disease. Can. J. Plant. Pathol. 17:336-341

Carson, J. K., Gonzalez-Quinones, V., Murphy, D. V., Hinz, C., Shaw, J. A., Gleeson, D. B. 2010. Low Pore Connectivity Increases Bacterial Diversity in Soil. Appl. Environ. Microb. 76:3936-3942

Chin, K. J., Liesack, W., Janssen, P. H. 2001. *Opitutus terrae* gen nov, sp nov, to accommodate novel strains of the division 'Verrucomicrobia' isolated from rice paddy soil. Int. J. Syst. Evol. Micr. 51:1965-1968

Claesson, M. J., Wang, Q.O., O'Sullivan, O., Greene-Diniz, R., Cole, J. R., Ross, R. P., O'Toole, P. W. 2010. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic. Acids. Res. 38

Cui, Z., Ojaghian, M. R., Tao, Z., Kakar, K. U., Zeng, J., Zhao, W., Duan, Y., Cruz, C. M. V., Li, B., Zhu, B. 2016. Multiplex PCR assay for simultaneous detection of six major bacterial pathogens of rice. J. Appl. Microbiol. 120:1357-1367

D'Argenio, V., Casaburi, G., Precone, V., Salvatore, F. 2014. Comparative Metagenomic Analysis of Human Gut Microbiome Composition Using Two Different Bioinformatic Pipelines. Biomed. Res. Int. doi:10.1155/2014/325340

Damm, U., Fourie, P. H., Crous, P. W. 2010. *Coniochaeta* (*Lecythophora*), *Collophora* gen. nov and *Phaeomoniella* species associated with wood necroses of *Prunus* trees. Persoonia 24:60-80

De Clercq, D., Van Trappen, S., Cleenwerck, I., Ceustermans, A., Swings, J., Coosemans, J., Ryckeboer, J. 2006. *Rhodanobacter spathiphylli* sp nov., a gammaproteobacterium isolated from the roots of *Spathiphyllum* plants grown in a compost-amended potting mix. Int. J. Syst. Evol. Micr. 56:1755-1759

Eo, J., Park, K. C. 2014. Effects of dazomet on soil organisms and recolonisation of fumigated soil. Pedobiologia 57:147-154 doi:10.1016/j.pedobi.2014.01.008

Franke-Whittle, I. H., Manici, L. M., Insam, H., Stres, B. 2015. Rhizosphere bacteria and fungi associated with plant growth in soils of three replanted apple orchards. Plant Soil 395:317-333

Hale, M. D., Eaton, R. A. 1985. The ultrastructure of soft root fungi. 1. Fine hyphae in wood cell-walls. Mycologia 77:447-463

Hayat, R., Ali, S., Amara, U., Khalid, R., Ahmed, I. 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. Ann. of. Microbiol. 60:579-598

Hoestra, H. 1968. Replant diseases of apple in the Netherlands. Meded. Landbouwhogeschool Wageningen Nederland 68-13

Huang, L. F., Soing, L. X., Xia, X. J., Mao, W. H., Shi, K., Zhou, Y.H., Yu, J. Q. 2013. Plant-Soil feedbacks and soil sickness: form mechanisms to application in agriculture. J. Chem. Ecol. 39(2): 232-242

Huang, X. Q., Liu, L. L., Wen, T., Zhang, J. B., Wang, F. H., Cai, Z. C. 2016. Changes in the soil microbial community after reductive soil disinfestation and cucumber seedling cultivation. Appl. Microbiol. Biot. 100:5581-5593

Inceoglu, O., Hoogwout, E. F. , Hill, P., van Elsas, J. D. 2010. Effect of DNA Extraction Method on the Apparent Microbial Diversity of Soil. Appl. Environ. Microb. 76:3378-3382

Jaffee, B. A., Abawi, G.S., Mai, W. F. 1982. Role of soil microflora and *Pratylenches penetrans* in apple replant disease. Phytopathology 72:247-251 doi:10.1094/Phyto-72-247

Koljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., Bates, S. T., Bruns, T. D., Bengtsson-Palme, J., Callaghan, T. M., Douglas, B., Drenkhan, T., Eberardt, U., Duenas, M., Grebenc, T., Griffith, G. W., Hartmann, M., Kirk, P. M., Kohout, P., Larsson, E., Lindahl, B. D., Luecking, R., Martin, M. P., Matheny, P. B., Nguyen, N. H., Niskanen, T., Oja, J., Peay, K. G., Peintner, U., Peterson, M., Poldmaa, K., Saag, L., Saar, I., Schuessler, A., Scott, J. A., Senes, C., Smith, M. E., Suija, A., Taylor, D. L., Telleria, M. T., Weiss, M., Larsson, K. H. 2013. Towards a unified paradigm for sequence-based identification of fungi. Mol. Ecol. 22:5271-5277 d

Liu, L. L., Kong, J. J., Cui, H. L., Zhang, J. B., Wang, F. H., Cai, Z. C., Huang, X. Q. 2016. Relationships of decomposability and C/N ratio in different types of organic matter with suppression of *Fusarium oxysporum* and microbial communities during reductive soil disinfestation. Biol. Control. 101:103-113

Manzanilla-Lopez, R. H., Esteves, I., Finetti-Sialer, M. M., Hirsch, P. R., Ward, E., Devonshire, J., Hidalgo-Diaz, L. 2013. *Pochonia chlamydosporia*: Advances and Challenges to Improve Its Performance as a Biological Control Agent of Sedentary Endo-parasitic Nematodes. J. Nematol. 45:1-7

Marsh, P. D. 1994. Microbial ecology of dental plaque and its significance in health and disease. Adv. Dent. Res. 8, 263–271.

Mazzola, M., Hewavitharana, S. S., Strauss, S. L. 2015. Brassica Seed Meal Soil Amendments Transform the Rhizosphere Microbiome and Improve Apple Production Through Resistance to Pathogen Reinfestation. Phytopathology 105:460-469

Mazzola, M., Manici, L. M. 2012. Apple Replant Disease: Role of Microbial Ecology in Cause and Control. In: VanAlfen NK, Leach JE, Lindow S (eds) Annual Review of Phytopathology, Vol 50, vol 50. Annu. Rev. Phytopathol. pp 45-65.

Meisel, J. D., Kim, D. H. 2014. Behavioral avoidance of pathogenic bacteria by *Caenorhabditis elegans*. Trends Immunol. 35:465-470 doi:10.1016/j.it.2014.08.008

Nicola, L., Vrhovsek, U., Soini, E., Insam, H., Pertot, I. 2016. Phlorizin released by apple root debris is related to apple replant disease. Phytopathol. Mediterr. 55(3): 432-442

Nicola, L., Turco, E., Albanese, D., Donati, C., Thalheimer, M., Pindo, M., Insam, H., Cavalieri, D., Pertot, I. 2017. Fumigation with dazomet modifies soil microbiota in apple orchards affected by replant disease. Appl. Soil. Ecol. 113:71-79

Nizampatnam, N. R., Schreier, S. J., Damodaran, S., Adhikari, S., Subramanian, S. 2015. microRNA160 dictates stage-specific auxin and cytokinin sensitivities and directs soybean nodule development. Plant J. 84:140-153

Peruzzi, E., Franke-Whittle, I. H., Kelderer, M., Ciavatta, C., Insam, H. 2017 Microbial indication of soil health in apple orchards affected by replant disease. In publication on Appl. Soil. Ecol.

Postma, J., Scheper, R. W. A., Schilder, M. T. 2010. Effect of successive cauliflower plantings and *Rhizoctonia solani* AG 2-1 inoculations on disease suppressiveness of a suppressive and a conducive soil. Soil. Biol. Biochem. 42:804-812

Prakash, O., Green, S. J., Jasrotia, P., Overholt, W. A., Canion, A., Watson, D. B., Brooks, S. C., Kostka, J. E. 2012. *Rhodanobacter denitrificans* sp nov., isolated from nitrate-rich zones of a contaminated aquifer. Int. J. Syst. Evol. Micr. 62:2457-2462

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glockner, F. O. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids. Res. 41:D590-D596

Renker, C., Alphei, J., Buscot, F. 2003. Soil nematodes associated with the mammal pathogenic fungal genus *Malassezia* (Basidiomycota : Ustilaginomycetes) in Central European forests. Biol. Fert. Soils 37:70-72

Rosier, B. T., De Jager, M., Zaura, E., Krom, B. P, 2014. Historical and contemporary hypotheses on the development of oral diseases: are we there yet? Front Cell Infecti Microbiol 4

Schloss, P. D, Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A, Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thalinger, G. G., Van Horn, D. J., Weber, C. F. 2009. Introducing mothr: open-sorce, platform-indipendent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75(23):7537-7541

Schloss, P. D., Handelsman, J. 2006. Toward a census of bacteria in soil. PLoS Comput. Biol. 2:786-793

Sun, J., Zhang, Q., Zhou, J., Wei, Q. P. 2014. Illumina Amplicon Sequencing of 16S rRNA Tag Reveals Bacterial Community Development in the Rhizosphere of Apple Nurseries at a Replant Disease Site and a New Planting Site. PLoS ONE 9

Tewoldemedhin, Y. T,, Mazzola, M., Botha, W. J., Spies, C. F. J., McLeod, A. 2011a. Characterization of fungi (*Fusarium* and *Rhizoctonia*) and oomycetes (*Phytophthora* and *Pythium*) associated with apple orchards in South Africa. Eur. J. Plant. Pathol. 130:215-229

Tewoldemedhin, Y. T., Mazzola, M., Labuschagne, I., McLeod, A. 2011b. A multi-phasic approach reveals that apple replant disease is caused by multiple biological agents, with some agents acting synergistically. Soil Biol. Biochem. 43:1917-1927

Timper, P., Parajuli, G. 2012. Suppression of *Meloidogyne incognita* by *Paecilomyces lilacinus* is enhanced by planting cover crops. J. Nematol. 44:494-495

Uroz, S., Oger, P., Tisserand, E., Cebron, A., Turpault, M. P., Puee, M., De Boer, W., Leveau, J. H J., Frey-Klett, P. 2016. Specific impacts of beech and Norway spruce on the structure and diversity of the rhizosphere and soil microbial communities. Sci. Rep. 6

van Bruggen, A. H. C., Narouei-Khandan, H. A., Gravel, V., Blok, W. J. 2016. Corky root severity, root knot nematode galling and microbial communities in soil, rhizosphere and rhizoplane in organic and conventional greenhouse compartments. Appl. Soil. Ecol. 100:112-123

van Schoor, L., Denman, S., Cook, N. C. 2009. Characterisation of apple replant disease under South African conditions and potential biological management strategies. Sci. Hortic. 119:153-162

Yim, B., Smalla, K., Winkelmann, T. 2013. Evaluation of apple replant problems based on different soil disinfection treatments-links to soil microbial community structure? Plant Soil 366:617-631

Yim, B., Winkelmann, T., Ding, G. C., Smalla, K. 2015. Different bacterial communities in heat and gamma irradiation treated replant disease soils revealed by 16S rRNA gene analysis - contribution to improved aboveground apple plant growth? Front. Microbiol. 6

Yin, C. M., Yiang, L., Wang, G. S., Wang, Y. F., Shen, X., Chen, X. S., Mao, Z.Q. 2016. How to plant apple trees to reduce replant disease in apple orchards: a study on the phenolic acid of the replanted apple orchard. PLoS ONE 11(12): e0167347

Yin, C. T., Hulbert, S. H., Schroeder, K. L., Mavrodi, O., Mavrodi, D., Dhingra, A., Schillinger, W. F., Paulitz, T. C, 2013. Role of Bacterial Communities in the Natural Suppression of *Rhizoctonia solani* Bare Patch Disease of Wheat (*Triticum aestivum* L.). Appl. Environ. Microb. 79:7428-7438

Zhou, J. Z., Xia, B. C., Treves, D. S., Wu, L. Y., Marsh, T. L., O'Neill, R. V., Palumbo, A. V., Tiedje, J. M. 2002. Spatial and resource factors influencing high microbial diversity in soil. Appl. Environ. Microb. 68:326-334

Zimerman-Lax, N., Shenker. M., Tamir-Ariel, D., Perl-Treves, R., Burdman, S. 2016. Effects of nitrogen nutrition on disease development caused by *Acidovorax citrulli* on melon foliage. Eur J Plant Pathol 145:125-137

Online resources

Meta-analysis of microbiomes in soils affected by Apple Replant Disease

Lidia Nicola[1,2]*, Heribert Insam[2], Ilaria Pertot[1], Blaz Stres[3]

[1]Research and Innovation Centre, Fondazione Edmund Mach (FEM), 38010 San Michele all'Adige (TN), Italy
[2] Institute of Microbiology, University of Innsbruck, Innsbruck, Austria
[3] Group for Microbiology and Microbial Biotechnology, Department of Animal Science, University of Ljubljana, Ljubljana, Slovenia

Online Resource 1:

Result of the literature searches taken place on May 3, 2016 with the key words: "apple replant disease" AND "soil" AND "microbiome", "apple replant disease" AND "soil" AND "454", "apple replant disease" AND "soil" AND "Illumina".

1. Mazzola M, Hewavitharana S, Strauss SL (2015). Brassica seed meal soil amendments transform the rhizosphere microbiome and improve apple production through resistance to pathogen reinfestation. Phytopathology, 105(4):460-469.

2. Mazzola M, Strauss SL (2014) Replant disease control and system resileance to pathogen re-infestation in response to Brassica seed meal amendment. Conference 8th International Symposium on Chemical and Non-Chemical Soil and Substrate Disinfestation Location: Torino (Italy). Book Series: Acta Horticulturae, 1044: 105-122

3. Yim B, Winkelmann T, Ding GC, Smalla K (2015). Different bacterial communities in heat and gamma irradiation treated replant disease soils reveald by 16S rRNA gene analysis - contribution to improved aboveground apple plant growth? Frontiers in Microbiology 6:1224

4. Franke-Whittle IH, Manici LM, Insam H, Stres B (2015). Rhizosphere bacteria and fungi associated with plant growth in soils of three replanted orchards. Plant Soil, 395:317-333.

5. Sun J, Zhang Q, Zhou J, Wei QP (2014). Illumina amplicon sequencing of 16S rRNA tag reveals bacterial community development in the rhizosphere of apple nurseries at a replant disease site and a new planting site. PLOS ONE, 9(10):e111744

6. Yang JI, Rueger PM, McKenry MV, Becker Jo, Borneman J (2012) Correlations between root-associated microorganisms and peach replant disease symptoms in a California soil. PLOS ONE, 7(10):e0046420

Online Resource 2

List of all the papers used in the meta-analysis

1. Franke-Whittle IH, Manici LM, Insam H., Stres B (2015). Rhizosphere bacteria and fungi associated with plant growth in soils of three replanted orchards. Plant Soil, 395:317-333. Bioproject SUB801699.
2. Mazzola M, Hewavitharana S, Strauss SL (2015). Brassica seed meal soil amendments transform the rhizosphere microbiome and improve apple production through resistance to pathogen reinfestation. Phytopathology, 105(4):460-469. Bioproject PRJNA266254.
3. Yim B, Winkelmann T, Ding GC, Smalla K (2015). Different bacterial communities in heat and gamma irradiation treated replant disease soils reveald by 16S rRNA gene analysis - contribution to improved aboveground apple plant growth? Front Microbiol 6:1224. Bioproject PRJNA276496.
4. Nicola L, Turco E, Albanese D, Donati C, Thalheimer M, Pindo M, Insam H, Cavalieri D, Pertot I (2017). Fumigation with dazomet modifies soil microbiota in apple orchards affected by replant disease. Appl Soil Ecol 113:71-79. Bioproject PRJNA374488.
5. Peruzzi E, Franke-Whittle IH, Kelderer M, Ciavatta C, Insam H. (2017). Microbial indication of soil health in apple orchards affected by replant disease. In publication on Appl Soil Ecol. Bioproject PRJNA377423.

