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"Sustainable Approaches to Improve Microbial Biodiversity in the Vineyard"

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“On the shoulders of giants”

To my supervisor and co supervisor,
with infinity love and gratitude.

Marco

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SUMMARY

Grapevine is one of the most important and globally widespread fruit species, with a high impact on the economy of many countries but with an intense environmental impact as well. Among all the different cultural systems, viticulture is considered one of the most treated cropping systems, with an intensive fungicide treatments schedule used to control the spread of pathogens. Modern agriculture (including viticulture) is now entering in a new 'green revolution' and therefore new environmentally friendly defence strategies against pathogens are needed to increase the sustainability and the biodiversity in agricultural system. The study of plant-microbiome interactions and the exploitation of plant-associated microorganisms in agriculture seems to be one of the most promising sustainable tools in agriculture. Indeed, the association between plants and microorganisms is known to be ancient and several studies showed the huge impact of plant-associated microorganisms on plant's health and resilience. The plant health has been demonstrated to be so related with its own microbiome that plants and associated microorganism are nowadays considered as a unique organism, the so-called "holobiont". The concept of holobiont requires a collective view of the functions and interactions existing between a plant host and its associated microorganism considering plants no longer as standalone entities but with a more holistic perception. Considering this, the subject of the proposed research is the elucidation of the microbial biodiversity in the vineyard with the aim of improving and enriching it, discovering, and testing new sustainable alternative strategies, also in the light of ongoing climate change. We focused our attention on unearthing the inner world within plant-microbiome interactions and improving microbial biodiversity in vineyard, favouring the beneficial ones and reducing the accumulation of pathogen-related species.

The first chapter is a brief and general introduction of the study to take an overview of the viticultural sector and to give the reader enough information on the object of the thesis.

The second chapter represents an initial literature review aiming at analysing in detail the effort of an anthropocentric point of view that, until now, has characterized the selection of elite plant genotypes and its effects on the plant-microbiome interactions. The final goal of this work is to propose the possibility of developing customized genotype- and environmental-specific synthetic community to boost plant resilience and improve holobiont functionality.

Although beneficial effects from grapevine and Arbuscular Mycorrhizal Fungi (AMF) interactions have been already reported, the impact on plant growth-defence trade-off features has still to be

elucidated and so in the third chapter of this study we evaluated it on young grapevine cuttings grafted onto two different rootstocks (1103P and SO4) known for having opposite growth and defence attitudes. In AM inoculated samples, it has revealed an involvement of the whole root microbiome in the growth-defence trade-off balancing and a significant stimulation of plant growth and defence pathways.

In the fourth chapter of our work, we aimed to enhance our understanding of the intricate interactions between plants and their associated microorganisms within a complex, natural system. We propose a metatranscriptomic study to explore whether the taxonomic composition and behaviour of microbial communities associated with grapevine plants are affected by the health status of the host. Simultaneously, we have identified several bacterial taxa in healthy samples. Once isolated from the original wood tissue, these taxa exhibited potential biocontrol activities against a wood-degrading fungal taxon.

The fifth chapter is focused on the isolation, characterization, and selection of the best performing microbial agent able to display positive features once inoculated, improving plant wellness, and reaching more resilient agricultural systems. We gave rise to a new database (ViMed-biomebank, https://www.revine-prima2020.org/vimed) consisting of more than 1000 isolates (fungi and bacteria) all of whom wood endophytes of *V. vinifera*. These microbial isolates have been then combined to be tested *in vitro* and *in vivo* with the aim to formulate the best performing customized synthetic communities (SynComs).

To underline the importance of the improvement of the whole holobiont instead of specific planttraits, in the chapter six is presented a perspective about the exploitation of synthetic microbial communities in agricultural systems to develop more sustainable breeding strategies based on the implementation of multiple interactions between plants and their beneficial associated microorganisms. We underline how the adoption of a holistic vision of plant breeding and the exploitation of 'holo-omics' techniques will lead to more resilient holobionts to abiotic and biotic stresses.

In the last chapter we present a global and comprehensive conclusion to contextualizes and finalizes the scientific findings of the work.

CHAPTER 1 | GENERAL INTRODUCTION TO THE STUDY

In the last decades, the demand of agricultural asset remarkably grew with a great increment in food production across the world since the beginning of the 1960s. Since then, food production rose by 140% in Africa, by almost 200% in Latin America, by 280% in Asia and by 68% in Western Europe (FAO 2005). During the same period, to meet the increasing demand, the intensity of production on agricultural lands has also risen substantially leading to higher consumption of water, fertilizers, and pesticides (Hazell & Wood 2008). Despite the clear and significant connection between the use of these agricultural input and the increment of food production is well known, it would be both simplistic and optimistic to assume that this relationship will remain linear in the future and that gains will continue at the previous rates (Tilman 1999). Indeed, the excessive use of pesticides, fertilizers and irrigation resulting from non-precision farming leads to inefficient use of feedstock and a big environmental impact. There is also growing evidence that this approach to agricultural production has reached critical environmental limits and that the aggregate costs in terms of lost or foregone benefits from environmental services are too great for the world to bear (Ruttan 1999; MEA 2005; Kitzes et al. 2008).

In the agricultural landscape, grapevine (*Vitis vinifera*) is one of the most important crops in the EU with 3.4 million cultivated ha in 2019, accounting for 50% of the grapevine cultivated area in the world (Faostat, http://www.fao.org/faostat/en/#data/QC). At the same time, among the different agricultural system, viticulture is also one of the most environmentally impactful since to meet high-quality standard of wine and fruit production and to prevent severe yield losses caused by pathogens spread as well, multiple fertilizer and pesticide applications are required across the growing season (Provost et al., 2016). Therefore, the development of viticulture practices that are more respectful for the environment, with reduced agrochemical needs, is globally acknowledged as a priority soon (Giudice et al., 2022). Giving birth to a new vision of viticulture that aims at optimization of the use of raw materials and a great reduction in the use of chemical molecules is necessary. Indeed nowadays, the European Union (EU) is fostering the research and the transition toward more sustainable viticulture, especially considering the ongoing climate change scenario. To achieve this goal, the EU adopted a strictly regulating framework for the use of agrochemicals, as well as fixing residue levels in food and feed products (Report (EC) 204/2020; Regulation (EC) 396/2005). For instance, the adoption of the Directive 2009/128/EC fostered the implementation of integrated pest management (IPM), encouraging the evaluation of all plant protection strategies and the adoption of

the most appropriated agricultural practices to control infection of plant pathogens, minimizing pesticides consumptions. This context of European limitations that will become increasingly restrictive, highlights the need to develop new alternative sustainable techniques (e.g., bio-control agents) and new environmentally friendly molecules that can adequately replace the use of chemical inputs for vineyard management.

Additionally, sustainability in agricultural systems incorporates also concepts of both resilience (the capacity of systems to buffer shocks and stresses) and persistence (the capacity of systems to continue over long periods) (Jules et al., 2008). This entails a sustainable agriculture focused not only on crop productivity but also on long-term environmental sustainability, leading to a sustainable system where productivity and profit are maximised, environmental damage is minimised, and natural resources are preserved (Sharuddin et al., 2022). However, modern cultivars are characterized by a growth-defence trade-off mainly shifted towards growth features and consequently they lost their ability to face up the multitude of abiotic and biotic stresses with whom they must deal with throughout the entire production cycle. In fact, it has been already showed that plants don't have an unlimited quantity of energy and so if the limited carbon sources produced by photosynthesis is mainly dedicated on growing, automatically they have less ability to deal with several stresses such as pathogen infection or harsh environmental conditions (Nerva et al., 2022; Hout et al., 2014). This means that the agricultural system lost their balance becoming more productive but less resilient as well. One of the main causes of the loss of these fundamental balances and the consequent loss of plants' ability to deal with the surrounding environment is to be found in the failure of modern cultivars to interact with the multitude of microorganisms that cohabit on their tissues (Nerva et al., 2022; Pérez-Jaramillo et al., 2018). It is well known that these microorganisms can form complex co-associations with plants and have important roles in promoting their productivity and health in natural environments. Indeed, nowadays plants and the members of its associated microbiota are considered as a single entity, the so called holobiont. It represents the "unit of selection" at which plant– microbiome interactions have probably co-evolved to maintain host functionality and fitness over ecological and even evolutionary timescales (Trivedi et al., 2020). However, there are evidence that modern cultivars, not needing to recruit beneficial microorganisms since they are constantly fertilized, irrigated, and protected by huge amount of pesticide, lost by the years the ability to interact with them (Romero et al., 2020; Smýkal et al 2018) and this results in a severe lack of resilience. For instance, it has been shown by recent studies that wild ancestors and primitive landraces of wheat (*Triticum aestivum*) and maize (*Zea mays*) can benefit more from mycorrhizal symbiosis (Hetrick et al. 1992; Sangabriel-Conde et al. 2014) and are characterized by richer and more biodiverse microbiomes (Sandrini et al., 2022).

Considering the importance of plant-microbiome interactions and the potential of beneficial microorganisms, the purpose of this thesis was studying and deepening the dynamics on which the plant-microbiome interactions are grounded on and the development of new alternative techniques that can improve microbial biodiversity in vineyard and viticulture sustainability and resilience.

The second chapter of this thesis is a literature review in which the possibility to develop a microbe-assisted crop improvement to restore the holobiont functionality and resilience is analysed in detail. In fact, in the past years, breeding programs focusing just on the improvement of specific commercial plants traits (*e.g.*, growth) led to low self-support production systems enhancing the need of external inputs such as substantial fertilization plans and a large use of pesticides (Matson et al., 1997). To improve viticulture sustainability and to realize a "green transition", the study and the development of alternative techniques able to mitigate this negative trend are mandatory. The altered allocation of carbon resources (*i.e.*, growth-defence trade-off) is one of the main features that can be restored by specific plant-associated microbiomes (Nerva et al., 2021; Bastías et al., 2021). Considering this we thought that restoring the ability of plants to interact with their own microbiomes recruiting beneficial microorganism can be pivotal to reach holobiont functionality in agricultural systems.

Among beneficial microorganisms, arbuscular mycorrhizal fungi (AMF) are known to establish symbioses with most land plants showing an important role in providing nutrients, water, and other elements to the host plant (Jacott et al. 2017; Balestrini and Lumini 2018). It has already been demonstrated that AMF are able to influence plant growth and productivity and enhance the tolerance to biotic and abiotic stresses appearing to be a promising tool to increase plants resilience (Alagna et al., 2020; Balestrini et al., 2018). However, the impact of AMF on growth-defence trade-off features has still to be elucidated and so we decided to focus the third chapter of this study on unearthing the capacity of this soil beneficial microorganisms to influence the allocation of plant carbon sources and the plant immunity system. The potential benefits of an inoculum formed by two AM fungal species, with or without a monosaccharide addition, was investigated on young grapevine cuttings grafted onto 1103P and SO4 rootstocks (knowing to have an opposite behaviour). We showed how AMF can affect the allocation of limited carbon sources of the plants leading them in a state of alertness—'primed state' or 'priming'—and enabling them to respond more quickly and robustly in case of the exposure to any stress (Beckers and Conrath 2007). In fact, in plants treated with AM the evaluation of gene expression, agronomic traits and metabolites production, revealed a positive impact on plants immunity balancing rootstock's trade-off features. The final purpose of the study was investigating whether the use of AMF inoculum can help modern viticulture in developing crops with improved

performance and better adaptability to any environmental scenario, achieving more resilient holobiont and thus less anthropocentric input need and more microbial biodiversity.

Given the limited data about the inner world of plant-microbiome interactions, the fourth chapter of this study was dedicated to the metatranscriptomic analysis to achieve a comprehensive understanding of the role and potential of beneficial microbes in agriculture (Nerva et al., 2022). Metatranscriptomic is defined as the assessment of a gene's expression in a population or a whole community and it is used to expand our knowledge of the microbial community's functions in terms of gene expression, regulations, and pathways together with the interacting plant host (Peimbert et al., 2016). We reported a metatranscriptome profiling from a complex woody tissue in a real environment, highlighting that this approach can define the microbial community better than referenced transcriptomic approaches. A comparison between the plant and inhabiting microbes transcripts of asymptomatic and symptomatic grapevines esca symptoms was assessed, highlighting a strong transcripts reprogramming in infected tissues. Additionally, we demonstrated that some fungal and bacterial taxa displayed significant differences in relative abundances between asymptomatic and symptomatic samples. In parallel, the bacterial taxa more abundant in healthy samples were isolated from the original wood tissue and displayed biocontrol activities against a wood-degrading fungal taxon *in vitro*. The aim of this chapter was to underline the importance of environmental metatranscriptomic applications in microbiome-based innovations and its possible contribution towards realising environmentally sustainable agriculture by harnessing the microbiome's potential with the interacting plant host (Sharuddin et al., 2022).

In chapter five we investigate the potential of synthetic communities (SynCom) as sustainable alternative in viticulture. There are several evidence in literature about the potential of microbial agents to control pathogen development and to promote plant growth and wellness (Babalola et al., 2010; Berendsen et al., 2018; Armanhi et al., 2021; Castrillo et al., 2017; Ferrigo et al., 2017). However, the exploitation in agriculture of commercial microbial agents is often disappointing and contrasting since non-specific additive microbial cocktails are sub-optimal for general application, and this can be related to genotype and environmental-dependent effects (Ownley BH et al., 2003; Rodriguez et al., 2019). Considering this, we decided to isolate bacteria directly from grapevine wood tissues characterizing a collection of 42 isolates, 27 of which belonging to the Actinobacteria phylum. All the isolates were tested in dual culture assay against the main grape pathogens such as *B. cinerea* and the etiological agents of esca syndrome and screened for different plant growth promoting traits (e.g., phosphorus solubilization). Subsequently, the best performing isolates were selected to

constitute a customized SynCom which was compared in a field trial with a commercial inoculum made by AMF fungi and different rhizobacteria. At véraison, physiological parameters, biochemical and molecular analyses were collected to evaluate the effects on plant performances. SynCom treatment shaped the plant growth-defence trade-off, thus moving the energy allocation through the defence pathways by affecting the photosynthetic performances. On the other hand, the AMF+B treatment revealed a more equilibrated allocation across the growth-defence trade-off even if with a milden activation of the defence pathways. The final goal of this chapter was giving a clearer picture of the responses occurring between the plant and its inhabiting microbiome, confirming that a holistic vision can make lights on SynCom application in natural environment.

Chapter six of my PhD thesis synthesizes the collective findings from the studies conducted. We delved into the potential of developing sustainable breeding strategies by harnessing the intricate interactions between plants and their associated microorganisms. Plant associated microorganisms are known to improve plant growth and wellness, and thus the plant's ability to interact and cooperate with microorganisms should be considered as a fundamental trait in modern breeding programs (Nerva et al., 2022). A holistic vision of plant breeding aiming at the improvement of the holobiont functionality as a sustainable tool to restore plant resilience against stressful factors have been discussed. This work represents a perspective aiming to setting up a new kind of breeding based on the development of SynComs to increase the agroecosystem resilience and sustainability.

In the last chapter, to conclude, we briefly discuss our results and how they provide significant data about the importance of deepening the knowledge of beneficial plant-microbiome interactions and the potential of microorganisms' exploitation in viticulture and in agriculture in general.
The improvement of holobiont performance seems to be an increasingly achievable goal and it will open a path to try to manage plant-microbiome interactions as a sustainable tool to restore plant resilience against stressful factors.

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CHAPTER 2 | MICROBE-ASSISTED CROP IMPROVEMENT: A SUSTAINABLE WEAPON TO RESTORE HOLOBIONT FUNCTIONALITY AND RESILIENCE

Review Article

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Abstract

In the past years, breeding programs have been mainly addressed on pushing the commercial features, forgetting important traits, such as those related to environmental stress resilience, that are instead present in wild relatives. Among the traits neglected by breeding processes, the ability to recruit beneficial microorganisms that recently is receiving a growing attention due to its potentiality. In this context, this review will provide a spotlight on critical issues of the anthropocentric point of view that, until now, has characterized the selection of elite plant genotypes. Its effects on the plant-microbiome interactions, and the possibility to develop novel strategies mediated by the exploitation of beneficial root-microbe interactions, will be discussed. More sustainable microbial-assisted strategies might in fact foster the green revolution and the achievement of a more sustainable agriculture in a climatic change scenario.

Introduction

Agriculture and climate change are closely linked, as the agricultural sector generates significant amounts of gas emissions that strongly influence the climate and, in turn, augment frequency and duration of stresses [1]. The relevant increase of greenhouse gases in the atmosphere, the small but constant increase in temperatures, and the changes in the precipitation regimes strongly affect the quality and stability of agricultural production [2]. In this scenario, people need to protect crops from increasing environmental stresses by limiting the use of chemicals and adopting sustainable approaches as promoted by various national and international regulations and programs (e.g. Paris Agreement on Climate Change, European Green Deal, etc.) [3]. In the past years, breeding programs have been focused mainly on promoting commercial traits, neglecting several other important ones, such as those related to environmental stress resilience, which are indeed present in wild relatives [4]. Among these neglected traits, the ability to recruit beneficial, and functional, microbiomes are receiving increasing attention for its potential. Plants in fact, together with their associated microorganisms, are now considered as unique biological entity called holobiont. To ensure their functionality, plants deploy their resources differently depending on environmental stimuli. Elite varieties, derived by long breeding programs, usually result into an unbalanced use of their resources, generally prioritizing growth and limiting the defence responses. The altered allocation of carbon resources (i.e. growth-defence trade-off) is one of the main features that can be restored by specific plant-associated microbiomes as recently demonstrated by few reports [5–7]. This negative trend is called "domestication syndrome" and it has been reported where intensively domesticated plants have lost their ability to survive on their own, away from the care of humans [8]. The domestication process has led to low self-support production systems with an enhanced need for external inputs such as substantial fertilization plans and a large use of pesticides [9]. Additionally, this process caused a dramatic reduction of plant genetic diversity leading to a significant impact of pathogens and pests on plant productivity and consequently an excessive use of chemical inputs to avoid excessive losses. Thus, the main side effect of plant domestication could be summarized as the loss of human neglected traits which are very important for wild plants fitness and their survival in natural environments [10], where plants live in association with thousands diverse microorganisms, with diverse outcomes depending on the interactions. Nowadays, most of the published articles about the agricultural application of microorganisms are focused on soil beneficial bacteria [11] or fungi [12]. Although many species of soil-bacteria or fungi capable of supporting plants have been identified, the next paragraphs will be focused on the plants interaction with two groups of microorganisms that are receiving growing attention for their potential in stressed environments: the mutualistic symbiosis formed by arbuscular mycorrhizal fungi (AMF) with the roots of almost all the terrestrial plants,

including several crops, and the associations with Actinomycetes. By now, AMF are known to be one of the most important plant's allies in the interaction with the surrounding environment, providing several ecosystem services to agricultural systems [13, 14]. Considering that plant roots in natural ecosystems are commonly colonized by AMF, the rhizosphere concept has been expanded to include the fungal component of the symbiosis, resulting in the term "mycorrhizosphere" [15]. The rhizosphere constitutes the microhabitat where fungal-bacterial interactions occur, with the fungi that affect the associated bacteria and vice versa (e.g. providing water and nutrients supply) [5]. However, there is a need to further strengthen the research to explore their potential to improve plant productivity and to restore the plant-microbiome equilibrium in agricultural system. For this reason, unearthing the mechanisms on which this fundamental cooperation is based on and trying to improve it by a more holistic view of breeding programs could be very promising. Additionally, domestication process has often modified the capacity of plants to interact with these fundamental soil microorganisms compared to the relative wild types [16–18]. Actinomycetes have been also shown to be very often part of the plant's core microbiome [19–21]. They can be considered among the protagonists in the hidden world of plant-microbiome interactions. The application of next-generation sequencing (NGS) approaches to study microbial communities allowed to find the Actinobacteria phylum as one of the five most dominant bacterial phyla in soils [19–21]. Thanks to the peculiar capacity to live in wide range of temperatures and pH, and to change their morphology adapting to extreme environments, they are an ecologically divergent groups which can occupy a huge range of environmental niches [22, 23]. Furthermore, although Actinomycetes are important representatives of microorganisms beneficial for plants, their plant growth-promoting (PGP) traits, as well as their potential as biocontrol agents, have not been studied like for some other beneficial bacterial species such as Bacillus spp. and Pseudomonas spp. [24–26]. Several studies about Actinomycetes and their important role in supporting plants growth and wellness have been performed, but the dynamic interactions between them and plants are not still fully known, limiting the possibility to exploit these microorganisms in agriculture. Notably, it has been reported that these bacteria can enter in a close association also with AMF, giving an additional reason to contemporaneously analyze the potentiality of both Actinomycetes and AMF in improving plant performances [27]. In the last years, the application of biochar as amendment in agriculture has been also proposed and, in addition to an impact on carbon sequestration, a positive influence on rhizospheric beneficial microorganisms, including AMF, and microbial community network complexity has been reported in diverse plant species [28, 29]. Accordingly, this review will focus on examining in depth the critical issues related to the possibility to develop novel microbial-assisted selection of plants, optimizing rhizosphere/root-microbiome beneficial relationships, with a particular emphasis on AMF and Actinomycetes.

Significant flaws of plant breeding: from domestication to new plant breeding techniques

As previously cited one of the most important traits that has been shelved is the ability of plants to interact with the thousands of microorganisms surrounding and supporting them in dealing with both biotic and abiotic stresses [30–34]. The domestication of plant populations is a co-evolutionary process, in which human selection of cultivated plant populations brings over changes in allele frequencies within these populations, making them more useful to human purposes and better adapted to the human-induced changes to the agro-environment [35]. It is now fundamental to underline how human-focused breeding has shaped plant traits involved in the interactions with microbiomes and how, in turn, the loss of these plant traits may negative influence the ability of current genotype to dealing with the surrounding environment. It is evident that anthropocentric breeding has profoundly altered the interactions between plants, insects, and their natural enemies. For instance, it has been reported that domestication process led to lower levels of volatile emissions during pest attacks as compared to wild relatives, thus affecting the attraction of natural enemies of pests and pathogens [8]. Production of resistant varieties through breeding programs is often slowed by the necessity to move polygenic resistances which requires several crossing cycles that, in case of woody plants, are laborious and time-consuming [35]. To overrun such issue, the common choice of breeders is to work with monogenic resistances which are easily manageable but likewise overcome by pathogens in short time [36, 37]. For example, the major resistance gene *Ty-1* was introduced to control tomato (yellow) leaf curl disease (TYLCV). However, plants showed differential responses to TYLCV strains [38] suggesting the importance of pyramiding multiple resistance genes improving the spectrum and resistance durability. This scenario was observed also in rice, indeed Qu and colleagues reported a time retained resistance level due to a rapid evolution of *M. grisea* [39]. Breeding programs for woody plants encounter additional limitations such as the high heterozygosity of elite cultivars, long juvenile stages that slow down the backcross steps and the movement of unwanted genes in the progeny that are linked to the gene(s) or QTL of interest (*i.e.* linkage drag) [40–42]. The latter aspect is often linked to modification of important commercial features, such as aromas, that generate new tastes and organoleptic profiles [43] that have to be acknowledged by consumers. For example, grapevine is characterized by different cultivars that are associated to the production of many aromas and therefore different commercial wines. The products of grapevine breeding programs lead to the production of new individuals with different characteristics from the parental cultivars that need to be registered as new varieties (with new commercial names) and in consequence the necessity of market acceptance [44, 45]. Thus, breeding programs on woody species result in laborious and time-consuming processes which can be quite easily overcame by pathogens and that in parallel negatively affect qualitative traits of fruits. The possibility to exploit genome editing and cisgenesis technologies,

based on the precise modification of DNA sequences, introduce a new shortcut for improving elite crop varieties [46–48]. The development and application of microbial-based products could be in more sustainable than classical and new genetic approaches, overcoming their limits, especially considering woody plants for which breeding programs are particularly slow and time consuming. Despite the interesting features of the new biotechnology approaches, diverse main problems should be in fact to be solved yet: i) strict regulatory rules are still diffuse [49], limiting the use of genome edited or cis-genesis varieties and ii) the ability in recruiting beneficial microbial consortia is not easily tackled. Additionally, these new genetic technologies may produce deleterious effects in crops by genome-wide off-target mutations, making the generation of novel tolerant/resilient crops by using them a little bit more complex than expected [50]. Beneficial microorganisms constitute an important target to enhance plant features, such as productivity and/or tolerance and resilience to environmental stresses thus reducing chemical inputs [51–56].

Root traits to improve microbe-mediated climate resilience

A holobiont-level breeding strategy, in which microbes are one of the direct targets of the selection process, can originate a range of new phenotypes without changing plant genomic information [57]. Particularly, it could be very useful unearthing the ability of crops to assemble useful and healthy microbial communities. Several studies have already shown that diverse plant species are able to recruit specific microorganisms, establishing active interkingdom interactions that could be perceived as a "cross talk" [58, 59]. The "cry out for help" concept has been recently exposed in literature [60], considering root exudation as an adaptive mechanism by which stressed plants assemble health-promoting soil microbiomes [61, 62]. It has been demonstrated that plants can recruit beneficial bacteria upon pathogen infections, mainly disease resistance-inducing and growth-promoting ones [63]. The selection of plant phenotypes that efficiently interact and recruit taxa suppressing pathogens may alleviate the need to introduce disease resistances into the plant genomes [64]. Another important concept within the hidden world of holobiont interactions is the "soil memory": from one plant generation to another, a given soil would hold its associated microbiota and thus, the wellness of plants can be improved, taking advantage of the pre-existing beneficial microbes for their development [65].This plant/soil feedback is strictly due to the microbial legacies, which plants leave in soil, and it could be applied to the time scales necessary for the renewal of a olive grove, a vineyard, or at least an orchard, and could be exploited to restore the soil's microbial functionality. Hannula et al. [66] have looked at the persistence and the impact of these legacies following a subsequent colonization by the same or different genotypes using six typical grassland plants [66]. The authors observed that microbial soil legacies, at the time of plant establishment, have a pivotal function in

plant growth, concluding that soil microbiome legacies, although reversible and versatile, can create plant/soil feedback through the alteration of the endophytic communities developed in the course of early ontogeny. Additionally, the host genome is highly conserved with slow genetic changes, especially in perennial plants. Genomes of microbiomes, instead, are dynamic (*e.g.* horizontal gene transfer, mutation) and can change rapidly by modifying microbial populations in response to environmental changes [67]. Considering the climate change scenario and the consequent increase in the incidence of both abiotic (*e.g.* drought and salinity) and biotic stresses (invasive pathogens), holobionts with functional and dynamic microbiomes can better adapt to the occurrence of different stresses when necessary. A full understanding of the mechanisms governing the selection of microbial communities by the plants will enhance the development of new strategies to improve the agriculture future. Since plants are sessile, they need to mine soil for finding important resources such as water and nutrients (*e.g.* phosphorous, nitrogen, potassium, etc.), whose distribution is patchy and change rapidly over time. Modulation of the root system architecture, root anatomy and chemistry are the plant responses to this challenging environment, allowing them to explore soil, detect and exploit nutrients and water [68]. Additionally, plants can also shape the root-associated microbiomes to improve their foraging activities. Reciprocally, soil microbes can trigger important adjustments in root development, physiology, and chemistry [69] (Figure 1), creating a dynamic interplay that impact on plant nutrition and health modulating the growth-defence trade-off [70].

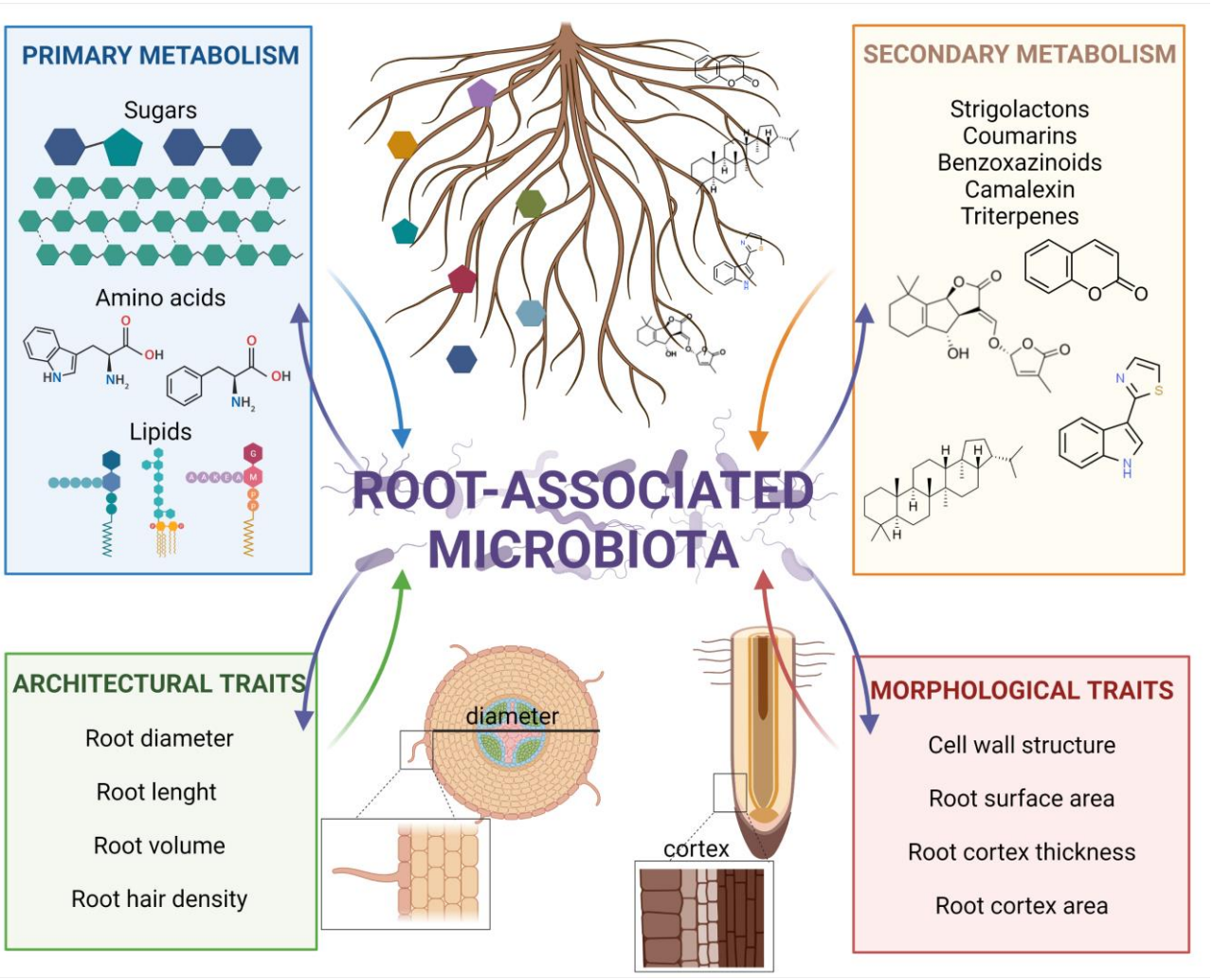

Figure 1. Representation of the reciprocal influence that root-associated microbiota and plant features exerts each other. In the upper part metabolic features usually associated to root exudates that interact with root-associated microbiota. In details, both molecules from the primary metabolism (*e.g.* sugars, amino acids, organic acids, etc.) and from the secondary metabolism (*e.g.* strigolactones, flavonoids, terpenoids, etc.) are involved in the recruitment of beneficial microbes as well as in shaping the soil microbiota. In the lower part, root architectural and morphological traits that influence the interaction with soil microbes and shape the composition of the root-recruited microbiota.