Online Resource 3 A-B-C-D

Characteristics of the studies taken into consideration in the meta-analysis. Legend: 1= Franke-Whittle et al. (2015); 2= Mazzola et al. (2015); 3= Yim et al. (2015); 4= Nicola et al. (2017); 5= Peruzzi et al. (2017)

A)

Paper	Study type	Rootstock	Treatments	Location	Samples
1	bioassay test in greenhouse	M9	replant vs fallow	3 orchards	6
2	field trial	M9 or G11	Replanted control – chemical fumigation – fumigation with Brassicaceae	1 orchard	60
3	biotest in greenhouse	M26 20 days old	Replanted control vs soil treated at 50°C vs gamma radiated soil	2 nurseries	24
4	field trial	M9	Replanted control vs fumigated	1 orchard	8
5	field trial	M9	6 soil treatments (different fertilizers) + replanted control	2 orchards	42

B)

Paper	DNA extraction	16S region	16S primers	ITS region	ITS primers	Sequencing technique	Data Analysis
1	NucleoSpin Soil Extraction kit	V1-V3	27F-534R	ITS1	ITS1F-ITS2	454	Mothur
2	UltraClean Mega Soil DNA Isolation Kit (MO BIO)	V1-V3	27F-519R	ITS1-2	ITS1F- ITS4	454	custom scripted bioinformatic pipelines
3	FastPrep +GENECLEAN SPIN kit	V3-V4	338F-802R	NP	NP	454	Mothur
4	FastDNA Spin Kit for Soil	V1-V3	27f-518r	ITS1	ITS1F-ITS2	454	Micca
5	NucleoSpin Soil Kit	V1-V3	27f - 534r	NP	NP	Illumina MiSeq	Mothur

C)

Paper	Location	Soil type	Year	Season	Altitude (m)
1	Nachtwieh (Germany)	Rhizosphere	2012	April -August (greenhouse)	179 m
	Haidegg (Austria)	Rhizosphere	"	"	883 m
	Egma - Neustift, South Tyrol (Italy)	Rhizosphere	"	"	223 m
2	Near Palisades (WA, USA)	Rhizosphere	2011	November (field)	299 m
3	Pinneberg area (Germany)	Soil loosely adhering to the roots	2012	November -January (greenhouse)	6 m
	"		"	"	10 m
4	Laimburg (Italy)	Bulk soil	2013	October (field)	225 m
5	Laimburg (Italy)	Bulk soil	2014	July (field)	220 m
	Latsch (Italy)	Bulk soil	"	"	700 m

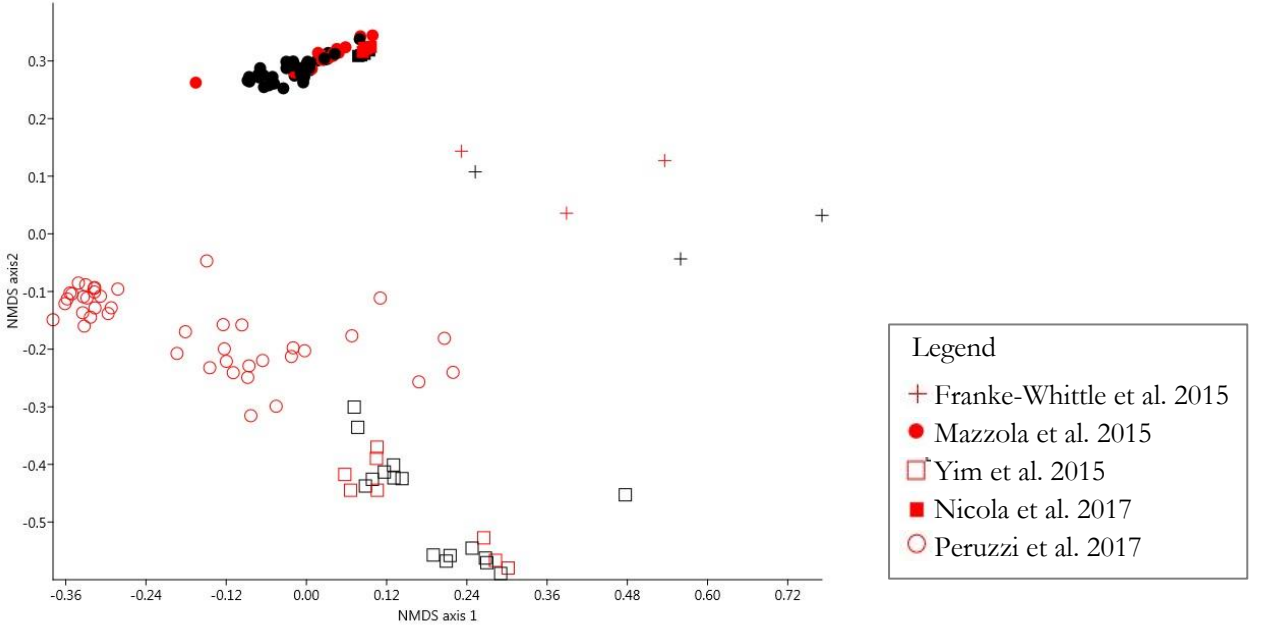
D)

Paper	Soil texture	pH	Organic matter	Latitude	Longitude
1	Clay loam	6,22-6,78	4,4% -6,2 %	50.6252	6.96336
	Sandy clay loam	5,3-5,78	8%	47.57806	13.45415
	Loam sand	7.5	5,4%-6,1 %	46.31159	11.27251
2	Pogue fine sandy loam	6.9	1.20%	47	-120
3	Sandy soil	5.2	4.20%	54	9.692778
	Slightly loamy soil	4.8	3.70%	54	10
4	sandy silt	7.3	1.9%-2.6%	46	11.28916
5	sandy silt	7.46	4.77%	46.35	11.3
	sandy loam	6.98	8.33%	47	10.86667

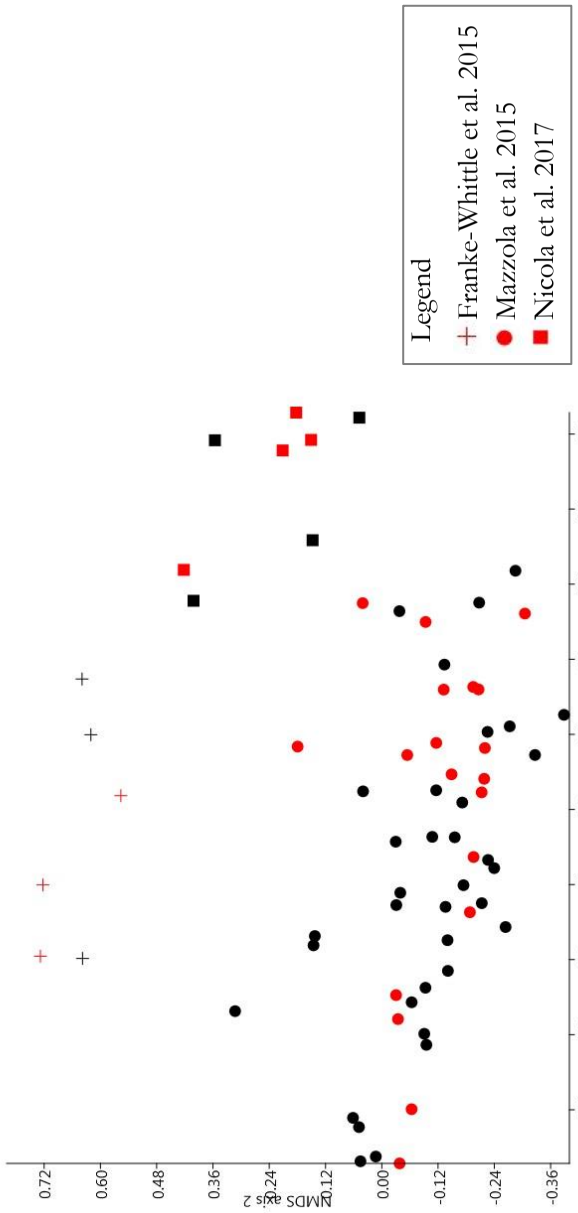
Online Resource 4

Non Metric Multidimensional Scaling (NMDS) of high throughput sequencing microbial data of soil samples taken from ARD-affected sites. The colour red corresponds to RG soils, the colour black to H soils. A) Bacteria, R^2=0.985, lowest stress=0.06; B) Fungi, R^2=0.879, lowest stress=0.15.

A)



B)


Legend
Franke-Whittle et al. 2015
Mazzola et al. 2015
Nicola et al. 2017
NMDS axis 1
NMDS axis 2
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
0.72
0.60
0.48
0.36
0.24
0.12
0.00
-0.12
-0.24
-0.36

V. Paper 4

Phlorizin released by apple root debris is related to apple replant disease

Phlorizin released by apple root debris is related to apple replant disease

Lidia Nicola[a,b*], Urska Vrhovsek[a], Evelyn Soini[a], Heribert Insam[b] and Ilaria Pertot[a]

[a] Research and Innovation Center, Fondazione Edmund Mach, 38010 San Michele all'Adige (TN), Italy

[b] Department of Microbiology, University of Innsbruck, 6020 Innsbruck, Austria

* Corresponding author.
Email address: lidia.nicola@fmach.it (L. Nicola).

Phytopathologia Mediterranea 2016, Volume 55, Issue 3: pp 432-442

DOI: 10.14601/Phytopathol_Mediterr-18480

Abstract:

Autotoxic compounds are likely to be among the causes of apple replant disease, but their secretion is low during plant life. Using targeted metabolomics, the changes in soil phenolic profile were analyzed after the addition of apple roots, and their potential autotoxicity was assessed on apple seedlings. The addition of apple roots severely damaged the plants, attributed to autotoxic action of the phenolic compound phlorizin. Prolonged residence time of the roots in the soil before planting reduced their negative action, probably due to the degradation of phlorizin.

Keywords: allelochemicals, phenolic compounds, soil, autotoxicity, continuous cropping obstacle.

1. Introduction

Apple replant disease (ARD) is a complex syndrome arising from the repeated replanting of apple trees in the same soil; the main symptom is reduced plant growth, particularly root biomass. This syndrome is related to biotic factors (i.e. increased concentrations of pathogenic fungi, decrease in plant growth promoting bacteria) and, possibly, abiotic factors in soil, although the precise aetiology is still unclear (Mazzola and Manici 2012). One of the possible biotic causes of ARD is autotoxicity, in which the phenolic compounds released by roots may play an important role (Huang et al. 2013). The roots of apple trees can release several different phenolic compounds and some of them (phlorizin, *p*-hydroxybenzoic acid, *p*-hydroxy hydrocinnamic acid, phloroglucinol) were found in liquid cultures (Börner 1959). However, root exudation of these substances is quite low during the lifespan of apple plants (Hofmann et al. 2009). On the other hand, phenolic compounds released from decomposing apple leaves and roots (1% in soil) may reach high concentrations, as demonstrated by Politycka and Adamska (2003). In the present study, we increased the quantity of root material added to soil by up to 20% of its volume.

In-field studies investigating the causes of ARD are of extremely difficult interpretation, because of the high number of factors that could be involved. We therefore studied the phenomenon with an artificial setup under controlled conditions, where only the factor 'effect of roots on new plants' varied. Sampling was performed at 0, 3 and 7 months at the most active temperature (20 °C), to specifically identify and quantify the phenolic

compounds released during the decay of apple roots, using Ultra High Performance Liquid Chromatography (UHPLC) coupled to a mass spectrometer. Furthermore, we tested root autotoxic potential on apple seedlings in soil.

2. Materials and methods

2.1 Experimental design and plant growth measures

Healthy roots (diam. < 3 mm) were collected from explanted apple trees (rootstock M26) in the Trentino-South Tyrol region (Italy) on 26 January 2015. They were ground and mixed (1:5, v:v) with sieved soil (loam; pH 7.7; 52 g kg^{-1} of organic matter) taken from an uncultivated area (treatment R3). The soil was divided into two portions that were used to repeat the experiment twice in the same conditions. Sieved soil without any addition of ground roots served as an untreated control (treatment C3). After gentle watering (20 mL kg^{-1} of soil), both soils (R3 and C3) were kept under controlled conditions (20 °C) in the greenhouse for 90 d. The same protocol was repeated three months later (4 May 2015) using the soil collected in January, which was kept in natural conditions in the meantime, and a soil mixed with root debris (treatment R0) and an untreated control soil (C0) were obtained. Apple seedlings, grown in peat from seeds of the cv. Fuji in peat, were transplanted at the age of 90 d into the four treated soils (R0, C0, R3, C3), with three soil samples being collected from each soil treatment for analysis of phenolic compounds before transplanting (time T1). The soil samples were also checked for absence of the three main apple tree pathogens, *Armillaria* spp., *Phytophthora cactorum* and *Rosellinia necatrix*, using diagnostic PCRs, according, respectively, Lochman *et al.* (2004), Bhat and Browne (2010) and Pasini et al. (2016). Fifteen replicates (pots) per soil treatment, having one seedling each, were held at 20±0.5 °C in a greenhouse. After 120 d, the chlorophyll content of the apple seedling leaves was measured (SPAD502, Spectrum Technologies) and the fresh weights of whole plants and roots were assessed. At the same time, three soil samples per treatment were taken from the pots and subjected to phenolic compound analysis (time T2). During the experiment the plantlets did not show any symptoms ascribable to root infections of microbial pathogens.

2.2 Analysis of phenolic compounds

Samples were extracted as described in Vrhovsek et al. (2012). After evaporation of methanolic fractions, samples were applied to a preconditioned ENV+ Isolute C18 SPE column. Preconditioning was performed by purging the column with 10 mL of methanol and 20 mL of water. After loading a sample onto the column, it was washed with 10 mL of water. Polyphenols, retained in the column, were eluted with 20 mL of methanol. Solvent was evaporated using a rotavapor and the residues were dissolved in 500 μL of a methanol/water mixture (2:1). Samples were injected before and after concentration using SPE. Phenolic compounds were analyzed according to Vrhovsek et al. (2012), with a method that allows the detection of a total of 135 different phenolic compounds. Briefly, UHPLC (Waters Acquity UPLC - Milford) coupled to a mass spectrometer (Waters Xevo TQMS - Milford) was used. Separation of the compounds was achieved on a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm (Milford), kept at 40°C. Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The chemicals used for the analysis were purchased from Sigma Aldrich.

2.3 Statistical analyses

Statistical analyses was performed with PAST, version 2.17 (Hammer et al. 2001) and Statistica 9 software (StatSoft). An F-test was used to demonstrate non-significant differences between the two repetitions of the experiment ($p > 0.05$) and data on plant growth were pooled. Since the distribution of data was not normal, statistically significant differences between treatments ($p < 0.05$) were assessed with the Kruskal-Wallis test with Mann Whitney pairwise comparisons (Bonferroni corrected). During analysis of the phenolic compounds, values below the Limit Of Detection (LOD) were substituted with $LOD/\sqrt{2}$ (Verbovšek 2011). Once homogeneity of variance assessed with Levene's test ($p > 0.05$) was satisfied, non-metric multidimensional scaling (NMDS), one-way analysis of similarities (ANOSIM), similarity percentage analysis (SIMPER) and the Wilcoxon test were employed to assess the difference in composition in the phenolic profile of soils. Pearson's correlation was calculated to determine the relationship between the concentrations of phenolic compounds and plant weights.

3. Results and Discussion

Diagnostic PCRs (*Armillaria* spp., *Phytophthora cactorum*, *Rosellinia necatrix*) did not amplify any products, therefore we excluded the presence of apple root pathogens in the soil treated with roots. The soil treatments affected seedling growth. In particular, seedlings planted in soil immediately after mixing with root debris (treatment R0) showed lower chlorophyll content and total seedling weight compared with all other treatments (Table 1, Kruskal-Wallis and Mann Whitney pairwise test, $p < 0.05$). The mean root weight in the R0 treatment was only significantly less than R3 and C3 treatments. The addition of apple roots to soil just before planting therefore significantly impaired the health of the seedlings, showing marked autotoxic effects on the plants and not just on their root systems.

Table 1: Means ± Standard Error of the vitality measurements for apple seedlings after four months of growth in soils amended with old apple roots at different times and in control soils. Significance at the 0.05 probability level ($p < 0.05$). R3= soil with roots amended three months before planting; C3= control soil of the R3 treatment; R0= soil with roots amended just before planting; C0= control soil of the R0 treatment. Columns: whole plant fresh weight, measured in g; fresh root weight of the seedlings, measured in g; chlorophyll content (SPAD).

	Whole plant weight (g)	**Root weight (g)**	**Chlorophyll content (SPAD)**
R3	5.53 ± 0.34 a	3.29 ± 0.26 a	33.9 ± 0.8 a
C3	6.70 ± 0.52 a	3.69 ± 0.31 a	38.0 ± 0.9 b
R0	3.19 ± 0.16 b	2.21 ± 0.14 b	24.5 ± 1.2 c
C0	5.90 ± 0.54 a	2.93 ± 0.24 ab	38.6 ± 1.1 b

Our results indicate that this autotoxic effect of roots on new plants was visible in the soil, and not only in water cultures (Börner, 1959). In contrast, Politycka and Adamska (2003) found a stimulating or slightly inhibiting effect of apple roots on radical growth of cucumber, results that could be due to the use of a different plant species and/or lower concentrations of apple roots in the soil. The artificial experimental set up allowed us to separate the effect of roots on new plants, without confounding effects from other factors.

Fourteen phenolic compounds were detected in soil samples at time T1 (preplanting). The concentrations of these compounds were generally low, with the exception of phlorizin, phloretin and narigenin (Table 2). An NMDS

Table 2: Means ± Standard Error of the phenolic compounds' concentrations in soil at planting time (T1), measured with UHPLC coupled with a mass spectrometer (unit of measure: μg g^{-1}). Values below the Limit Of Detection (LOD) were substituted with LOD/√"2. R3= soil with roots amended three months before planting; C3= control soil of the R3 treatment; R0= soil with roots amended just before planting; C0= control soil of the R0 treatment.

	R3	**C3**	**R0**	**C0**
anthranilic acid	0.0015 ± 0.010	0.0009 ± 0.0004	0.0040 ± 0.0024	0.0023 ± 0.0014
4-aminobenzoic acid	0.0004 ± 0.002	0.0002 ± 0.0000	0.0003 ± 0.0001	0.0002 ± 0.0000
p-hydroxybenzoic acid	0.0086 ± 0.0037	0.0160 ± 0.0074	0.0235 ± 0.0096	0.0053 ± 0.0027
Cinnamic acid	0.0736 ± 0.0616	0.0734 ± 0.0726	0.0957 ± 0.0796	0.0173 ± 0.0170
vanillin	0.0048 ± 0.0005	0.0040 ± 0.0004	0.0056 ± 0.0005	0.0050 ± 0.0002
vanillic acid	0.0008 ± 0.0002	0.0009 ± 0.0001	0.0010 ± 0.0002	0.0009 ± 0.0002
2,6-diOH-benzoic acid	0.0217 ± 0.0114	0.0109 ± 0.0021	0.0551 ± 0.0416	0.0436 ± 0.0348
p-coumaric acid	0.0479 ± 0.0334	0.0496 ± 0.0465	0.0916 ± 0.0553	0.0211 ± 0.0185
caffeic acid	0.0010 ± 0.001	0.0058 ±0.0023	0.0036 ± 0.0011	0.0034 ± 0.0016
ferulic acid	0.0707 ± 0.0433	0.0392 ± 0.0381	0.1339 ± 0.0852	0.1092 ± 0.1081
phloretin	0.0107 ± 0.080	0.0024 ± 0.0016	3.6734 ± 0.8509	0.0104 ± 0.0091
phlorizin	0.0707 ± 0.0000	0.0707 ± 0.0000	77.4076 ± 8.0480	0.0707 ± 0.0000
naringenin	0.1536 ± 0.1275	0.0230 ± 0.0195	0.1752 ± 0.1153	0.1683 ± 0.1648
quercetin-3-rhamnoside	0.0124 ± 0.0059	0.0243 ± 0.0097	0.1562 ± 0.0786	0.0119 ± 0.0084

(stress = 0.078, R^2 axis 1 = 0.992, axis 2 = 0.085) on Euclidean distances of the dataset indicated that data points representing the samples from R0 soil clustered together, separated from the other cluster, which comprised samples from the R3, C0 and C3 treatments (Figure 1A). A one-way ANOSIM with Bonferroni-corrected pairwise comparisons, confirmed the difference between the phenolic profile of R0 samples and all the other samples ($p < 0.05$).

The concentration of four phenolic compounds, *p*-coumaric acid, quercetin-3-rhamnoside, phloretin and phlorizin, significantly increased in R0 treatment soils, compared to C0 (Wilcoxon test, $p < 0.05$). These compounds are all considered to be allelochemicals in apple and in other plants (Huang et al. 2013; Inderjit and Dakshini 1995). In the R0 treatment, the concentrations of all these compounds, but not *p*-coumaric acid, were also significantly greater than those in R3, meaning that after 3 months of roots in the soil, these substances had degraded. A significant negative correlation was found between the sum of the concentrations of the single phenolic compounds measured at T1 and total plant weight (Pearson correlation $r = -0.89$, $p < 0.05$), so a high concentration of polyphenols at planting corresponded to diminished plant growth. In order to detect which phenolic compounds were most responsible for the difference in R0 soils, SIMPER was used. This indicated phlorizin as the phenolic compound contributing to more than 90% of inter-group dissimilarity between R0 and the other treatments, and phloretin as the second most important compound (approximately 5%). In the R0 samples, phlorizin and phloretin reached average concentrations, respectively, of 77.4 ($\pm$ 8.0) and 3.7 ($\pm$ 0.9) $\mu g\ g^{-1}$, while in the other samples phorizin concentrations were $<0.1\ \mu g\ g^{-1}$ and phloretin $<0.06\ \mu g\ g^{-1}$.

We therefore confirm the trend for polyphenol concentrations observed by Politycka and Adamska (2003), although they measured total phenolic content, which also comprises other high molecular weight polyphenols, such as proanthocyanidins. Phlorizin and phloretin are the main flavonoids produced by apple plants and are usually stored in bark and roots (Gosch et al. 2010). These polyphenols inhibit root and shoot growth in water culture (Börner 1959), and phlorizin can specifically inhibit the respiratory rate and enzyme activities of the tricarboxylic acid cycle in apple roots (Wang et al. 2012, Yin et al. 2016). The concentration of phlorizin and phloretin in R3 treatment soils was comparable with that in control soils, indicating that the

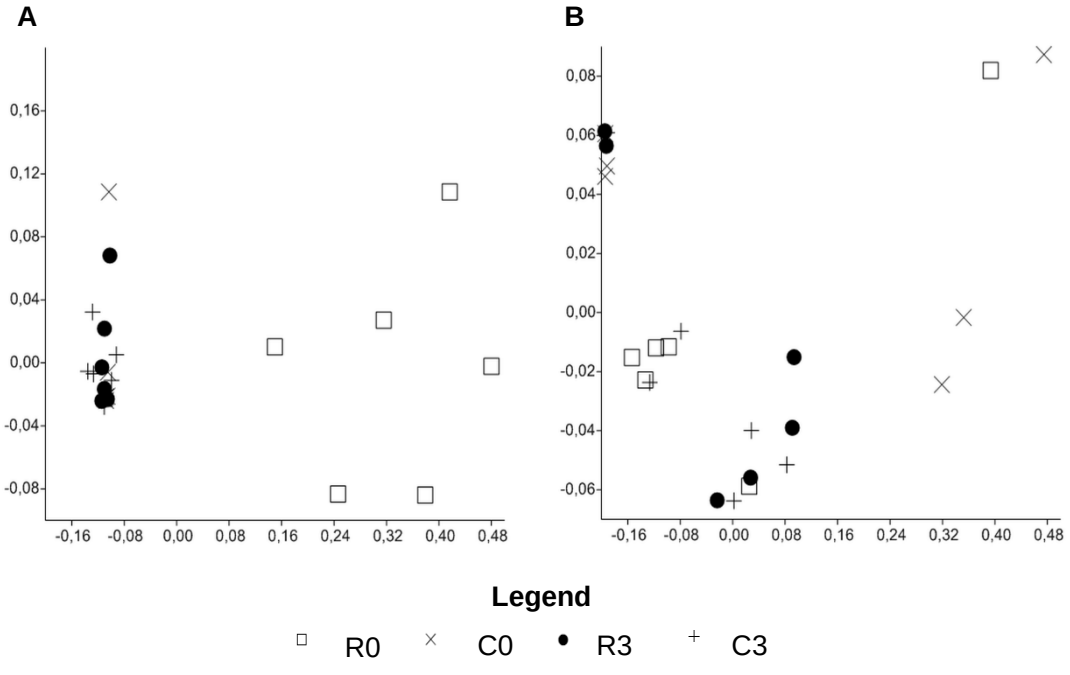


Figure 1: Non Metric Multidimensional Scaling (NMDS) based on Euclidean distances of soil samples amended with old apple roots at different times and control soils. R3= soil with roots amended three months before planting; C3= control soil of the R3 treatment; R0= soil with roots amended just before planting; C0= control soil of the R0 treatment. Each point represents the phenolic profile of one sample. a) at planting time (T1); b) after 4 months of seedlings growth (T2).

Table 3: Means ± Standard Error of the phenolic compounds' concentrations in soil after 4 months of seedlings growth (T2), measured with UHPLC coupled with a mass spectrometer (unit of measure: $\mu g\ g^{-1}$). Values below the Limit Of Detection (LOD) were substituted with LOD/√2. R3= soil with roots amended three months before planting; C3= control soil of the R3 treatment; R0= soil with roots amended just before planting; C0= control soil of the R0 treatment.

	R3	**C3**	**R0**	**C0**
p-hydroxybenzoic acid	0.0206 ± 0.0018	0.0265 ± 0.0063	0.0271 ± 0.0054	0.0275 ± 00.67
vanillin	0.0032 ± 0.0005	0.0027 ± 0.0004	0.0040 ± 0.0007	0.0026 ± 0.0004
vanillic acid	0.0222 ±0.0025	0.0266 ± 0.0024	0.0296 ± 0.0043	0.0316 ± 0.0058
syringaldehyde	0.0012 ±0.0003	0.0009 ± 0.0002	0.0008 ± 0.0002	0.0009 ± 0.0002
esculin	0.0004 ±0.0000	0.0007 ± 0.0003	0.0018 ± 0.0010	0.0004 ± 0.0000
p-coumaric acid	0.0028 ±0.0008	0.0020 ± 0.0003	0.0041 ± 0.0005	0.0033 ± 0.0005
ferulic acid	0.0020 ±0.0005	0.0012 ± 0.0001	0.0023 ± 0.0005	0.0012 ± 0.0002
Phloretin	0.0054 ±0.0020	0.0042 ± 0.0029	0.0105 ± 0.0023	0.0064 ± 0.0029
Phlorizin	1.2200 ± 0.3982	1.1454 ± 0.3057	1.4584 ±0.6136	2.0692 ± 0.9222
taxifolin	0.0073 ± 0.0029	0.0069 ± 0.0025	0.0081 ±0.0030	0.0073 ± 0.0030
dihydrokaempferol	0.0049 ± 0.0008	0.0040 ± 0.0003	0.0057 ± 0.0021	0.0141 ± 0.0062

three months when the ground roots remained in the soil were sufficient to allow degradation of these compounds. These results suggest that in orchards the concentration of phlorizin in soil should be measured before replanting to assess the level of autotoxicity, using this compound as an indicator of soil health.

We ascertained that concentrations of 77 μg g-1 in soil were detrimental for apple seedlings. Leaving several months between explanting and replanting is also recommended, especially because the degradation of phenolic compounds is much slower in winter, when the soil temperatures are low (Politycka and Adamska, 2003), and the release of phenolic compounds from intact roots could be gradual.

Eleven phenolic compounds were detected in soils from sampling at time T2. Again in this case, the concentrations were low (Table 3). As compared to T1, a lower number of benzoic acid derivatives was found. At this time, the NMDS on Euclidean distances (stress = 0.01, R2 axis 1 = 0.99, axis 2 = 0.1) did not show any clustering of the samples (Figure 1B), a fact that was confirmed by one-way ANOSIM, which found no significant differences in the phenolic profile in the different treatments ($p>0.05$). The only phenolic compound that significantly increased in all soil treatments at T2 as compared to T1 was vanillic acid (Wilcoxon test, $p<0.05$), suggesting possible exudation from seedling roots, as happens in other plant species (Kong et al., 2006). Four months after planting the seedlings, the concentrations of phlorizin and phloretin in R0 soils, which were very high in T1, dropped significantly (Wilcoxon test, $p<0.05$), although weights of seedlings planted in this soil were reduced. This suggests that the initial stress caused by high concentration of phlorizin can impair plant health for long periods, as the plants remained stunted even when the concentration of the compound decreased significantly. In conclusion, this study confirmed that the presence of apple root debris in soil can significantly impair the growth of apple seedlings, and that this negative effect disappears when phenolic compounds (mainly phlorizin and phloretin) have degraded. If the seedlings are planted just after the addition of roots, the initial negative impact on subsequent growth persists over time, despite the reduction in concentrations of phenolic compounds. Assessment of phlorizin could therefore be the basis for developing an indicator of ARD risk in orchard soils, or to determine the appropriate time for replanting to avoid ARD.

References

Bhat R.G., and G.T. Browne, 2010. Specific detection of *Phytophthora cactorum* in diseased strawberry plants using nested polymerase chain reaction. Plant Pathol. 59, 121-129 doi:10.1111/j.1365-3059.2009.02147.x.

Börner H., 1959. The apple replant problem. I. The excretion of phlorizin from apple root residues. Contrib. Boyce Thompson Inst. 20, 39-56.

Gosch C., H. Halbwirth and K. Stich, 2010. Phloridzin: Biosynthesis, distribution and physiological relevance in plants. Phytochemistry 71, 838-843 doi:10.1016/j.phytochem.2010.03.003.

Hammer, O., D.A.T. Harper and P.D. Ryan, 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4.

Hofmann, A., L. Wittenmayer, G. Arnold, A. Schicber and W. Merbach, 2009. Root exudation of phloridzin by apple seedlings (*Malus* x *domestica* Borkh.) with symptoms of apple replant disease. J. Appl. Bot. Food Qual. 82, 193-198.

Huang, L.F., L.X. Song, X.J. Xia, W.H. Mao, K. Shi, Y.H. Zhou and J.Q. Yu, 2013. Plant-Soil Feedbacks and Soil Sickness: From Mechanisms to Application in Agriculture. J. Chem. Ecol. 39, 232-242 doi:10.1007/s10886-013-0244-9.

Inderjit A. and K.M.M. Dakshini, 1995. Quercetin and quercitrin from *Pluchea lanceolata* and their effect on growth of asparagus bean. In: Allelopathy: Organisms, Processes, And Applications 582, 86-93 doi: 10.1021/bk-1995-0582.ch006.

Kong C.H., H.B. Li, F. Hu, X.H. Xu and P. Wang, 2006. Allelochemicals released by rice roots and residues in soil. Plant Soil 288, 47-56 doi:10.1007/s11104-006-9033-3.

Lochman J., O. Sery and V. Mikes, 2004. The rapid identification of European *Armillaria* species from soil samples by nested PCR. Fems Microbiol. Lett. 237, 105-110 doi:10.1016/j.femsle.2004.06.019.

Mazzola M. and L. M. Manici, 2012. Apple Replant Disease: Role of Microbial Ecology in Cause and Control. Annu. Rev. Phytopathol. 50, 45-65 doi: 10.1146/annurev-phyto-081211-173005.

Pasini, L., D. Prodorutti, S. Pastorelli and I. Pertot, 2016. Genetic Diversity and Biocontrol of *Rosellinia necatrix* Infecting Apple in Northern Italy. Plant Dis. 100, 444-452 doi:10.1094/pdis-04-15-0480-re.

Politycka B., and D. Adamska, 2003. Release of phenolic compounds from apple residues decomposing in soil and the influence of temperature on their degradation. Pol. J. Environ. Stud. 12, 95-98.

Verbovšek T., 2011. A comparison of parameters below the limit of detection in geochemical analyses by substitution methods. RMZ – Materials Geoenviron. 58, 393-404.

Vrhovsek U., D. Masuero, M. Gasperotti, P. Franceschi, L. Caputi, R. Viola and F. Mattivi, 2012. A Versatile Targeted Metabolomics Method for the Rapid Quantification of Multiple Classes of Phenolics in Fruits and Beverages. J. Agr. Food Chem. 60, 8831-8840 doi:10.1021/jf2051569.

Wang Q., Y. Hu, H. Zhou, X. Zhan, Z. Mao and S. Zhu, 2012. Effects of phloridzin on the tricarboxylic acid cycle enzymes of roots of *Malus hupehensis* Rehd. seedlings. Sci. Agr. Sin. 45, 3108-3114.

Yin C.M., Y.L. Hu, G.S. Wang, X.F. Zhang, H. Zhou, X. Shen, X.S. Chen and Z.Q. Mao, 2016. Effect of main phenolic acids of the apple replanted soil on the roots of *Malus hupehensis* Rehd. seedlings. Sci. Agr. Sin. 49, 961-969.

VI. Paper 5

Soil microbiota respond to green manure in vineyards managed using environmentally friendly methods

Soil microbiota respond to green manure in vineyards managed using environmentally friendly methods.

Claudia M.O. Longa[a], Lidia Nicola[a,b], Livio Antonielli[c], Enzo Mescalchin[d], Roberto Zanotti[d], Elena Turco[a], Ilaria Pertot[a,e].

[a] Research and Innovation Centre, Fondazione Edmund Mach (FEM), 38010 San Michele all'Adige (TN), Italy
[b] Department of Microbiology, University of Innsbruck, 6020 Innsbruck, Austria
[c] Bioresources, Center for Health & Bioresources, Austrian Institute of Technology GmbH, Tulln, Austria
[d] Technology Transfer Centre, Fondazione Edmund Mach (FEM), 38010 San Michele all'Adige (TN), Italy
[e] Center Agriculture Food Environment, University of Trento

Manuscript in preparation to be submitted to Journal of Applied Microbiology.

Abstract:

The microbiological diversity of soil, including bacterial and fungal composition and distribution, is used as a sensitive indicator of soil quality, considering the major role played by microorganisms in organic matter decomposition and nutrient cycling. In this work, microbiological characterisation of soil from vineyards was performed to investigate the effects of biodynamic viticulture with and without the addition of green manure, in comparison with organic management using high throughput sequencing. Our results showed that green manure was the greatest source of soil microbial biodiversity, and significantly changed microbial richness and community composition compared to other soils, while microbial communities associated with biodynamic and organic farming systems were very similar. Green manure also significantly enriched bacterial taxa involved in the soil nitrogen cycle (e.g. *Microvirga* sp., *Pontibacter* sp. and *Nitrospira* sp.). Evidence of increased nitrogen-fixing and nitrite-oxidising bacteria populations in soil as a response to green manure incorporation suggests they could potentially be used to increase nitrogen availability. The extension of organic/biodynamic farming, associated with green manure application, could contribute to maintaining microbial biodiversity in vineyard soils.