It is evident that, over the time, breeding programs aimed at improving productive features, often not considering the root traits as an important aspect to be characterized and associated to beneficial and functional microbiome structures [71]. Remarkably, root architecture as well as morphology are deeply involved in resource acquisition, and breeding for different root ideotypes have been suggested as promising targets for climate resilient crops also thanks to an improved rhizosphere microbiome [72]. Root architecture encompasses the spatial configuration of the whole root system including pivotal traits such as root length, density, branching, angle, and total biomass [73]. Root morphology, on the other hand, encompasses physical traits of each single root, such as cell wall structure, root hairs, diameter and surface area [73]. Interestingly, hints of reciprocal relationship between soil

microbes and root architecture/morphology were already reported. Inoculation with single strains highlighted the ability of specific rhizosphere bacteria to modify root architecture and morphology through the production and the release of key plant phytohormones (*e.g.* auxins and cytokinins) [74]. Furthermore, recent reports demonstrated an increase of root length, volume and branching in wheat and soybean when inoculated with specific microbial isolates [75–77]. Despite these interesting reports on root architecture, effects of microbial inoculations on root morphological traits are less clear, as demonstrated by the inoculation experiments in rice, wheat, or soybean, where the same isolates displayed increased, decreased, or no effects on root diameter respectively [76, 78, 79]. Thus, sustainable agriculture through inoculation of microbial consortia is a feasible route, but it still remains a gap that have to be filled by studying root traits and the effects on soil and root-associated microbiomes. This knowledge will result in pivotal information exploitable for breeding program aimed to restore the precious ecological services offered by beneficial microbiomes. In addition to architecture and morphology, root exudates, both from primary (particularly sugars, amino acids, and organic acids) as well as secondary metabolism (*e.g.* flavonoids and strigolactones), play key roles in defining symbiotic relationships [80] and, consequently, changes in exudates composition might limit or negatively influence these positive interactions. Considering survival of root-associated microbial communities, plants can support the proliferation of soil microbiota releasing carbon substrates through the root system [81]. Different studies also highlighted that a high plant diversity was associated with high microbial diversity [82, 83], confirming that when exudate mix from several plants were added to monocultures, an increase in microbial diversity was observed [84].

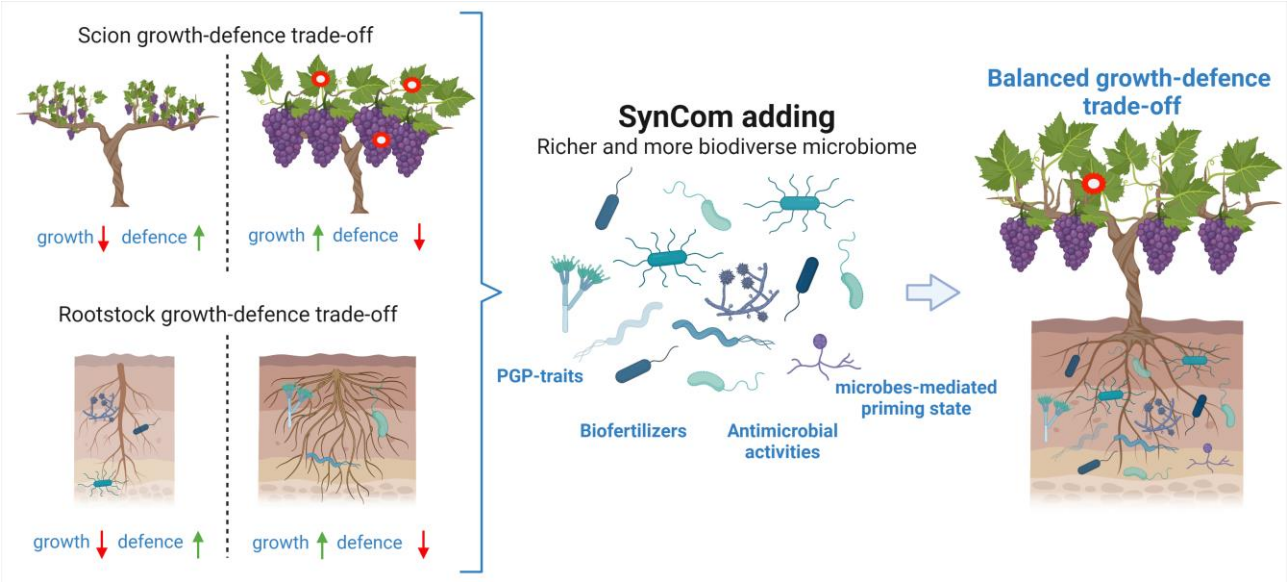

Figure 2. Schematic representation of SynCom adding traits for balancing the growth-defence trade-off in grafted crops. Depicting different (culturable) microbial populations, associated to diverse environments, can allow the development of SynCom that can in turn modulate the growth-defence trade-off, leading to more resilient plants showing balanced growth-defence features.

In this respect, it is worth noting to consider the potential ecosystem services conferred by root system of cover crops in the agro-ecosystems. Cover crops species are often ignored since most of them do not provide a direct economic income to growers underestimating their positive roles for belowground features (*e.g.* resources capture, improvement of microbial biodiversity and soil physico-chemical characteristics [85]). Although research investigating the relationships between cover cropping and soil microbiomes are still limited, promising results in terms of positive effects on soil microbial abundance, diversity and functionality have been reported in many agricultural systems [86]. Furthermore, emerging studies outlined the ecosystem services provided by roots of diverse cover crops such as: i) improved soil structure and stability thus limiting water runoff and topsoil displacing (*e.g.* roots of grasses prevented soil erosion [87] while tap-rooted plants as forages, alfalfa or chicory can easily penetrate compacted soil layers favouring soil aeration and water infiltration [88, 89]); ii) enhanced soil resource capturing, improving soil nutrition and fertilizers use efficiency (*e.g.* increase of soil nutrients by tall fescue or chicory [89]; nitrogen increase by leguminous cover crops such as pea, vetch or alfalfa thus reducing chemical fertilizers needs [90]); iii) improved soil microbiome biodiversity and organic matter content mediated by root exudates of which composition greatly varied among cover crops species, positively influencing soil microbiome structure and functionality [91]. Taken together these findings highlight the importance to enhance cover crop root traits selecting those able to enhance ecosystem services in the agricultural contexts as (near) future

challenge for breeders. Even if increasingly attention were recently posed by several scientists, to date cover crops have been subjected only to minimal domestication and breeding selection with respect to cultivated crops [92]. However, in the frame of a holistic view about microbe-assisted improvement of crop (and agro-ecosystems) resilience, breeding programs, or the use of novel genetic tools able to exploit superior root traits of specific cover crops (*e.g.* targets to improve rooting depth or root exudates to increase beneficial microbes' recruitment/biodiversity), will be able to provide high impact to the environment and farmers at low cost. Further efforts are needed to develop novel breeding approaches more focused to protect and improve the interactions between plants and the associated microbial communities, also restoring growth-defence trade-off balance in host plants (Figure 2).

Customized genotype- and environmental-specific SynComs to boost plant resilience

The use of multi-omics approaches (*i.e.* so called holoomics [93]) to study the functionality of plant-microbiome ecosystems led to the generation of data on multiple levels [92], focusing on diverse targets (DNA, RNA, protein or metabolites), as well as to the characterization the plant associated microbiota [62, 94]. These data are particularly relevant, resulting in obtaining information on the ability of specific genotypes to recruit specialized microbial strain(s) or consortia and providing information that might be useful in the manipulation of these interactions. The development of next-generation DNA sequencing platforms, and the integration of data from diverse omics approaches, have facilitated the exploration of the complexity of the plant associated microbial communities in a wide range of environments. Corbin et al. [95] proposed a framework to identify genes involved in plant-microbe interactions *via* stochastic perturbation of DNA methylation patterns. Exogenously induced DNA demethylation can randomly generate new epialleles in a plant population, that can subsequently alter gene expression of genes and thus the plant phenotype (including the associated microbiomes). The combination between individual changes in DNA methylation (novel epialleles) and phenotype (novel microbial community composition and functions) can be determined using epigenome wide association studies (EWAS) and plant gene expression analysis followed by the evaluation of metabolites production as a validation step. Interestingly, Huang et al. [96] showed how *Arabidopsis thaliana* can assembly and shape its root-associated microbial community producing a variety of specialized triterpenes [96]. Bulgarelli et al. [97] suggested a prevailing recruitment model for root microbiota assembly based on the relative abundance of specific microbial taxa. Mainly basing on 16S rRNA sequencing, the relative abundance of bacterial taxa in soil suggested that bacterial root community forms by two-step or multiple-step selection process, being dense in bulk soil and becoming more differentiated and enriched for specific phyla from rhizosphere to root. In

contrast, Wang et al. [98] suggested a novel amplification-selection model useful to quantify rhizosphere microbiota assembly, sustaining that the relative abundance of microbial 16S rRNA gene sequences does not correctly reflect the absolute abundance of bacteria. The microbial communities were quantified in bulk soil, rhizosphere and roots of two different plants (*Medicago truncatula* and *Oryza sativa*), showing all the dominant bacterial phyla more abundant in the rhizosphere than in bulk soil, and an additional host specific selection of bacterial phyla in roots. The augmentation of diverse phyla in the rhizosphere reflected an increase in nutrient availability in this compartment, while lack of some bacterial taxa might depend on several factors such as nutrient availability, growth rate and the interactions with other microorganisms. Looking at the tolerance and resilience to abiotic stresses, Zolti et al. [99] described the taxonomic variations and the functional responses upon long-term irrigation with water differing for its quality (freshwater *vs* treated wastewater). As previously mentioned, an interesting aspect uncovered by the multi-omics approach is the so called "soil memory", namely the opportunity to alter the soil microbial communities planting specific plant species [100]. Thanks to computational approaches it is possible to verify the most important microbial taxa that can influence the composition of a specific environment-associated microbiome, that can be identified as the soil core microbiota [65]. In this line, it is possible defining the impact that specific taxa have on the recruiting of others that in turn influence diverse plant functions [101]. The SynCom approach can be defined as an interesting laboratory approach to study plant-microbes interaction excluding other environmental effects, limiting the complexity of the experimental system [102]. To formulate a valuable SynCom it is necessary to collect several information from the holo-omics and from the microbe behavioural side. In details, the formulation of a core microbiota [101] is grounded on the identification of keystone species: a group of well described microbes with known PGP-traits (see next paragraph) and antagonistic activities that have no human or animal pathogen features [103]. Zhuang and colleagues [104], have adopted high-efficiency top-down approaches based on high-throughput technology and synthetic community approaches to find plant-growth promoting bacteria (PGPB) in garlic rhizosphere. They have found out that bacteria belonging to the Pseudomonas genus were key PGPB in the rhizosphere of garlic and, subsequently, SynCom with six Pseudomonas strains isolated from the garlic rhizosphere was assembled, showing the ability to promote plant growth. Such microorganisms are fundamental since they have naturally evolved in close cooperation with a specific plant's genotype and phenotype and so they can be considered in a breeding program grounded on the improvement of the holobiont [105]. Furthermore, Paredes et al. [106] have recently developed a new method, based on synthetic communities approach, −omics techniques (e.g. RNAseq) and neural network (NN) prediction, to design and test bacterial communities altering the plant response to phosphate starvation [106]. As a first step, a bacterial

collection has been classified (plant-bacterium binary association assays) according to the effect on plant Pi content achieving the design of bacterial synthetic communities. Then, the Arabidopsis phenotypes with the synthetic communities have been evaluated (e.g. Pi content, roots elongation and plant transcriptional profile) and the prediction of Pi content for new hypothetical synthetic communities has been achieved by using NN. Finally, they validated the NN predictions evaluating the performances of A. thaliana with the new developed synthetic communities. Thus, this strategy allowed to design and test small consortia of bacteria with predictable host phenotypic outputs, discovering the best synthetic community for a specific host genotype. Nowadays the synthetic community approach has been exploited mainly as reductionist model to understand the plant microbiome assembly and the output obtained from field applications of experimental SynComs has been often contrasting, since non-specific additive microbial cocktails are sub-optimal for general application [107, 108]. This can be related to genotype and environmental-dependent effects. Additionally, only limited data on the mechanisms at the basis of the interactions with beneficial microorganisms in natural conditions are available, rendering still unstable their exploitation in agriculture [109, 110]. Nowadays, however, thanks to the emergence of next-generation sequencing, the application of complementary omic-tools, and considering the differences among their outputs, deep insights into the diversity and composition of the bacterial communities associated with diverse host, the characterization of plant-microbiome interactions and the selection of the best performing SynCom for a specific genotype and environment could be reached (Figure 3).

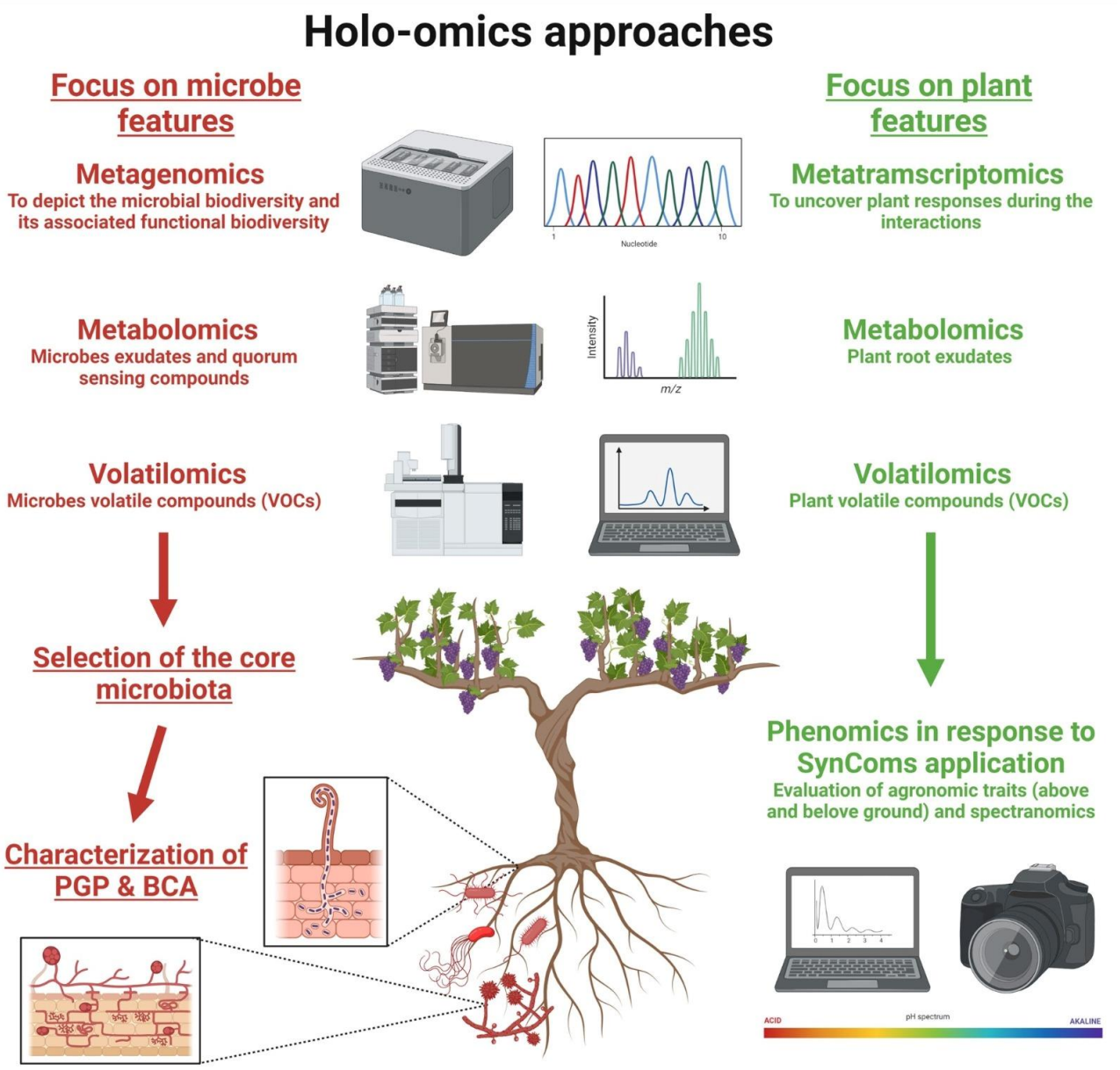

Figure 3. Workflow for the characterization of customed SynComs. The same -omics approaches can be exploited for the characterization of both plant and microbe features. From the top, sequencing techniques can be used to characterize the microbial profile and/or specific isolates (*e.g.* microbiome profiling, microbial genome sequencing, etc . . .) as well as plant responses under specific conditions. Then, metabolomics and volatilomics approaches can be used to identify key metabolites involved in the interactions between microbes and its host. Once the core microbiota has been selected, the characterization of plant growth-promoting features and biological control potential will be evaluated. Finally, phenomics approaches (applied both on root and canopy) can be exploited to detect plant responses when exposed to the developed core microbiome.

Specific examples of SynCom formulations developed during the last 10 years for several purposes and their relative outcomes have been summarized in Supplementary Table S1. SynComs plant-customized with high-throughput methods (such as metagenomics and metatranscriptomics) can address problems commonly faced with microbial field applications [111]. It is thus possible to screen beneficial microorganisms for predicting their establishment and functioning in different natural environments, defining a range of microbial functions associated to diverse strains in different conditions (under a gradient of pH, temperature, and water and nutrient concentration, etc.) [106]. Additionally, the compatibility between host plants and microorganisms may be evaluated in different pairwise combinations. Furthermore, this approach may offer a flexible and powerful tool suiting the needs of individual farmers. The same reference plant genotype could be combined with different microbiota to generate easily customized phenotypes [57]. Starting from modern phenomics approaches (highthroughput plant phenotyping), several traits related to growth, yield, and adaptation to stress can be precisely evaluated and the screening of eco-physiological and agronomical traits can be simultaneously performed [112]. From these collected data, the selection of the best performing phenotypes living in a specific environment can be easily viable. Once phenotypes selection has been done, it is possible to screen the related hologenome by complementing the data obtained from metagenomics and other omics techniques (*e.g.* metatracriptomics and metaproteomics), achieving thus a detailed hologenome picture leading to define the core beneficial microbiome strictly linked with the involved genotype growing in a particular environment [113].

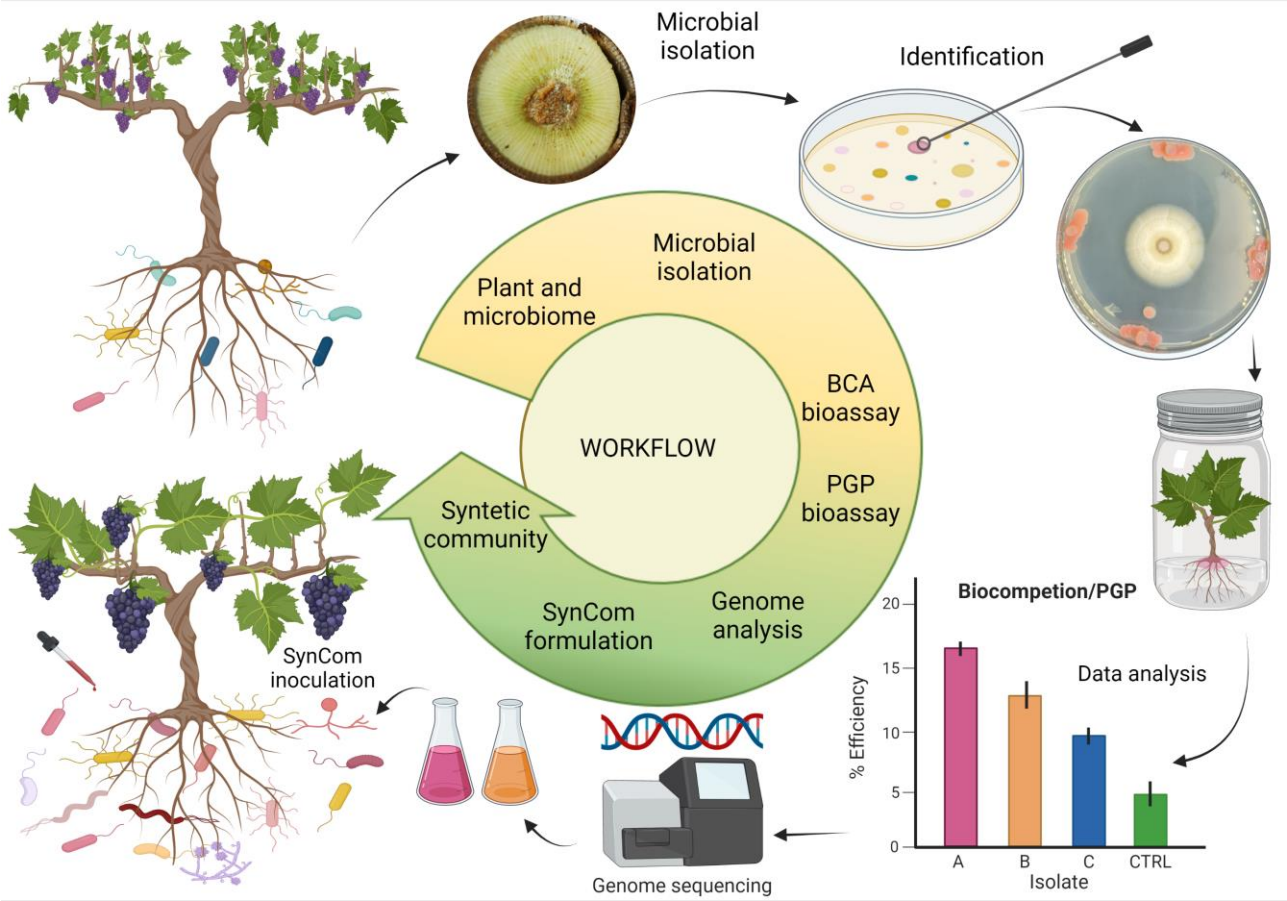

Figure 4. Workflow for the development of a SynCom. Starting from a specific environment and/or wild well-adapted plant population, the first step is to isolate and identify the culturable microbial endophytes. Thus, the identification and selection of potentially beneficial microbes occur through several *in-vitro* tests (*e.g.* biocompetition against phytopathogens and assessment of plant growth-promoting traits). Finally, prior to SynCom formulation, it is highly desirable to perform genomes sequencing of the best performing microbes (at least for bacteria) to have a clear picture of the biosynthetic pathways present in their genomes and to avoid the selection of isolates which can potentially produce metabolites with detrimental effects on animals and humans.

After a customized SynCom development and application, the evaluation of different plant physiological parameters and the dissection of plant responses at molecular level (through RNA-seq, proteomics, metabolomics and volatilomics) are necessary to confirm the ability of these customized SynCom [114]. A crucial still open question is how to implement this strategy on an industrial scale. As a first point, industries have to keep records of microbial characteristics such as name and function, etc. Similarly, they should collect data on ecological features, *e.g.* survival in different types of physical environments, compatibility with crop variety, and mutual antagonism [115]. If these data will be available, then customizing personalized microbial consortia will be feasible [116]. Collected information could be also analysed using specific software (*e.g.* decision supporting system - DSS) that will further minimize the need of experts for such customizations [115]. In addition, looking to wild growing species, it is feasible to find microorganisms that can confer important plant traits lost during the domestication and/or breeding programs [117, 118]. After the identification and characterization of target microorganisms, building a personalized SynCom and inoculating it in agricultural systems might be a very promising tool to restore and improve beneficial microbial communities which have been previously damaged due to long time of anthropocentric breeding [119, 120] (Figure 4).

Soil beneficial bacteria: Focus on Actinomycetes, promising allies to support holistic breeding programs

During the last few years, a great number of studies have been dedicated to searching out for soil beneficial plant associated bacteria (Supplementary Table S2) [121], mainly focusing the interest on *Bacillus* and *Pseudomonas* species that have showed promising attitude to enhance plant growth and wellness. Diverse aspects of *Pseudomonas* and *Bacillus* spp. as elicitors of Induced Systemic Resistance (ISR) and as direct antagonists towards different pathogens have been largely described, suggesting that some strains might achieve significant reductions in the incidence/severity of diverse diseases on several plants [122, 123]. These species are characterized by several PGP traits, *i.e.* the production of phytohormones or siderophores, the solubilization of nutrients, *i.e.* phosphorus, and the capacity to fix atmospheric nitrogen [124, 125]. Moreover, both these genera have showed great potentiality in alleviating damages of abiotic stresses like extreme temperatures [126, 127], water stress [128] or high salinity [129]. As an example, *Bacillus cereus* KTMA4 has been reported to produce molecules involved in growth-promoting and tolerance such as Indole-3-acetic acid (IAA), ammonia, siderophore and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. It has been demonstrated that tomato plants obtained from seeds treated with this strain showed an increasing in seed germination percentage and an inhibition against major tomato phytopathogens [130].

Considering *Pseudomonas* species, Noori et al. [131] showed the potentiality of *Pseudomonas fluorescens* in enhancing plant growth and in dealing with the dangerous cereals' pathogen *Pyricularia oryzae*. Twenty *Pseudomonas* strains, isolated from the rhizosphere soils of paddy areas in Malaysia, were screened for their plant growth promoting activity, showing the ability for siderophores' production. Additionally, fifteen strains were positive for IAA production and eighteen isolates for phosphate (Pi) solubilisation. All the twenty bacterial isolates also inhibited the pathogen *Pyricularia oryzae* in an *in vitro* experiment [131]. In addition to this well-studied bacteria, diverse species belonging to Actinobacteria phylum have shown to be very often a promising part of the plant's core microbiome [19–21] and they can be thus considered as main actors in the hidden world of plant-microbiota interactions. The use of NGS technologies to describe the microbial communities allowed to verify that this bacterial group is one of the five most dominant bacterial group in soils [19–21]. Moreover, differently from other phyla, Actinomycetes present the capacity to live under the most diverse conditions such as aerobic and anaerobic environments as well as different temperatures and pH. Furthermore, they are involved inthe catabolism of complex molecules (*e.g.* diverse plant cell wall components, proteins and lignin), achieving a nutritional advantage and giving them a high chance to survive and compete for the colonization of natural ecological niches [23, 132, 133]. These bacteria also present other exploitable features such as the production of a wide range of secondary metabolites of agricultural values [134–136] and are important for the plant health, forming associations with some non-leguminous plants and fixing atmospheric N (*Frankia* genus) that is then available to both the host and other nearly plants [137]. Overall, the results already present suggest that Actinomycetes have several properties that make them good candidates for the biotechnological exploitation in agriculture, mainly in light of the climate change already ongoing [138, 139]. Regarding the allocation of carbon sources in plants, Actinomycetes seem to be a promising tool to modulate the plant growth-defence trade-off since they are able to improve both the growth and the resilience of plants to stress conditions [140]. They can also be considered as biofertilizers thanks to their PGP-traits and so they may be considered for improving yield in genotypes characterized by low productivity [141, 142]. Kim et al. [143] highlighted the effects of two microbial inocula, one containing two *Methylobacterium oryzae* strains (CBMB20 and CBMB110) and one with the addition of three species of arbuscular mycorrhizal fungi (AMF), on the growth of red pepper (*Capsicum annum L.*). The use of *Methylobacterium oryzae* strains led to a significant increasing in root length and root fresh weight with respect to untreated control plants [143]. Additionally, the inoculation of *M. oryzae* strains and AMF significantly increased diverse growth parameters and chlorophyll content in comparison with uninoculated control plants [143]. Several studies have shown the Actinomycetes capacity to inhibit the growth of different pathogens, thus limiting disease incidence and severity

[144, 145] (Supplementary Table S1). They can act through both a direct antagonism towards pathogens [144, 146] and by activating a state of priming in the plants [147]. In this respect, Zothanpuia et al. [148] used dual culture *in vitro* assay to screen twenty-two actinobacterial strains against diverse fungal, including diverse *Fusarium* species, and bacterial pathogens, such as *Staphylococcus aureus, Pseudomonas aeruginosa, Micrococcus luteus, Bacillus subtilis and Escherichia coli*, in addition to one yeast pathogen (*Candida albicans*), providing information on the most promising strains for antimicrobial activity against both bacterial and fungal pathogens. An ability to enhance plant responses to face up some of the main abiotic stresses such as extreme temperature, drought and salinity was also reported [149] (Supplementary Table S2). Confirming the role of actinobacteria to increase drought tolerance in plants, Yandigeri et al. [150] demonstrated that the co-inoculation of three different endophytic actinobacteria (*Streptomyces coelicolor* DE07, *Streptomyces olivaceus* DE10 and *Streptomyces geysiriensis* DE27) will lead to significant enhancement of seedling features, growth and yield in wheat upon water limitation. Additionally, actinobacteria seems to have a potential role being able to live in environment characterized by extreme temperature. Kurapova et al. [151] conducted a study in Mongolian desert soils observing that actinomycetes strains with thermotolerant and thermophilic characteristics were present in abundance and, among the thermotolerant individuals, members of the order *Actinomycetales, Streptomyces, Micromonospora, Actinomadura,* and *Streptosporangium* genera. However, although many works about the efficiency of *in vivo* applications of Actinomycetes bio-inoculants, both as biofertilizers and biocontrol agents, have been already performed [152, 153], their potentiality has not yet been adequately explored. Thus, it would be very useful unearthing the full potential of this bacterial phylum and implementing their application in agricultural environments, also considering the possibility to formulate them in SynComs (see previous paragraph).