1. Introduction

Since there are vineyards dating back to pre-Roman times in Europe, the grapevine offers a unique opportunity to study extremely long-term monocultures (Schlegel 1973). The physical and chemical properties of soil generally have a major effect in shaping the microbial population of vineyard soil (Corneo et al. 2013). Compared to other agro-ecosystems, vineyard soils receive lower nitrogen fertiliser input and are subjected to relatively infrequent tilling and fewer herbicide applications, which may be reflected in the composition of microbial communities (Steenwerth et al. 2008). On the other hand, in recent years grape-growing areas have been subjected to cropping intensification. Thus traditional vineyards planted along the contours of hills on small terraces have often been abandoned and replaced by larger plots on low to moderate slopes, with chemical fertilisation and weed control, which increases grape production, but also intensifies soil degradation (Lopez-Pineiro et al. 2011). Indeed, in terms of biochemical properties, vineyard soils are usually highly degraded (Miguens et al. 2007). Long-term use of certain

inorganic pesticides, in particular copper-based fungicides, has resulted in increased concentrations of heavy metals in the soil, affecting the environmental compartments of soil (Komarek et al. 2010). Although the grapevine is an important crop worldwide and preserving the biological quality of soil is mandatory for sustainable agriculture, knowledge about soil microbiological processes in vineyards is generally limited (Probst et al. 2008).

The addition of organic substances is of vital importance for soil quality and health (Baldi et al. 2010). Organic fertilisers can be a good solution for maintaining soil health, since the release of nutrients in soil is slower than for chemical fertilisers and often better matches plant needs in the growing season. Of the different organic fertilisers, green manure consists of growing specific crops in the inter-rows of the vineyard and then ploughing them into the terrain to improve soil quality. Green manure crops usually include grass mixtures and legume plants, such as vetch, clover, barley and others. Multiple benefits are produced by green manure. The physical structure of soil is improved, because green manure tends to reduce soil erosion and leaching (Ingels et al. 2005). This practice can also help to provide ecological niches supporting predators/parasitoids, improving pest control in the vineyard (Irvin et al. 2014). Green manure influences the grapevine plant and its fruit by enhancing the organoleptic characteristics of the grapes (Rotaru et al. 2011). Moreover, the organic carbon available for soil microorganisms is significantly increased by green manure, which also enhances the activity of numerous soil enzymes, especially those involved in the N cycle (Okur et al. 2016). Although the advantages of using green manure have been recognised, little is known about the possible modifications that it could cause in soil microbial communities.

Only organic fertilisers and green manure are allowed in organic agriculture, where the use of chemical products is forbidden. Organic agriculture, also referred to as biological agriculture, is based on a substantial decrease in pesticide use and soil management having a lower impact, without using mineral fertilisers and compounds for weed control (Lotter 2003). The principles of biodynamic agriculture were established in Germany by Rudolf Steiner in the 1920s. Biodynamic agriculture can be regarded as a pioneer version of organic agriculture (Kirchmann 1994). This type of management adopts a holistic approach to the exploitation of natural resources, taking into consideration the sustainability of different elements, such as the crops themselves, animal life preservation or the maintenance of high quality soil,

in order to recover, preserve or improve ecological harmony. The biodynamic system has a strong metaphysical component to its farm practices and uses a set of specific compost preparations to be applied to crops to aid fertilisation, and the application of other homoeopathic treatments based on infusions or plant extracts (Lotter 2003).

Knowledge of how soil management affects soil microbial species richness and abundance is important because microbial diversity and stability determine the soil's ability to react to external changes, the impact and degradation (Munoz et al. 2007). Beneficial microbial processes are essential for crop production as they determine the soil's ability to supply nutrients to the plant; they retain nutrients in the profile, contribute to the formation of soil structure, suppress plant pathogens, and contribute to soil humus formation (Ingels et al. 2005).

The main goal of this study was to investigate the effects of biodynamic viticulture on the microbial community structure and diversity of vineyard soils, with or without the addition of green manure, using high throughput sequencing, in comparison to organic management.

2. Materials and methods

2.1 Sampling site and vineyard management

The study site was located at an experimental site (1.0 ha) in the Trentino-South Tyrol region in northern Italy (San Michele all'Adige, 46.19 N, 11.14 E). Two vineyards were selected (Field 1 and Field 2), which were then divided into replicated plots (n=12). The vineyards were both planted with Cabernet franc variety (clones 214, 331 and 327) on SO4 rootstock in 2002 and the Guyot vine training system was adopted (2.0 m × 1.0 m). Starting from the autumn of 2011, each plot was managed according to organic (O), biodynamic (BD) or biodynamic with green manure (BDGM) principles (2 plots per field for each type of management). In the O plots, pneumatic leaf removal and mechanical hedging were adopted for canopy management, while in the BD and BDGM plots pneumatic leaf removal was substituted with manual removal of lateral shoots, and instead of hedging, the shoots were rolled onto the last couple of wires in the vegetative wall. Chemical fertilisers were not applied to any of the plots, but copper and sulphur were used to control fungal disease in the O plots. In the BD and BDGM plots,

biodynamic preparations 500 and 501 were used to aid fertilisation. The BDGM plots received green manure in autumn and spring (Table 1, 181 Kg ha^{-1}, dry weight of green manure 0.58 Kg m^{-2}). Specifically, in the BDGM plots, a chisel plough set at 50 cm was used before cover crop seeding, followed by a rotating harrow. Mechanical weed control was performed during inter row mowing in all the plots.

Table 1: Composition of the cover crop seed mixture (planted on 5/10/2011) for green manure and average productivity of the cover crops and natural grass measured on 15/05/2012 as dry weight.

Cover crops	**% weight**
Vicia sativa	11.0
Pisum sativum	22.1
Vicia Faba	55.2
Secale cereale	11.1
Brassica Napus	0.6

2.2 Soil sampling and processing

Soil sampling was carried out in autumn 2012. Three sampling points were chosen along two grapevine rows (at the two ends and a central point) in each field and for each type of vineyard management. For each sampling point, three-soil cores (Ø 5 cm, depth 19 cm) were collected from the topsoil and transferred into sterile bags, after removing the first 5 cm of the soil layer (mostly humus). The soil samples were sieved separately to a < 2 mm particle size, and an equal amount of soil from each sampling point was transferred into a 50-ml sterile falcon tube (Sarsdedt, Germany), lyophilized and stored at -80°C for metagenomic analysis. A total of 18 soil samples per field were collected (n= 36).

Physical and chemical analysis was carried out on the remaining soil, after pooling by field, vineyard management and row (n = 12): sand, silt, clay, total soil organic matter (SOM), pH, total macro and microelements were determined, following the official methods for soil chemical analysis (DM 11/05/92 and DM 13/09/99).

2.3 DNA extraction, amplification and pyrosequencing

Total genomic DNA was extracted from 0.5 g of lyophilised soil using a FastDNA® Spin kit (MP Biomedicals, France), following the manufacturer's instructions, and quantified using a NanoDrop 8000 spectrophotometer. For bacterial identification, the V1-V3 hypervariable region of 16S rRNA was PCR amplified using the primer set 27f (Weisburg et al. 1991) and 518r (Muyzer et al. 1993). At the 5' end, the forward primer carried the 454-adaptor A with a specific Roche-10 nt multiplex identifier (MID) for each soil sample. Each sample was amplified in triplicate in a 25 μl reaction, following the amplification protocol by Nicola et al (2017). The 18S rRNA - 5.8S rRNA internal spacer (ITS) of fungal rRNA was amplified using the primer pair ITS1F (Gardes and Bruns 1993)- ITS2 (White et al, 1990). One way amplicon sequencing was carried out as in Nicola et al. (2017). Three independent PCR reactions (technical replicates) were performed for each sample and pooled together. All the PCR products were then analysed with gel electrophoresis and cleaned using an AMPure XP beads kit (Beckman Coulter, Brea, CA). Two final and distinct libraries (16S and ITS) were constructed from the 36 PCR products. Pyrosequencing was performed on a GS FLX+ system (Roche, Mannheim, Germany) using XL+ chemistry, following the manufacturer's instructions.

2.4 16S rRNA gene and ITS sequence processing

Pyrosequence quality was checked in PRINSEQ (Schmieder and Edwards 2011) and flowgrams were filtered and denoised using FlowClus (Gaspar and Thomas 2015). Denoised microbial reads were processed using Metaxa2 v2.1.3 (Bengtsson-Palme et al. 2015) to target the extraction and to verify the 16S rRNA variable regions. Similarly, but for fungal reads, ITSx v1.0.11 (Bengtsson-Palme et al. 2013) was used to target the ITS1. USEARCH v7 (Edgar 2013) was used to de-replicate, sort and cluster the extracted regions with 97% pairwise sequence identity. Chimeras were removed by adopting both *de novo* and reference based methods as features of the above mentioned tool. The RDP classifier train set n.15 (2015/09) was used as a reference database for microbial chimeras, whereas the UNITE reference sequences version n. 7.0 (2016/01) were chosen for fungal ITS chimera detection in UCHIME (Edgar et al. 2011). Taxonomy assignment was performed by employing naïve Bayesian RDP classifier v2.10 (Wang et al. 2007) in QIIME (Caporaso et al. 2010b) with a minimum confidence of 0.6 against the SILVA

database, release 123 (2016/05) (Quast et al. 2013) and the UNITE database, version n. 7.1 (2016/08) (Abarenkov et al. 2010) for 16S rRNA-based and ITS-based sequences respectively. Sequence data were made available in the NCBI SRA database under BioProject number PRJNA381189.

2.5 16S rRNA gene-based microbial and ITS-based fungal community analysis and statistics

OTU-based analysis was carried out in QIIME to calculate richness and diversity after multiple rarefaction. The OTUs observed were counted and the diversity within each individual sample was estimated using Simpson's diversity index. Richness and diversity values were separately analysed in R, fitting all the factors in Generalized Linear Models (GLMs) assuming a Gamma distribution and validated via graphical representation of residuals vs. fitted values. The statistical significance of the GLMs was inferred by adopting the chi-squared test and *post hoc* pairwise comparisons were calculated using Tukey's HSD test in the multcomp R package (Hothorn et al. 2008). Microbial and fungal richness and diversity values were graphically represented as bar plots using the ggplot2 R package.

Multivariate analysis of community structure and diversity was performed according to the recommendations of Anderson and Willis (Anderson and Willis 2003): 1) unconstrained ordination offered by Principal Coordinate Analysis (PCoA) (data not shown); 2) constrained multidimensional scaling using Constrained Analysis of Principal Coordinates (CAP) as re-implemented in the vegan R package (Oksanen et al. 2017); 3) permutation test to assess the significance of the constraints and permutational multivariate analysis of variance (PERMANOVA); 4) identification and correlation of OTUs responsible for shaping the diversity structure. The effects of rare species were downweighted by applying Hellinger transformation to the rarefied OTU tables.

In more detail, the differences between bacterial communities were investigated using the Bray–Curtis dissimilarity distance and the ordination methods applied to the same distance matrices. All the ordination analyses were computed and CAP plotted in phyloseq (points 1 and 2). The significance of the treatment grouping factor used as a constraint in CAP was assessed via the permutation test in the vegan R package. The null hypothesis of no differences between *a priori* defined groups was investigated using the

PERMANOVA approach, implemented in vegan as the ADONIS function and applied to the Bray–Curtis dissimilarity distances.

Permutational pairwise comparisons between the treatments were carried out with the RVAideMemoire R package (Hervé 2017) and P values were FDR-adjusted (point 3). Indicator OTU analysis was applied for calculation of differential OTU abundance in treatments using the indicspecies R package (De Caceres and Legendre 2009) and P values were FDR-adjusted. Procrustes analysis (Lisboa et al. 2014) was then applied to CAP ordinations to correlate bacterial and fungal beta-diversity in response to different farming practices (point 4). Differential OTU abundance for treatments at genus level was assessed via permutation ANOVA (RVAideMemoire R package) for both the bacterial and fungal dataset. Significantly different genera (FDR-adjusted p-values) were then shown as bar plots (mean ± standard deviation of number of reads) and for each genus the pairwise permutation t-test was applied to all treatment combinations.

3. Results

The physical and chemical analysis of soil revealed subtle differences between the different types of management. For example BDGM plots had higher SOM, MgO, Cu and Zn concentrations, while O plots had a higher C/N ratio and BD plots had higher K_2O and Pb concentrations (Table 2).

Pyrosequencing yielded a total of 401,824 raw pyrotags reads for bacteria and 305,990 reads for fungi. After quality filtering and chimera removal, a total of 314,910 16S rRNA sequences and 164,227 ITS sequences remained for community analysis, corresponding to an average ± standard deviation of 8,997 ± 1,726 reads and 4,562 ± 1,367 reads per sample for bacteria and fungi respectively. A total of 4,809 bacterial OTUs and 633 fungal OTUs were detected.

The most abundant bacterial phyla, in all soil samples, were Actinobacteria (31.71%), Proteobacteria (21.96%), Acidobacteria (12.78%) and Gemmatimonadetes (8.29%). A total of 32 phyla, 116 classes, 255 orders, 505 families and 850 genera were detected. As regards genera, the most abundant in vineyard soil were *Gaiella* sp. (5.66%), *Bacillus* sp. (1.99%), *Arthrobacter* sp. (1.74%) and *Nitrospira* sp. (1.26%) for bacteria. The fungal communities were instead dominated by Ascomycota (77%), Basidiomycota (16%) and

Zygomycota (7%). Overall, a total of six phyla, 22 classes, 61 orders, 124 families and 220 genera were found in the soil samples. The Ascomycota mostly consisted of Sordariomycetes, followed in decreasing order of relative abundance by Dothideomycetes and Eurotiomycetes. More than 37% of the Sordariomycetes reads belonged to the Hypocreales order, and within this order Nectriaceae were the most abundant family. *Chlonostachys* sp. (13.29%), *Coprinellus* sp. (8.13%), *Exophiala* sp. (4.15%) and *Fusarium* sp. (4.08%) were the most abundant genera of fungi.

Table 2: Physical-chemical analysis of soil samples, divided according to the management system applied. O = samples from organically managed soil; BD = samples from biodynamically managed soil; BDGM = samples from biodynamically managed soil with the addition of green manure as fertiliser.

	O	**BD**	**BDGM**
pH	7.97	7.97	7.97
Total limestone (g kg-1 CaCO3)	367.50	365.50	371.00
Active limestone (g kg-1)	13.50	13.00	12.50
Organic substance (g kg-1)	24.00	22.50	26.00
N (g kg-1)	1.10	1.10	1.40
C/N	12.51	11.82	11.00
P2O5 (mg kg-1)	59.50	54.00	61.00
K2O (mg kg-1)	229.00	233.00	214.00
MgO (mg kg-1)	417.00	446.00	466.50
CSC (meq/100g)	14.40	15.05	15.80
Cu DTPA (mg kg-1)	27.65	27.90	32.85
Fe DTPA (mg kg-1)	10.70	11.13	11.00
Mn DTPA (mg kg-1)	10.85	10.13	11.05
Zn DTPA (mg kg-1)	4.65	4.89	5.71
Pb DTPA (mg kg-1)	21.42	26.48	23.45
Cd DTPA (mg kg-1)	0.10	0.10	0.10
Sand (g kg-1)	295.50	263.50	285.00
Loam (g kg-1)	534.50	556.50	550.00
Clay (g kg-1)	170.00	180.00	165.00

The Glomeromycota phylum, an important soil microbial group that forms one of the most common types of symbiosis (arbuscular mycorrhizal fungi; AMF), presented a low abundance in all soils and the management systems did not influence its diversity. Three classes - Archaeosporales, Glomerales and Paraglomerales - represented this phylum. The Glomeraceae family was more abundant compared to the Ambisporaceae and Paraglomeraceae families. The *Glomus* and *Funneliformis* genera were common with all the management systems, while the genus *Septoglomus* was present in O and BD soils and was almost absent in BDGM soils.

The alpha (within-sample) diversity (observed OTUs) found in bacterial communities in biodynamic soils with green manure (BDGM) was significantly higher than that in organic (O) and biodynamic (BD) soils (Fig. 1a; Supplementary Material 1). Moreover, the bacterial richness in Field 1 was significantly greater than in Field 2, and the same trend was observed in fungal communities (Fig. 1b). On the other hand, the different types of soil management did not influence fungal alpha diversity.

When beta (between-sample) diversity was analysed using PERMANOVA, both fungal and bacterial communities were significantly different according to the type of soil management, the field of origin and the interaction between the two ($p<0.05$, Supplementary Material 2). With permutational pairwise comparisons, it was ascertained that the microbiome of BDGM soils was significantly different from those in O and BD soils ($p<0.05$). Building on these results, Constrained Analysis of Principal Coordinates (CAP) was performed on bacteria and fungi (Fig. 2), using the factors that appeared to be significant in PERMANOVA as constraints. The samples were divided according to the type of soil management (BDGM vs O and BD) and the field of origin, both for bacteria and fungi. Procrustes correlation testing for CAP analysis was performed and a correlation of 0.51 ($m12=0.74$) with a significance $p<0.05$ was found, meaning that bacterial and fungal diversity reacted in a similar way to soil management. Bacterial and fungal indicator OTUs significant for soil management were identified. In BDGM and BD soils the bacterial indicator species were mainly genera associated with the soil nitrogen cycle, such as *Nitrospira* sp., *Pontibacter* sp. and *Frankia* sp. (Table 3). The fungal indicator species were instead mainly saprobic fungi in each type of soil management (Table 4). *Exophiala* sp., a black yeast often associated with soil enriched with organic waste, was the indicator species in O soils. In BD soils, *Mortierella* sp., *Mortierella Antarctica, Humicola nigrescens* and the

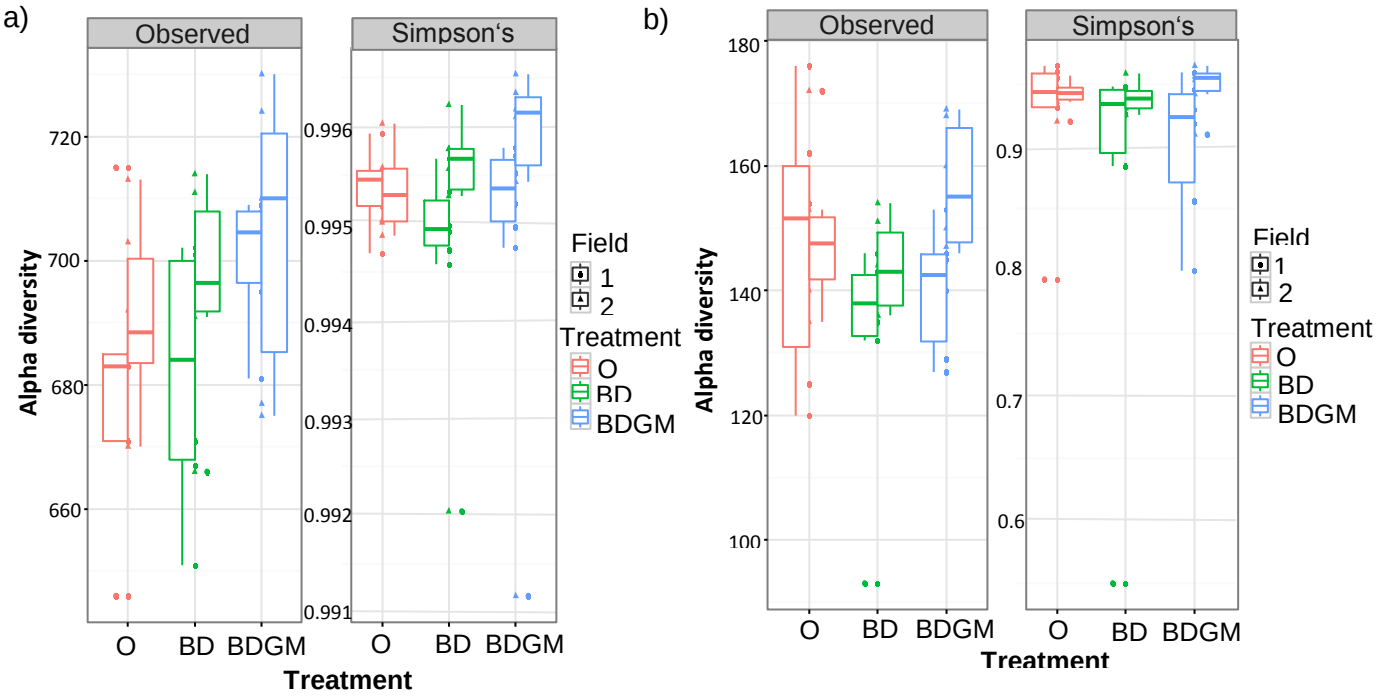


Fig. 1. Box plots representing observed OTUs and Simpson indices of bacterial (a) and fungal (b) communities in vineyard soils managed with different sustainable approaches. O = samples from organically managed soil; BD = samples from biodynamically managed soil; BDGM = samples from biodynamically managed soil with the addition of green manure as fertiliser; 1 = soil samples from Field 1; 2 = soil samples from Field 2.

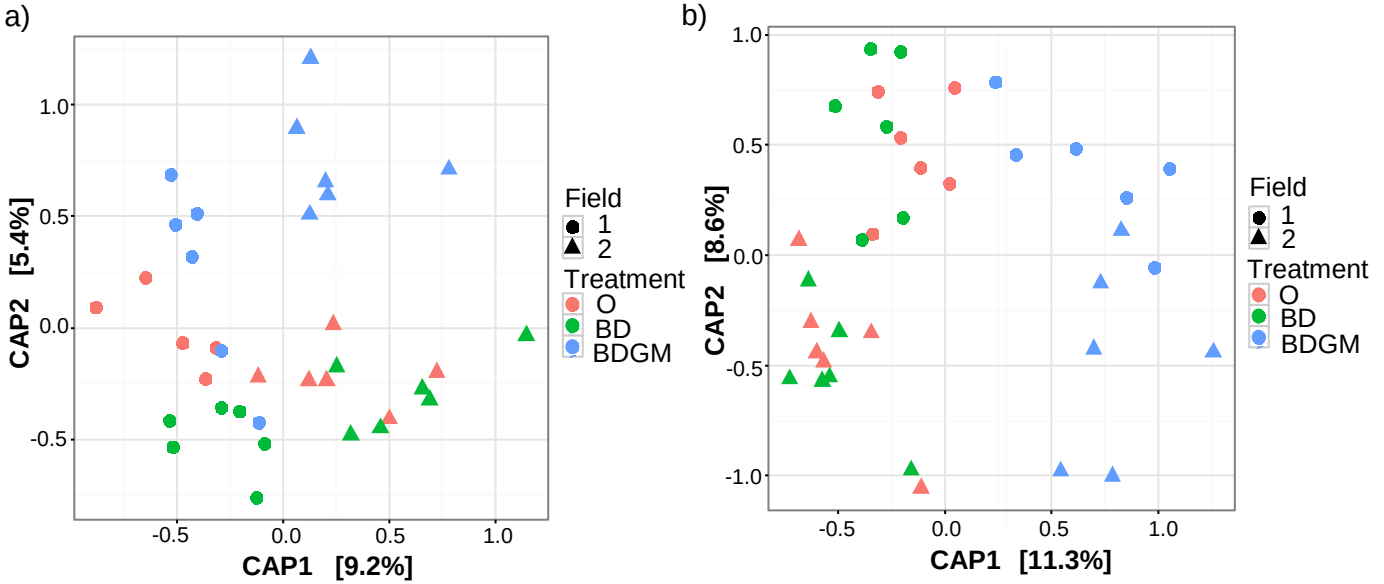


Fig. 2. Constrained Analysis of Principal Coordinates (CAP), based on the Bray–Curtis dissimilarity distance of 454 sequencing bacterial (a) and fungal (b) data for soil samples from vineyard soils managed with different sustainable approaches. O = samples from organically managed soil; BD = samples from biodynamically managed soil; BDGM = samples from biodynamically managed soil with the addition of green manure as fertiliser; 1 = soil samples from Field 1; 2 = soil samples from Field 2.

antagonistic fungus *Acremonium persicinum* were the indicator OTUs. On the other hand, BDGM soils contained the biocontrol agent and plant-growth promoting *Cladorrhinum* sp., *Capnobotryella* sp., a black-pigmented fungi, *Cystofilobasidium capitatum*, a pectinolytic yeast and *Exophiala* sp.

Table 3: Bacterial indicator OTUs for the different types of vineyard soil management obtained with the indicspecies R package (p values corrected using FDR). O = samples from organically managed soil; BDGM = samples from biodynamically managed soil with the addition of green manure as fertiliser.

Soil management	Bacterial OTUs	P values
O	*Lactobacillus* sp.	0.0496
BDGM	*Nitrospira* sp.	0.0037
	Catelliglobosispora sp.	0.0037
	Planosporangium sp.	0.0396
	Paenibacillus sp.	0.0496
	Pontibacter sp.	0.0496

Table 4: Fungal indicator OTUs for the different types of vineyard soil management obtained with the indicspecies R package (p values corrected using FDR). O = samples from organically managed soil; BD = samples from biodynamically managed soil; BDGM = samples from biodynamically managed soil with the addition of green manure as fertiliser.

Soil management	Fungal OTUs	P values
O	*Exophiala* sp. 1	0.044
BD	*Mortierella antarctica*	0.0069
	Acremonium persicinum	0.0092
	Mortierella sp. 04M 158	0.0069
	Humicola nigrescens	0.0166
BDGM	*Exophiala* sp. 2	0.0104
	Capnobotryella sp. MA 4775	0.0173
	Cystofilobasidium capitatum	0.027
	Cladorrhinum sp.	0.0303

As regards the differences in OTU abundance in O soil and BD and BDGM soil, we found two nitrogen-fixing bacterial genera (*Microvirga* sp. and *Pontibacter* sp.) to be significantly more abundant in BDGM soils, together with *Actinoplanes* sp., which has an important role both in the degradation of fallen leaves and as an antagonist of oomycetes like *Pythium* and *Phytophthora* (Fig. 3). On the other hand, the genus *Terrimonas*, involved in S cycling in soil, was significantly more abundant in O soil than in BD and BDGM soil. In fungal analysis, the genera *Cladorrhinum*, *Cystofilobasidium* and *Myrmecridium* and the psychrophilic basidiomycetous yeast *Mrakiella* sp. were significantly more abundant in BDGM soils (Fig. 4). In addition, the genera *Colletotrichum, Gibberella* and *Leptosphaeria,* which include pathogenic species of plants, were abundant where green manure was applied. In contrast, *Clonostachys* sp. and *Pyrenochaeta* sp., associated with biocontrol and plant pathogens respectively, were more abundant in O and BD samples than in BDGM samples.

4. Discussion

Scientific studies on biodynamic management in vineyards and its effect on soil microbiota are rare (Burns et al. 2016), since most works tend to concentrate on the effects on plants or grapes. Recent work on biodynamic viticulture has affirmed that in terms of grape health, the microbiological and chemical characteristics in these vineyards were comparable or better to those in vineyards cultivated using conventional methods (Guzzon et al. 2016).

There are several theories regarding the way in which the biodynamic preparations may interact with crops, and may include hormonal stimulation, enhancing crop growth, especially at root level (Villanueva-Rey et al. 2014). As regards the effects of biodynamic preparations, according to Chalker-Scott (2013), the addition of these products did not affect the yield of the crops analysed, and other authors have also stated that biodynamic preparations had little influence on plant biotic parameters (Baskar and Shanmugham 2016; Doring et al. 2015).

We used the high-resolution power of 454-pyrosequencing to investigate soil microbial biodiversity in sustainably managed vineyards, specifically studying the short-term effects of two types of farming management (O, BD) and green manure application (BD, BDGM) on the diversity, richness and

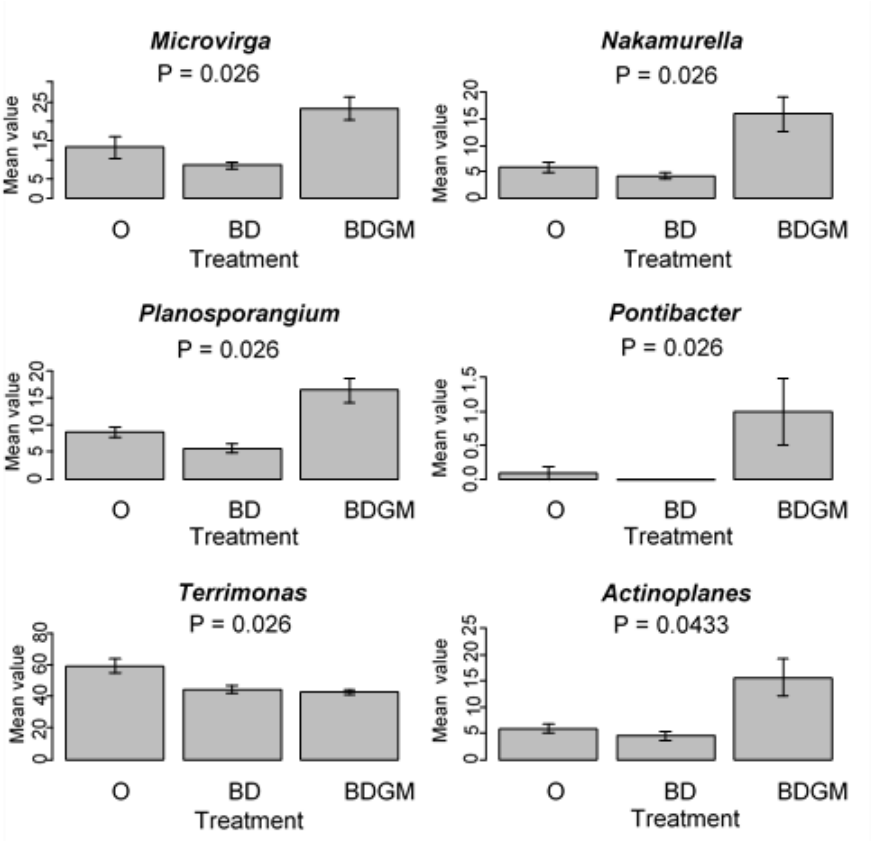


Fig. 3: Bar plots of the different bacterial OTU abundance at genus level for different types of vineyard soil management obtained via permutation ANOVA. Only significantly different genera (p-values corrected using FDR) are shown (mean ± standard deviation of number of reads). O = samples from organically managed soil; BD = samples from biodynamically managed soil; BDGM = samples from biodynamically managed soil with the addition of green manure as fertiliser.

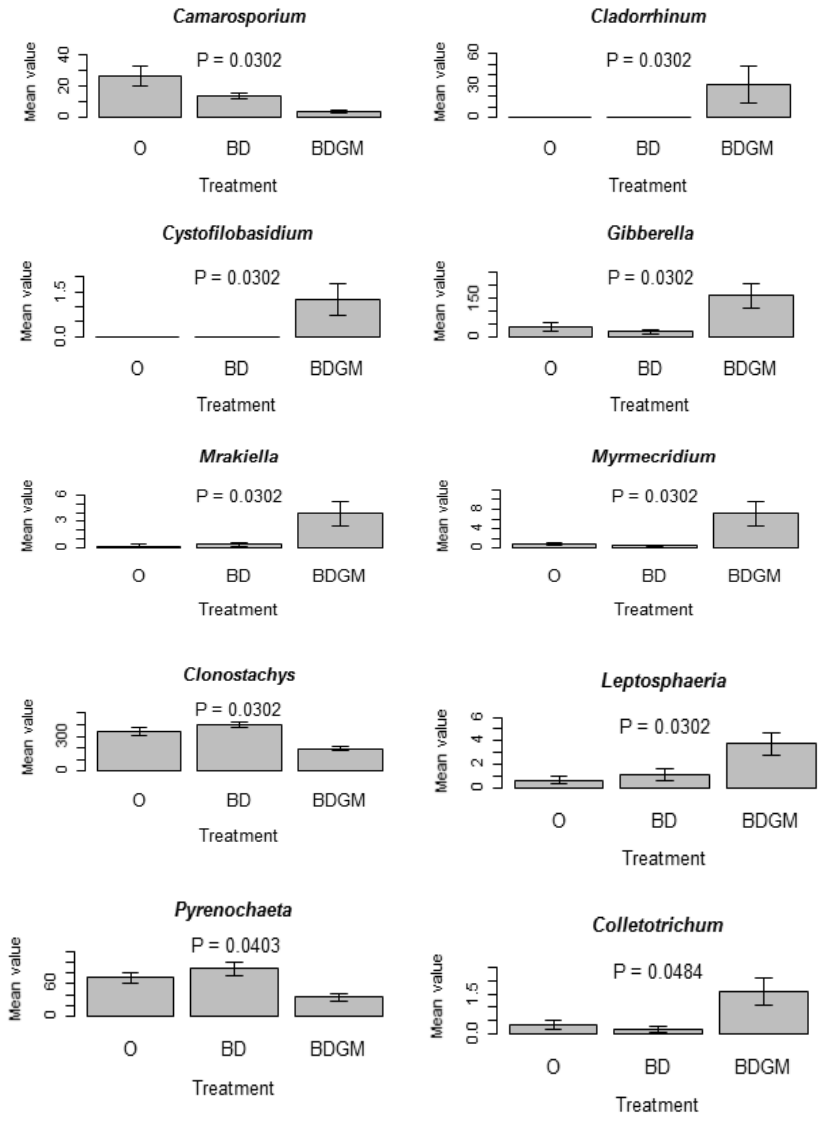


Fig. 4: Bar plots of the different fungal OTU abundance at genus level for different types of vineyard soil management obtained via permutation ANOVA. Only significantly different genera (p-values corrected using FDR) are shown (mean ± standard deviation of number of reads). O = samples from organically managed soil; BD = samples from biodynamically managed soil; BDGM = samples from biodynamically managed soil with the addition of green manure as fertiliser.

composition of soil microbial communities. In our study, no difference in alpha or beta diversity was noticed between O and BD soil samples. This is in agreement with previous studies on biodynamic management, which have indicated similar behaviour for organic and biodynamic farming systems in terms of microbial soil composition and diversity. Carpenter-Boggs et al. (2000) found that organically and biodynamically managed soils had a similar microbial composition, but they were more biotically active than soils that did not receive organic fertilisation. Moreover, organic management enhanced soil biological activity, but additional use of biodynamic preparations did not significantly affect the soil biotic parameters tested.