Focus on arbuscular mycorrhizal symbiosis responsiveness as a trait for breeding

Among beneficial root-associated microorganisms, AMF are considered the most important bio-fertilizers. These microorganisms, as symbiotic fungi, colonize plant roots of several crop species and help the host plants in the uptake of water and nutrients, by receiving in turn carbohydrates and lipids compounds [154, 155]. Additionally, these molecules are thought to be exported out of the root cells across the peri-arbuscular membrane to be exploited by the fungus [156]. Besides an enhanced nutrition, mainly related to an improvement in phosphate (Pi) uptake that particularly occur in limiting nutrient conditions, several papers have described the impact of AMF on plant tolerance under abiotic stresses such as drought, salinity, and cold conditions [157]. Since AM associations are broadly present in cultivated soil from diverse environments and they form symbiosis with the roots of major

crop species, their potential to improve crop productivity is an opportunity for plant breeding that should be more exploited. In addition, it is worth noting that developing crops with higher P-use efficiency is an important goal for breeders [158]. Recently, it has been suggested that both direct and indirect pathways (this last *via* AM symbiosis) that most plant species utilize to ensure phosphate are regulated by the same phosphate sensing-centered pathway. These findings, leading to the recognition of several actors of the phosphate starvation response-centered regulatory network involved in AM symbiosis, might be useful to assist breeding in the generation of plants that use P more efficiently [159]. However, this goal is generally addressed mainly focusing root traits on diverse genotypes without considering the interactions with soil microorganisms. As recently suggested [160], breeding approaches to improve the results from beneficial plant-fungus interactions should be obtained through the selection of traits of both symbionts (*i.e.* the plant and the fungus) involved in the association establishment and functioning. It will be important that future breeding strategies takes in account the interaction of root traits with symbiosis-related ones, with the aim to achieve optimal production also reducing application of fertilizers (mainly P-based products). An increasing number of studies report that AM responsiveness varies among plant accessions [161, 162]. An important point that should be developed is related to the characterization of additional host genotypes, including landrace and wild-relative whose diversity should be more explored [163]. The evaluation of mycorrhizal dependency in diverse plant species accessions has been performed since a long time. A comparison among varieties of wheat generated before and after 1990 suggested that the oldest varieties were more responsive to AM colonization than those obtained later [164]. Thus, plant breeding under high nutrient conditions has selected wheat lines with an increased phosphorous demand contrary to the capacity to form AM interactions. However, the impact of breeding on symbiosis effectiveness is still under debate [165], it is incontrovertible that plant breeding inadvertently selected for a reduction in dependence on AM symbiosis and not for a loss of compatibility, leading to modern cultivars with reduced but still retained ability to form AM symbioses. Genotype-dependent plant responses to AMF colonization have been demonstrated on biomass, yield and physiological features, while less is known when it comes to those for AMF-mediated disease resistance [166]. It has been proposed to include disease resistance as a trait for mycorrhizal responsiveness and it is worth noting that, to observe differences in the efficiency, genotype selection needs to occur in environments that do not suppress the plant–microbe interaction [166]. As for the classical breeding, novel breeding protocols evaluating a genotype responsiveness to AMF colonization could takes advantage from the development of protocols for the high-throughput phenotyping platforms, allowing to test many plants contemporaneously. The combination with high-throughput genotyping systems already led to the identification of quantitative

trait loci (QTLs) linked to host benefit, supporting the feasibility of breeding crops to maximize profit from symbiosis with AMF [167]. In addition, QTLs with a role in colonization have been reported in several crops [167, 168]. A relevant bottleneck that should be considered in field studies is the lack of appropriate AMF free controls when an exogenous AM fungal inoculum is applied to soil, rendering difficult the evaluation of the efficiency of the AM symbiosis in agriculture. Although in the last twenty years great advancement have been done in the ecology and biology of these interactions, most of the experiments were carried out in greenhouses or growth chambers, while only limited studies have been conducted in open-field conditions [169]. Additionally, some plant and AMF combinations are more productive than others, and the nutrient status of soils also affects the species composition of AMF and the success for the symbiotic interaction, complicating the real application of these beneficial microorganisms [6]. In parallel to breeding protocols that consider the potential to form AM symbiosis as a priority trait, a successful strategy could be to maintain and improve the soil AMF potential with the use of soil managements with a low impact on soil microbial communities [170]. Interestingly, the *Rhizophagus irregularis* non-symbiotic growth and spore production were reported in the presence of an external supply of certain fatty acids, *i.e.* myristates [171]. In this line a useful application for agriculture could be the developing of crop plants for myristate production with the aim to have AM fungi-friendly crops [172]. Additionally, the application of myristates could enhance the AM fungal biomass *in loco*, leading to a reduction in an external inoculation. This should be particularly relevant, and directly applicable in agriculture, often where AM fungal abundance is suppressed by a range of invasive agricultural management practices [170].

Conclusion

The ongoing climate change is seriously threatening the food access for billions of people and the neglect of environmental or ecosystems health and associated loss of biodiversity is a critical issue further worsening the health of the agricultural systems. Thus, the challenge of maintaining adequate yield and quality of food and feed under unrelenting climate changes is formidable. Improving or developing new eco-friendly management strategies, able to restore part of the loss biodiversity, and selecting stress-adapted genotypes represent sustainable approaches that are now under scrutiny. Notwithstanding, an essential step to face this challenge should be to consider the roles played by root associated microbes and exploit the hidden potential that is starting to unearth. Development of SynComs adapted to specific agro-environmental conditions is the beginning of a regenerative path that will not consider the plant as a stand-alone entity but as a complex organism composed also by the associated microbiota (*i.e.* the holobiont). In the light of what discussed, exploiting the potential

of microbes to improve wellness, resilience and product safety of crops seem to be a promising path to overcome the ongoing climate change and preserve food yield and quality for the future.

Supplementary data:

Table S1. Examples of SynCom formulations developed and tested *in vivo* during the last 10 years.

Plant species and condition	SynCom source and formulations	Main outcomes	Reference
Maize (three commercial hybrids grown under glasshouse conditions)	17-strain community formed by bacteria isolated from Sugarcane root and stalk tissues	Upon severe drought SynCom in inoculated plants positively increase resilience to drought modulating sap flow and improving the water use efficiency as well as other real-time phenotyping parameters analyzed	Armanhi et al. (2021)[1]
A. thaliana (plants grown in sterilized substrate)	Two SynComs formed by 218 leaf-derived bacteria and 188 root+soil-derived bacteria	Inoculation with leaf- or root+soil-derived SynComs showed that specific SynCom can colonize their respective organs displaying competitive advantages	Bai et al., (2015)[2]
A. thaliana (55 mutant plants grown in axenic conditions)	7-strain community representing the core microbiota composed by 4 Alphaproteobacteria, 2 Actinobacteria and 1 Betaproteobacteria	The authors concluded that SynCom composition is influenced by host genetic variation helping to identify novel host genes directly involved in phyllosphere microbiota structure and dynamics	Bodenhausen et al. (2014)[3]
A. thaliana (gnotobiotic plants)	62-strain community assembled composed by 32 Proteobacteria, 20 Actinobacteria, 6 Bacterioidetes, 4 Firmicutes	In this study the authors deciphered the principles determining the community development and its structure dynamics after inoculation. The early timing of microbiome manipulation is essential to obtain a stable *in planta* SynCom	Carlström et al. (2019)[4]
A. thaliana (mutants with altered phosphate starvation responses. For SynCom experiments plants were	35-strain community isolated from roots of *Arabidopsis* and Brassicaceae species	The inoculated SynCom positively influenced plant responses under phosphate-limiting conditions establishing a direct link between plant nutrition and	Castrillo et al. (2017)[5]

grown in axenic conditions)		expression of genes related to the immunity responses	
Sorghum (4 genotypes with contrasting N-use efficiency. Potted plants grown under glasshouse conditions)	Five SynComs formed by a total of 36-strain community bacteria isolated from sorghum roots and bulk soil grown in high- and low-Nitrogen field contents	Plants inoculated with the diverse SynComs showed growth features that are genotype- and plant N status-dependent. The same factors determined the colonization pattern by bacterial SynComs	Chai et al. (2021)[6]
A. thaliana (gnotobiotic plants)	Seven SynComs formed by 148 bacteria, 34 fungi and 8 oomycetes mixed in diverse combination	SynComs were used to re-populate gnotobiotic plants at multi-kingdom level showing maximal plant growth and survival. These findings demonstrated that over the evolution plant as host favoured interkingdom microbial assemblage rather than with a single microbial group	Durán et al. (2018)[7]
A. thaliana (plants grown in axenic conditions)	Two SynComs formed by 218 leaf-derived bacteria and 188 root+soil-derived bacteria	This study provides new insights about plant microbiome modulation by pathogen (powdery mildew) which affect host source-sink relationships and immune responses. SynComs applied did not altered the powdery mildew infection.	Durán et al. (2021)[8]
A. thaliana (plants grown in axenic conditions under diverse P concentrations)	3 SynComs formed by bacteria isolated from Brassicaceae roots	The authors dissected plant-microbe interactions under different P concentrations, linking phenotypic shifts with changes in microbiota composition	Finkel et al. (2019)[9]
A. thaliana (plants grown in axenic conditions)	185-strain community formed by bacteria isolated from Brassicaceae roots	The authors demonstrated that a single genus, *Variovorax*, is the key for maintaining root growth by manipulating host hormone balance	Finkel et al. (2020)[10]
A. thaliana (plants grown in axenic conditions)	14 partially overlapping SynComs	In this study the complex interactions among host phenotypes, microbiota and environment have been investigated. The presented approach defines the rational design and deployment of microbes to improve host performances	Herrera Paredes et al. (2018)[11]
Tomato (plants grown in natural soil)	Community of 8-*Pseudomonas* strains with proved biocontrol activities against the	*Pseudomonas* SynComs survival in natural soil is enhanced with increased *Pseudomonas* community	Hu et al. (2016)[12]

	bacterial pathogen *Ralstonia solanacearum*	diversity and, in turn, decreasing *R. solanacearum* disease incidence	
Maize (plants grown in pot with sterilized substrate under glasshouse conditions)	Two SynComs formulated using bacterial strains isolated from i) bulk soil (15-strain community) and ii) maize roots (12-strain community)	In this study the SynCom approach was used to understand bacterial colonization and assembly as well as plant-microbe interactions under organic (phthalate) pollution	Huang et al. (2022)[13]
A. thaliana (mutants plants defecting of defence-signalling sectors grown in pot with calcined clay sterilized substrate)	38-strain community identified as core microbiota after 16S rRNA sequencing	The authors established that immune signalling (mainly by salicylic acid) drives root colonization of the available soil microbial communities	Lebeis et al. (2015)[14]
Tomato (potted experiments using sterilized soil under glasshouse conditions)	4-strain community formed by rhizosphere isolated bacteria	The inoculated SynCom strongly activate host immune responses against *R. solanacearum* with greater extent than the individual strain. Dysbiosis of protective Gram-positive rhizosphere community can promote the disease incidence	Lee et al. (2021)[15]
Wheat (potted experiments using autoclaved soil under glasshouse conditions)	8-strain community formed by PGPR isolated from wheat rhizosphere	The inoculated SynCom increased wheat yield and biomass. Additionally, a soil-borne pathogen *Fusarium pseudograminearum* load in soil was significantly reduced by SynCom-mediated alteration of soil microbiome structure	Liu et al. (2022)[16]
A. thaliana and Barley (plants grown in axenic conditions)	Two SynComs composed of two fungal models *Serendipita vermifera* and *Bipolaris sorokiniana* for both plants + 4-bacterial strain community in Arabidopsis and 26-bacterial strain community for Barley	Using SynCom with a fungal endophyte and core bacteria microbiota members for each plant, the authors demonstrated the hypothesis that the establishment of beneficial inter-kingdom interactions in the plant microbiota is an evolutionary and conserved trait leading to a synergistic protection against a soilborne fungal pathogen	Mahdi et al. (2022)[17]
Alfalfa (plants grown in axenic conditions under different Nitrogen concentrations)	10-strain community formed by Alfalfa leaves and flowers isolated bacteria	Researchers demonstrated that plants could shape the microbiome assembly depending on nitrogen concentration	Moccia et al. (2020)[18]

Rice (seedlings grown in axenic conditions)	10-strain community formed by root isolated PGPR bacteria	Inoculated seedlings showed higher growth rates than the uninoculated. Authors concluded that the use of simplified SynCom is useful to better understand microbe-microbe and microbe-plant synergistic interactions	Moronta-Barrios et al. (2018)[19]
Maize (SynCom experments were performed on seedlings grown in axenic conditions)	7-strain community representing three of the most dominant phyla found in maize roots	In this study the authors demonstrated the roles that each strain plays during the community assembly. Furthermore, they highlight the SynCom potential as useful approach to investigate how bacterial interspecies interactions affect both root microbiome assembly and beneficial hosts effects	Niu et al., (2017)[20]
Sorghum (plants grown in axenic conditions)	Three SynComs by differently mixed a total of 53-strain community isolated from Brassicaceae roots (Finkel et al., 2020)	In this study, the SynCom approach allowed to identify that the *Variovorax* strains can protect sorghum growth from drought and from the activity of root growth inhibition bacterial strains. Additionally, data were compared with field experiments with some convergent results	Qi et al. (2022)[21]
A. thaliana (plants grown in axenic conditions)	185-strain community formed by bacteria isolated from Brassicaceae roots	In this study the effects of sublethal dose of Glyphosate (hormesis induction) on plant microbiome was observed using a SynCom approach. SynCom reduces hormesis effects due to some strains known as root growth inhibitors. In sum, glyphosate hormesis phenomenon is completely dependent by the microbiome composition	Ramirez-Villacis et al. (2020)[22]
Nicotiana attenuata (grown in axenic conditions)	5-strain community formed by native bacterial isolates isolated from the plant's natural habitat	The inoculated SynCom protects its host against fungal pathogens because of complementary traits of the five strains forming the multitaxa consortia	Santhanam et al. (2019)[23]
Tomato (plants grown in pots with unsterilized substrate)	Two SynCom: 1) 15-strain community and 2) the simplified SynCom 1 to 5-strain community	The authors used non-sterile soil to mimic a realistic agricultural setting. Both SynComs successfully	Schmitz et al. (2022)[24]

	formed by root-derived bacteria isolated from the desert plant *Indigofera argentea*	protected tomato plants against salt stress. This was coupled with a differential expression of salt stress-related genes and ion accumulation in inoculated tomato plants	
A. thaliana (plants grown in axenic conditions) and Tomato (plants grown in pots under controlled growth chamber)	Two SynComs formulated using 25-strain community each isolated from the rhizosphere of tomato plants grown in a suppressive compost	Both SynComs positively affected Tomato growth and suppressed *Fusarium* wilt symptoms. Conversely no or negative effects were observed for *Arabidopsis in vitro*. The authors concluded that the application of SynComs on poor substrates can yield reproducible plant phenotypes	Tsolakidou et al. (2019)[25]
A. thaliana (mutants with absence of root-secreted phytoalexins, flavonoids and coumarins)	22-strain community isolated from *Arabidopsis* roots	In this study, mechanisms of microbiome shaping by plant derived molecules were elucidated as mainly mediated by secreted coumarins	Voges et al. (2019)[26]
Maize (grown *in vitro* and in gnotobiotic bags)	7-strain community formed by maize root-colonizing bacteria	The authors demonstrated an ecological phenomenon where belowground microorganisms can influence the early growth of inbred and hybrid maize plants	Wagner et al. (2021)[27]
Rice (*indica* and *japonica* varieties)	Two SynComs: 1) 16 bacteria from *indica*-enriched OTUs; 2) 3 bacteria from *japonica*-enriched OTUs	In this study the authors used SynComs to explain the historical observation of higher Nitrogen Use Efficiency (NUE) in *indica* varieties respect to *japonica* ones. The *indica* SynCom conferred higher growth effects respect the *japonica*-derived SynCom likely due to diverse bacteria-mediated transformation of organic nitrogen	Zhang et al. (2019)[28]
Rape (potted experiments under controlled conditions)	7-strain community formed by inorganic-phosphate-solubilizing bacteria (iPSBs) isolated from bulk soil	The SynCom was co-inoculated using biochar as carrier and mixed in substrate of potted rape. iPSB community was effective in rape growth features, P content and uptake	Zheng et al. (2019)[29]
Radish (axenic seedlings)	6-*Pseudomonas* strain community isolated	The authors used a top-down approach able to	Zhuang et al. (2021)[30]

	from garlic rhizosphere in two growth periods (bolting and maturation) and different growth condition (diverse soil characteristics)	identify *Pseudomonas* as PGPR providing insights in how plants affect their microbial community assembly and how the microbiome influence growth and defence features, highlighting the SynCom exploitability for a sustainable agriculture	
Maize (grown in mesocosms with unsterilized soil under semi-controlled conditions)	3-strain community formed by microbes isolated from tomato rhizosphere cultivated in arid and saline soil	SynCom increased tolerance to water stress improving ecophysiological parameters, biomass and yield production highlighting that SynCom selected for a particular stress could be suitable also for agronomical applications	Zoppellari et al. (2013)[31]

Table S2. Examples of beneficial soil-microorganism applications to mitigate abiotic and biotic plant stresses during the last 15 years.

Beneficial Soil-microorganism	Plant	Abiotic Stress	Biotic Stress	Reference
Streptomyces rochei IT20*S ; Streptomyces vinaceusdrappus* SS14	Pepper (*Capsicum annuum* L.)		*Phytophthora capsici*	Abbasi et al. (2020)[32]
Pseudomonas fluorescens	Mung bean (*Vigna radiata*)	Salinity		Ahmad et al. (2013)[33]
Pseudomonas sp. AKM-P6	Sorghum (*Sorghum vulgare*)	Heat		Ali et al. (2009)[34]
Streptomyces sp.	Wheat (*Triticum aestivum*)	Salinity		Aly et al. (2012)[35]
Pseudomonas PS01	Arabidopsis *(Arabidopsis thaliana)*	Salinity		Chu et al. (2019)[36]
Arthrobacter arilaitensis; Streptomyces pseudovenezuelae	Maize (*Zea mais*)	Drought		Chukwuneme et al. (2020)[37]
Bacillus amyloliquefaciens B9601-Y2	Maize (*Z. mais*)		*Bipolaris maydis*	Cui et al. (2019)[38]
Streptomyces albidoflavus H12*; Nocardiopsis aegyptica* H14	Tomato (*Solanum lycopersicum*); Carrot (*Daucus carota*)		*Fusarium oxysporum f. sp. 42 adices-lycopersici; Rhizoctonia solani*	Djebaili et al. (2021)[39]
Streptomyces sp.	Sugar beet (*B. vulgaris*)		*Sclerotium rolfsii*	Errakhi et al., (2009)[40]
Bacillus tequilensis SSB07	Soybean (*Glycine max*)	Heat		Kang et al. (2019)[41]
Bacillus cereus	Tomato (*S. lycopersicum*)		*Fusarium* o*xysporum; Alternaria solani*	Karthika et al. (2020)[42]
Bacillus sp. BS061	Cucumber (*Cucumis sativus*) Strawberry (*Fragaria sp.*)		*Botrytis cinerea; Podosphaera xanthii*	Kim et al. (2013)[43]
Streptomyces sp.	Guava (*Psidium guajava*)		*Fusarium oxysporum; Alternaria solani.*	Mohandas et al. (2013)[44]
Streptomyces sp. PGPA39	Tomato (*S. lycopersicum*)	Salinity		Palaniyandi et al. (2014)[45]
Pseudomonas putida PCI2	Tomato (*S. lycopersicum*)		*Fusarium oxysporum*	Pastor et al. (2016)[46]
Bradyrhizobium japonicum; Bacillus thuringiensis	Soybean (*G. max*)	Drought		Prudent et al. (2014)[47]
Streptomyces sp.	Wheat (*T. aestivum*)	Salinity		Sadeghi et al. (2012)[48]
Amycolatopsis sp.	Apple *(Malus domestica)*		*Colletotrichum gloeosporioides*	Sadeghian et al. (2016)[49]

Citricoccus zhacaiensis B–4	Onion (*Allium cepa*)	Drought		Selvakumar et al. (2015)[50]
Streptomyces sp.	Noce metella (*Datura metel)*		*Tobacco Mosaic Virus* (TMV)	Sonya et al., (2012)[51]
Streptomyces rochei SM3	Chickpea (*Cicer arietinum*)	Salinity		Srivastava et al. (2015)[52]
Streptomyces coelicolor DE07 ; *Streptomyces olivaceus* DE10 ; *Streptomyces geysiriensis* DE27	Wheat (*T. aestivum*)	Drought		Yandigeri et al. (2012)[53]

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Conflict of interest

The authors declare no conflicts of interest.

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CHAPTER 3 | MYCORRHIZAL SYMBIOSIS BALANCES ROOTSTOCK-MEDIATED GROWTH-DEFENCE TRADEOFFS

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Abstract

It is well known that AM symbiosis provides several ecosystem services leading to plant adaptation in different environmental conditions and positively affects physiological and production features. Although beneficial effects from grapevine and Arbuscular Mycorrhizal Fungi (AMF) interactions have been reported, the impact on growth-defence tradeoffs features has still to be elucidated. In this study, the potential benefits of an inoculum formed by two AM fungal species, with or without a monosaccharide addition, were evaluated on young grapevine cuttings grafted onto 1103P and SO4 rootstocks. Inoculated and non-inoculated plants were maintained in potted vineyard substrate under greenhouse conditions for three months. Here, agronomic features were combined with biochemical and molecular techniques to assess the influence of the different treatments. Despite the opposite behaviour of the two selected rootstocks, in AM samples the evaluation of gene expression, agronomic traits and metabolites production, revealed an involvement of the whole root microbiome in the growth-defence tradeoffs balancing. Noteworthy, we showed that rootstock genotypes and treatments shaped the root-associated microbes, stimulating plant growth and defence pathways. Progresses in this field would open new perspectives, enabling the application of AMF or their inducers to achieve a more sustainable agriculture also considering the ongoing climate change.

Keywords AMF, trade-off, plant priming, stress tolerance, N, growth-defence balance

Introduction

Grapevine is one of the most cultivated crop worldwide since its great economic importance resulting from grape and wine production, and commercialization (Chitarra *et al*. 2017). For this reason, over the years viticulture industry has selected several cultivars showing different traits (*i.e.*, flavour, yields, colour) influenced by geology, soil-scape and climate features, driving some major wine peculiarities (Priori *et al*. 2019). These components, and their interactions, concur to define the *terroir* of a particular environment (Resolution OIV/VITI 333/2010). Besides scion variety features, rootstocks are able to strongly affect scion performances by means of water transport, biochemical and molecular processes, impacting the whole plant functions and its response to biotic/abiotic stress factors (Chitarra *et al*. 2017). In the last decade, research on scion/rootstock interactions strongly increased, aiming to develop more sustainable practices against pests and ameliorating plant adaptability to the ongoing climate change (Lovisolo *et al*. 2016; Warschefsky *et al*. 2016; Zombardo *et al*. 2020). Key drivers influencing defence features and adaptive traits are thought to be the microbial communities residing in plant tissues. To date, several studies reported evidence about their influence on physiological performances (*e.g.*, production of flavours, hormones, VOCs) in many plants, including grapevine, where residing microbiota contribute to defining the *microbial terroir* (Gilbert *et al*. 2014).

According to the Intergovernmental Panel on Climate Change (IPCC 2014), an increase in the global surface temperature is expected over the next years, affecting crop production as a consequence of the predicted occurrence of biotic and abiotic stresses (Mittler and Blumwald 2010). To achieve resilience to stress, numerous efforts have been done over the years, such as the adoption of specific breeding programs and genetic engineering approaches (Cushman and Bohnert 2000). Researchers have been focusing just recently their attention on the exploitation of 'native' plant defence mechanisms (e.g. hormone signalling, plant immunity activation) against biotic and abiotic stressful factors (Feys and Parker 2000; Jones and Dangl 2006; Hirayama and Shinozaki 2007). The triggering of these responses can occur using chemical treatments (Balestrini *et al*. 2018), root-associated microorganisms and RNA interference technologies (Alagna *et al*. 2020), leading plants in a state of alertness - 'Primed state' or 'Priming' – and enabling them to respond more quickly and robustly in case of the exposure to a stress (Beckers and Conrath 2007).

Among soil beneficial microorganisms, Arbuscular Mycorrhizal Fungi (AMF) establish symbioses with the majority of land plants showing an important role in providing nutrients, particularly phosphate and N, but also water and other elements to the host plant (Jacott *et al*. 2017; Balestrini and Lumini 2018). Mycorrhizal symbiosis is able to influence plant growth and productivity and enhance the tolerance to biotic and abiotic stresses as demonstrated in many crops (Balestrini and

Lumini 2018; Balestrini *et al*. 2018; Alagna *et al*. 2020). In addition, AMF are able to increase aggregation of soil surrounding roots, improving soil matrix stability and physicochemical characteristics (Uroz *et al*. 2019). Grapevine roots are naturally colonized by native AMF with a great impact on growth, yield, quality and development performances (Deal *et al*. 1972; Karagiannidis *et al*. 1995; Linderman and Davis 2001; Trouvelot *et al*. 2015). Thanks to the application of metagenomics approaches to soil and roots, new insights about the AMF living in symbiosis with grapevine have been discovered (Balestrini *et al*. 2010; Holland *et al*. 2014; Balestrini and Lumini 2018).

Rootstocks-mediated adaptation to a specific environment is based on the growth-defence trade-offs-mediated mechanisms (Chitarra *et al*. 2017). Trade-off phenomenon was firstly observed in forestry plants-insect interaction studies and is based on the idea that the limited carbon resources produced by photosynthesis are allocated toward growth or defence processes in order to maximize the adaptation strategies and fitness costs in diverse environments (Huot *et al*. 2014; Chitarra *et al*. 2017; Züst and Agrawal 2017). Stresses impair plant growth, redirecting energy and carbon sources toward defence, reducing growth and reproduction performances (Bandau *et al*. 2015; Züst and Agrawal 2017). Recently, it was suggested that through a meta-analysis, that the increased plant resistance promoted by *Epichloë* fungal endophytes does not compromise plant growth, eliminating the trade-off between growth and defence (Bastías *et al*. 2021). A role in tradeoffs balance has been demonstrated also for AM symbioses, improving nutrient uptake, disease tolerance and abiotic stress resilience (Jacott *et al*. 2017).

In this study, we aimed to evaluate if AMF and rootstocks can concomitantly contribute to fine-tuning growth-defence tradeoffs features in grapevine, thus enabling plants to trigger earlier and enhanced defence responses against a potential stressor. The use of specific molecules that can promote the AM fungal colonization have been proposed to improve mycorrhizal inoculum applications under practical field condition (Bedini *et al*. 2018). In this context, an affordable strategy is the application at low doses of oligosaccharides (*i.e.*, glucose, fructose, and xylose) that have a stimulant effect on AM symbiosis colonization (Lucic and Mercy 2014 - Patent application EP2982241A1). These compounds, initially called as elicitors, in relation to the impact on plant defense, can promote mycorrhizal performances and, for this reason, the term “inducer” was proposed (Bedini *et al*. 2018). In this work, the impact of an inoculum formed by two AMF species (*Funneliformis mosseae* and *Rhizophagus irregularis*), already reported among the species present in vineyards (Berruti *et al*. 2018), with or without the addition of a monosaccharide (D-glucose) at low dose (the so called inducer), has been evaluated on young grapevine cuttings cv. Glera grafted onto 1103 Paulsen and SO4 rootstocks, well known to trigger an opposite growth-defence behaviour in the scion. The effect

of the several treatments on the root-associate microbiota has been also evaluated, to verify the response mediated by the AM and its recruited mycorrhizosphere.