Green manure application had a major impact on soil microorganisms. Our results showed the crucial importance of green manure for soil microbiota, since it promoted higher bacterial richness and significant changes in the microbial communities found in BDGM soils. Bacteria and fungi responded in a similar way to green manure application, with the same degree of change in both communities. These results are in accordance with Ingels et al. (2005), who analysed microbial communities using phospholipid fatty acid (PLFA) analysis, showing that biodynamic management associated with green manure application increased the taxonomic and phylogenetic richness, diversity and heterogeneity of soil microbiota compared with other farming systems. Furthermore, Wittwer et al. (2017) highlighted others benefits of cover crops, such as providing various ecological services to agro-ecosystems, protection against soil erosion, reduction of nutrient losses, improvement of soil and water quality, and to some extent, a reduction in weeds and pests.

Moreover, the addition of green manure significantly enriched the population of bacteria active in the soil nutrient cycle, such as *Microvirga* sp., *Pontibacter* sp., and *Actinoplanes* sp. *Microvirga* sp. is a nitrogen-fixing bacterium that is often found in symbiosis in the root nodules of legumes (Ardley et al. 2012; Reeve et al. 2014). *Pontibacter* sp. is a Gram-negative genus isolated from different environments, such as different kinds of soil, muddy water and marine water (Srinivasan et al. 2014), and some strains carry out nitrogen-fixing activity in soil (Xu et al. 2014). On the other hand, *Actinoplanes* sp. is often found in leaf litter (Nurkanto et al. 2016) and may have an important role in the degradation of fallen leaves and organic matter (Hop et al. 2011), in addition to exercising antagonistic activities against several soil-borne pathogens, such as *Pythium* spp. and *Phytophthora megasperma* (El-Tarabily et al.

2010; Filonow and Lockwood 1985). Green manure also increased the presence of some fungal OTUs, such as the genus *Cladorrhinum*, a fungal group of prime importance for agriculture and livestock, since some species have biocontrol potential or have been shown to promote plant growth and produce phytases (Carmaran et al. 2015), and the cold-adapted heterobasidiomycetous genus *Cystofilobasidium*, which can utilise D-glucuronate and inositol as sole carbon sources and the assimilation of nitrate as sole nitrogen source (Linkind et al. 2009). Other increased fungal OTUs in BDGM were *Myrmecridium,* a fungal genus whose members are either saprobes or plant endophytes (Peintner et al. 2016) and the psychrophilic basidiomycetous yeast *Mrakiella*. The genera *Colletotrichum*, *Gibberella* and *Leptosphaeria*, which include important phytopathogens of many economically significant plants cultivated around the world, were also more abundant where green manure was applied.

As regards bacterial composition, the two most abundant bacterial phyla in these soil samples, Actinobacteria and Proteobacteria, are copiotrophs in soil and they are plentiful in conditions of high nutrient availability, exhibiting high growth rates (Fierer et al. 2007). The third phylum in order of abundance was Acidobacteria, which instead comprises oligotrophic soil bacteria (Fierer et al. 2007; Schimel and Schaeffer 2012). As regards fungi, OTUs belonging to the phylum Ascomycota were dominant in all types of soil management, which is common in cultivated soil (Abujabhah et al. 2016; Franke-Whittle et al. 2015; Sugiyama et al. 2010), and were followed by those of Basidiomycota, Zygomycota and Chytridiomycota. A similar trend was observed by Orgiazzi et al. (2012) when analysing ITS fragments from different soil types with 454 pyrosequencing.

In this work, almost all the microbial genera were found indiscriminately in each of the management systems at both sites. However, differences in abundance could be detected and some OTUs could be associated with specific types of soil management as their indicator OTUs. In O soils, only two indicator OTUs were found, the bacterium *Lactobacillus* sp. and the fungus *Exophiala* sp. *Lactobacillus* sp. often grows on grape skin (Bae et al. 2006; Nisiotou et al. 2015), but it can also be isolated from soil and it shows antifungal activity against several fungi, among which *Fusarium* spp. (Baffoni et al. 2015; Gajbhiye and Kapadnis 2016). According to Franke-Whittle et al. (2015), the genus *Exophiala* includes black yeasts that had negative correlations with apple plant growth and that were significant due to the high

abundance in the soil. Black yeasts is a *terminus technicus* describing a heterogeneous group of fungi that have in common melanised cell walls and the formation of cells by yeast-like budding (Sterflinger 2006). While no bacteria were significantly associated with this treatment, four common saprotrophic soil fungi were indicator OTUs in BD soils. These were: *Mortierella* sp. and *Mortierella antarctica,* which occur mainly in the soil of different ecosystems, including terrestrial habitats of Antarctica (Adams et al. 2006), *Humicola nigrescens,* a thermophilic mould capable of efficiently degrading organic materials by secreting thermostable enzymes (Singh et al. 2016), and *Acremonium persicinum*, an endophytic fungus of the grapevine with antagonistic activity against both the asexual and sexual spores of *Plasmopara viticola* (Burruano et al. 2016). *Acremonium persicinum* also hydrolyses cellulose and produces cephalosporin C, which is a major precursor of semisynthetic cephalosporin antibiotics used to treat a wide range of bacterial infections (Sarookhani and Moazzami 2007).

As regards the bacterial indicator species of BDGM soils, there were three bacterial genera involved in the soil nitrogen cycle (*Nitrospira* sp., *Paenibacillus* sp. and the aforementioned nitrogen-fixing *Pontibacter* sp.). *Nitrospira* sp. belongs to nitrite-oxidising bacteria (NOB) (Hayatsu et al. 2008) and is widely distributed in many habitats, including soil, oceans, freshwater and wastewater treatment plants (Koch et al. 2015). In soil, it is often associated with an increased supply of nitrogen from mineral fertilisation (Zhou et al. 2015). Another nitrogen-fixing bacterium is *Paenibacillus* sp., which is also considered a plant growth promoter due to its production of IAA (indole-3-acetic acid) and it also has biocontrol potential against grapevine pathogens such as *Botrytis cinerea* and *Neofusicoccum parvum* (Grady et al. 2016; Haidar et al. 2016). As regards fungi, four black yeasts (*Cladorrhinum* sp., *Capnobotryella* sp., *Cystofilobasidium capitatum* and *Exophiala*) were fungal indicator OTUs in BDGM soils. Most of these genera are found as saprobes colonising inert surfaces, or in hydrocarbon- or heavy-metal-polluted habitats, and several are potential human pathogens (Seyedmousavi et al. 2014). Currently, little information about the ecophysiology of other detected indicator OTUs (*Catelliglobosispora* sp., *Planosporangium* sp. and *Capnobotryella* sp.) is available in order to deduce any putative ecological role in the soil system.

Arbuscular mycorrhizal fungi occur in the roots of most plants and are an ecologically important component of the soil microbiome. Analysis of the OTUs belonging to Glomeromycota showed a low level of AMF relative

abundance. According to Orgiazzi et al. (2012), ectomycorrhizal phylotypes are numerous in natural sites covered by trees, but they are almost completely lacking in anthropogenic and grass-covered sites. Ciccolini et al. (2016), on studying the community of AMF with 454 pyrosequencing, reported a low level of AMF richness in intense cropping systems. However, we should also consider the limited coverage by the primers used in this work to be partly responsible for the few Glomeromycota observed (Stockinger et al. 2010). The genus *Glomus* was most abundant and present in all soils, in accordance with other studies, which have found this genus to be the most abundant AMF in the grapevine (Schreiner and Mihara 2009).

Soil is a non-renewable resource and most vineyard soils are considered to be highly degraded in terms of loss of organic carbon, as a result of a decrease in nutrient content, an accumulation of metals and organic pollutants (Coll et al. 2011). The effect of agricultural management systems on soil microorganisms is generally studied with plants undergoing rotation, but less is known about soils used for perennial plants, such as the grapevine. To our knowledge, this is the first work comparing the microbial communities of soil in organic and biodynamic vineyards using 454 pyrosequencing. Overall, our results showed that the diversity and composition of the microbial communities associated with biodynamic and organic farming systems were similar, indicating that the use of biodynamic preparations 500 and 501 did not cause any significant detectable changes to the soil microbial community in the short term, while the effects of green manure were significant in soil microbiota. The increase in soil microbial diversity associated with the use of green manure could have possible benefits for plant nutrition, considering that in organic farming systems mineralisation of organic matter depends on soil microorganism activity. The incorporation of green manure was shown to increase the diversity of microorganisms in soil, particularly the abundance of specific bacteria and fungi. Evidence of increased nitrogen-fixing and nitrite-oxidising bacteria populations in soil as a response to the use of green manure, suggests they can potentially be adopted to increase nitrogen availability. An extension of organic/biodynamic farming associated with green manure application could contribute to maintaining higher microbial biodiversity in vineyard soil and consequently positively influence overall soil quality.

References

Abarenkov, K., Nilsson, R. H., Larsson, K. H., Alexander, I. J., Eberhardt, U., Erland, S., Hoiland, K., Kjoller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A. F. S., Tedersoo, L., Ursing, B. M., Vralstad, T., Liimatainen, K., Peintner, U., and Koljalg, U. 2010. The UNITE database for molecular identification of fungi - recent updates and future perspectives. New Phytol. 186:281-285.

Abujabhah, I. S., Bound, S. A., Doyle, R., and Bowman, J. P. 2016. Effects of biochar and compost amendments on soil physico-chemical properties and the total community within a temperate agricultural soil. Appl. Soil Ecol. 98:243-253.

Adams, B. J., Bardgett, R. D., Ayres, E., Wall, D. H., Aislabie, J., Bamforth, S., Bargagli, R., Cary, C., Cavacini, P., Connell, L., Convey, P., Fell, J. W., Frati, F., Hogg, I. D., Newsham, K. K., O'Donnell, A., Russell, N., Seppelt, R. D., and Stevens, M. I. 2006. Diversity and distribution of Victoria Land biota. Soil Biol. Biochem. 38:3003-3018.

Anderson, M. J., and Willis, T. J. 2003. Canonical analysis of principal coordinates: A useful method of constrained ordination for ecology. Ecology 84:511-525.

Ardley, J. K., Parker, M. A., De Meyer, S. E., Trengove, R. D., O'Hara, G. W., Reeve, W. G., Yates, R. J., Dilworth, M. J., Willems, A., and Howieson, J. G. 2012. *Microvirga lupini* sp nov., *Microvirga lotononidis* sp nov and *Microvirga zambiensis* sp nov are alphaproteobacterial root-nodule bacteria that specifically nodulate and fix nitrogen with geographically and taxonomically separate legume hosts. Int. J. Syst. Evol. Micr. 62:2579-2588.

Bae, S., Fleet, G. H., and Heard, G. M. 2006. Lactic acid bacteria associated with wine grapes from several Australian vineyards. J. Appl. Microbiol. 100:712-727.

Baffoni, L., Gaggia, F., Dalanaj, N., Prodi, A., Nipoti, P., Pisi, A., Biavati, B., and Di Gioia, D. 2015. Microbial inoculants for the biocontrol of *Fusarium* spp. in durum wheat. Bmc Microbiol. 15.

Baldi, E., Toselli, M., Marcolini, G., Quartieri, M., Cirillo, E., Innocenti, A., and Marangoni, B. 2010. Compost can successfully replace mineral fertilizers in the nutrient management of commercial peach orchard. Soil Use Manage. 26:346-353.

Baskar, P., and Shanmugham, P. M. 2016. Performance of chillies-sunflower cropping system under organic nutrient management. Int. J.Agric. Stat. Sci. 12:193-196.

Bengtsson-Palme, J., Hartmann, M., Eriksson, K. M., Pal, C., Thorell, K., Larsson, D. G. J., and Nilsson, R. H. 2015. metaxa2: improved identification and

taxonomic classification of small and large subunit rRNA in metagenomic data. Mol. Ecol. Resour. 15:1403-1414.

Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A., De Wit, P., Sanchez-Garcia, M., Ebersberger, I., de Sousa, F., Amend, A. S., Jumpponen, A., Unterseher, M., Kristiansson, E., Abarenkov, K., Bertrand, Y. J. K., Sanli, K., Eriksson, K. M., Vik, U., Veldre, V., and Nilsson, R. H. 2013. Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods Ecol. Evol. 4:914-919.

Burns, K. N., Bokulich, N. A., Cantu, D., Greenhut, R. F., Kluepfel, D. A., O'Geen, A. T., Strauss, S. L., and Steenwerth, K. L. 2016. Vineyard soil bacterial diversity and composition revealed by 16S rRNA genes: Differentiation by vineyard management. Soil Biol. Biochem. 103:337-348.

Burruano, S., Mondello, V., and Conigliaro, G. 2016. Endophytic fungi in asymptomatic *Vitis vinifera* L. and their effects on *Plasmopara viticola*. Page 233 in: Biocontrol of Major Grapevine Diseases: Leading Research. M. F. Compant S, ed. CAB International, Oxfordshire, UK. .

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Tumbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335-336.

Carmaran, C. C., Berretta, M., Martinez, S., Munaut, V. B. F., and Gasoni, L. 2015. Species diversity of Cladorrhinum in Argentina and description of a new species, *Cladorrhinum australe*. Mycol. Prog. 14.

Carpenter-Boggs, L., Kennedy, A. C., and Reganold, J. P. 2000. Organic and biodynamic management: Effects on soil biology. Soil Sci. Soc. Am. J. 64:1651-1659.

Chalker-Scott, L. 2013. The Science Behind Biodynamic Preparations: A Literature Review. Horttechnology 23:814-819.

Ciccolini, V., Ercoli, L., Davison, J., Vasar, M., Opik, M., and Pellegrino, E. 2016. Land-use intensity and host plant simultaneously shape the composition of arbuscular mycorrhizal fungal communities in a Mediterranean drained peatland. Fems Microbiol. Ecol. 92.

Coll, P., Le Cadre, E., Blanchart, E., Hinsinger, P., and Villenave, C. 2011. Organic viticulture and soil quality: A long-term study in Southern France. Appl. Soil Ecol. 50:37-44.

Corneo, P. E., Pellegrini, A., Cappellin, L., Roncador, M., Chierici, M., Gessler, C., and Pertot, I. 2013. Microbial community structure in vineyard soils across altitudinal gradients and in different seasons. Fems Microbiol. Ecol. 84:588-602.

De Caceres, M., and Legendre, P. 2009. Associations between species and groups of sites: indices and statistical inference. Ecology 90:3566-3574.

Doring, J., Frisch, M., Tittmann, S., Stoll, M., and Kauer, R. 2015. Growth, Yield and Fruit Quality of Grapevines under Organic and Biodynamic Management. Plos One 10.

Edgar, R. C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10:996-+.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194-2200.

El-Tarabily, K. A., Hardy, G. E. S., and Sivasithamparam, K. 2010. Performance of three endophytic actinomycetes in relation to plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber under commercial field production conditions in the United Arab Emirates. Eur. J. Plant Pathol. 128:527-539.

Fierer, N., Bradford, M. A., and Jackson, R. B. 2007. Toward an ecological classification of soil bacteria. Ecology 88:1354-1364.

Filonow, A. B., and Lockwood, J. L. 1985. Evaluation of several actinomycetes and the fungus *Hyphochytrium catenoides* as biocontrol agents for phytophthora root-rot of soybean. Plant Dis. 69:1033-1036.

Franke-Whittle, I. H., Manici, L. M., Insam, H., and Stres, B. 2015. Rhizosphere bacteria and fungi associated with plant growth in soils of three replanted apple orchards. Plant Soil 395:317-333.

Gajbhiye, M. H., and Kapadnis, B. P. 2016. Antifungal-activity-producing lactic acid bacteria as biocontrol agents in plants. Biocontrol Sci. Techn. 26:1451-1470.

Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113-118.

Gaspar, J. M., and Thomas, W. K. 2015. FlowClus: efficiently filtering and denoising pyrosequenced amplicons. Bmc Bioinformatics 16.

Grady, E. N., MacDonald, J., Liu, L., Richman, A., and Yuan, Z. C. 2016. Current knowledge and perspectives of *Paenibacillus*: a review. Microb. Cell Fact. 15.

Guzzon, R., Gugole, S., Zanzotti, R., Malacarne, M., Larcher, R., von Wallbrunn, C., and Mescalchin, E. 2016. Evaluation of the oenological suitability of grapes grown using biodynamic agriculture: the case of a bad vintage. J. Appl. Microbiol. 120:355-365.

Haidar, R., Deschamps, A., Roudet, J., Calvo-Garrido, C., Bruez, E., Rey, P., and Fermaud, M. 2016. Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine. Biol. Control 92:55-65.

Hayatsu, M., Tago, K., and Saito, M. 2008. Various players in the nitrogen cycle: Diversity and functions of the microorganisms involved in nitrification and denitrification. Soil Sci. Plant Nutr. 54:33-45.

Hervé, M. 2017. Diverse Basic Statistical and Graphical Functions. R package version 0.9-64. https://CRAN.R-project.org/package=RVAideMemoire.