Materials and methods

Biological materials and experimental set-up

Two hundred one year-old dormant vines of 'Glera' cultivar grafted onto 1103 Paulsen (1103P) and SO4 rootstocks certified as 'virus free' were purchased from an Italian vine nursery (Vivai Cooperativi Rauscedo, Italy; http://www.vivairauscedo.com). Vine roots were washed with tap water and cut to about 4 cm before plantation in 2 L pot containers filled with not sterilized substrate mixture of vineyard soil/*Sphagnum* peat (8:2, v:v) to better simulate the field conditions. The substrate composition was a sandy-loam soil (pH 7.8; available P 10.4 mg kg^{-1}; organic matter 1.80 %; cation exchange capacity 20.11 mew 100 g^{-1}).

Grapevine cuttings were inoculated with AMF mixed inoculum (INOQ GmbH, Germany, 238.5 million propagule per kg inoculum) at planting time by placing it in the hole and in contact with the roots following the manufacturer's instructions. Mycorrhizal inoculum, a powder based mycorrhizal root fragment (Advantage Grade II, 2016 - INOQ GmbH) contained 50% *Rhizoglomus irregulare* (syn. *Rhizophagus irregularis*; 450 million propagules per Kg) and 50 % *Funneliformis mosseae* (27 million propagules per Kg). The fungal lines were produced *ex vitro*, on *Zea mays* and *Plantago lanceolata* (sand/vermiculite, v/v). Both AMF inoculum and D-glucose at low dose (i.e., the Inducer) were prepared by Louis Mercy (INOQ GmbH; patent EP2982241A1). The containers were prepared according to treatments as follow: i) 25 plants for each rootstock as uninoculated control plants (C); ii) 25 plants for each rootstock inoculated with 50 mg/L of AMF mixed inoculum (M); iii) 25 plants for each rootstock inoculated with 50 mg/L of AMF mixed inoculum + inducer (M+I); iv) 25 plants for each rootstock amended with 50 mg/L of inducer to stimulate the exploitation of native AMF symbiosis (I). Daily watered grapevine plants were kept under partially climate-controlled greenhouse, under natural light and photoperiod conditions for three months.

After three months, at the end of the experiment, engraftment, growth index and chlorophyll content were recorded. Leaf and root samples for molecular and biochemical analysis were collected from at least three randomly selected plants and immediately stored at -80°C. A part of the root apparatus was used to estimate the level of mycorrhiza formation as described (Balestrini *et al*. 2017).

Morphological observations in the colonized fragments of thin roots allowed to identify the presence of the typical structures of the symbiosis, regardless of the thesis. However, the patchy level of colonization, and the quality of the root segments after the staining, made morphological quantification difficult, and therefore the AMF presence has been assessed by molecular analyses (see below).

Growth index, engraftment, and chlorophyll content

At the end of the experiment, phenological stages were recorded and classified according to Biologische Bundesanstalt, bundessortenamt und CHemische industrie (BBCH) scale (from 00 to 12, from dormancy to 9 or more leaves unfolded, respectively). BBCH scales have been developed for many crops, including grapevine, and it is based on a decimal code system that identify the growth stage (Lancashire *et al*. 1991), engraftment % (i.e. rooting %) were visually determined for each plant and treatment. Chlorophyll content was determined using a portable chlorophyll meter SPAD (Konica Minolta 502 Plus). Readings were collected from the second or third leaf from the top on at least three leaves per plant on five randomly selected vines for each experimental condition (Chitarra *et al*. 2016).

Targeted metabolite analyses

Contents of *trans*-resveratrol, viniferin and abscisic acid (ABA) were quantified on at least three biological replicates per condition according to the protocol previously described (Pagliarani *et al*. 2019, 2020; Mannino *et al*. 2020). Leaves and roots from two randomly selected plants were pooled to form a biological replicate, immediately frozen in liquid nitrogen, freeze-dried and stored at -80°C until use. Briefly, about 100 mg of freeze-dried sample (leaf or root) were transferred with 1 mL of methanol:water (1:1 v/v) acidified with 0.1 % (v/v) of formic acid in an ultrasonic bath for 1 h. Samples were centrifuged for 2 min at 4°C and 23.477 *g*, and the supernatant was analysed by high-performance liquid chromatography (HPLC). Original standards of resveratrol (purity ≥ 99 %), viniferin (purity ≥ 95 %) and ABA (purity ≥ 98.5%, Sigma-Aldrich) were used for the identification by comparing retention time and UV spectra. The quantification was made by external calibration method. The HPLC apparatus was an Agilent 1220 Infinity LC system (Agilent R, Waldbronn, Germany) model G4290B equipped with gradient pump, auto-sampler, and column oven set at 30°C. A 170 Diode Array Detector (Gilson, Middleton, WI, United States) set at 265 nm (ABA and IAA) and 280 nm (for stilbenes) was used as detector. A Nucleodur C18 analytical column (250x4.6 mm i.d., 5 μm, Macherey Nagel) was used. The mobile phases consisted in water acidified with formic

acid 0.1% (A) and acetonitrile (B), at a flow rate of 0.500 mL min^{-1} in gradient mode, 0-6 min: from 10 to 30 % of B, 6-16 min: from 30 % to 100 % B, 16-21 min: 100% B. Twenty µL was injected for each sample.

Total N, soluble carbohydrate content in leaf and net nitrate uptake in root

The Kjeldahl method was performed according to method 981.10 of the AOAC International (2016), using VELP Scientifica DKL 20 Automatic Kjeldahl Digestion Unit and UDK 159 Automatic Kjeldahl Distillation and Titration System. Approximately 0.2 g of leaf raw material was hydrolyzed with 15 mL concentrated sulfuric acid (H_2SO_4) containing one catalyst tablets (3.47 g K_2SO_4 + 0.003 Se, VELP Scientifica, Italy) in a heat block (DK Heating Digester, VELP Scientifica, Italy) at 300°C for 2 h. After cooling, H_2O was added to the hydrolysates before neutralization with NaOH (30%) and subsequently distilled in a current of steam. The distillate was collected in 25 mL of H_3BO_3 (1%) and titrated with HCl 0.1 M. The amount of total N in the raw materials were calculated. Leaf soluble carbohydrate content was quantified (Chitarra *et al*. 2018). At the end of the experiment, white non-lignified roots (0.5 – 1 g) were collected from four randomly selected plants for each treatment and rootstock. Root samples were washed in 0.5 mmol L^{-1} $CaSO_4$ for 15 min, then transferred to a 20 mL aerated uptake solution containing 0.5 mmol L^{-1} $Ca(NO_3)_2$ and 0.5 mmol L^{-1} $CaSO_4$. Net uptake of NO_3^- was measured removing samples of uptake solution (aliquot of 200 µL) for its determination every 2 min for 10 min(Tomasi *et al*. 2015). The aliquots were carefully mixed with 800 µL of salicylic acid (5% w/v in concentrated H_2SO_4) and incubated for 20 min at room temperature following the addition of 19 ml of 2 mol L^{-1} NaOH. After cooling, nitrate concentration was measured at the absorbance of 410 nm (Shimadzu UV Visible Spectrophotometer UVmini-1240. Kyoto, Japan) and the net nitrate uptake was expressed as µmol (g FW h^{-1}).

RNA isolation and RT-qPCR

Expression changes of target transcripts were profiled on root and leaf samples (three independent biological replicate for each treatment) by quantitative real-time PCR (RT-qPCR) (Chitarra *et al*. 2018). Total RNA was isolated from the same lyophilized samples (leaves and roots) used for HPLC-DAD analysis and cDNA synthesis was performed as previously reported (Chitarra *et al*. 2016). The absence of genomic DNA contamination was checked before cDNA synthesis by qPCR using *VvUBI* specific primers of grapevine. RT-qPCR reactions were carried out in a final volume of 15 µL containing 7.5 µL of Rotor-Gene™ SYBR® Green Master Mix (Qiagen), 1 µL of 3 µM specific

primers and 1:10 of diluted cDNA. Reactions were run in the Rotor Gene apparatus (Qiagen) using the following program: 10 min preincubation at 95°C, followed by 40 cycles of 15 s at 95°C, and 30 s at 60°C. Each amplification was followed by melting curve analysis (60–94°C) with a heating rate of 0.5°C every 15 s. All reactions were performed with at least two technical replicates. The comparative threshold cycle method was used to calculate relative expression levels using plant (elongation factors, actin and ubiquitin, *VvEF* and *VvUBI* for root and *VvACT* and *VvEF* for leaf tissue) reference genes. While *R. irregularis* and *F. mosseae* elongation factors (*RiEF1*, *FmEF,* respectively) were used to normalize the expression of the AMF phosphate transporter (*PT*) genes. Oligonucleotide sequences are listed in Supplementary Table 1. Gene expression data were calculated as expression ratio (Relative Quantity, RQ) to Control 1103P plants (C 1103P).

Root DNA isolation and sequencing

Root samples were lyophilized prior to DNA extraction. About 30 to 40 mg of freeze-dried and homogenized material were used to extract total DNA following manufacturer instruction of plant/fungi DNA isolation kit (Norgen Biotech Corp., Thorold, ON, Canada) as previously reported (Nerva *et al*. 2019). Total DNA was quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific,Waltham, MA, USA), and DNA integrity was inspected running the extracted samples on a 1% agarose electrophoretic gel. Before sending DNA to sequencing a further quantification was performed using a Qubit 4 Fluorometer (Thermo Fisher Scientific,Waltham, MA, USA). To inhibit plant material amplification, we added a mixture of peptide nucleotide acid (PNA) blockers oligos (Kaneka Eurogentec S.A., Belgium) targeted at plant mitochondrial and chloroplast 16S rRNA genes (mitochondrial and plastidial) and plant 5.8S nuclear rRNA. Mitochondrial sequence was derived from (Lundberg *et al*. 2013) with a 1bp mismatch, mitochondrial sequence was derived from (Cregger *et al*. 2018). PNA was custom-designed for *V. vinifera* (VvpPNA: GGCTCAACCCTGGACAG; Vv-ITS-PNA: CGAGGGCACGCCTGCCTGG; Vv-mPNA: GGCAAGTGTTCTTCGGA). Thermal cycler conditions were maintained as suggested by the Illumina protocol as previously reported (Nerva *et al*. 2019).

Sequences were deposited in NCBI database under the BioProject PRJNA718015, BioSamples SAMN18520793 to SAMN18520808 and SRR14089924 to SRR14089939.

Rhizoplane metaphylogenomic analyses, taxonomic distributions

A first strict quality control on raw data was performed with PrinSeq v0.20.4 (Schmieder and Edwards 2011) and then processed with Qiime2 (Bolyen *et al*. 2019). A previously reported and specific pipeline was used for fungal analysis: retained reads were used to identify the start and stop sites for the ITS region using the hidden Markov models (HMMs) (Rivers *et al*. 2018), created for fungi and 17 other groups of eukaryotes, which enable the selection of ITS-containing sequences. Briefly, the software allows to distinguish true sequences from sequencing errors, filtering out reads with errors or reads without ITS sequences. To distinguish true sequences from those containing errors, sequences have been sorted by abundance and then clustered in a greedy fashion at a threshold percentage of identity (97%). Trimmed sequences were analyzed with DADA2 (Callahan *et al*. 2016) and sequence variants were taxonomically classified through the UNITE (Abarenkov *et al*. 2010) database (we selected the reference database built on a dynamic use of clustering thresholds). For graphic representation, only genera with an average relative abundance higher than the settled threshold (1%) were retained.

A 16S specific pipeline was used for bacteria: quality filtering was performed with DADA2 which is able to perform chimera removal, error-correction and sequence variant calling with reads truncated at 260 bp and displaying a quality score above 20. Feature sequences were summarized and annotated using the RDP classifier (Cole *et al*. 2014) trained to the full length 16S database retrieved from the curated SILVA database (v132) (Quast *et al*. 2012).

Statistics

Metagenome analyses were performed using R version 3.6.3 (2020-02-29). Fungal and bacterial data were imported and filtered with Phyloseq package (version 1.28.0) (McMurdie and Holmes 2013), keeping only the operational taxonomic units (OTUs) with a relative abundance above 0.01 in at least a single sample. Differential abundance of taxa due to the effects of rootstock-treatment interaction was then tested using DESeq2 (version 1.24.0) (Love *et al*. 2014) package.

For phenotypic, biochemical, and RT-qPCR data, when ANOVA indicated that for either Rootstock (R, 1103P and SO4), Inducer (I, NI) and Myc inoculum (M, Myc and NMyc) factors or their interaction was significant, mean separation was performed according to Tukey's HSD test at a probability level of $P \leq 0.05$. ANOVA and Tukey's HSD test were also used to analyze the treatments effects for each rootstock individually. The standard deviation (SD) or error (SE) of all means were calculated.

Results

Growth, primary metabolism and N uptake and accumulation

The impact of an AM inoculum, an inducer and a combination of both was evaluated on growth parameters (both rooting % and growth stages coded by BBCH scale) in two grapevine rootstock genotypes (R, 1103P and SO4). Four conditions for each genotype were considered: C, not inoculated plants; I, plants treated with the inducer (I); M, AM-inoculated plants; M+I, AM-inoculated plants + inducer.

Results showed a similar impact of the three treatments on the cutting growth parameters (Fig. 1, Table S2), independently from the genotype. Particularly, in SO4 genotype both the rooting % and the BBCH values were higher in treated plants with respect to the control (Fig. 1a, b). Chlorophyll Content Index (CCI) has been evaluated at the end of the experiment, showing no strong differences among the genotypes and treatments (Fig. 1c), although it was significantly influenced by root colonization (M), the inducer (I) and the M x I interaction in both rootstock genotypes.

Treatments generally led to slightly lower values of carbohydrates content in leaves except for M, and only R and I factors significantly influenced this measurement (Fig. 1d). In detail, for each rootstock I and M+I plants showed significant lower levels of carbohydrates (Fig. 1d). Net nitrate uptake (NNU) was evaluated (Fig. 2a Table S2), showing that it was significantly affected by M factors and the interaction M × I with lower values in treated samples for both genotypes, particularly in M SO4 plants with respect to C SO4 ones (Fig. 2a). As for the CCI, only slight differences in total N content in leaves were evident among genotypes and treatments, although it was significantly affected by the M factor and the M × I interactions (Fig. 2b).

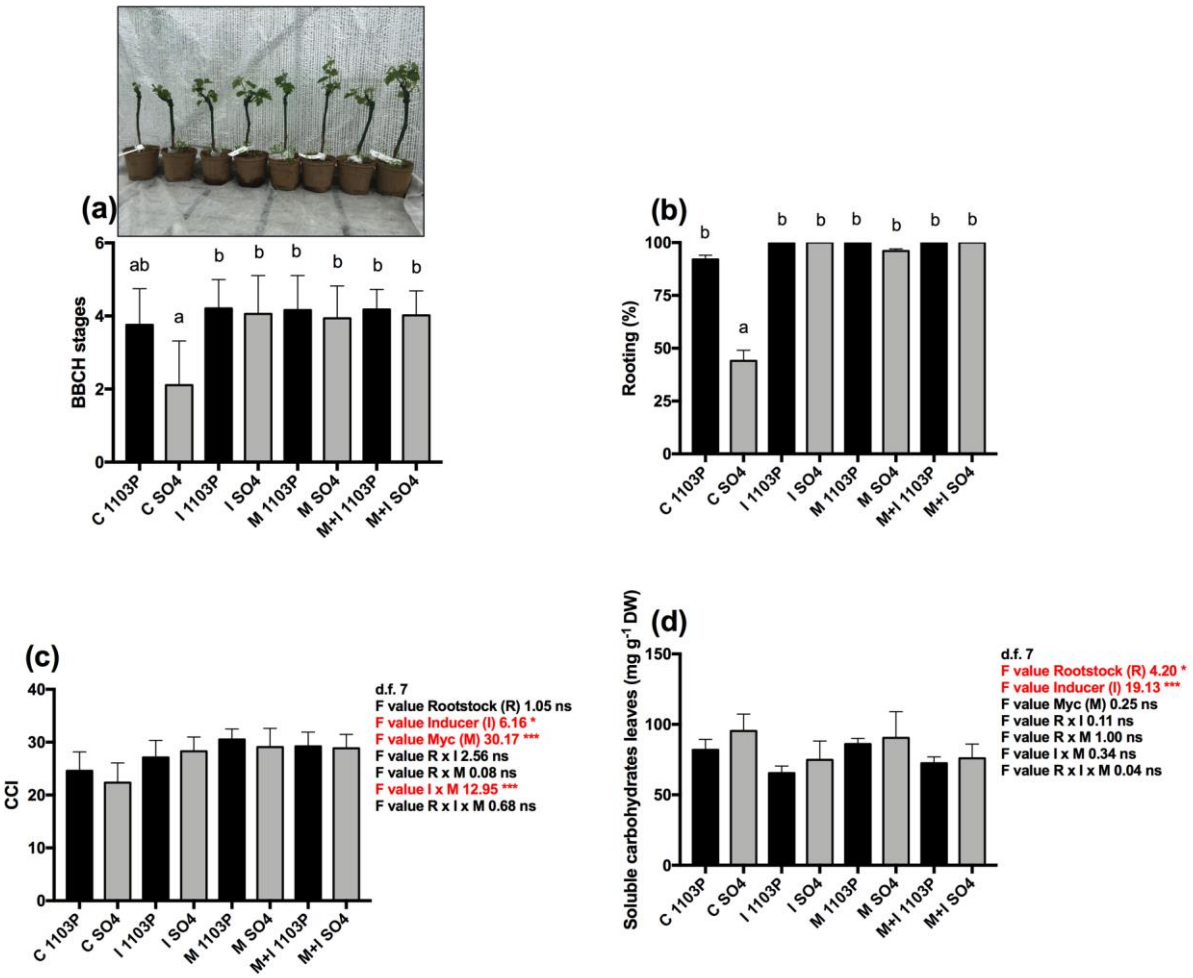


Fig. 1 Growth-related traits and metabolites. **a** Growth index according to BBCH scale recorded for each treatment at the end of the experiment (n = 25). Upper picture showed an overview of the cuttings' development in response to the treatments at the end of the experiment. **b** Rooting % of cuttings at the end of the experiment (n = 25). **c** Chlorophyll Content Index (CCI) measured at the end of the experiment (n = 25). **d** Quantification of soluble carbohydrates contents in leaves at the end of the experiment (n = 4). All data are expressed as mean • } SD. ns, *, ** and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R × I × M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C, control plants; I, inducer-treated plants; M, AMF mixed inoculum-treated plants; M + I AMF mixed inoculum + inducer-treated plants for 1103P and SO4 selected rootstocks.

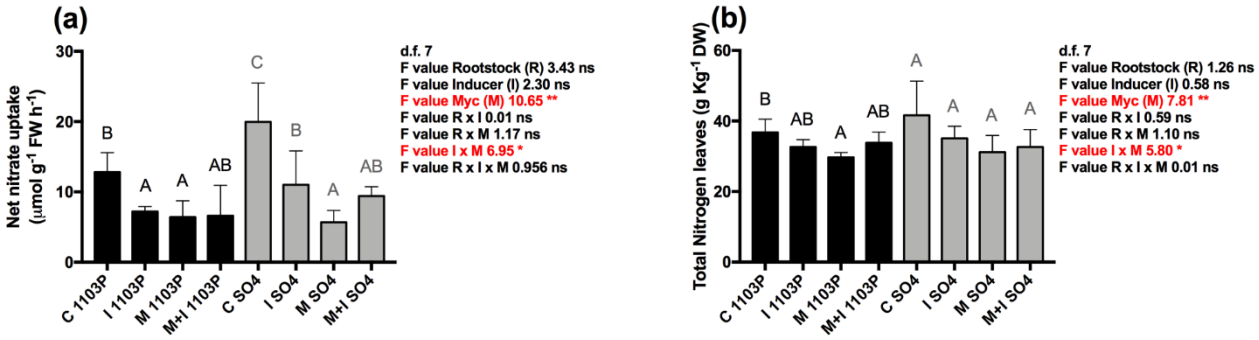


Fig. 2 Net nitrate uptake in roots and total N in leaves. **a** In vivo Net nitrate uptake. **b** Total N in leaves (g kg− 1 DW). All data are expressed as mean • } SD (n = 3). ns, *, ** and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R × I × M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C, control plants; I, inducer-treated plants; M, AMF mixed inoculum-treated plants; M + I AMF mixed inoculum + inducer-treated plants for 1103P and SO4 selected rootstocks.

ABA Content and the Expression of ABA-related Genes

To complete the physiological characterization of the two genotypes in response to treatments, the concentration of ABA was quantified in roots and leaves (Fig. 3, Table S2). ABA levels showed a complex scenario in roots where all treatments led to higher ABA levels with respect to the control with the greater significant increase recorded in M SO4. Statistical analyses showed that factors influencing its level were R and M, alone or in the interactions with I (R x I, M x I, R x M x I) (Fig. 3a). ABA content in leaves was under the detection limit among the treatments (data not shown). To better understand the role of ABA in our system, the expression of ABA-related genes was analyzed in both leaves and roots. Relative expression of: i) a gene encoding for a 9-cis-epoxycarotenoid dioxygenase potentially involved in ABA biosynthesis (*VvNCED3,* VIT_19s0093g00550 previously reported as *VvNCED1*); ii) a gene coding for an enzyme involved in conversion of ABA to 8′-hydroxy ABA (*VvABA8OH1*); iii) a β-glucosidase (BG) involved in free ABA biosynthesis *via* hydrolysis of ABA glucose ester to release the ABA active form (*VvBG1*; Jia *et al*. 2016); iv) a gene encoding an ABA glucosyltransferase (*VvGT*; Sun *et al*. 2010) were evaluated in leaves and roots. In leaves, *VvNCED3* expression was not affected by rootstock genotype whereas M samples showed significantly higher expression levels with respect to the other samples (Fig. 3b). No significant difference was detected for *VvABA8OH1* expression in leaves although 1103P generally showed higher values with respect to SO4 (Fig. 3c). By contrast, *VvNCED3* expression in roots was influenced by R, M and I factors as well as by R x I interaction, and values for each rootstock genotype were lower in all treatments when compared to C plants (Fig. 3d). Similar to that observed in leaves,

M+I treatment led to the significant lowest *VvNCED3* transcripts level in root samples (Fig. 3d). Two pathways promote free ABA accumulation: (1) NCED-mediated *de novo* synthesis (Qin and Zeevaart 1999) and (2) BG-mediated hydroxylation (Lee *et al*. 2006). Looking at *VvBG1* gene, its expression was significantly influenced by R and I in leaves, while the presence of the AMF was not significantly relevant. In roots all the factors and interactions, significantly affected *VvBG1* expression level, with the highest level in C SO4 samples (Fig. 3e, g). Finally, *VvGT* showed a trend similar to *VvBG1* in leaves where its expression was significantly influenced by R, I and I x M with the exception of SO4 samples where its expression was significantly higher only in M SO4 with respect to C SO4 (Fig. 3f). Conversely, in roots *VvGT* transcript levels were significantly lower in all the conditions with respect to the C 1103P plants (Fig. 3h).

Although *VvABA8OH1*, coding for an enzyme involved in ABA conversion, was not significantly regulated among genotypes and treatments in leaves, it results to be affected by all the considered factors and interactions in roots (Fig. 3i) where it appeared significantly upregulated in M 1103P, M SO4 and M+I SO4 plants with respect to their C (Fig. 3i). It is worth noting the low expression in I root samples, suggesting that the inducer may affect ABA catabolism independently from the genotype and the presence of the AM inoculum.

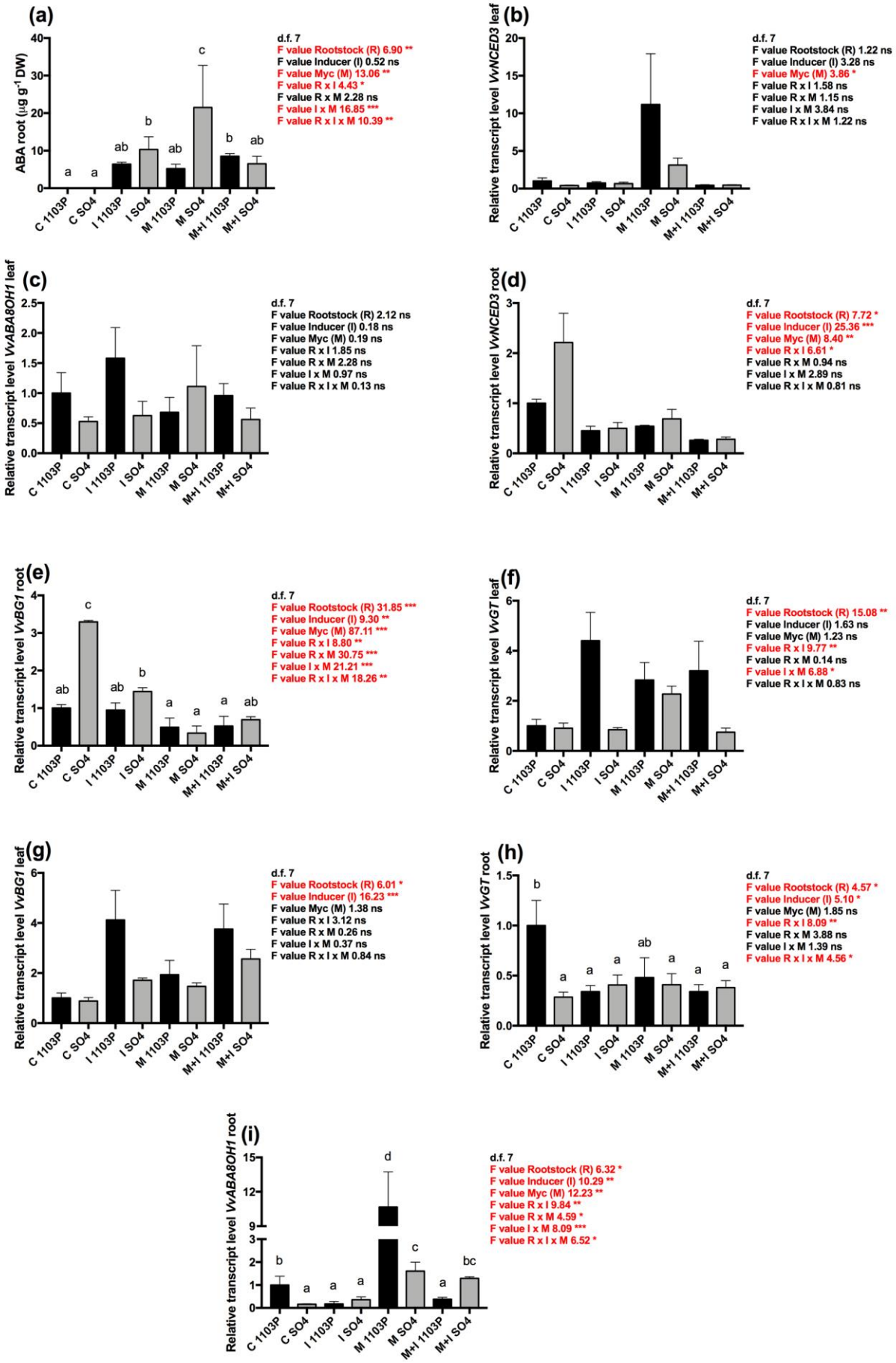

(a)
ABA root (µg g-1 DW)
d.f. 7
F value Rootstock (R) 6.90 **
F value Inducer (I) 0.52 ns
F value Myc (M) 13.06 **
F value R x I 4.43 *
F value R x M 2.28 ns
F value I x M 16.85 ***
F value R x I x M 10.39 **
(b)
Relative transcript level VvNCED3 leaf
d.f. 7
F value Rootstock (R) 1.22 ns
F value Inducer (I) 3.28 ns
F value Myc (M) 3.86 *
F value R x I 1.58 ns
F value R x M 1.15 ns
F value I x M 3.84 ns
F value R x I x M 1.22 ns
(c)
Relative transcript level VvABA8OH1 leaf
d.f. 7
F value Rootstock (R) 2.12 ns
F value Inducer (I) 0.18 ns
F value Myc (M) 0.19 ns
F value R x I 1.85 ns
F value R x M 2.28 ns
F value I x M 0.97 ns
F value R x I x M 0.13 ns
(d)
Relative transcript level VvNCED3 root
d.f. 7
F value Rootstock (R) 7.72 *
F value Inducer (I) 25.36 ***
F value Myc (M) 8.40 **
F value R x I 6.61 *
F value R x M 0.94 ns
F value I x M 2.89 ns
F value R x I x M 0.81 ns
(e)
Relative transcript level VvBG1 root
d.f. 7
F value Rootstock (R) 31.85 ***
F value Inducer (I) 9.30 **
F value Myc (M) 87.11 ***
F value R x I 8.80 **
F value R x M 30.75 ***
F value I x M 21.21 ***
F value R x I x M 18.26 **
(f)
Relative transcript level VvGT leaf
d.f. 7
F value Rootstock (R) 15.08 **
F value Inducer (I) 1.63 ns
F value Myc (M) 1.23 ns
F value R x I 9.77 **
F value R x M 0.14 ns
F value I x M 6.88 *
F value R x I x M 0.83 ns
(g)
Relative transcript level VvBG1 leaf
d.f. 7
F value Rootstock (R) 6.01 *
F value Inducer (I) 16.23 ***
F value Myc (M) 1.38 ns
F value R x I 3.12 ns
F value R x M 0.26 ns
F value I x M 0.37 ns
F value R x I x M 0.84 ns
(h)
Relative transcript level VvGT root
d.f. 7
F value Rootstock (R) 4.57 *
F value Inducer (I) 5.10 *
F value Myc (M) 1.85 ns
F value R x I 8.09 **
F value R x M 3.88 ns
F value I x M 1.39 ns
F value R x I x M 4.56 *
(i)
Relative transcript level VvABA8OH1 root
d.f. 7
F value Rootstock (R) 6.32 *
F value Inducer (I) 10.29 **
F value Myc (M) 12.23 **
F value R x I 9.84 **
F value R x M 4.59 *
F value I x M 8.09 ***
F value R x I x M 6.52 *
C 1103P
C SO4
I 1103P
I SO4
M 1103P
M SO4
M+I 1103P
M+I SO4

Fig. 3 Expression changes of ABA-related genes and metabolite quantification in both root and leaf tissues. **a** ABA content in roots. **b** *VvNCED3* in leaf. **c** *VvABA8OH1* in leaf. **d** *VvNCED3* in root. **E** *VvBG1* in root. **f** *VvGT* in leaf. **g** *VvBG1* in leaf. **h** *VvGT* in root. **I** *VvABA8OH1* in root. All data are expressed as mean • } SD ($n = 3$). ns, *, ** and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R × I × M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C, control plants; I, inducer-treated plants; M, AMF mixed inoculum treated plants; M + I AMF mixed inoculum + inducer-treated plants for 1103P and SO4 selected rootstocks.

Defence

Stilbenes are the main defense-related metabolites synthesized in grapevine. In this study *trans*-resveratrol and viniferin levels were measured in leaves among the several conditions tested (Fig. 4, Table S2). Particularly, resveratrol was only affected by the MxI interaction, showing in parallel significantly higher levels in I and M plants, independently from genotype, with respect to M+I and C plants (Fig. 4a). Viniferin, which was not detectable in C plants, was affected by the M x I interaction and by the I factor alone. I, M and M+I treated plants presented in fact significantly higher values of viniferin than C plants in both rootstocks (Fig. 4b). To correlate biochemical data with molecular responses, expression levels of genes coding for two stilbene synthases (*VvSTS1* and *VvSTS48*) were assessed. Results showed that in both rootstocks *VvSTS1* was upregulated mainly in M 1103P whereas in SO4 plants was observed an upregulation in both I and M with respect to the other treatments (Fig. 4c). *VvSTS48* expression was influenced by all the factors and their interactions, with the highest expression value in leaves of I-treated SO4 plants (Fig. 4d). Looking independently at each rootstock, in 1103P only I and M induced significant overexpression of *VvSTS48* while in SO4 plants all the treatments showed enhanced gene expression compared to their controls (Fig. 4d). RT-qPCR was also applied to detect the expression levels of several target genes as markers of diverse defense response pathways (Fig. S1, Table S2). Two genes were studied both in leaves and roots (a sugar transporter, *VvSPT13* and a class III chitinases, *VvChitIII*), three genes only in leaves (a callose synthase, *VvCAS2*; a lipoxygenase *VvLOX,* and the Enhanced Disease Susceptibility 1, *VvEDS1*) (Fig. S1a-g). Expression of all the considered genes were influenced by I factor, while influence by M was more variable, suggesting a different impact of the treatments on plant metabolism. Among these genes, *VvSTP13*, encoding a sugar transporter, in leaves of both rootstocks was significantly upregulated in all treatments with respect to their C plants (Fig. S1a) while in root only M-treated plants showed significantly higher expression values (Fig. S1). *VvChitIII* showed a different pattern in leaves and roots. In leaves, *VvChitIII* transcript was significantly induced in M- and M+I-treated plants (Fig. S1c) while in roots an upregulation was observed only in M-treated ones (Fig. S1d).

VvCAS2, coding for a callose synthase (Santi *et al*. 2013), showed a downregulation in all the treatments, while *VvLOX* gene, encoding a lipoxygenase involved in the jasmonic acid biosynthesis, was upregulated in all the treatments: among them, the lowest value was observed in M SO4 plants (similar to the C 1103P leaves), suggesting a different response to symbioses in the two genotypes (Fig. S1e-f). *VvEDS1*, selected as marker of Systemic Acquired Responses (SAR) mediated by Salicylic Acid (SA), was influenced by I and M, showing an upregulation trend in I-treated leaves. Conversely, this gene was downregulated in M-treated plants (Fig. S1g).

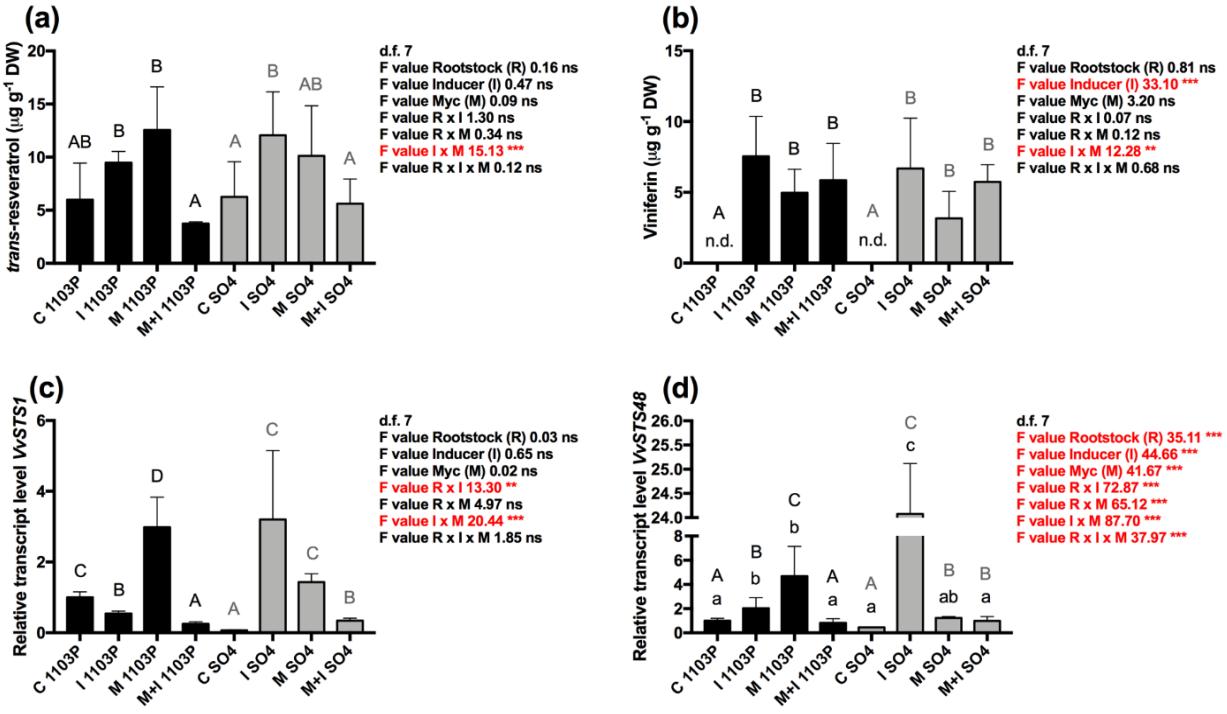


Fig. 4 Expression changes of stilbenes-related genes and metabolites quantification in leaf tissues. **a** *trans*-resveratrol quantification. **b** Viniferin quantification. **c** *VvSTS1* gene expression changes. **d** *VvSTS48* gene expression changes. All data are expressed as mean • } SD ($n = 3$). ns, *, ** and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R × I × M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C, control plants; I, inducer-treated plants; M, AMF mixed inoculum treated plants; M + I AMF mixed inoculum + inducer-treated plants for 1103P and SO4 selected rootstocks.

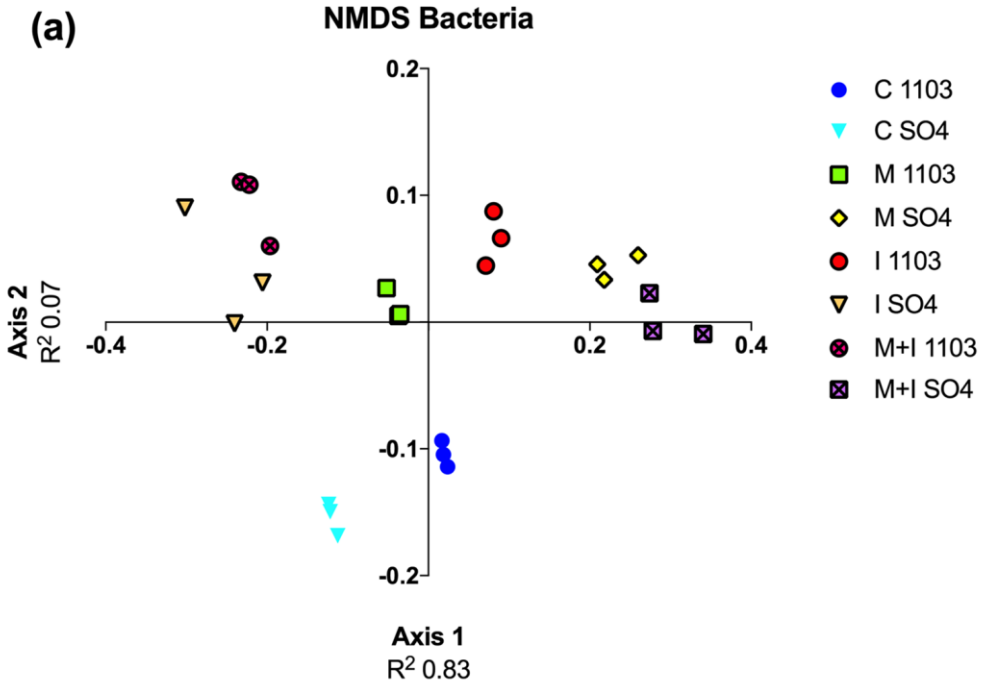
Rhizoplane metaphylogenomic analyses

Bacterial community was analyzed at both order and genus level: the number of retained sequences after chimera removal and taxonomical assignment was always above 35,000 (detailed results of sequencing are reported in Table S3). Shannon index diversity indicated that the only significant difference was observed for the I SO4 samples which show higher index values (Table S4). No significant differences were observed among samples comparing the Shannon index on the fungal community (Table S5). Similar to Shannon index, non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity matrixes showed that the bacterial community (Fig. 5a) is more affected by treatments than the fungal one (Fig. S2).

The bacteria community composition for each sample type at order and genus levels are reported in Table S6. Statistical results of pairwise comparisons among genera are reported in Table S7. To simplify, results are described for the orders and genera that represent at least the 1% of the bacterial community (Fig. 5b). Comparison of the bacterial community between the two rootstocks (1103P *vs* SO4) revealed that 1103P has a significant higher relative abundance of *Pseudomonas* species whereas SO4 has a significant higher relative abundance of *Bacillus* species. Among the bacterial genera, which display significant differences among the treatments, M 1103P vines stimulated the presence of *Bacillus* species but repressed the interaction with *Pseudomonas* ones. In parallel, when comparing treatments on SO4 rootstock, a positive interaction between the mycorrhizal inoculation and the *Pseudomonas* abundance was observed, whereas the inducer treatment showed a negative impact on *Flavobacterium* abundance.

The fungal community composition for each sample type at order and genus levels are reported in Table S6. Statistics of the pairwise comparisons among genera are reported in Table S8. Results for the fungal orders and genera that represent at least the 1% of the fungal community are reported in Fig. S3. Focusing on AMF, results confirm the presence of *Rhizophagus* and *Funneliformis* in inoculated plants. However, AMF were detected also in the I-treated plants (Fig. 6a). Despite the presence of AMF associated to these roots, gene expression analysis on fungal PT genes showed the presence of *RiPT* and *FmPT* transcripts only in M-inoculated plants. Surprisingly, absent or low expression levels were detected in I-treated plants (Fig. 6b, c; Table S2). Indeed, fungal *PT* genes were expressed in a different way in the two genotypes, suggesting a different symbiosis efficiency of the two rootstocks. This finding was further confirmed by a plant PT gene (*VvPT1-3*), which expression level was mainly affected by R and M factors, and by 'R x I' interaction. It was up-regulated in 1103P roots, independently by treatment, with respect to C 1103P and strongly up-regulated in M SO4 ones (Fig. 6d, Table S2). Comparing the fungal composition in C, 24 genera with

significant differences of relative abundance were observed. Among the analysed genera, *Clonostachys* displayed a significant negative correlation with all the treatment in both rootstock genotypes. Focusing on significant genera, usually involved in pathogenic interaction, such as *Fusarium*, *Rhizoctonia* and *Ilyonectria* (Fig. S4), the concomitant use of mycorrhizal inocula with the inducer brought to a significant reduction of *Ilyonectria* in both rootstocks. Conversely, *Fusarium* abundance was stimulated in all treatments except for the inoculation with AMF in the 1103P rootstock. Finally, *Rhizoctonia* genus was positively influenced by the inducer, but only in the SO4 rootstock.


(a)
NMDS Bacteria
0.2
0.1
-0.1
-0.2
-0.4
-0.2
0.2
0.4
Axis 2
R2 0.07
Axis 1
R2 0.83
C 1103
C SO4
M 1103
M SO4
I 1103
I SO4
M+I 1103
M+I SO4

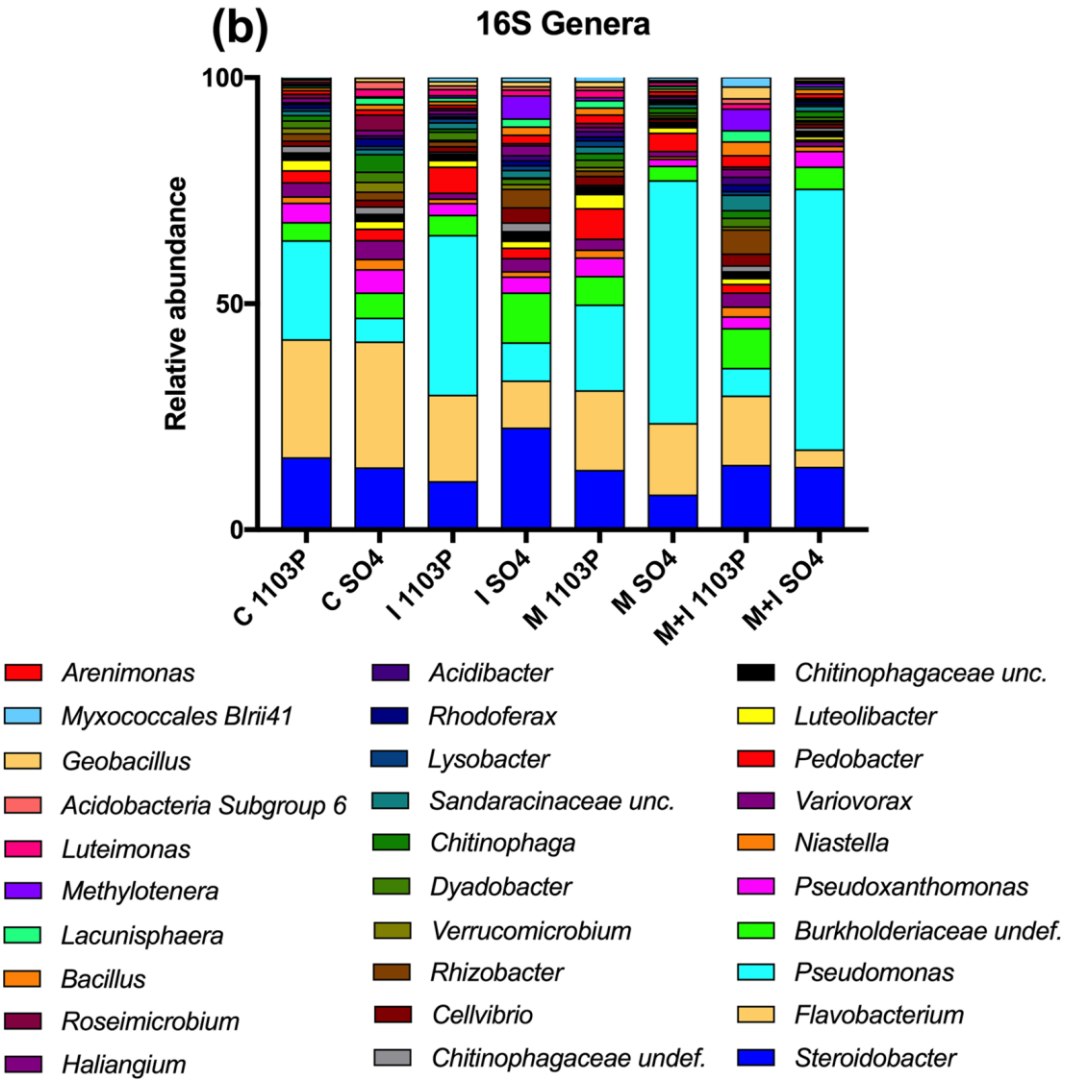

(b)
16S Genera
100
50
0
Relative abundance
C 1103P
C SO4
I 1103P
I SO4
M 1103P
M SO4
M+I 1103P
M+I SO4
Arenimonas
Myxococcales Blrii41
Geobacillus
Acidobacteria Subgroup 6
Luteimonas
Methylotenera
Lacunisphaera
Bacillus
Roseimicrobium
Haliangium
Acidibacter
Rhodoferax
Lysobacter
Sandaracinaceae unc.
Chitinophaga
Dyadobacter
Verrucomicrobium
Rhizobacter
Cellvibrio
Chitinophagaceae undef.
Chitinophagaceae unc.
Luteolibacter
Pedobacter
Variovorax
Niastella
Pseudoxanthomonas
Burkholderiaceae undef.
Pseudomonas
Flavobacterium
Steroidobacter

Fig. 5 Distinct root-associated bacteria community composition among treatments. NMDS algorithm based on Bray–Curtis distances matrixes was used to reduce into a bi-dimensional scaling data obtained for bacteria community (**a**). Relative abundance of bacterial genera (**b**) among treatments. Only genera representing at least the 1% over the total number of classified amplicons were retained (n = 3). C, control plants; I, inducer-treated plants; M, AMF mixed inoculum-treated plants; M + I AMF mixed inoculum + inducer treated plants for 1103P and SO4 selected rootstocks.

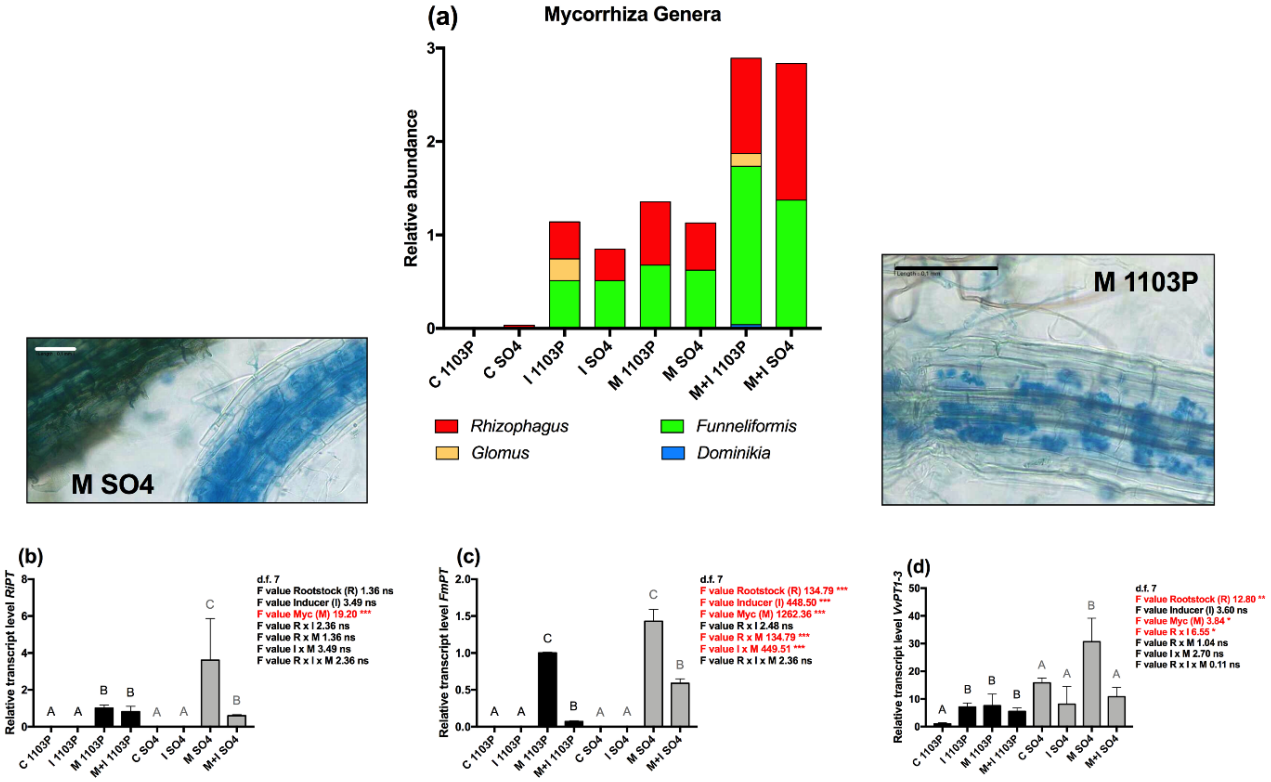


Fig. 6 Mycorrhiza genera and expression changes of plant and fungus phosphate transporter (PT) genes as markers of functional symbioses. **a** Relative abundances of mycorrhiza genera (n = 3). **b** *RiPT*. **C** *FmPT*. **d** *VvPT1-3*. Gene expression data are expressed as mean • } SD (n = 3). ns, *, ** and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R × I × M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C, control plants; I, inducer-treated plants; M, AMF mixed inoculum treated plants; M + I AMF mixed inoculum + Inducer-treated plants for 1103P and SO4 selected rootstocks. Insets: microscope images of typical AM symbioses structures in 1103P and SO4 M-colonized roots.

Discussion

Treatments and genotypes differently shape the root-associated bacterial and fungal communities

The importance of root-associated microbes was extensively demonstrated in several crops including grapevine, with the potential to exploit biocontrol strategies that rely on the beneficial traits of plant growth-promoting microorganisms (PGPBs) naturally associated with plants (Verbon and Liberman 2016; Marasco *et al*. 2018; Yu *et al*. 2019). Among them, AMF and their impacts on diverse plant species, including economically important crops, have been largely studied highlighting the importance of this relationship that can positively affect both growth and defense traits (Jacott *et al*. 2017). However, despite these advantages, grapevine breeders normally focus their attention more on phenotypic or metabolic peculiarities rather than on the improvement of the interactions with root-associated microbes (Marín *et al*. 2021).

Grapevine roots are commonly colonized by different AMF taxa depending on the considered environment, season and soil management making them relevant in defining the 'microbial terroir' of a specific grape cultivar (Massa *et al*. 2020). Svenningsen *et al*. (2018) reported that AMF ecosystem services might be suppressed by some bacterial groups belonging to Acidobacteria, Actinobacteria, Firmicutes, Chitinophagaceae, and Proteobacteria. Our results showed an inverse correlation between the presence of some of these bacteria (i.e. Acidobacteria, genus *Vicinamibacter* and Actinobacteria genus *Gaiella*) and AMF 'functionality', although ITS sequencing showed a similar level in terms of AMF abundance. It is also necessary to consider that, ITS was used in the present work as universal fungal marker (Schoch *et al*. 2012; Lindahl *et al*. 2013) to better define the overall fungal population despite ribosomal large subunit (LSU) region consistently shows greater utility for taxonomic resolution for AMF (Xue *et al*. 2019a). Despite the latter approach can give better results, it has rarely been used in environmental studies of AMF because of sequencing and bioinformatics challenges (Delavaux *et al*. 2021). Similarly, for a better description of the AMF population, it was recently reported that, the use of AMF specific primers, coupled to nested PCR, can greatly help in better define the AMF population (Suzuki *et al*. 2020). Additionally, results obtained from the microbiome analysis confirm that the response of microbial communities to the different treatments are genotype dependent (Marasco *et al*. 2018). This is particularly clear for the bacterial community, where the addition of the mycorrhizal inoculum promoted the *Pseudomonas* genus in 1103P and the *Bacillus* genus in SO4. It is important to remind that both these genera were largely investigated in grapevine because of their ability to protect vine plants against several fungal pathogens. *Pseudomonas* genus was studied for its ability to impair *Botrytis*, *Neofusiccocum*,

Ilyonectria, *Aspergillus*, *Phaeomoniella* and *Phaeoacremonium* genera, which are all well-known grape fungal pathogens (Andreolli *et al*. 2019; Niem *et al*. 2020). On the other hand, *Bacillus* species were studied for their ability to reduce the impact of black foot disease (mainly due to infection by *Cylindrocarpon* and *Ilyonectria* species) and downy mildew on grapes (Zhang *et al*. 2017; Russi *et al*. 2020). These studies well fit with our data where we observed the lower *Ilyonectria* abundance in M+I 1103P and concomitantly the higher abundance of *Bacillus* species. Looking at the fungi, all the treatments promoted the presence of different AMF species, suggesting the recruitment of native AM fungal communities by the I-treated roots, independently from the rootstock genotypes. In detail, it is worth noting a higher diversity in AMF colonization in I 1103P with respect to I SO4 plants, independently from the presence of the AMF inoculum, confirming a diverse recruitment pattern for the two genotypes. Interestingly, *Clonostachys* genus negatively correlated with all the treatments. This genus was extensively studied for its promising exploitation as biological control agents against soil and root pathogens (Nygren *et al*. 2018; Sun *et al*. 2020). Considering that in all treatments the *Rhizophagus* genus was more abundant than in C, we can confirm that a mutual exclusion between *Clonostachys* and *Rhizophagus* genera is present. Although a full explanation for this reciprocally inhibitory interaction is still missing, the complex microbial community modulation mediated by the AMF could impair the ability of *Clonostachys* to endophytically colonize the host plant (Ravnskov *et al*. 2006; Akyol *et al*. 2018; Xue *et al*. 2019b). These findings, in accordance with the increase in defense-related metabolites and the expression data on defense-related genes, well fit with the concept of mycorrhizal-induce resistance (MIR) (Cameron *et al*. 2013) as a cumulative effect of direct and indirect (i.e. mediated by mycorrhizosphere associated microorganisms) defense responses. Recently, Emmett *et al*. (2021) also demonstrated that a conserved community is associated to AMF extraradical hyphae, suggesting an influence on the plant-fungal symbiosis.

AMF and root-associated microbes balance rootstocks growth traits showing a different pattern of functional symbioses

The impact of the different treatments on two different rootstock genotypes was evaluated. The selected rootstocks (i.e. 1103P and SO4) were well characterized at both agronomic and molecular level (Chitarra *et al*. 2017), showing opposite growth and defense attitudes. Among rootstock features, fine root development and density, imparting vigor to the scion, varied considerably with an impact on water and nutrient uptake as well as on the interaction with soil microorganisms. AM

colonization showed that SO4 consistently presented higher levels of root colonization, together with Kober 5BB and Ruggieri 140, with respect to the others (Chitarra *et al*. 2017). This is in agreement with previous works (Bavaresco and Fogher 1996; Bavaresco *et al*. 2000), who showed a variation in the range of AM-colonized grape rootstocks among genotypes, which could be considered the main factor driving AM recruitment. However, functional symbiosis was strongly influenced also by scion requirements, soil fertility and soil pH (Bavaresco and Fogher 1996; Bavaresco *et al*. 2000). Here, both rooting and growth parameters, and partially the CCI, clearly showed a compensation effect in the less vigorous SO4 with respect to 1103P, reaching similar values in all the treatments. A role could be attributed to AMF particularly in SO4. To attest this hypothesis, considering that high-affinity PTs in AM have been characterized and it has clearly been demonstrated that plants possess a symbiotic Pi uptake pathway (Berruti *et al*. 2016), AM fungal PT genes (*RiPT* and *FmPT*) have been tested showing a highly expression in M SO4 for both, and also in M+I SO4 for *FmPT*. Similarly, the plant gene *VvPT1-3*, homolog of mycorrhiza-inducible inorganic phosphate transporters such as *LePT4* and *OsPT11* (Balestrini *et al*. 2017), was significantly up-regulated in M SO4. The positive effects exerted by AM symbiosis in growth and physiological features were largely documented in several plants (*e.g.*, Chitarra *et al*. 2016; Balestrini *et al*. 2020). Surprisingly, although the ITS sequencing showed a certain abundance of AM genera in both I and M+I, the inducer seemed to lower the expression of plant and fungal genes generally involved in symbiosis functioning. This should be related to presence of bacteria reported to diminish AMF functionality (Svenningsen *et al*. 2018). As well, an impact of the inducer on the number of fine roots, which are those colonized by AMF, cannot be excluded also considering that IAA was not detectable in I samples. Looking at the whole microbial community, in addition to a selection based on the rootstock genotype, it is worth noting that I treatment (particularly I SO4) was able to significantly increase diversity of the microbiota (Table S4). Samples treated with the inducer showed higher bacterial diversity hosting many groups of PGPBs such as *Burkholderiaceae* that might be linked to potassium (K) and phosphorous (P) solubilization and availability (Gu *et al*. 2020); *Pseudomonas* and *Bacillus* spp. able to produce siderophores, auxin, cytokinins and characterized as phosphate-solubilizing bacteria (Saad *et al*. 2020; Subrahmanyam *et al*. 2020) (Table S7). These findings could explain the bacteria-mediated growth effects in I treatments particularly for the SO4 genotype. By contrast, the whole fungal diversity was not significantly affected among the treatments.