Hop, D. V., Sakiyama, Y., Chu, T. T. B., Otoguro, M., Hang, D. T., Miyadoh, S., Luong, D. T., and Ando, K. 2011. Taxonomic and ecological studies of actinomycetes from Vietnam: isolation and genus-level diversity. J. Antibiot. 64:599-606.

Hothorn, T., Bretz, F., and Westfall, P. 2008. Simultaneous inference in general parametric models. Biometrical J. 50:346-363.

Ingels, C. A., Scow, K. M., Whisson, D. A., and Drenovsky, R. E. 2005. Effects of cover crops on grapevines, yield, juice composition, soil microbial ecology, and gopher activity. Am. J. Enol. Viticult. 56:19-29.

Irvin, N. A., Pinckard, T. R., Perring, T. M., and Hoddle, M. S. 2014. Evaluating the potential of buckwheat and cahaba vetch as nectar producing cover crops for enhancing biological control of *Homalodisca vitripennis* in California vineyards. Biol. Control 76:10-18.

Kirchmann, H. 1994. Biological dynamic farming - an occult form of alternative agriculture. J. Agr. Environ. Ethic. 7:173-187.

Koch, H., Lucker, S., Albertsen, M., Kitzinger, K., Herbold, C., Spieck, E., Nielsen, P. H., Wagner, M., and Daims, H. 2015. Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus *Nitrospira*. P. Natl. Acad. Sci USA 112:11371-11376.

Komarek, M., Cadkova, E., Chrastny, V., Bordas, F., and Bollinger, J. C. 2010. Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. Environ. Int. 36:138-151.

Linkind, D., Gadanho, M., van Broock, M., and Sampaio, J. P. 2009. *Cystofilobasidium lacus-mascardii* sp nov., a basidiomycetous yeast species isolated from aquatic environments of the Patagonian Andes, and Cystofilobasidium macerans sp nov., the sexual stage of *Cryptococcus macerans*. Int. J. Syst. Evol. Micr. 59:622-630.

Lisboa, F. J. G., Peres-Neto, P. R., Chaer, G. M., Jesus, E. D., Mitchell, R. J., Chapman, S. J., and Berbara, R. L. L. 2014. Much beyond Mantel: Bringing Procrustes Association Metric to the Plant and Soil Ecologist's Toolbox. Plos One 9.

Lopez-Pineiro, A., Albarran, A., Nunes, J. M. R., Pena, D., and Cabrera, D. 2011. Long-term impacts of de-oiled two-phase olive mill waste on soil chemical properties, enzyme activities and productivity in an olive grove. Soil Till. Res. 114:175-182.

Lotter, D. W. 2003. Organic agriculture. J. Sustain. Agr. 21:59-128.

Miguens, T., Leiros, M. C., Gil-Sotres, F., and Trasar-Cepeda, C. 2007. Biochemical properties of vineyard soils in Galicia, Spain. Sci. Total Environ. 378:218-222.

Munoz, A., Lopez-Pineiro, A., and Ramirez, M. 2007. Soil quality attributes of conservation management regimes in a semi-arid region of south western Spain. Soil Till. Res. 95:255-265.

Muyzer, G., Dewaal, E. C., and Uitterlinden, A. G. 1993. Profiling of Complex Microbial-Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S Ribosomal-RNA. Appl. Environ. Microbiol. 59:695-700.

Nicola, L., Turco, E., Albanese, D., Donati, C., Thalheimer, M., Pindo, M., Insam, H., Cavalieri, D., Pertot, I. 2017. Fumigation with dazomet modifies soil microbiota in apple orchards affected by replant disease. Appl. Soil. Ecol. 113:71-79

Nisiotou, A. A., Dourou, D., Filippousi, M. E., Diamantea, E., Fragkoulis, P., Tassou, C., and Banilas, G. 2015. Genetic and Technological Characterisation of Vineyard- and Winery-Associated Lactic Acid Bacteria. Biomed Res. Int. id. 508254

Nurkanto, A., Lisdiyanti, P., Hamada, M., Ratnakomala, S., Shibata, C., and Tamura, T. 2016. *Actinoplanes bogoriensis* sp nov., a novel actinomycete isolated from leaf litter. J. Antibiot. 69:26-30.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens M. H. H., Szoecs, E., and Wagner, H. 2017. vegan: Community Ecology Package. R package version 2.4-2. https://CRAN.R-project.org/package=vegan.

Okur, N., Kayikcioglu, H. H., Ates, F., and Yagmur, B. 2016. A comparison of soil quality and yield parameters under organic and conventional vineyard systems in Mediterranean conditions (West Turkey). Biol. Agric. Hortic. 32:73-84.

Orgiazzi, A., Lumini, E., Nilsson, R. H., Girlanda, M., Vizzini, A., Bonfante, P., and Bianciotto, V. 2012. Unravelling Soil Fungal Communities from Different Mediterranean Land-Use Backgrounds. Plos One 7.

Peintner, U., Knapp, M., Fleischer, V., Walch, G., and Dresch, P. 2016. *Myrmecridium hiemale* sp nov from snow-covered alpine soil is the first eurypsychrophile in this genus of anamorphic fungi. Int. J. Syst. Evol. Micr. 66:2592-2598.

Probst, B., Schuler, C., and Joergensen, R. G. 2008. Vineyard soils under organic and conventional management - microbial biomass and activity indices and their relation to soil chemical properties. Biol. Fert. Soils 44:443-450.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glockner, F. O. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41:D590-D596.

Reeve, W., Ardley, J., Tian, R., De Meyer, S., Terpolilli, J., Melino, V., Tiwari, R., Yates, R., O'Hara, G., Howieson, J., Ninawi, M., Zhang, X. J., Bruce, D., Detter, C., Tapia, R., Han, C., Wei, C. L., Huntemann, M., Han, J., Chen, I. M., Mavromatis, K., Markowitz, V., Szeto, E., Ivanova, N., Pagani, I., Pati, A., Goodwin, L., Woyke, T., and Kyrpides, N. 2014. Genome sequence of the *Listia angolensis* microsymbiont *Microvirga lotononidis* strain WSM3557(T). Stand. Genomic Sci. 9:540-550.

Rotaru, L., Stoleru, V., and Mustea, M. 2011. Fertilization with green manure on Chasselas Dore grape vine as an alternative for sustainable viticulture. J. Food Agric. Environ. 9:236-243.

Sarookhani, M. R., and Moazzami, N. 2007. Isolation of *Acremonium* species producing cephalosporine C(CPC) from forest soil in Gilan province, Iran. Afr. . Biotechnol. 6:2506-2510.

Schimel, J. P., and Schaeffer, S. M. 2012. Microbial control over carbon cycling in soil. Front. Microbiol. 3.

Schlegel, W. 1973. Der Weinbau in der Schweiz. Steiner, Wiesbaden:24-92.

Schmieder, R., and Edwards, R. 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27:863-864.

Schreiner, R. P., and Mihara, K. L. 2009. The diversity of arbuscular mycorrhizal fungi amplified from grapevine roots (*Vitis vinifera* L.) in Oregon vineyards is seasonally stable and influenced by soil and vine age. Mycologia 101:599-611.

Seyedmousavi, S., Netea, M. G., Mouton, J. W., Melchers, W. J. G., Verweij, P. E., and de Hoog, G. S. 2014. Black Yeasts and Their Filamentous Relatives: Principles of Pathogenesis and Host Defense. Clin. Microbiol. Rev. 27:527-542.

Singh, B., Pocas-Fonseca, M. J., Johri, B. N., and Satyanarayana, T. 2016. Thermophilic molds: Biology and applications. Crit. Rev. Microbiol. 42:985-1006.

Srinivasan, S., Lee, J. J., Lee, S. S., and Kim, M. K. 2014. *Pontibacter humi* sp nov., Isolated from Mountain Soil. Curr. Microbiol. 69:263-269.

Steenwerth, K. L., Drenovsky, R. E., Lambert, J. J., Kluepfel, D. A., Scow, K. M., and Smart, D. R. 2008. Soil morphology, depth and grapevine root frequency influence microbial communities in a Pinot noir vineyard. Soil Biol. Biochem. 40:1330-1340.

Sterflinger, K. 2006. Black Yeasts and Meristematic Fungi: Ecology, Diversity and Identification. Pages 501-514 in: Biodiversity and Ecophysiology of Yeasts Part of the series The Yeast Handbook. C. R. G. Peter, C. Leao (Eds.), ed. Springer Berlin Heidelberg, Berlin.

Stockinger, H., Kruger, M., and Schussler, A. 2010. DNA barcoding of arbuscular mycorrhizal fungi. New Phytol. 187:461-474.

Sugiyama, A., Vivanco, J. M., Jayanty, S. S., and Manter, D. K. 2010. Pyrosequencing Assessment of Soil Microbial Communities in Organic and Conventional Potato Farms. Plant Dis. 94:1329-1335.

Villanueva-Rey, P., Vazquez-Rowe, I., Moreira, M. O. T., and Feijoo, G. 2014. Comparative life cycle assessment in the wine sector: biodynamic vs. conventional viticulture activities in NW Spain. J. Cleaner Prod. 65:330-341.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microb. 73:5261-5267.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. 1991. 16S Ribosomal DNA Amplification for Phylogenetic Study. J. Bacteriol. 173:697-703.

White, T., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: a guide to methods and applications. Academic Press, New York, USA, pp. 315–322.

Wittwer, R. A., Dorn, B., Jossi, W., and van der Heijden, M. G. A. 2017. Cover crops support ecological intensification of arable cropping systems. Sci. Re. 7.

Xu, L. H., Zeng, X. C., Nie, Y., Luo, X. S., Zhou, E. M., Zhou, L. L., Pan, Y. F., and Li, W. J. 2014. *Pontibacter diazotrophicus* sp nov., a Novel Nitrogen-Fixing Bacterium of the Family Cytophagaceae. Plos One 9.

Zhou, X., Fornara, D., Wasson, E. A., Wang, D. M., Ren, G. D., Christie, P., and Jia, Z. J. 2015. Effects of 44 years of chronic nitrogen fertilization on the soil nitrifying community of permanent grassland. Soil Biol. & Biochem. 91:76-83.

Supplementary Materials

Microbial communities respond to green manure in vineyards managed using environmentally friendly methods.

Claudia M.O. Longa[a], Lidia Nicola[a,b], Livio Antonielli[c], Enzo Mescalchin[d], Roberto Zanotti[d], Elena Turco[a], Ilaria Pertot[a,e].

[a] Research and Innovation Centre, Fondazione Edmund Mach (FEM), 38010 San Michele all'Adige (TN), Italy
[b] Department of Microbiology, University of Innsbruck, 6020 Innsbruck, Austria
[c] AIT Austrian Institute of Technology, Health and Environment Department, Konrad-Lorenz-Straße 24, A-3430 Tulln, Austria
[d] Technology Transfer Centre, Fondazione Edmund Mach (FEM), 38010 San Michele all'Adige (TN), Italy
[e] Center Agriculture Food Environment, University of Trento

Supplementary Material 1

Alpha-diversity analysis

Generalized linear model (GLM): bacteria -> observed OTUs values

Analysis of Deviance Table
Model: Gamma, link: inverse
Response: observed
Terms added sequentially (first to last)

	Df	Deviance Resid	Df Resid.	Dev	Pr(>Chi)
NULL			34	0.108383	
Treatment	2	0.0262747	32	0.082109	3.813e-05 ***
Field	1	0.0083812	31	0.073727	0.0108417 *
Treatment:Field	2	0.0016453	29	0.072082	0.528802
Field:Line	2	0.0048036	27	0.067279	0.155649
Treatment:Field:Line	4	0.0251098	23	0.042169	0.0006419 ***
Field:Line:Point	4	0.0051843	19	0.036984	0.403961
Treatment:Field:Line:Point	8	0.0228832	11	0.014101	0.0234049 *

---Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Posthoc analysis for treatment: bacteria -> observed OTUs values

Simultaneous Tests for General Linear HypothesesSupplementa
Multiple Comparisons of Means: Tukey Contrasts
Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(>|z|)
B - A == 0	8.52E-06	1.46E-05	0.583	0.8292
BS - A == 0	-3.47E-05	1.42E-05	-2.446	0.0382 *
BS - B == 0	-4.32E-05	1.39E-05	-3.114	0.0053 **

---Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
(Adjusted p values reported -- single-step method)

Generalized linear model (GLM): fungi -> observed OTUs values

Analysis of Deviance Table
Model: Gamma, link: inverse
Response: observed
Terms added sequentially (first to last)

	Df	Deviance Resid	Df Resid.	Dev	Pr(>Chi)
NULL			35	0.43538	
Treatment	2	0.028557	33	0.40682	0.24551
Field	1	0.057438	32	0.34939	0.01746 *
Treatment:Field	2	0.020305	30	0.32908	0.36838
Field:Line	2	0.026611	28	0.30247	0.27016
Treatment:Field:Line	4	0.071851	24	0.23062	0.13237
Field:Line:Point	4	0.042897	20	0.18772	0.37713
Treatment:Field:Line:Point	8	0.057667	12	0.13005	0.6839

---Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Supplementary material 2

Beta-diversity analysis

#PERMANOVA on Bray-Curtis dissimilarities: bacteria (Hellinger transformed)

Call:
Permutation: free
Number of permutations: 9999

Terms added sequentially (first to last)

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Treatment	2	0.09732	0.04866	1.7971	0.08406	0.0003 ***
Field	1	0.10174	0.101736	3.7574	0.08787	0.0001 ***
Treatment:Field	2	0.09601	0.048005	1.773	0.08293	0.0004 ***
Field:Line	2	0.04996	0.024979	0.9225	0.04315	0.6778
Treatment:Field:Line	4	0.11809	0.029522	1.0903	0.10200	0.2186
Field:Line:Point	4	0.15197	0.037993	1.4032	0.13127	0.0047 **
Treatment:Field:Line:Point	8	0.24481	0.030602	1.1302	0.21146	0.1261
Residuals	11	0.29784	0.027076		0.25726	
Total	34	1.15774			1.00000	

---Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Permutational pairwise comparisons of bacterial dissimilarities between treatments

Pairwise comparisons using permutational MANOVAs on a distance matrix
9999 permutations

	O	BD
BD	0.131	-
BDGM	0.045	0.019

P value adjustment method: fdr

PERMANOVA on Bray-Curtis dissimilarities: fungi (Hellinger transformed)

Call:
Permutation: free
Number of permutations: 9999
Terms added sequentially (first to last)

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Treatment	2	0.5173	0.258629	3.3631	0.15020	0.0001 ***
Field	1	0.2533	0.253322	3.2941	0.07356	0.0001 ***
Treatment:Field	2	0.2226	0.111321	1.4476	0.06465	0.0207 *
Field:Line	2	0.1238	0.06188	0.8047	0.03594	0.8871
Treatment:Field:Line	4	0.3614	0.090342	1.1748	0.10493	0.1194
Field:Line:Point	4	0.4425	0.110618	1.4384	0.12848	0.0036 **
Treatment:Field:Line:Point	8	0.6002	0.07503	0.9757	0.17429	0.5889
Residuals	12	0.9228	0.076902		0.26796	
Total	35	3.4439			1.00000	

---Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

VII. Summary and conclusions

This thesis explored the microbial and biochemical complexity of agricultural soils, both at laboratory and field scale, using next generation technologies. Particularly, it focused on factors affecting soil health on two important and profitable cultivations: apple and grape. The main questions were related to the aetiology of apple replant disease (ARD) and the effect of green manure on soil microbial populations (Chapters II to VI). Specifically Chapters II, III and IV investigated the role of soil microorganisms in the aetiology of ARD, finding that soil microbial communities and the associations between their members were significantly different in ARD-affected soils. In Chapter V, a change in the phenolic profile of soil amended with apple root debris was assessed, indicating that specific substances produced by old trees could contribute to ARD development. Finally, in Chapter VI, the focus was on vineyards, where green manure significantly increased the biodiversity of soil microbial communities in sustainably managed plots.

Role of soil microorganisms and phenolic compounds in the aetiology of ARD

The presence of a complex of fungal pathogens in soil is one of the most accredited hypotheses regarding the onset of ARD (Mazzola and Manici 2012). In several studies, fungi belonging to the genera *Cylindrocarpon*, *Rhizoctonia* and *Pythium* were isolated from ARD affected soils (Jaffee et al. 1982; Mazzola 1998), but their recovery was not consistent in every orchard. The advent of next-generation sequencing (NGS) allowed a more comprehensive look at whole soil communities, permitting to understand if there were any changes in taxa composition in ARD-affected soils. In the first step in the study of ARD, soil microbial communities were analysed in an orchard where fumigation significantly improved ARD symptoms at the end of the second growing season (Chapter II). High-throughput sequencing revealed differences in the soil microbial community composition caused by fumigation, an effect that persisted 19 months after the application of the product. This modification consisted mainly in a slight imbalance between beneficial and pathogenic microorganisms for plants in fumigated and untreated soils. Specifically, a complex of potentially pathogenic fungi (*Ilyonectria* sp., *Pyrenochaeta* sp. and *Mortierella* sp.) was found to be negatively correlated with apple tree growth, while a strong positive correlation was

found with plant growth promoting microorganisms (*Chaetomium* sp. *Microbacterium* sp., *Micromonospora* sp., *Streptomyces* sp., *Bacillus* sp., and *Pseudomonas* sp.), suggesting that ARD might be the result not only of an increase in soil-borne pathogens, but also of a reduction in plant beneficial microorganisms, also confirming some of the results by Yim et al. (2015).

In Chapter III, the different hypotheses on the aetiology of ARD (microbial origin, presence of toxins, nutrient imbalance in soil) were tested through the application of specific soil treatments (fumigation, addition of fungal biocontrol agent, soil washing, compost amendment) in greenhouse conditions during two consecutive years, and the effects on apple plants and on soil microbial communities were evaluated. Soil microbial communities were quite resilient to the soil treatments applied, in fact only fumigation significantly modified both bulk soil and rhizosphere communities. The variability of the effects on plants was assessed between two years: soil washing was the most successful treatment in 2013, while in 2014, it was fumigation. This change in effectiveness did not correspond to any significant change in the microbial communities in the two years. However, as in Chapter II, a number of OTUs with plant growth promotion potential was found to be positively correlated with plant growth (*Pseudoxanthomonas* sp.*, Sphingobium* sp.*, Dyadobacter* sp.*, Sphingopyxis* sp.*, Bosea* sp.*, Nocardioides* sp.). This different success of intensive irrigation and fumigation in the two years could be due to the participation of additional factors besides microorganisms for the onset of ARD, specifically water-soluble substances, produced by old apple trees, which could have been washed away by the soil washing, especially when close to replanting.

Since the advent of NGS, this technique has been increasingly used to investigate the dynamics of soil microbial communities in ARD-affected soils (Franke-Whittle et al. 2015; Mazzola et al. 2015; Yim et al. 2015). Each study discovered different aspects concerning soil microorganisms and ARD, but their results were difficult to generalize, since each used different approaches. To overcome this impasse, a meta-analysis was developed, using a taxonomic binning approach, and analyzing together all the deep-sequencing data sets available on microbial communities in soils with a reduction in growth due to ARD (Chapter IV). Together with other four studies, the study in Chapter II was also involved in this meta-analysis. This approach allowed the simultaneous analysis of the largest cohort of microbial community data on

ARD from different parts of the world. This broad analysis confirmed the difference in the microbial communities in healthy and diseased soils, as already indicated in other studies (Mazzola et al. 2015; Nicola et al. 2017; Yim et al. 2015). In addition to a higher presence of phytopathogenic and nematophagous microorganisms in ARD-affected soils, there was also a change in the microbial associations, that could be due to a change in the soil environmental chemistry and metabolome. As already hinted in Chapter III, there are indications that soil microorganisms might not be the sole causal agent of ARD, but, most likely, a number of environmental parameters affects microbial physiology and from their mutual interplay ARD arises, therefore defining this syndrome as an opportunistic microbial infectious disease. In this Chapter, also considering the results of the previous chapters, an "ecological ARD hypothesis" is proposed, together with a possible cycle, where a change in key soil factors can alter the equilibrium in soil ecosystem, leading to the disease.

In order to investigate other parameters that could be involved in the onset of ARD, the soil metabolic profile was analysed after the addition of apple roots debris and the effect of this addition was also monitored on apple seedlings' health. Indeed, the presence of apple root debris in soil significantly impairs the growth of apple seedlings. The addition of the roots caused a significant increase in specific phenolic compounds (mainly phlorizin and phloretin) and, if the seedlings were planted just after the addition of roots, the initial negative impact on seedlings persisted over time, despite the reduction in concentration of phenolic compounds. Therefore, the presence of toxic phenolic compounds in soil could be one of the factors contributing to create a damaging soil environment for the new plant, and probably also affecting the microbial communities. In other studies, these compounds were also detected in the holes of explanted old apple trees (Yin et al. 2016) and their permanence in soil is dependent on the temperature (Politycka and Adamska 2003).

Effect of green manure on soil microbial populations

As assessed in the previous chapters, soil is an extremely complex environment, where numerous parameters can influence its mechanisms. Intensive monoculture can modify the soil, often leading to a depletion in

nutrients and biodiversity. In ARD-affected soils, the effect of compost was studied among other treatments, but it did not decrease ARD symptoms in the short period. Beside compost, green manure is another agronomic practice that can improve soil quality and increase organic matter. In vineyards, instead, green manure was applied in plots managed organically or biodynamically. No significant differences were found in microbial diversity between the plots with organic or biodynamic management, while green manure revealed itself as a great resource to improve soil health and microbial diversity. This fertilization technique, in fact, increased the abundance of bacteria involved in soil nutrients cycle, such as *Microvirga* sp., *Pontibacter* sp., *Actinoplanes* sp. Hence, green manure is recommended in cultivations that do not allow for crop rotation, especially fruit trees, where beneficial herbaceous and leguminous plants can be cultivated between tree rows, to maintain a biodiverse microbial community in soil.

Outlook

This thesis increases our understanding of the composition and dynamics of microbial communities in agricultural soils, with particular focus on apple orchards and vineyards. It also provides insights in the fields of soil targeted metabolomics, soil-borne diseases, fertilization, thus contributing to unravel the complex picture of the soil ecosystem.

It is crucial that future studies keep focusing on investigating agricultural soil, since soil is a key factor for a fruitful production and healthy crops. Specifically, studies that link the newest research discoveries to the development of new applications and indications for farmers are needed, i.e. to actively promote and apply measures that preserve soil health (high biodiversity and high nutrient concentration), and, at the same time, to maintain an intensive crop production.

As regards apple cultivation and replant disease, future studies should pay particular attention to measure the widest set of soil and environmental parameters while conducting experiments, to clearly describe the soil environment and the possible changes happening. Specifically, it would be useful to pinpoint which environmental parameters are involved in ARD development, which could be used later as indicators of the risk or presence

of the disease. For example, soil connectivity was never taken into consideration in relation to ARD. In fact, soil bacteria usually experience a situation of low pore connectivity when under normal field conditions in soils, whereas the decaying root systems in ARD-affected soils could represent a source of continuous substrate flush and moisture buildup, increasing physical connectivity. It would be interesting to understand if soil connectivity actually changes in ARD-affected soils and how this modification could affect soil microbial communities and apple plants. Another aspect that could be examined in depth is the involvement of nitrogen metabolism in ARD development. Indeed, in this work we found indications that ARD-affected soils hosted a reduced population of microorganisms involved in the nitrogen cycle. Reduced nitrogen availability in ARD-affected soils could contribute to impaired plant growth, so specific essays could be set up to monitor the activity on these microorganisms and their enzymes in diseased soils.

Finally, the new "omics" techniques, like metabolomics, transcriptomics and shotgun sequencing, could be exploited to know not only the taxonomic composition of the soil microbial communities, but also the changes in the expression of their genes and in the metabolites they produce: linking all these pieces of information together will result in a clearer picture of all the possible factors involved in ARD and how to fight it.

References

Franke-Whittle, I. H., Manici, L. M., Insam, H., and Stres, B. 2015. Rhizosphere bacteria and fungi associated with plant growth in soils of three replanted apple orchards. Plant Soil 395:317-333.

Jaffee, B. A., Abawi, G. S., and Mai, W. F. 1982. Role of soil microflora and Pratylenches penetrans in apple replant disease. Phytopathology 72:247-251.

Mazzola, M. 1998. Elucidation of the microbial complex having a causal role in the development of apple replant disease in Washington. Phytopathology 88:930-938.

Mazzola, M., and Manici, L. M. 2012. Apple Replant Disease: Role of Microbial Ecology in Cause and Control. Pages 45-65 in: Annu. Rev.

Phytopathol., Vol 50, vol. 50. N. K. VanAlfen, J. E. Leach and S. Lindow, eds.

Mazzola, M., Hewavitharana, S. S., and Strauss, S. L. 2015. Brassica Seed Meal Soil Amendments Transform the Rhizosphere Microbiome and Improve Apple Production Through Resistance to Pathogen Reinfestation. Phytopathology 105:460-469.

Nicola, L., Turco, E., Albanese, D., Donati, C., Thalheimer, M., Pindo, M., Insam, H., Cavalieri, D., and Pertot., I. 2017. Fumigation with dazomet modifies soil microbiota in apple orchards affected by replant disease. Appl. Soil Ecol. Accepted.

Politycka, B., and Adamska, D. 2003. Release of phenolic compounds from apple residues decomposing in soil and the influence of temperature on their degradation. Pol. J. Environ. Stud. 12:95-98.

Yim, B., Winkelmann, T., Ding, G. C., and Smalla, K. 2015. Different bacterial communities in heat and gamma irradiation treated replant disease soils revealed by 16S rRNA gene analysis - contribution to improved aboveground apple plant growth? Front. Microbiol. 6.

Yin, C. M., Xiang, L., Wang, G. S., Wang, Y. F., Shen, X., Chen, X. S., and Mao, Z. Q. 2016. How to Plant Apple Trees to Reduce Replant Disease in Apple Orchard: A Study on the Phenolic Acid of the Replanted Apple Orchard. Plos One 11.

VIII. Acknowledgements

I would like to express my gratitude to all the people who have contributed to this thesis and helped me along this journey.

First of all, I would like to thank my PhD supervisors Univ. Prof. Ilaria Pertot and Univ. Prof. Heribert Insam, for giving me the chance to start this adventure. You gave me new perspectives and guided me safely across the perilous maze of Research. Thank you for your advice and support along the years.

A special thanks goes to Univ. Prof. Blaž Stres for teaching me about data analysis and statistics and clearing all my (very many) doubts. Collaborating with you was an enriching and eye-opening experience.

I would like to thank Dr. Elena Turco for welcoming me at Edmund Mach Foundation and providing guidance when I was a young, inexperienced and brand-new PhD student. Thank you for believing in me and supporting me even from far away.

I would like to express my gratitude also to all the co-authors, who collaborated with me in the papers implemented in this Thesis. Thank you for your precious input.

A huge thanks goes to my partners in crime during these four years: the group "Dottorate e Dottorande", alias Luisa, Valentina, Giulia, Martina and Selena. I am so glad we shared our days inside and outside the lab. Thank you for the laughters, the endless talks, and the support. Meeting you was the highlight of these years.

Thanks to my former room-mate Chiara, for sharing the apartment with me for three long years and for being my family far from home.

Thanks to everyone the "ex-PDP" building at Edmund Mach Foundation, especially the people (past and present) of the "Plant Pathology and Applied Microbiology" group: Oscar, Gerardo, Claudia, Francesca, Elena, Livia, Carmela, Denise, Luca, Franca, Christian, Daniele and Pierluigi and many others. Thank you for all the lunches and good times together.

I would like also to thank all the people of the Microbial Ecology Group at Innsbruck University. Thank you for welcoming me during my periods in

Innsbruck and for always making me feel at home with your kindness and happy spirit.

I will always owe endless gratitude to my family. My father Antonio, my mother Flavia, my sister Marta and my grandparents showered me with love since the day I was born and they taught me the value of hard work and the passion for science.

To my friends scattered all over Italy and Europe: Alberto, Giulia, Bianca, Alessandra, Tambu, Elena, Arianna, Anna and Maria, thank you for making me laugh even from far away.

Finally, I will never be thankful enough to Jacopo: you are the cornerstone of my life, thank you for the endless love, patience and support during these years.

GRAZIE

IX. Curriculum Vitae

Lidia Nicola, MSc.

Work adress:	Plant Pathology and Applied Microbiology Group, Fondazione Edmund Mach, via Edmund Mach 1, 38010 San Michele all'Adige (TN), Italy
Email addresses	lidia.nicola@fmach.it Lidia.Nicola@student.uibk.ac.at
Current Home address:	via Biasi 2, 38010 San Michele all'Adige (TN), Italy.
Date and Place of Birth:	1st August 1988, Voghera, Italy
Nationality:	Italian

Education and training

Since May 2013

PhD student in Microbiology on the project "ReSoil – the living soi metagenome project", a collaboration between Edmund Mach Foundation (Plant Pathology and Applied Microbiology Group, supervisor: Prof. Ilaria Pertot) and Innsbruck University (Microbial Ecology Group, supervisor: Prof. Heribert Insam).

February 2013 – November 2012

Internship at Edmund Mach Foundation to study the changes in fungal biodiversity in soils subjected to different agronomic managements using a molecular approach, sponsored by IUSS Pavia – Institute of Advanced Studies.

September 2012 – October 2010

Master's Degree in Experimental and Applied Biology, course in Environmental Biology and Biodiversity at the University of Pavia (Italy), 110/110 cum laude, after the discussion of the thesis entitled "The effects of temperature and soil moisture on lag phase of bacterial growth in soil after substrate addition" (supervisors: Prof. Solveig Tosi and Prof. Erland Bååth)

January 2012 – August 2011

Erasmus exchange period at Lunds Universitet (Sweden), with research at the Microbial Ecology Group on the influence of environmental factors on the length of soil bacterial lag period, under the supervision of Prof. Erland Bååth

September 2010 – October 2007

Bachelor's Degree in Biology at the University of Pavia (Italy), 110/110 cum laude, after the discussion of the thesis entitled "In vitro evaluation of nematophagous activity of fungal isolates" (supervisor: Prof. Solveig Tosi)

Peer-reviewed publications

Nicola L., Tosi S., Savini D. 2014. *In vitro* evaluation of nematophagous activity of fungal isolates. J. Basic Microbiol., 54(1):1-5.

Nicola, L., Vrhovsek, U., Soini, E., Insam, H., and Pertot., I. 2016. Phlorizin released by apple root debris is related to apple replant disease. Phytopathol. Mediterr. 55:432-442.

Nicola, L., Turco, E., Albanese, D., Donati, C., Thalheimer, M., Pindo, M., Insam, H., Cavalieri, D., and Pertot., I. 2017. Fumigation with dazomet modifies soil microbiota in apple orchards affected by replant disease. Appl. Soil Ecol. 113: 71-79.

Nicola, L., Turco, E., Thalheimer, M., Pindo, M., Insam, H., Pertot, I. 2017. Apple replant disease is not caused solely by soil microorganisms. Submitted to Plant. Dis.

Nicola, L., Insam, H., Pertot, I., Stres, B. 2017. Meta-analysis of microbiomes in soils affected by Apple Replant Disease. Submitted to Plant Soil.

Participation to conferences and summer schools

Conferences

Tosi, S., Dalla Valle, E., Nicola, L., Oro, V., Curto, G. 2012. *In Vitro* Nematophagous Activity of Fungi Isolated from Soil and Some Heteroderidae (Nematoda: Tylenchida). Poster presented at World Congress of Microbes-2012, Guangzhou (China). Book of Abstracts p. 310.

Longa, C., Turco, E., Nicola, L., Zanzotti, R., Mescalchin, E., Pertot, I. 2013. The soil microbial ecology and sustainability of organically and conventionally managed vineyards. Poster presented at Future IPM in Europe, 19-21 March 2013, Riva del Garda (TN), Italy.

Turco, E., Longa, C., Nicola, L., Pertot, I. 2013. Soil microorganisms vs. pesticides: potential bioeffector molecules for an environmental-friendly viticulture. Poster presented at Future IPM in Europe, 19-21 March 2013, Riva del Garda (TN), Italy.

Nicola, L., Turco, E., Albanese, D., Donati, C., Thalheimer, M., Pindo, M., Insam, H., Cavalieri, D., and Pertot., I. 2015. Fumigation with Dazomet modifies soil bacteria and fungal communities of apple orchards affected by Specific Replant Disease. Poster presented at Ecology of Soil Microorganisms, 29 November – 3 December 2015, Prague, Czech Republic.

Nicola, L., Insam, H., Pertot, I., Stres, B. 2016. Meta-analysis of microbiomes in soils affected by Apple Replant Disease. Poster presented at 3rd Thünen Symposium on Soil Metagenomics, 14-16 December, Braunschweig, Germany.

Summer schools and courses

July 1-5 2013. Summer school Bioinformatics tools for genomics and network analysis. EMBL-EBI at Edmund Mach Foundation (San Michele all'Adige, Italy).

November 12-15 2013. Genome reading & writing towards a natural genetic engineering – James A. Shapiro lectures at Edmund Mach Foundation (San Michele all'Adige, Italy).

June 26-30 2016 Joint International Summer School in Advanced methods and new integrated approaches to study soil processes in mountain ecosistems. Società Italiana della Scienza del Suolo, SensFor and COST (European Cooperation in Science and Technology) at the Alpine Study Center in Pieve Tesino (Italy).

Scholarships

May 2013 –May 2017

Scholarship for the PhD project "ReSoil – The living soil metagenome project" funded by FIRS>T (FEM International Research School Trentino).

October 2007 – May 2013

Scholarship from IUSS Pavia – Institute of Advanced Studies, for promising undergraduate students in the class of studies "Biomedical Sciences".

October 2007 – July 2012

Scholarship from MINTAS Foundation for the stay at Santa Caterina da Siena University College, Pavia

"I don't need no persuading

I'll trip, fall, pick myself up and

Walk unafraid"

R.E.M.