Nitrogen (N) is an essential element for all grapevine processes and N transporters were found among the genes upregulated by both a single AMF and a mixed bacterial-fungal inoculum through transcriptomics in grapevine roots (Balestrini *et al*. 2017). However, although AMF may positively influence plant N compound uptake and transport (Balestrini *et al*. 2020), negative, neutral or positive

AMF effects on N nutrition has been reported (Bücking and Kafle 2015). Due to the fact that several nitrate transporters were found to be regulated by an AMF inoculum (Balestrini *et al*. 2017), the attention was mainly focused on nitrate uptake. Lower values of nitrate uptake with respect to controls were observed among all treatments, independently from the considered genotypes. Furthermore, any relevant effect on N accumulation in leaves was observed, suggesting that a positive correlation between N content and growth is not relevant in our system or likely due to a biomass dilution effect since the higher growth index recorded particularly in SO4-treated plants. AMF have been reported to show NH_4^+ preference to be assimilated in extraradical mycelium and translocated to plant roots after completion of the GS-GOGAT cycle (Balestrini *et al*. 2020). In this respect, to the plants side the lower NNU observed in M inoculated plants suggest a role of AMF in regulating root N uptake strategies helping plants in acquire N. The plant hormone ABA is a chemical signal involved in the plant response to various abiotic environmental factors, but it can also play a role in interactions with phytopathogens by modulating tissue colonization depending on microorganism type, site and time of infection (Ton *et al*. 2009). An impact of ABA on AMF colonization has been also reported at diverse colonization stages (Bedini *et al*. 2018). A role for ABA in the mechanisms by which AM symbiosis influences stomata conductance under drought stress was also suggested (Chitarra *et al*. 2016). Here, ABA levels were affected by both the genotype and the AMF inoculum. A significant effect of the M treatment was found on the expression of a key gene involved in the ABA synthesis in leaves *(VvNCED3*), showing a positive correlation with the ABA levels in roots. Our result is in accordance with the fact that ABA produced in leaves is then translocated in roots where it might act as a signal to promote root growth (McAdam *et al*. 2016). AMF presence led to higher ABA content in M SO4 roots, despite the fact that generally SO4 rootstock was reported to have a low endogenous content (Chitarra *et al*. 2017), suggesting a potential enhanced tolerance to abiotic stresses in M SO4. As already reported by (Ferrero *et al*. 2018), the relationship between biosynthetic and catabolic processes may be complex and diverse in the different plant organs. Our results showed a different expression pattern of most of the considered genes involved in ABA synthesis and catabolism in leaves and roots. A gene coding for an ABA 8'-hydroxylase (*VvABA8OH1*), belonging to the CYP707A gene family and with a primary role in ABA catabolism, showed an opposite trend in M and I root apparatus, in agreement with the ABA root accumulation.

Overall, obtained data are in accordance with that reported by Martín-Rodríguez *et al*. (2016) showing that both ABA biosynthesis and catabolism are finely tuned in AM-colonized roots. Although with the activation of different mechanisms depending on the treatment, an impact on ABA homeostasis can be suggested particularly in SO4 genotype.

AM symbiosis triggers defence-related transcripts and metabolites more in 1103P than in SO4 rootstock

Plants finely tune the immune system to control both pathogen infection and beneficial microorganism accommodation. Soil bacteria and fungi play a double role in promoting growth and defense response, helping in maintaining the homeostasis in the whole microbial communities associated to the roots through the Induced Systemic Resistance (ISR) pathways (Liu *et al*. 2020). In grapevine, stilbenes are phytoalexins with proved antifungal activities (Chalal *et al*. 2014). Here, resveratrol content was higher in I and M leaves with respect to untreated controls, while viniferin, that is highly toxic for grape foliar pathogens such as downy and powdery mildew (Chitarra *et al*. 2017), has a similar trend in all the treatments while it was not detected in C plants. These patterns clearly highlight a stimulating effect mediated by root-associated microbes (native or inoculated), with differences that might be related to the diverse microbiome composition. Among the genes involved in stilbene synthesis, *VvSTS48*, coding for a stilbene synthase reported as induced by downy mildew infection, showed the highest expression value in I SO4 plants, suggesting a different modulation among treatments and genotypes.

Carbohydrate metabolism is also involved in plant defense responses against foliar pathogens (Sanmartín *et al*. 2020). In tomato, AM symbiosis was reported to be involved in *Botrytis cinerea* resistance through the mycorrhiza-induced resistance (MIR) mediated by callose accumulation. A tomato callose synthase gene (*PMR4*) was in fact upregulated by mycorrhization mainly upon biotic infection (Sanmartín *et al*. 2020). In the present study, attention has been focused on the homolog grape gene *VvCAS2*. Conversely to that previously observed, *VvCAS2* showed a downregulation trend in all the treatments with respect to control plants. These findings suggest a primary role in microbe-mediate stimulating of defense responses against biotic factors in grape. Since a correlation between MIR and sugar signaling pathway was reported (Sanmartín *et al*. 2020), the expression of a grapevine sugar transporter gene (*VvSTP13*), homolog to the *Arabidopsis STP13*, involved in intracellular glucose uptake and in *B. cinerea* resistance, was followed in leaves and roots. Although total soluble carbohydrates were not affected by treatments in leaves, *VvSTP13* expression showed an upregulation trend in all the treatments, particularly in both I sample and M 1103P leaves, suggesting an effect of AMF inoculum in the susceptible genotype.

Looking at the roots, *VvSTP13* upregulation trend was observed mainly in mycorrhizal roots, in agreement with the fact that expression of genes from the STP family was revealed in arbuscule-containing cells of *Medicago truncatula* (Hennion *et al*. 2019). The same trend observed for *VvSTP13* was also found for a gene coding for a class III chitinase (*VvChitIII*). Class III chitinases have been

already reported to be markers of functional symbioses (Balestrini *et al*. 2017), being localized in arbuscule-containing cells (Hogekamp *et al*. 2011). Finally, the expression of two target genes (*VvLOX* and *VvEDS1*), respectively involved in ISR mediated by jasmonate and SAR mediated by salicylic acid, although differently modulated by the inducer and AMF, confirmed the role of the whole microbiome on the plant immunity system in the scion of both rootstock genotypes (Cameron *et al*. 2013).

Conclusion

Overall, our results allowed to provide new insights into growth-defense tradeoffs responses in a model fruit crop (Fig. 7). Although molecular mechanisms at the basis of plant priming are still matter of debate, several hypotheses have been proposed. In this study, a finely tune regulation of growth and defence traits have been highlighted considering three main influencing factors, *i.e.,* the plant genotype, an AM inoculum and an oligosaccharide described as involved in AMF colonization induction. The attention has been focused on two rootstocks characterised by opposite trade-offs. Growth traits have been improved mainly in the low vigour genotype (SO4) by all the treatments probably through the activation of diverse pathways by the root associated microbes. It is worth noting that all the treatments shaped the microbial communities associated to the roots in both the genotypes. Looking at the defence response, a positive impact on immunity system has been revealed both by the AMF inoculum and the oligosaccharide, although with the activation of different pathways. Results suggest that AM symbiosis triggers a mycorrhiza-induced resistance (MIR) also in a model woody plant such as grapevine.

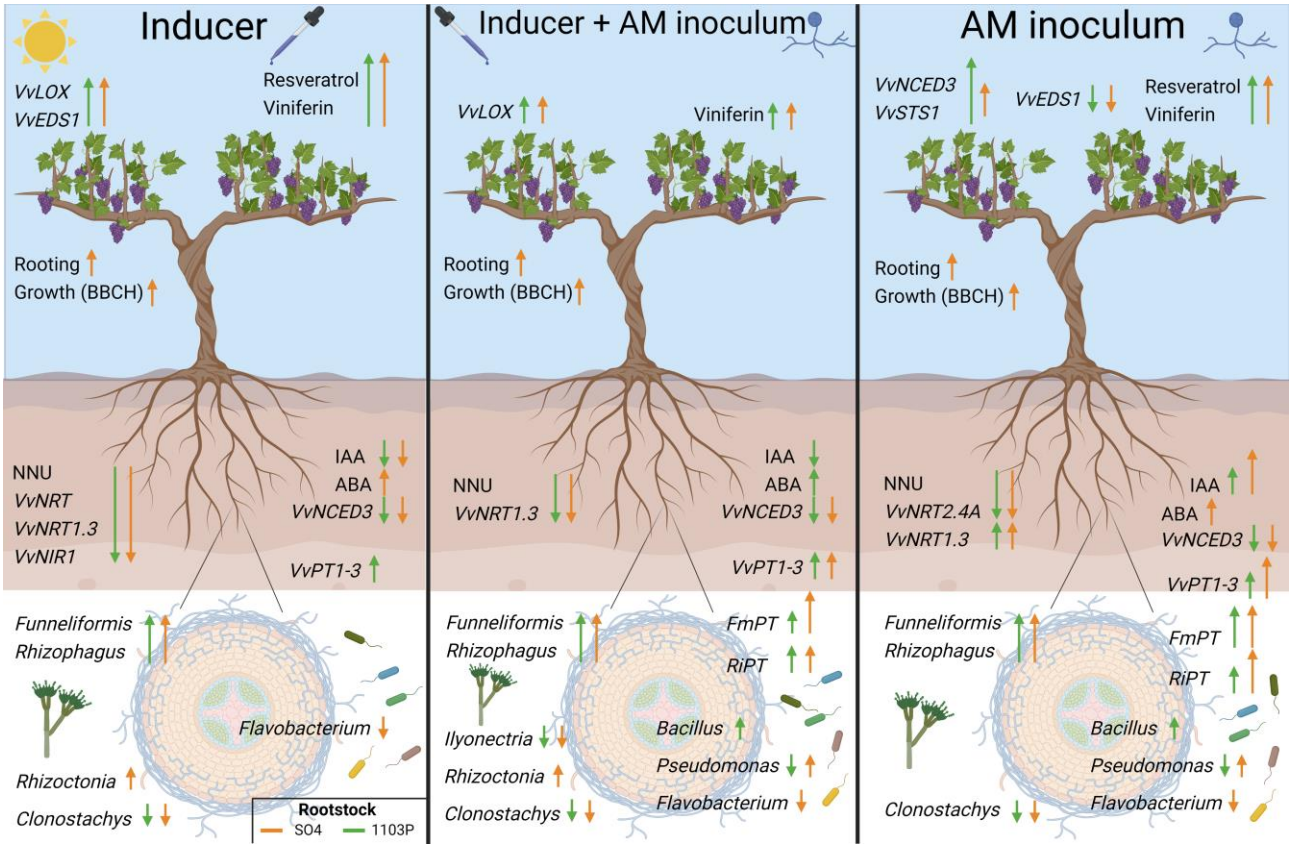


Fig. 7 Overview of phenotypic, biochemical and molecular changes induced by the treatments. Green arrows indicate responses in 1103 Paulsen (1103P) rootstock whereas orange ones are referred to SO4 genotype. Upward arrows indicate an increase whereas downward arrows represent a decrease in content of metabolites or gene relative expression or relative abundance of microbial taxa with respect to control (C) plants. NNU, net nitrate uptake; ABA, abscisic acid.

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Author contribution WC, RB and LN designed the experimental system. LN, GQ, GG, LM, NB, LL, RP, MGV, MS, FG, RB and WC conducted the wet lab experiments and performed data elaboration. LN, GQ, RB and WC performed RT-qPCR analyses. LN, GG and WC performed the microbiome data analysis of root endophytes. LN, RB and WC wrote the first draft of the manuscript. All the authors carefully revised the final version.

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Data availability Sequences were deposited in NCBI database under the BioProject PRJNA718015, BioSamples SAMN18520793 to SAMN18520808 and SRR14089924 to SRR14089939.

Declarations

Conflict of interest The authors declare no competing interests.

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CHAPTER 4 | THE HIDDEN WORLD WITHIN PLANTS: METATRANSCRIPTOMICS UNVEILS THE COMPLEXITY OF WOOD MICROBIOMES

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Abstract

The importance of plants as complex entities influenced by genomes of the associated microorganisms is now seen as a new source of variability for a more sustainable agriculture, also in the light of ongoing climate change. For this reason, we investigated through metatranscriptomics whether the taxa profile and behaviour of microbial communities associated to the wood of 20-year-old grapevine plants are influenced by the health status of the host. We report for the first time a metatranscriptome from a complex tissue in a real environment, highlighting that this approach is able to define the microbial community better than referenced transcriptomic approaches. In parallel, the use of total RNA enabled the identification of bacterial taxa in healthy samples that, once isolated from the original wood tissue, displayed potential biocontrol activities against a wood degrading fungal taxon. Furthermore, we unveiled an unprecedented high number of new viral entities (about 120 new viral species among 180 identified) associated to a single and limited environment and with potential impact on the whole holobiont. Taken together, our results suggest a complex multitrophic interaction in which also the viral community plays a crucial role in raising new ecological questions for the exploitation of microbial assisted sustainable agriculture.

Highlights

We adopted for the first time a multidisciplinary approach to describe the whole microbial community associated to the wood tissue of plants, identifying the major players associated to a “healthy” wood tissue or to a necrotic one, including an unprecedented wide viral community.

Keywords

bacterial endophytes, holobiont, microbial ecology, metatranscriptome, virome, *Vitis vinifera*

Introduction

In nature, plants live in a microbe-rich environment and must interact with a wide spectrum of pathogenic, commensal and beneficial microorganisms. How plants recognize the beneficial functions provided by microbes and, at the same time, tackle microbial pathogens has attracted the attention of scientists for many years. In this light, plants are no longer seen as single organisms but rather as an assemblage of species, which leads to the establishment of a complex biomolecular network composed by the host and its associated microbes, termed holobiont (Guerrero *et al.*, 2013; Hassani *et al.*, 2018). More specifically, plant tissues can provide microorganisms with a source of easily available nutrients and ecological niche spaces to microorganisms, while beneficial microbes increase growth and resistance to biotic and abiotic stresses (O'Banion *et al.*, 2020). Each plant microbiome leads to unique interactions at the tissue interface, in turn influencing the overall assemblage and functioning of the microbial community (Shahid, 2020).

Significant advances in sequencing technologies have been made in the last few decades, revolutionizing the conducting of biological experiments, particularly where they concern the study of complex plant microbiomes. However, most of the high throughput sequencing techniques have focused on DNA sequencing using either targeted approaches, like PCR-amplicon sequencing of ITS and 16S rRNA genes (or other marker genes) (Wagner *et al.*, 2016; Nerva *et al.*, 2019*b*) or metagenomics through sequencing of all available DNA from the sample (Kwak *et al.*, 2018). On the contrary, RNA sequencing (RNASeq) allows expressed transcripts within a microbiome to be recorded at a given time-point and under a set of environmental conditions that provide a closer look at the active biomolecular network (Dubey *et al.*, 2020).

The composition and structure of plant microbial communities are influenced by a number of factors, including host genotypes, environment, and interactions within plant microorganisms (Neelakanta and Sultana, 2013; Hardoim *et al.*, 2015; Lebeis, 2015). Although some indications of the microbial community associated to plants have been reported (Cordovez *et al.*, 2019), to date there is a lack of data presenting the microbial community associated to the woody tissues of perennial plants. Specifically, we describe the microbial community in the woody tissue of grapevine, a plant that has important socio-economic impacts (Nerva *et al.*, 2019*b*). Moreover, thanks to the description of its genomes (Jaillon *et al.*, 2007; Velasco *et al.*, 2007; Roach *et al.*, 2018; Minio *et al.*, 2019*a*; Vondras *et al.*, 2019; Zhou *et al.*, 2019), which is also well annotated (Grimplet and Cramer, 2019; Minio *et al.*, 2019*b*), it can be regarded as a model species for woody plants. To better understand how the microbial community can shift its behaviour according to the plant health status, we conducted our experiments using both apparently healthy and esca symptomatic 20-year-old grapevine plants. Esca of grapevine is a complex syndrome that causes extensive degradation of the woody tissue (Bruno

and Sparapano, 2006; Claverie *et al.*, 2020) and which is associated to distinctive foliar symptoms (named tiger-striped leaves) (Del Frari *et al.*, 2021) and, with the worsening of the disease, to the sudden death of plants during the summer (apoplexy) (Mugnai *et al.*, 1999; Fontaine *et al.*, 2016; Magnin-Robert *et al.*, 2017; Fischer and Peighami-Ashnaei, 2019). The causal agents associated to esca are a broad range of plant pathogenic fungi, such as the most frequently associated *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*, and to wood-rot fungi such as *Fomitiporia mediterranea* and *Diplodia seriata* which usually accelerate the wood degradation process (Bruez *et al.*, 2014, 2015, 2021; Del Frari *et al.*, 2021; Pacetti *et al.*, 2021). Apart from the well-known fungal pathogens, a recent study suggested that the whole microbial community could be involved in the syndrome onset (Bruez *et al.*, 2020), but to date no data on the complete microbial community (including plant viruses and viruses of microbes) have been reported.

In more detail, to deepen our knowledge of the wood-microbiota interactions we chose to analyse esca asymptomatic and symptomatic vines using an untargeted approach to detect all the symbionts, including fungi, bacteria, viruses and viroids and to check any possible remodelling among the microbial communities. We tested the hypothesis that the interactions occurring within the plant, studied as a holobiont, involve diverse trophic interactions between microorganisms belonging to different kingdoms that in turn influence the health status of the host plant.

Materials and Methods

Vineyard location and sampling

This study was conducted in the experimental vineyard of the Council for Agricultural Research and Economics - Research Centre for Viticulture and Enology in Spresiano (TV) (under a conventional management schedule, Supplementary Table 1), Veneto Region, Italy (elevation is 56 m a.s.l, with a warm temperate climate as reported in Nerva *et al.*, 2019*d*). Samples were collected from 20-year-old grapevine plants cultivar Glera grafted onto SO4 rootstock and Sylvoz-trained.

The vineyard was monitored visually for 4 years, plant by plant, to determine health status and presence of esca-related symptoms. Taking into consideration what was suggested by (Del Frari *et al.*, 2021) wood tissue was collected from 9 continuously asymptomatic plants (AS) and from 9 plants that displayed clear symptoms (tiger striped leaves and/or apoplexy) at least twice over the monitoring period and were symptomatic (SY) on the collection date (09-16-2017). The sampling method was partially destructive: a sterile corer was used to sample three to four transversal wood fragments from each plant at the crown ramification level from each plant. The wood fragments were collected

individually from each plant then, for total RNA extraction (see next section), three replicates were generated by pooling the wood from three plants (samples from 9 plants for each condition divided into 3 independent samples from 3 separate plants). This operation was undertaken to avoid an excess of individual variability, due to the interaction of the single plant with the surrounding environment, and to focus the attention on shared responses among esca SY or AS plants. Wood tissues were collected in sterile 50 ml conical tubes and immediately frozen at -80 °C, then lyophilized (less than 2 hours after collection). To confirm the wood health status plant were harvested at the end of the season and the wood was visually inspected for necrosis and rots (Nerva *et al.*, 2019*c*).

Total RNA extraction and RNAseq

To proceed with total RNA extraction from the woody tissue we used a combination of manual and commercial kit extraction methods. First, the wood was ground to a very fine powder using sterile mortars and pestles with the aid of liquid nitrogen. The powder obtained from samples of a single plant was then mixed in equal amount (500 mg) with the wood powder obtained from the other two plants (in total 1500 mg per biological replicate). Each biological replicate was used for RNA extraction following the protocol of Gambino *et al.* (2008) with some minor modifications. First 5 mL of extraction buffer (2% CTAB, 2.5% PVP-40, 2 M NaCl, 100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0 and 2% of β-mercaptoethanol) was heated at 65 °C and poured into a 15 mL conical tube together with the wood powder. The samples were mixed for 1 minute on a vortex and then incubated for 10 minutes at 65 °C. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added, vortexed for 1 minute and centrifuged at 12,000g for 10 minutes at 4 °C. The recovered supernatant was subjected to a second extraction with chloroform:isoamyl alcohol. The supernatant was then collected, an equal volume of isopropanol added, incubated on ice for 10 minutes and then centrifuged at 20,000g for 15 minutes. The supernatant was discarded, and the pellet was resuspended in 500 µL of SSTE buffer pre-heated to 65 °C. To clean polysaccharides and proteins, a Spectrum plant total RNA kit (Sigma-Aldrich, Saint Louis, MO, USA) was used following the manufacturer's instructions. RNA quantification and spectrophotometric parameters were tested using a NanoDrop 2000 Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA) and RNA integrity was tested on an agarose gel.

To proceed with RNAseq analysis at least 6 µg per sample was sent to Macrogen Inc. (South Korea) for rRNA depletion (Ribo-Zero™ Gold Kit, Epicentre, Madison, USA), cDNA libraries construction (TrueSeq total RNA sample kit, Illumina) and sequencing by Illumina Novaseq technology with an output of 100M paired-end reads of 100 bp for each sample.

Bioinformatics

Raw sequences were deposited in NCBI under the project PRJNA703377 (SRR13754975-SRR13754976). For all the bioinformatics analyses RNAseq data from three samples for each condition were used. To ensure that contamination from rRNA was below an acceptable threshold (less than 1%) the hidden Markov models (HMMs) (Rivers *et al.*, 2018) were run on the whole dataset. De novo assembly of the sequenced total RNA libraries was performed using only the high quality and cleaned sequences selected using Trimmomatic (Bolger *et al.*, 2014). For assembly operation we used Trinity (version 2.3.2) (Haas *et al.*, 2013). For virus identification blastx (version 2.6.0+) from the BLAST suite was used to search conserved viral protein among the assembled contigs using a custom made viral database (Nerva *et al.*, 2018*b*) as previously described (Nerva *et al.*, 2019*a*). Subsequently, alignments of reads against viral contigs were performed using BWA 0.7.15-r1140(Li and Durbin, 2010) and SAMtools 1.3.1(Li *et al.*, 2009). Coding open reading frames (ORFs) were detected with ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) and then blasted again to NCBI databases against the nr database. To produce the viral heatmap, data were normalized and then processed with GraphPad Prism (www.graphpad.com).

To determine gene expression of grapevine, cleaned reads were aligned against the grape reference coding sequence database (Jaillon *et al.*, 2007) (PN40024 CDS, http://www.grapegenomics.com/) using the built in DESeq2 (Love *et al.*, 2014*b*) R package in the Trinity differential expression analysis pipeline. Transcripts were annotated using annotation V1 of the grapevine genome (Grimplet *et al.*, 2012) and grouped into functional gene classes according to the VitisNet GO. The BiNGO 3.0 plug-in tool in Cytoscape (v3.2, U.S. National Institute of General Medical Sciences (NIGMS), Bethesda, MD, USA) was used for GO enrichment analysis, as described by Maere *et al.* (2005). Over-represented Plant GO slim categories were identified using a hypergeometric test with a significance threshold of 0.05. Differentially expressed genes (DEGs) were identified in a pairwise comparison (SY *vs* AS) using a p-value of 0.05% adjusted with the Benjamin-Hochberg method.

Fungal gene expression analysis started with the trimming and quality filtering of the raw reads using Trimmomatic v0.36 (Bolger *et al.*, 2014) with paired-end mode, phred33, a sliding window of 5:20, and a minimum length of 50 bp. Quality filtered reads were mapped to the reference of grapevine trunk-associated fungi using Bowtie2 (Langmead and Salzberg, 2012) as described by Morales-Cruz *et al.* (2018). This reference was updated with the transcriptome of *Aureobasidium pullulans* EXF-150 v.1.0.

The database of the grapevine trunk-associated fungi was annotated using functional databases of signal peptides, carbohydrate-active enzymes, secondary metabolism biosynthetic clusters, Pfam

domains, P450s, families of cellular transporters, and fungal peroxidases. The version of the databases and parameters used are presented in Supplementary Table 2. These annotations were used to assess the abundance of each mapped transcript. The comparison of the fungal species transcript abundance was executed on the log2 transformed counts using pairwise comparisons (t-test $\alpha = 0.05$) of each species between the asymptomatic and symptomatic samples.

Fungal and bacterial gene expression profiling were performed using R version 3.6.3 (2020-02-29) and DESeq2 (version 1.24.0) package (Love *et al.*, 2014*a*). Heatmaps were obtained using the Pheatmap package (version 1.0.12) (Kolde and Kolde, 2015). Differential expression of fungal genes between symptomatic and asymptomatic samples was tested using an alpha of 0.05. To analyze differential expression in selected strains across the experimental conditions, data were filtered retaining only those counts accounting for *A pullulans*, *F. mediterranea*, *Neofusicoccum parvum*, *P. minimum* and *P. chlamydospora,* the differential expression was then tested using an alpha of 0.05. Principal component analysis was performed using the plotPCA function from the DESeq2 package. The rlog transformed reads counts attributed to *Micromonosporaceae* were then used to build the heatmap.

To further analyse reads which were not assigned to grapevine or to the selected fungal genomes, we used MEGAN (Mitra *et al.*, 2011; Huson and Weber, 2013). Briefly, unassigned reads were de-novo assembled using MEGAHIT v.1.2.5 – beta (Li *et al.*, 2015) then each obtained contig was blasted using DIAMOND aligner (Buchfink *et al.*, 2015). Results tables from DIAMOND were then used in MEGAN for taxonomic analysis using the NCBI taxonomy based on GI and then also functionally classified using the KEGG database. Further data analysis was performed by normalization in DESeq2.

Biological validation of* in-silico *identified viruses

Viral sequences were deposited in NCBI – BankIt under accession MW648427 to MW648545. Since RNAseq can produce chimera sequences, which can lead to the false identification of new viruses, all the viral contigs were checked by reverse-transcription quantitative PCR (RT-qPCR). For each sample, cDNA was synthesized following the manufacturer's instructions provided for the High-Capacity cDNA Reverse Transcription kit (Thermo-Fisher Scientific, Waltham, MA, USA). iTaq universal SYBR Green supermix (Bio-Rad, Hercules, CA, USA) and specific primers were used to amplify viral contigs in a CFX-96 (Bio-Rad) thermal cycler. To further confirm the RNA nature of identified viruses and avoid identification of endogenized viral or viral-derived sequences we performed the RT-qPCR protocol using RNase-treated DNA as template.

RNA samples were also analysed to determine the transcript levels of a selection of genes (Supplementary Table 3), following a previously reported protocol (Chitarra *et al.*, 2017). Ubiquitin and Actin1 were used as internal controls and three independent biological replicates, and three technical replicates were run for each RT-qPCR. For each gene, statistically significant differences between SY and AS were attested by the Student's t-test. Significant differences were highlighted at $p < 0.05$, 0.01 and 0.001.

Phylogenetic analysis of viral sequences and statistics

The conserved part of the RNA dependent RNA polymerase (RdRP) coding ORF of each identified virus in the RNAseq analysis was used for multiple sequence alignments, using MUSCLE (Edgar, 2004) with default parameters, and the phylogenetic inference was carried out using the maximum likelihood methodology in IQ-TREE (Trifinopoulos *et al.*, 2016), using default parameters. Statistical analysis for each clade was performed through bootstrap analysis with 1,000 replicates. Sequences for each of the phylogenetic trees are reported in Supplementary Table 4 (leviviruses, narnaviruses, mitoviruses and botourmiaviruses), Supplementary Table 5 (other +ssRNA), Supplementary Table 6 (dsRNA) and Supplementary Table 7 (-ssRNA).

Elaboration of viral abundance data was performed using PAST (Hammer *et al.*, 2001) to produce PCoA (based on the Bary-Curtis dissimilarity matrix), taxa abundance and hierarchical clustering using paired group (UPGMA) with the Bray-Curtis matrix (1000 bootstrap replicates).

Actinobacteria isolation and identification

Actinomycetes were isolated from the inner wood tissue of the same *V. vinifera* cv Glera plants that didn't show any esca symptoms. Isolation of Actinomycetes was performed as previously suggested (Zhang and Zhang, 2011; Dhanasekaran and Jiang, 2016; Jiang *et al.*, 2016). Briefly, wood tissue was collected using a manual corer at the crown ramification level (one wood fragment from each esca asymptomatic plant). Wood fragments were stored in sterile 50 ml tubes and immediately (less than 2 hours after harvest) processed for Actinomycetes isolation. The central part of each wood fragment was separated from the outer part and cut into small pieces (less than 1 mm thickness), three pieces coming from three different plants were pooled together in order to maximize the isolation of different species.

To isolate the Actinomycetes from the small wood pieces, we added 5 ml of water amended with 0.1% SDS and shook the tube for 30 minutes on a vortex apparatus. The extract was then diluted 1:5

with sterile water and plated on sodium propionate media amended with cycloheximide to inhibit fungal growth (El-Nakeeb and Lechevalier, 1963; Zakharova *et al.*, 2003). Actinomycetes were left to grow for 2 weeks and then transferred on Czapek Yeast Agar (CYA) plates, selecting colonies with different morphologies as dereplication strategy. Small colony fragments were used to extract DNA using a classic phenol – chloroform approach, 16S sequence was amplified using the universal primers (Marchesi *et al.*, 1998) and PCR products were sequenced using the Sanger method (Sanger *et al.*, 1977) (at BioFab Research srl, Italy). All isolates were then stored in the CREA – Research Centre for Viticulture and Enology microbial bank both lyophilized and in glycerol stocks at -80 °C.

Evaluation of antagonistic activity against esca pathogens

To determine the potential antagonistic activity of the isolated Actinobacteria against esca pathogens, a previously characterized virus-free and virulent *P. minimum* isolate coming from esca symptomatic plants was employed (Nerva *et al.*, 2019*c*). The assay was performed as previously suggested in other studies (Solans *et al.*, 2016; Kshetri *et al.*, 2018; Torabi *et al.*, 2019). Briefly, four agar plugs (4 mm diameter) with actively growing mycelium of 7 to 10-day-old Actinobacteria grown on CYA were placed on 9 cm CYA plates, 2 cm from the edge. After 7 days, 5 µl of fungal conidia (10^6 conidia/ml) from growing fungal cultures were placed in the center of each plate, left to grow at 28 °C in the dark and checked for fungal growth after 4, 7, 14 and 21 days. Three biological replicates were measured for each dual culture. Colony growth inhibition expressed as a percentage (PI%) was calculated by using the formula PI% = [(C-T)/C] * 100, where C is the pathogen colony growth in the control, and T is the pathogen colony growth in dual culture with Actinobacteria (Khamna *et al.*, 2009).

Results

Plant genes expression profiling

The wood health status was confirmed as previously reported (Nerva *et al.*, 2019*c*), collecting the plants and visually inspecting the wood integrity by cutting the main trunk with a band saw. Results showed that symptomatic (SY) plants had compromised wood tissue, with wide necrotic and rotted areas. On the contrary, the appearance of the wood from asymptomatic (AS) plants looked much more intact and without obvious necrosis or rots. Total RNA-seq analysis from AS and SY samples produced an average of 66 million reads and 40 million reads per sample that were aligned to the PN40024 reference genome with a mapping rate of 87±5% and 65±10% for AS and SY, respectively. Distribution of reads mapping among plant, fungal, bacterial and viral communities is reported in Supplementary Table 8. Of the 29,970 annotated genes, 3,396 were significantly differentially expressed in SY and AS, and we adopted a fold-change (FC) cut-off to analyse only the genes whose expression was $|\log2FC| \geq 1$, thus obtaining 2,095 DEGs (Supplementary Table 2). Interestingly, 88% of these DEGs (1,849) were overexpressed in SY (Figure 1a) and the Gene Ontology (GO) enrichment analysis conducted on these transcripts indicated that response to stress, to abiotic and biotic stimuli, carbohydrate and secondary metabolisms were the overrepresented functional categories (Figure 1b). A selection of more interesting genes up-regulated in SY was analysed by RT-qPCR (Figure 1c, Supplementary Table 3), confirming the RNA-seq data (Supplementary Figure 1).

Several genes, markers of defence responses to pathogens, such as *VvPR-1*, thaumatin-like protein (*VvTHAU*), β-1-3 glucanase (*Vvβgluc*), disease resistance protein, R protein and WRKY transcription factors were strongly overexpressed in SY samples. In particular, *VvWRKY18* enhances defence responses in the plant by positively regulating the transcriptions of stilbene phytoalexins and PR genes in grapevine and its interaction with *VvNPR1* promotes the activation of systemic acquired resistance defence (Wang *et al.*, 2021). Interestingly, several wound-induced genes were overexpressed in SY samples; these genes produce small wound-induced polypeptides involved in the response to pathogen by promoting pattern-triggered immunity (Yu *et al.*, 2018). The transcriptional activation in SY samples of genes involved in H_2O_2 scavenging such as peroxidases, catalases (*VvCAT* and *VvPOX*), and glutathione S-transferases (Supplementary Table 2), a large family of proteins that prevent oxidative stress, suggest an accumulation of reactive oxygen species (ROS) in infected wood. The up-regulation in SY samples of 38 laccases (*VvLac*) is noteworthy, a group of genes known as positive regulators in lignin polymerisation playing an important role in cell wall lignin biosynthesis (Supplementary Table 2, Figure 1c).

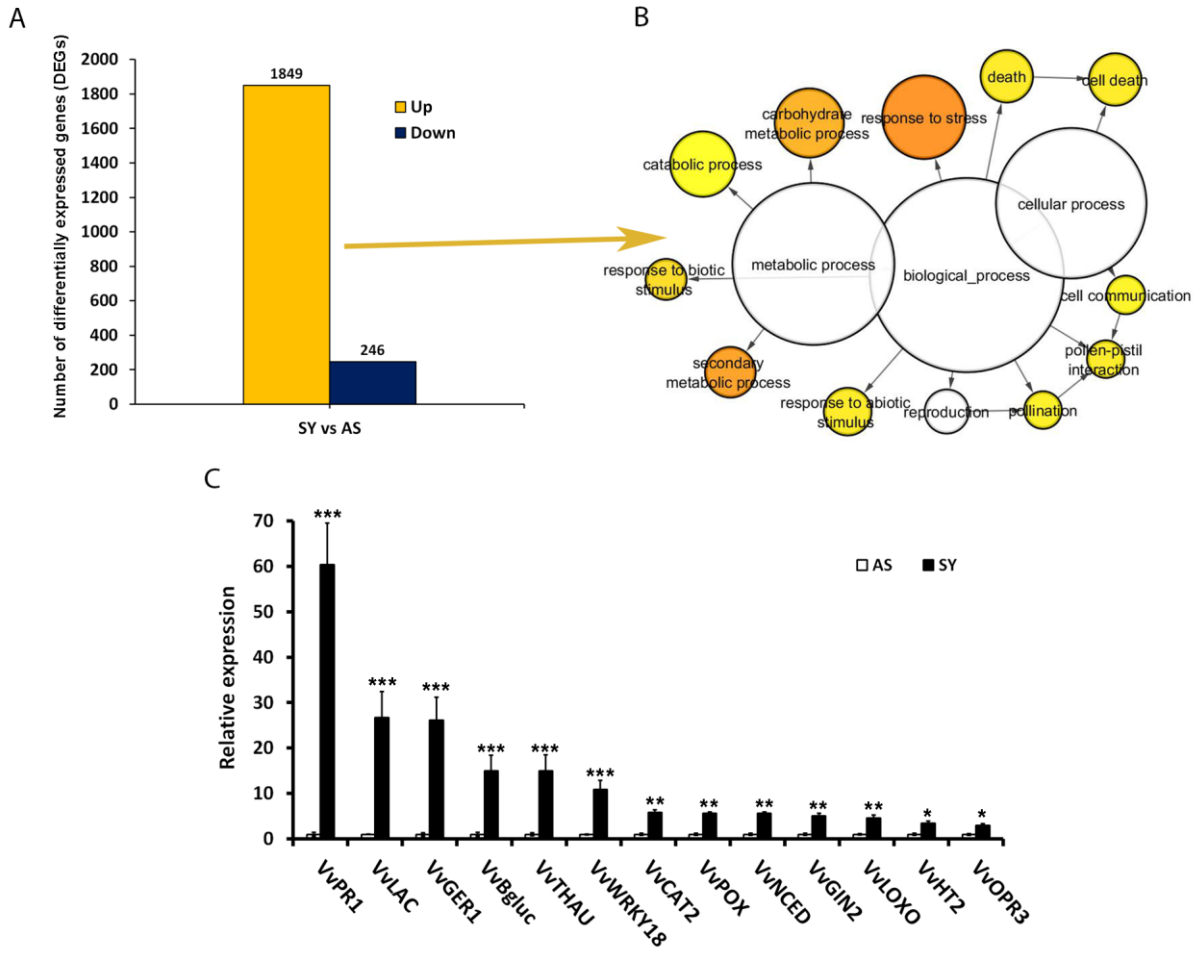

Fig. 1. Transcriptome analysis of wood collected from grapevine showing esca symptoms (SY) and asymptomatic (AS) wood. (A) Number of differentially expressed genes (DEGs), up- (UP) or down- (DOWN) regulated, in the comparison SY versus AS. (B) Significantly enriched GO biological functional categories identified for DEGs belonging to up-regulated genes in SY using Cytoscape with the BINGO plug-in according to enrichment *P*-value <0.05. (C) Results of candidate gene expression analysis performed by RT–qPCR assay. Ubiquitin and actin genes were used as endogenous controls for the normalization of transcript levels. Three independent biological replicates with three technical replicates each were used for analysis. For each gene (listed in Supplementary Table S2), statistically significant differences between SY and AS were determined by the Student's *t*-test (∗$P \leq 0.05$,∗∗$P \leq 0.01$, and ∗∗∗$P \leq 0.001$).

In addition to laccases, the metabolism of cell wall was in general altered in infected tissues, with the overexpression of several genes codifying for cellulose synthases and xyloglucan endotransglucosylase/hydrolases.

The carbohydrate transport and metabolism (e.g., sucrose synthase, hexose transporter *VvHT2*, vacuolar invertase *VvGIN2*), as well as hormonal metabolism were modulated in infected tissues. In particular, the hormonal metabolism, with relevant genes for the biosynthesis of abscisic acid, 9-cis-epoxycarotenoid dioxygenase (*VvNCED*), jasmonic acid, 12-oxophytodienoate reductase 2-like (*VvOPR*), auxins and ethylene, was strongly involved in the grapevine responses to esca disease (Supplementary Table 9, Figure 1c). The overexpression of lipoxygenases involved in hormonal metabolism and in the response to pathogens, as previously reported in grape infected by *Botrytis cinerea* (Podolyan *et al.*, 2010), was another interesting response of woody tissues to fungi responsible of esca disease. Furthermore, among the genes most overexpressed in SY there are several nitrilases (Supplementary Table S1) encoding enzymes that catalyse the hydrolysis of nitrile compounds to carboxylic acid and ammonia (Howden and Preston, 2009). These genes are involved in the auxin biosynthesis and in the hydrolysis of nitriles produced during the metabolism of cyanogenic glycosides and glucosinolates. Nitrilases are induced upon pathogen attack and involved in defence response (Choi *et al.*, 2016).

To summarize, the plant gene expression profiling revealed a general overexpression in the SY samples, with a significant number of genes involved in biotic and abiotic stress responses and in specific an overexpression of genes involved in the remodelling of the cell wall and of genes involved in the ROS scavenging pathway.

Fungal gene expression profiling using the cross-referenced approach

To profile the fungal behaviour and the virulence function expression in the two sample types we selected 11 fungal taxa that represent the major wood fungal pathogens in grapevine, and mapped the reads not belonging to the plant on their transcriptome assemblies. The average reads mapped on the 11 genomes in AS samples are 784,000 whereas in SY samples they are 8,272,000. Eight out of 11 taxa showed a significant overrepresentation in SY samples as reported in Table 1 and represented in Supplementary Figure 2. Two of the fungi usually considered the core of esca disease development, namely *P. minimum*, *P. chlamydospora*, and one of the most commonly esca-associated wood-rot fungus *F. mediterranea*, showed significant overexpression in the SY samples. The fungal taxon that displayed the most enhanced increase in abundance is *Neofusicoccum parvum*, which is almost 300 times more abundant in SY samples (Table 1).

Firstly, we focused our attention on the functional analysis of the transcript in order to understand the differences in fungal behaviour between the two sample types. As a first general analysis we produced a comparison of functional categories and single genes considering the 5 fungi all together (Figure 2a). A plot showing the results of dispersion estimation is also reported (Supplementary Figure 3). The results showed that fungal genes encoding for transporters and enzymes involved in the synthesis, metabolism, and transport of carbohydrate (CAZymes) are more represented in the SY samples. Although the overall functional categories showed no significant differences, some peroxidases and cytochrome P450 are overexpressed in SY samples (Figure 2a).

Table 1. T-test comparison of the log2 transcript counts per species between the asymptomatic (AS) and symptomatic (SY) samples.

Organism	**AS**	**SY**	***P*-value**
A. pullulans	9.007988	13.597155	0.00324
Ilyonectria sp.	4.833614	8.247705	0.04077
B. dothidea	8.457124	12.576797	0.14310
D. ampelina	5.357496	11.408915	0.08773
D. seriata	8.203022	13.289514	0.01591
F. mediterranea	18.79482	22.67565	0.02584
N. parvum	8.859677	17.190268	0.00025
P. chlamydospora	16.83924	18.6007	0.04497
S. hirsutum	5.473704	9.597275	0.01921
P. minimum	16.34658	17.46177	0.03129
Eutypa lata	14.07218	15.62605	0.17850

We then decided to focus our attention on the 5 (out of 11) fungal species, with attention to the three above-mentioned esca-related pathogens, to *N. parvum* which displays the wider difference in terms of abundance and *A. pullulans*, a fungal taxon generally considered as a positive symbiont for grapevine plants. Results showed that *P. minimum* presents more genes encoding for CAZymes and secondary metabolism in the AS samples than in SY ones, whereas some transporters are overexpressed in SY samples (Figure 2b). Similarly, *P. chlamydospora* displays overexpression of genes encoding secondary metabolism in the AS samples, whereas some transporters display an overexpression in SY samples (Figure 2c). As expected, *F. mediterranea* displayed overexpression

of the functional category CAZymes and some peroxidases, together with secondary metabolism (Figure 2d). Looking at *N. parvum* we observed a general downregulation of the secondary metabolism in SY samples, in parallel some CAZymes also show a downregulation in SY samples whereas many transporters are upregulated in SY samples (Figure 2e). Finally, *A. pullulans* displayed a general overexpression of genes with significant emphasis on the functional categories of CAZymes and transporters and with a large number of P450 genes (Figure 2f).

A final PCA analysis was produced to display the general behaviour of each fungal taxon (Supplementary Figure 4), which confirmed the possibility of separating the fungal gene expression pattern between AS and SY samples for *A. pullulans*, *F. mediterranea* and *N. parvum*. On the contrary, *P. minimum* and *P. chlamydospora* gene expression patterns are quite similar between the two sample types and cannot be clearly separated.

In summary, we found a general overexpression of genes belonging to the functional categories CAZymes and transporters, which is mostly due to the contribution of *F. mediterranea* and *A. pullulans*. On the contrary, *P. minimum* and *P. chlamydospora*, which represent two of the cores of esca-associated fungi, display a significant overexpression of CAZymes and secondary metabolites genes in the AS samples. More in general, looking at the PCA analysis (Supplementary Figure 4) the wider behavioural shift is observed for *N. parvum*.

Fungal and bacterial reads from the **de-novo** *assembly*

After analysing reads belonging to grapevine and to the most important fungal taxa involved in esca symptoms development or wood deterioration, we decided to focus our attention on the remaining contigs which were not assigned to any of the previous reference genome. These so called "unassigned contigs" were taxonomically assigned and quantified among samples in order to define which fungal (differently from the previously mentioned) and bacterial species are still detectable.

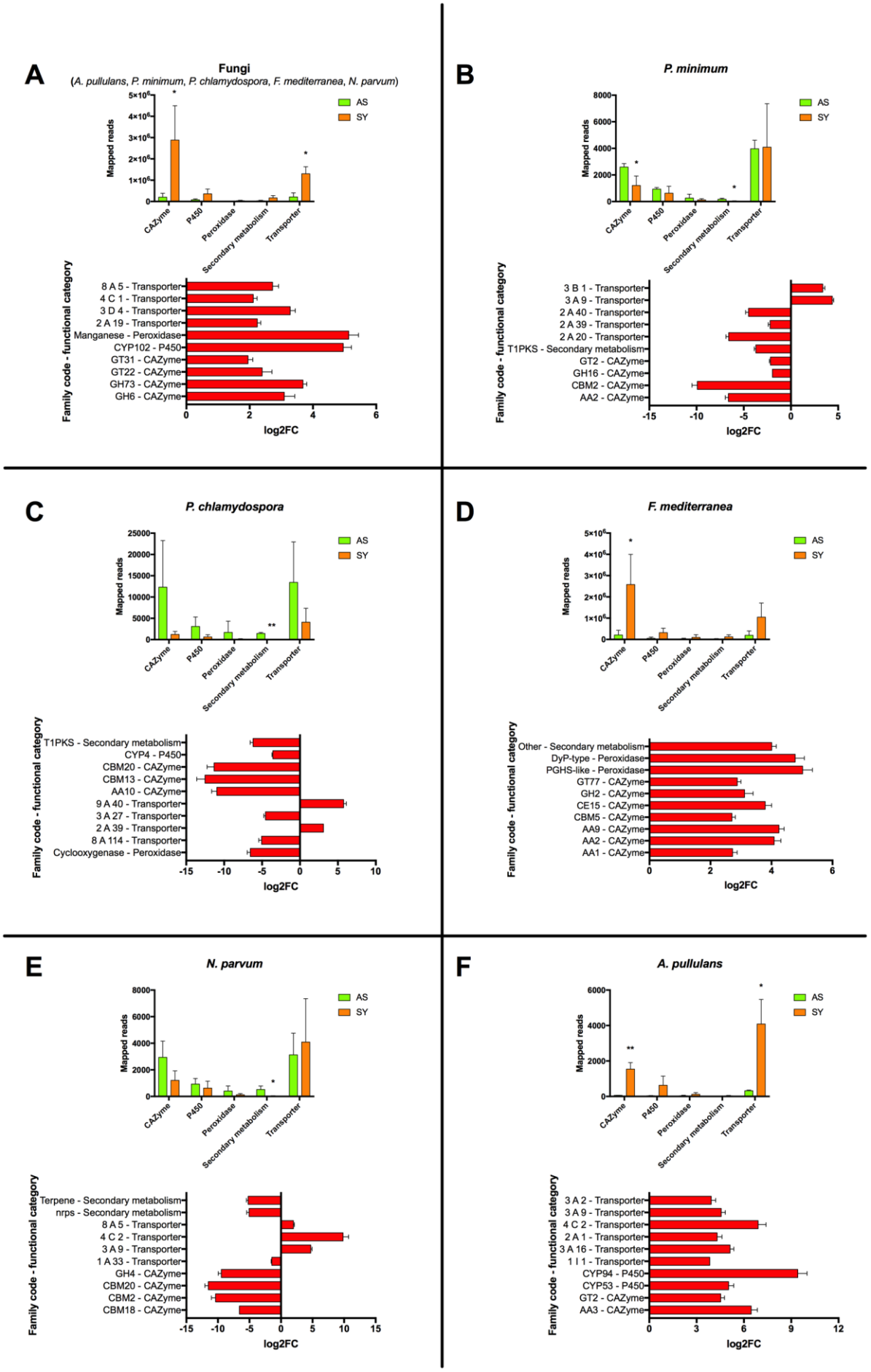
A
Fungi
(A. pullulans, P. minimum, P. chlamydospora, F. mediterranea, N. parvum)
Mapped reads
AS
SY
CAZyme
P450
Peroxidase
Secondary metabolism
Transporter
Family code - functional category
8 A 5 - Transporter
4 C 1 - Transporter
3 D 4 - Transporter
2 A 19 - Transporter
Manganese - Peroxidase
CYP102 - P450
GT31 - CAZyme
GT22 - CAZyme
GH73 - CAZyme
GH6 - CAZyme
log2FC
B
P. minimum
3 B 1 - Transporter
3 A 9 - Transporter
2 A 40 - Transporter
2 A 39 - Transporter
2 A 20 - Transporter
T1PKS - Secondary metabolism
GT2 - CAZyme
GH16 - CAZyme
CBM2 - CAZyme
AA2 - CAZyme
C
P. chlamydospora
T1PKS - Secondary metabolism
CYP4 - P450
CBM20 - CAZyme
CBM13 - CAZyme
AA10 - CAZyme
9 A 40 - Transporter
3 A 27 - Transporter
2 A 39 - Transporter
8 A 114 - Transporter
Cyclooxygenase - Peroxidase
D
F. mediterranea
Other - Secondary metabolism
DyP-type - Peroxidase
PGHS-like - Peroxidase
GT77 - CAZyme
GH2 - CAZyme
CE15 - CAZyme
CBM5 - CAZyme
AA9 - CAZyme
AA2 - CAZyme
AA1 - CAZyme
E
N. parvum
Terpene - Secondary metabolism
nrps - Secondary metabolism
8 A 5 - Transporter
4 C 2 - Transporter
3 A 9 - Transporter
1 A 33 - Transporter
GH4 - CAZyme
CBM20 - CAZyme
CBM2 - CAZyme
CBM18 - CAZyme
F
A. pullulans
3 A 2 - Transporter
3 A 9 - Transporter
4 C 2 - Transporter
2 A 1 - Transporter
3 A 16 - Transporter
1 I 1 - Transporter
CYP94 - P450
CYP53 - P450
GT2 - CAZyme
AA3 - CAZyme

Fig. 2. Analysis of functional categories and log2 fold change of the 10 most representative gene families considering the five fungi together (A) or singly (B–F). (B) *Phaeoacremonium minimum*, (C) *Phaeomoniella chlamydospora*, (D) *Fomitiporia mediterranea*, (E) *Neofusicoccum parvum*, and (F) *Aureobasidum pullulans*. Asterisks above the functional category graphs indicate significant differences as calculated with DESeq2 (∗P<0.05, ∗∗P<0.01). The 10 most up- or down-regulated gene families with a P-value <0.05 are reported in the log2 fold change graphs.

The average number of reads mapped on the unassigned contigs in AS samples was 507,000, whereas an average of 4,716,000 reads were observed in SY samples. Several fungal groups displayed differential accumulation in the two sample types (Supplementary Figure 5b): *Aspergillaceae* and *Xylariomycetidae* are more abundant in AS samples whereas the *Botryosphaeriaceae* group is more represented in SY ones (Supplementary Table 10).

Interestingly, looking at the bacterial community (Supplementary Figure 5a) we observed that in the AS samples the *Micromonosporaceae* represent the most abundant group, accounting for more than 23% of the bacterial community (Supplementary Figure 5a). The same family in SY samples represent only 3% of the bacterial community, and together with other bacterial groups show significant differences between the two sample types (Supplementary Table 11). In addition, several classes of proteins involved in both primary and secondary metabolisms of Actinobacteria show differential expression between the two groups of samples (Supplementary Figure 6). Finally, the majority of reads in both AS and SY samples were not classified and hence it was not possible to define which bacterial group was producing them.

Viral genomes form the de novo *assembly*

Assembly from the total RNA of the 6 metatranscriptomics samples was used to search viral sequences. Information about the assembly is summarized in Supplementary Table 12. Among the contigs not assigned to grapevine, fungi or bacteria we found a considerable number of virus-like sequences. To exclude such sequences from being just artefacts, we tested their presence using a specific primer in the cDNA derived from the same sequenced RNA samples. Interestingly, retaining only transcripts of at least 700 bp, we found 177 sequences of viral origin (Supplementary Table 13). Considering all the viruses (already known plant viruses and the newly identified ones), an average of 5,955,149 reads were detected in AS samples, whereas 11,628,472 reads were observed in SY samples. Considering the 177 viral sequences we found that 119 contigs represent new viral segments that have never been reported before. The details of the new viral contigs are reported in Supplementary Table 14.

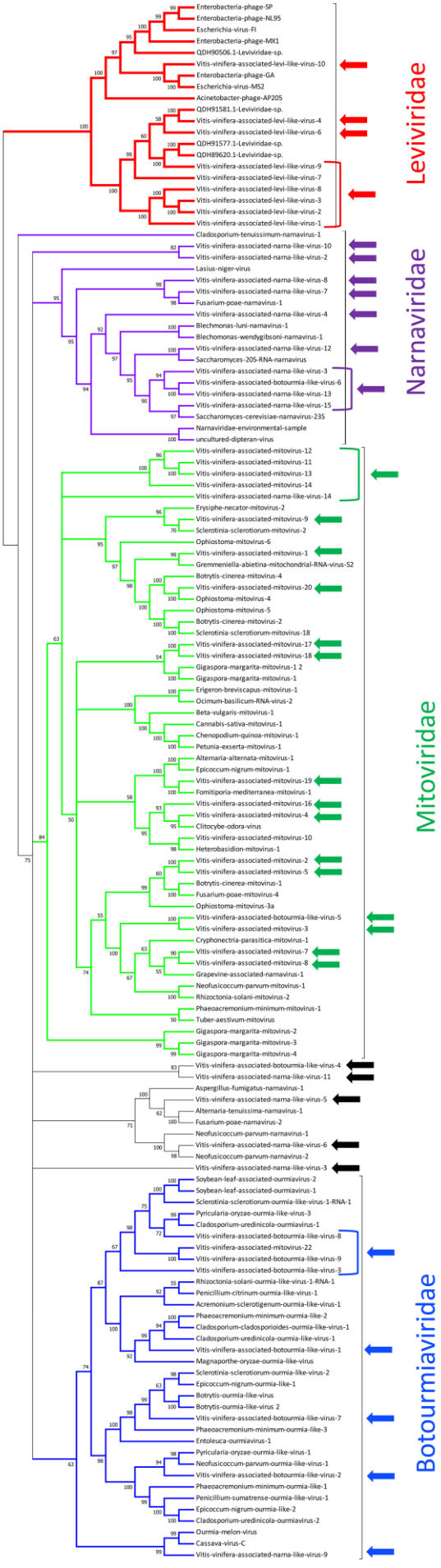

Fig. 3. Phylogenetic placement of putative viruses belonging to the *Lenarviricota* phylum. Amino acid sequences of RNA-dependent RNA polymerases (RdRps) were aligned using MUSCLE, and phylogeny were then derived using likelihood methodology in IQTREE. Numbers above branches represent statistical support based on bootstrap analysis (1000 replicates). Viruses identified in this work are indicated by coloured arrows.

All RNA genome types were detected: 87 viral segments belong to the +ssRNA group, 30 belong to the -ssRNA group and only 10 to the dsRNA group. The majority of +ssRNA (60 out of 87) belong to the Lenarviricota phylum: 15 segments show similarity to the *Narnaviridae* family, 22 segments show similarity to the *Mitoviridae* family, 11 segments to the *Leviviridae* family and 9 segments to *Botourmiaviridae*. Phylogenetic analysis of RdRP sequences belonging to this group is reported in Figure 3. The remaining viral sequences with similarities to +ssRNA viruses belong to: Tymovirales, *Tombusviridae*, *Virgaviridae*, *Nodaviridae*, *Solemoviridae, Hypoviridae* and *Potyviridae*, plus 12 sequences which show similarity to viruses that do not belong to any known +ssRNA viral group. Phylogenetic analysis of these sequences is reported in Supplementary Figure 7.

Viruses that belong to the dsRNA clade show similarities to *Partitiviridae* and *Endornaviridae* families, plus one virus belonging to the *Botybirnavirus* genus. Phylogenetic analysis of these sequences is reported in Supplementary Figure 8.

Finally, among viruses belonging to the -ssRNA clade we identified sequences belonging to Bunyavirales, Mononegavirals, Serpentovirales and Jingchuvirales plus 5 segments showing similarities to viruses that do not belong to any known viral group. Phylogenetic analysis of these sequences is reported in Supplementary Figure 9.

A further elaboration to highlight the diversity of viral ecology among samples was done through PCoA (Supplementary Figure 10a), taxa abundance (Supplementary Figure 10b) and hierarchical clustering (correlation of 0.953) using paired groups on Bray-Curtis matrix (Supplementary Figure 10c). All the analyses display a clear differentiation between AS and SY samples. Among all the viral species identified, Botryosphaeria dothidea fusarivirus 1 and Grapevine associated mitovirus 1 are present only in SY samples (with a good number of reads, covering more than 90% of genomes in all three samples). Furthermore, yellow speckle viroid 1, an already known and characterized grape viroid, is more abundant in AS samples than in SY ones. To better visualize the differences and have a comprehensive view of the virome communities in both AS and SY sample we produced a heatmap (Figure 4).

To sum up, a considerable number of new viral entities have been described, the majority of which belong to Lenarviricota, but with representative of all the RNA genome types (+ssRNA, -ssRNA and dsRNA). In parallel, many already known plant and fungal viruses were detected. Interestingly, bioinformatics analysis displayed a higher taxa representation in the SY samples.

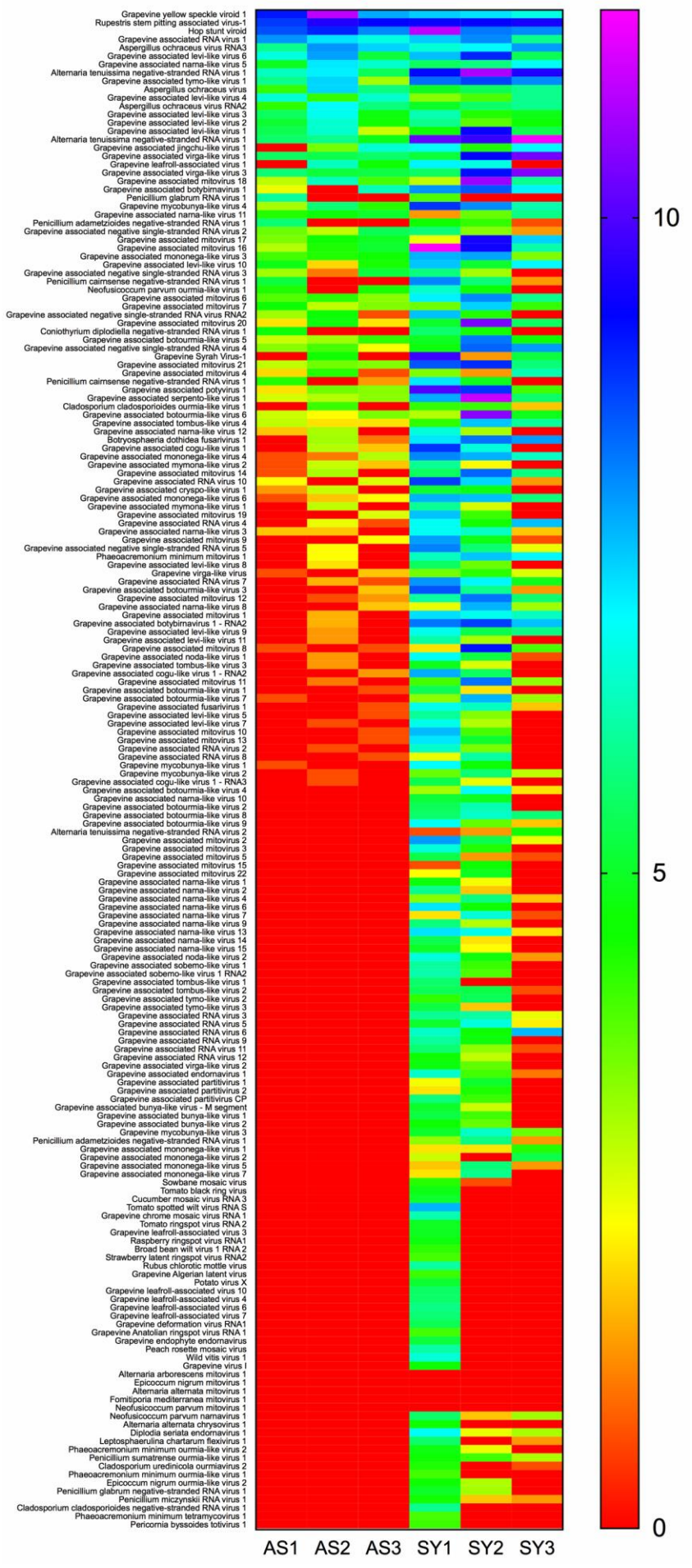

Fig. 4. Heatmap showing the differential abundance pattern of viruses in the three asymptomatic (AS) and three symptomatic (SY) samples represented as log2 of normalized reads per sample.

Potential biocontrol activity of isolated Actinobacteria

Due to the significant differences observed in the previous analysis we decided to isolate Actinobacteria from both sample types. Isolation of bacteria from wood fragments provided 38 different bacterial isolates (selected upon morphological characteristics). Each bacterial isolate was subjected to the 16S sequencing for identification (Supplementary Table 15). Among them 24 isolates belong to the Actinobacteria group whereas 13 belong to Rhizobiales and 1 to Pseudomonadales. All the identified isolates were tested in dual culture plates to assess their potential biocontrol activity against *P. minimum*, as representative of the esca core group, and the results are reported in Figure 5. Interestingly, the results showed that several isolates display the ability to impair fungal growth, three were the most effective and among these a *Micromonospora* sp. isolate was also identified.

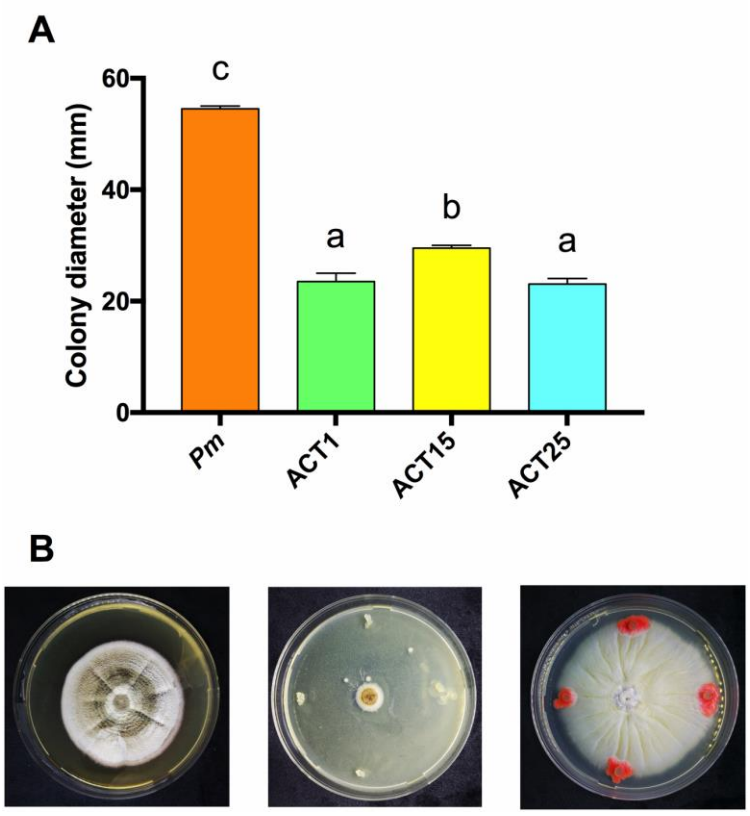

Fig. 5. Results of co-culture experiments for *P. minimum* and isolated bacteria. (A) Colony diameter of *P. minimum* grown alone or in co-culture with three different isolates of bacteria. Different letters indicate significant differences as attested by Tukey's HSD test. (B) Example of co-culture experiments at 10 d post-inoculation, in order from left to right: *P. minimum* alone, *P. minimum* with an inhibiting isolate (ACT1), and *P. minimum* with a non-inhibiting isolate (ACT10).

Discussion

Endophytes, which include bacteria, fungi and viruses, are microbes that inhabit plant tissues for at least a part of their life cycle (Kaul *et al.*, 2016). They have already been studied as a valuable source of novel metabolites, industrially important enzymes as well as stress relievers of the host plant (Wang and Dai, 2011; Mousa and Raizada, 2013; Toghueo *et al.*, 2017), but many aspects of their biology, behaviour and the complex interactions with their hosts still remain to be fully understood. In more detail, functions of individual endophytes are the result of their continuous and complex interactions with the host plant as well as with other members of the host microbiome, driving the composition of the so-called phytomicrobiome (Uroz *et al.*, 2019). For these reasons, in view of a more sustainable agriculture and considering the ongoing green revolution, understanding plant-microbiome interactions as a complex system studied in real agricultural environments becomes pivotal. This approach will enable the analysis and integration of these complex interactions for future effective field applications. Furthermore, we need to consider that microbe interactions among plants are very diverse, ranging from mutualistic to commensal, and up to pathogenic (Saikkonen *et al.*, 1998). Indeed, some endophytes are present in a latent phase, living with their hosts for some time, which show no symptoms of disease (Marsberg *et al.*, 2017). Border crossing eventually occurs when the environment surrounding the host or ecological niche (i.e., the plant tissue) becomes favourable for disease development (Abdullah *et al.*, 2017; Songy *et al.*, 2019). With the objective of this study, samples from two extreme "phenotypes" have been collected to study the interaction occurring among microorganisms inhabiting the wood plant tissues and clarify whether these interactions can in turn impact the host physiological state.

Metatranscriptomics enabled researchers to understand the responses occurring during the interaction of plant-microbes (both with pathogens and symbionts or commensalists), unveiling the complex molecular machinery activated from both sides (Nuss *et al.*, 2017; Massonnet *et al.*, 2018; Naidoo *et al.*, 2018; Wolf *et al.*, 2018; Mateus *et al.*, 2019). To date, despite several examples in the literature of dual RNA-seq experiments conducted in controlled conditions, very few data have been reported on complex samples such as wood from an open-air environment or in field conditions (Massonnet *et al.*, 2018; Morales-Cruz *et al.*, 2018). For this reason we decided to study grapevine, an economically important crop as well as a model for woody plants, applying the metatranscriptomics approach to the woody tissue of 20-year-old vines grown in the same environment, with the same cultural practices, but with a different health status influencing their physiological and metabolomic behaviour (Magnin-Robert *et al.*, 2017; Fischer and Peighami-Ashnaei, 2019; Ouadi *et al.*, 2019; Labois *et al.*, 2020). In this respect we observed that, as previously demonstrated (Pierron *et al.*, 2016; Morales-Cruz *et al.*, 2018), a multi-species closed-reference mapping approach is able to provide

quantitative assessments of species composition and transcriptional profiles that highlight the over-expression of virulence factors in SY samples, particularly for some fungal taxa. Indeed, an overexpression of functional categories related to wood decay was observed in SY samples, with *F. mediterranea* showing the most remarkable increase. In this line, a recent review gathered the knowledge on white rot fungi (*Hymenochaetales*), suggesting that their presence is pivotal for the expression of severe symptoms (Del Frari *et al.*, 2021) confirming the results reported in this study. In parallel, the abundance and behaviour of *N. parvum* and *A. pullulans* suggest that both these fungi can have a greater involvement during syndrome aggravation than previously suggested (Fischer and Peighami-Ashnaei, 2019) and also shift their behaviour from neutral (Slippers and Wingfield, 2007) or beneficial endophytes (Di Francesco *et al.*, 2020) to pathogens when the health status of the ecological niche becomes compromised. This observation is also supported by a recent work in which the study of the mycobiome associated to a vineyard with esca symptomatic plants revealed that a shift in the fungal community is not directly associated to the expression of leaf symptoms suggesting a more complex behavioural adjustment (Del Frari *et al.*, 2019).

The comparison between the plant transcriptome of grapevine wood showing esca symptoms and asymptomatic highlighted a strong overexpression of genes in infected tissues. The samples were collected in the advanced stage of disease development when symptoms were clearly evident in the wood, and the transcriptome of the infected tissues was reprogrammed almost exclusively by stimulating the defence responses. In particular, the overexpression of genes involved in ROS metabolism (*VvGER*, *VvCAT*, *VvPOX*, *VvGST*) suggested that ROS production, an essential component in the signal transduction cascade (Smirnoff and Arnaud, 2019), leads to defence responses in tissues infected by esca, and if not properly controlled by the scavenging systems of the plant it causes strong oxidative damage with cell death. Likewise, genes codifying PR proteins and WRKY transcription factors were overexpressed in infected wood confirming the importance of these pathways in the grapevine responses to biotic stresses (Wang *et al.*, 2014; Chitarra *et al.*, 2017; Mestre *et al.*, 2017). Likewise, the modification of hormonal responses (nitrilases and auxins) and lipid and fatty acid metabolism in SY through the lipoxygenase up-regulation involved in the production of signalling molecules (e.g. methyl jasmonate) and antimicrobial compounds (Farmer *et al.*, 2014) played an important role in the responses to pathogens of trunk disease. However, the main novelty is the demonstration that, in esca infected wood, many laccase genes were strongly overexpressed. Plant laccases are known to lignify the secondary cell walls with an important role in maintaining the structural strength of the plant (Bao *et al.*, 1993; Ranocha *et al.*, 2002). The overexpression of plant laccases can induce an increase of lignin content and enhanced resistance (e.g. pathogen(s) compartmentalizing within xylem tissues) against pathogens, as reported in *Malus hupehensis*, cotton

and *Arabidopsis* (Hu *et al.*, 2018; Zhang *et al.*, 2019*b*; Yu *et al.*, 2020). In our experimental conditions, in the woody tissues affected by esca, the strong overexpression of laccases can be interpreted both as a defence response against the pathogens and as an attempt to produce new lignified tissues to replace those affected by esca fungi. This second hypothesis is also supported by the activation of several genes linked to membrane, cell wall and cytoskeleton metabolisms (cellulose synthases, xyloglucan endotransglucosylase/hydrolases, tubulins, myosins).

A fascinating result was obtained for the reads that were assigned neither to grapevine nor to the selected group of fungi. Although some other fungal taxa displayed significant differences between the two sample types, bacteria showed the wider differences between AS and SY samples. In this respect, previous studies suggested that esca symptoms could be influenced by organisms other than fungi, suggesting a possible pathogenic role (Hofstetter *et al.*, 2012; Bruez *et al.*, 2015) or as biocontrol agents (Niem *et al.*, 2020). In our specific conditions, through the metatranscriptome approach we were able to identify several taxa of bacteria that showed different relative abundance between the sample types. Although we used the complete NCBI nr database, the vast majority of bacterial reads as well as fungal ones were not classified taxonomically, suggesting that the woody tissue of plants could be a source of new and never described endophytic bacteria and fungi. We therefore decided to focus our attention on the *Micromonospora* genus for several reasons: i) they showed a great reduction in SY samples (from about 24% in terms of relative abundance in AS to about 3% in SY); ii) they showed a wide gene expression rearrangement between the two sample types; iii) they belong to Actinobacteria, a group of well-known beneficial bacteria and iv) as previously demonstrated they can act as biocontrol agents (Hirsch and Valdés, 2010; Martínez-Hidalgo *et al.*, 2015). In light of this, we re-sampled wood tissues from asymptomatic vines and isolated Actinobacteria using a classic approach (see Methods section). *In vitro* trials led to identifying three isolates (including a *Micromonospora* sp.) able to limit the growth of *P. minimum* (selected as representative of esca pathogens). This result further highlights the potential of the metatranscriptome approach compared to dual-transcriptomics or metabarcoding studies which focus their attention only on specific microbial taxa or on the whole microbial community without looking at the functional activity. Furthermore, this analysis provided interesting information on the bacterial unknown fraction of the reads and contigs that didn't find any taxonomic classification highlighting that most of them probably belong to taxa isolated here that don't have any reference genome deposited in publicly available databases.

In addition to the valuable ecological data obtained particularly for the bacterial community, with the identification of several possible biocontrol agents, the other impressive ecological insight retrieved from the metatranscriptome concerns the virus population. Viruses are ubiquitous in all environments,

able to infect all cellular organisms, even to parasitize other viruses and can account for a substantial proportion of RNA within their hosts (Hisano *et al.*, 2018; Krupovic *et al.*, 2019; Zhang *et al.*, 2019*a*). In our experimental system we observed that the phytosanitary status, intended as the expression of esca symptoms accompanied to a compromised wood tissue, is associated with differences in viral abundance and diversity. Because of the close relationship in terms of distance, environmental conditions and microorganism populations we expected an almost identical virome across samples. Instead, SY samples displayed a wider virome population when compared to AS ones, suggesting that the ecology of viral species is strongly influenced by the tissue health status. Almost all the viruses detected are most probably viruses of microbes (mycoviruses or bacteriophages). Some of them have already been reported as mycoviruses of endophytic fungi (Nerva *et al.*, 2019*c*), which were isolated from the same plants as those analysed here. Two viruses identified were also reported as potential new plant viruses (Bertazzon *et al.*, 2020). These results suggest that, despite the ubiquitous nature and economic importance of grapevine, to date we have a poor understanding of the natural viral diversity that is part of the phytomicrobiome and of the plant as a holobiont. To this end, we employed an unbiased metatranscriptomics approach to reveal wood-associated viromes, comprising more than 150 viral species, of which more than 100 were new, in the framework of a microbial ecology study. In comparison to other ecological studies focused only on virus discovery (Shi *et al.*, 2016), our results highlight an unprecedented viral diversity associated to a limited ecological niche.

An intriguing issue arising from the virome data is how the microbial community can tolerate such a high level of virus diversity and abundance without compromising the biological activity, raising new questions about their role(s) in driving the microbial structure and dynamics in natural as well as anthropic environments. We can hypothesize that viral co-infection seems to be the rule rather than the exception, and that co-infection is also how viruses shape the host ecology and the interaction between them. This concept was already proposed in other works (Wille *et al.*, 2018; Nickbakhsh *et al.*, 2019; Thapa and Roossinck, 2019), but never observed in any agricultural system and quite unexpected for a such limited environment like the one reported here. Indeed, another key observation linked to the viral population is that, despite some differences in the composition of fungal and bacterial communities being observed, the taxonomy composition of the microbial communities does not seem to drive the virome structure. On the contrary, several studies already demonstrated the pivotal role of viruses in driving the microbial community composition (Roux *et al.*, 2016; Fernández *et al.*, 2018; Kuzyakov and Mason-Jones, 2018). To deepen the virome potential role(s) *ad hoc* wider metatranscriptomic studies will be necessary. Although the sequencing techniques allowed a much wider comprehension of the viral community inhabiting some important ecological niches, we still

have a surprisingly poor understanding of virus-virus interactions. For example, viruses may have synergistic or antagonistic effects within or across infections by other viral species (Díaz-Muñoz, 2017). Additionally, viral infection may play a fundamental role in the behaviour of their microbial host, enhancing or inhibiting the expression of genes involved in the interaction between them and with the host plant (Chun *et al.*, 2018; Nerva *et al.*, 2018*a*; Okada *et al.*, 2018).

In conclusion, we demonstrated that metatranscriptomics can better define the composition of the microbial community associated to a complex ecological niche, expanding our understanding of microbial and viral diversity and laying the foundation for future studies concerning the factors that can influence the microbial and viral structure in natural or semi-natural environments. Although from an ecological perspective our study is of limited scale, we have successfully isolated potential beneficial microbes starting from sequencing data, identified an unprecedented viral diversity and found evidence of differences across factors shaping the microbial interactions. Finally, we demonstrated that our understanding of viral ecology needs to be boosted and that we should consider both viruses and their hosts as complex ecological communities functioning as a whole unique organism.

SUPPLEMENTARY DATA

The following supplementary data available at *JXB* online

Supplementary Figure 1. Correlation between RNAseq (FPKM) and RT-qPCR results

Supplementary Figure 2. Relative abundance of the 11 fungal species selected for the closed-reference transcriptome analysis.

Supplementary Figure 3. Plot of the dispersion estimate and fitted dispersion-mean relationship obtained by the deseq function.

Supplementary Figure 4. Principal component analysis (PCA) of the five selected fungi considering their whole transcriptomes one by one.

Supplementary Figure 5. Relative abundance of bacterial (a) and fungal (b) taxa identified using the diamond-Megan pipeline analysis in AS and SY samples.

Supplementary Figure 6. Heatmap showing the differential expression pattern of genes encoded by Micromonosporaceae.

Supplementary Figure 7. Phylogenetic relationship among viruses displaying a positive single-stranded RNA (+ssRNA) genome.

Supplementary Figure 8. Phylogenetic placement of putative viruses belonging to double-stranded RNA clade.

Supplementary Figure 9. Phylogenetic placement of putative viruses belonging to the negative single-stranded RNA (-ssRNA) viruses.

Supplementary Figure 10. Data of viral communities were used to calculate principal coordinate (PCoA) graph (a), abundance of viral taxa (b) and highlight the similarity of samples using hierarchical clustering based on Bray-Curtis matrix (c).

Supplementary Table 1. Active ingredients and average doses used for the control of the main diseases over the monitoring period.

Supplementary Table 2. Databases and methods used to annotate transcriptomes of the grapevine trunk-associated fungi reference database.

Supplementary Table 3. List of the oligonucleotides used in this study.

Supplementary Table 4. List of accessions used for alignment and phylogenetic inference of *Leviviridae*, *Narnaviridae* and *Botourmiaviridae* viruses.

Supplementary Table 5. List of accessions used for alignment and phylogenetic inference of positive single-stranded RNA viruses.

Supplementary Table 6. List of accessions used for alignment and phylogenetic inference of double-stranded RNA viruses.

Supplementary Table 7. List of accessions used for alignment and phylogenetic inference of negative-stranded RNA viruses belonging to *Bunyavirales*, *Serpentovirales* and *Mononegavirales* orders.

Supplementary Table 8. Distribution of reads mapping among plant, fungal, bacterial and viral communities.

Supplementary Table 9. Functional categories of genes differentially expressed in the comparison between grapevine wood showing esca symptoms (SY) and asymptomatic (AS).

Supplementary Table 10. Relative transcripts abundance for the fungal groups which significantly differ between asymptomatic (AS) and symptomatic (SY) plants.

Supplementary Table 11. Relative transcripts abundance for the bacterial groups which significantly differ between asymptomatic (AS) and symptomatic (SY) plants.

Supplementary Table 12. Statistical metrics describing the Trinity assembly.

Supplementary Table 13. Summary of reads mapping on the 182 viral contigs detected among samples.

Supplementary Table 14. Viral segments detected, with description, first hit in database, putative function and viral name.

Supplementary Table 15. Identified bacterial colonies from the wood isolations using Actinobacteria-specific media.

Conflict of Interests

The authors declare that they have no conflicts of interest. This article does not contain any studies with human or animal participants.

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Data Availability Statement

The GenBank accession numbers of the sequences reported in this paper are:
Study: PRJNA703377 (SRR13754975-SRR13754976)
Viral sequences: from MW648427 to MW648545.

Author contributions

LN and WC designed the experimental system. LN, FF, LM, GGi, MS and WC conducted the wet lab experiments and performed data elaboration. GGa performed the metatranscriptome data analysis of plant transcripts. DC and JFG performed part of the data analysis of microbial community in the metatranscriptome dataset. LN, GGi, GGa, MS, WC, RV, AZ and MG analysed the whole data and wrote the first draft of the manuscript. All the authors contributed to the writing and carefully revised the final version.

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CHAPTER 5 | BACK TO THE HOLOBIONT: ECOPHYSIOLOGICAL PERFORMANCES OF SYNTHETIC COMMUNITY-PRIMED ROOTED-CUTTINGS

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Abstract

Despite microbe-based products for grapevine protection and growth improvement are already available, few of them contain microbes directly isolated from vines tissues. For this reason, a collection of endophytic bacterial isolates obtained directly from grapevine woody tissues has been exploited for producing an *ad-hoc* inoculum. Selected bacterial isolates were tested in biocontrol assays against some of the main grapevine pathogens (*e.g.*, *Botrytis cinerea*, *Guignardia bidwellii*, *Neofusicoccum parvum*) and the best performing isolates were then screened for plant growth promoting (PGP)-traits (*e.g.*, phosphorous solubilization, indole-acetic acid and siderophore production). Before being planted, rooted cuttings were inoculated with two different synthetic communities: the first one was an *ad-hoc* developed microbial community (SynCom), and the second one was a commercial consortium formed by arbuscular mycorrhizal fungi (AMF) and rhizosphere bacterial strains (AMF+B). At véraison, physiological parameters, biochemical and molecular analyses have been collected to evaluate the effects on plant performances. Thanks to the holistic approach, we showed that SynCom treatment shaped the plant growth-defence trade-off, thus moving the energy allocation through the defence pathways by affecting the photosynthetic performances. Our findings suggest that an approach considering both the bacterial features as well as their impacts on plant growth and defence could shade light on the "dark-side effects" of SynCom application, enabling their exploitation with a refined awareness. On the other hand, the AMF+B treatment revealed a more equilibrated allocation across the growth-defence trade-off even if with a mitigated activation of the defence pathways.

Keywords

SynCom, growth-defence tradeoffs, grapevine, AMF, endophytes

Introduction

Plants naturally share their environment with a multitude of microbes, some of which can colonize their inner tissues becoming endophytes and strongly influencing plants life cycle and responses to the environmental stimuli (Nerva et al., 2022b). The recruitment of microbes by plants (*i.e.*, microbiome assembly) depends on interactions among plants and the surrounding environment and, once the relationship is established, the plant and its microbiome behave as a unique super organism, the so-called holobiont (Vandenkoornhuyse et al., 2015). Considering the global climate changing effects and the resulting prediction of more frequent biotic and abiotic stressful events, new sustainable alternatives are increasingly urgent to improve agricultural resilience (Chitarra et al., 2015; Giudice et al., 2021; Giudice et al., 2022). In this context, a better understanding of plant-microbiome interactions could help to dissect key factors involved in beneficial microbes recruiting by the host (Nerva et al., 2022b). Thanks to the great advances in microbial biotechnology, metagenomics and the extensive collections of microbes recently developed, we have a treasure at our disposal to manipulate bacterial communities on a large scale (Zou et al., 2019; Sandrini et al., 2022a). In this fashion, it is possible cultivate pure characterized strains and develop synthetic microbial communities (SynComs) to mimic natural microbiome functions and to study, through multi- and interdisciplinary approaches, the plant microbiome assembly and its effects on plant performances under diverse environmental conditions (Sandrini et al., 2022b). Interestingly, the assembly rules for establishing plant microbiota have been revealed in gnotobiotic *Arabidopsis* plants using a drop-out and a late introduction approach with SynComs composed by 62 native strains. The authors found that community assembly has historical contingency with priority effects and a certain degree of resistance to late microbe arrivals (Carlström et al., 2019). These findings provided new insights in plant-microbiome interactions and suggested that successful SynComs applications should be at the early stages of host life cycle, when the microbiome is still under development. As cited before, many beneficial endophytes-inhabiting plant tissues (*e.g.*, Plant Growth-Promoting microorganisms, as for example Actinomycetes and Arbuscular Mycorrhizal Fungi – AMF) showed key roles in protecting plants against biotic and abiotic stresses (Chitarra et al., 2016; Carrión et al., 2019). Besides basic research to study the complex host-microbiome interactions, SynComs can be exploited to promote growth and other beneficial traits in the host. During the last decade, many SynComs approaches have been reported in literature (most of them conducted in axenic conditions), highlighting the increased attention to this topic for future sustainable agricultural practices (Sandrini et al., 2022a). Plant diseases cause significant losses in agricultural production that 83 lead not only to lower yields and decreased quality but also to loss of biodiversity, mitigation costs due to control measures, and an important downstream impact on human health (Ristaino et al., 2021). Plants

possess natural defence mechanisms that are finely regulated by several phytochemicals. Once infected by pathogens, plants promptly react activating defence innate mechanisms that are tightly mediated by jasmonic acid (JA), ethylene (Et) and salicylic acid (SA) hormones. These molecules orchestrate several hormonal-mediated pathways from cells to systemic routes by means of the so-called systemic acquired resistance (SAR, mediated by SA) or induced systemic resistance (ISR, mediated by JA and Et) (Burketova et al., 2015). The latter is activated not only in consequence to pathogen attacks but also by soil-inhabiting beneficial microbes (*e.g.*, PGPB, AMF) recruited by plants thanks to the modulation of signalling root exudates abundantly released under stressful conditions (Berendsen et al., 2018). Thus, the development of tailored SynComs could represents a powerful tool to prime plants against biotic (and/or abiotic) stresses preventing food losses. Berendsen et al. (2018), developed a simplified SynCom formed by three bacterial species that synergistically protected *Arabidopsis* plants against downy mildew. In another study, Lebeis (2015) using a SynCom composed by 38 bacterial strains, demonstrated that immune signalling is the driver of microbiome development in *Arabidopsis*. Recently, Li et al. (2021), assembled two SynComs (one complex and another one simplified to 4-species forming community) with disease controlling functions against *Fusarium* sp., the causal agent of root rot disease in *A. mongholicus*. These authors observed that both SynComs controlled the disease development *via* synergistic cooperation by activating ISR in the host. It is worth noting that the native microbiome is continuously modulated depending on the host genotype as well as the environmental stimuli. Similarly, SynComs structure and functionality can be strongly influenced by the same variables although further studies are needed. A better understanding of SynCom functionality in "natural" environments would allow to fully exploit their potentials, especially considering that many of the studies available to date were conducted in axenic conditions (Wei et al., 2018; Veach et al., 2019). Grapevine represents one of the most important fruit crops worldwide, hardly affected by many pathogens (mainly fungal ones) both in pre- and post-harvest (*e.g.*, powdery mildews, gray mold, esca syndrome). These pathogens cause important damages, usually controlled by massive pesticides application that strongly impact agroecosystems, beneficial microbiota and human health, making urgent the development of new sustainable alternatives (Armijo et al., 2016; Nerva et al., 2019; Giudice et al., 2022; Nerva et al., 2022a). Thus, in this study we aimed to investigate whether the plant inoculation with a simplified SynCom formed by biocontrol agents could confer a priming status to the host. To address the above question, a SynCom formed by 117 seven bacterial isolates retrieved from the inner grapevine woody tissues (Nerva et al., 2022a) was developed and inoculated in grapevine rooted cuttings prior to planting them in field. The newly developed SynCom was compared with a commercial one formed by a mixed inoculum of AMF and bacterial strains. Afterward, combined ecophysiological,

biochemical and molecular approaches were used to investigate the SynCom effects on the host physiological performances, plant growth promotion and ISR activation. Criticisms and suggestions for the implementation of SynCom protocols for future scale up and field applications have also been discussed.

Results

Isolation and molecular characterization of bacterial collection

Forty-three bacterial isolates were collected, and the molecular identification was achieved by 16S rRNA gene sequencing analysis. The sequences obtained were submitted to NCBI nr database using BLAST (Basic Local Alignment Tool). The BLASTn analysis revealed that the 44 isolates belonged to different genera, most of which belonging to Actinobacteria phylum (Table 1). In fact, although a specific protocol for Actinobacteria isolation was adopted, 22 out of 44 isolates were found to be Actinobacteria and 22 out of 44 Proteobacteria (Table 1). Sequences of 16S from each isolate was deposited in NCBI GenBank under accessions OP994307-OP994344. Checking in literature, no one bacterial isolate showed similarities to those harmful for humans or plants. Not being the sole purpose of establishing a collection of grapevine endophytes, the whole 44 bacterial isolates were *in vitro* assessed for biocontrol activity against some of the most important and widespread grapevine pathogens (Table 1) and for PGP-traits (Table 2) as below reported.

Table 1. Biological control activity of the whole bacterial collection. Data for biological control activity are referred to the pathogen growth inhibition rate (%) toward each fungal pathogen. The reported values of pathogen growth inhibition rate are the mean values of three replicates ± SD for each isolate. Selected isolates forming the SynCom are reported in bold.

Code	Phylum	Specific Epithet	*B. cinerea*	*P. minimum*	*N. parvum*	*G. bidwelli*
			Pathogen Growth Inhibition Rate (%)			
ACT1	Actinobacteria	*Micromonospora sp.*	-	-	-	27.9±3.25
ACT2	**Actinobacteria**	***Actinomadura glauciflava***	**17.85±0.10**	**26.15±0.10**	**-**	**49.6±1.77**
ACT3	Actinobacteria	*Saccharopolyspora sp.*	96.42±0.10	7.69±0.10	-	52.1±1.06
ACT4	Actinobacteria	*Actinomadura glauciflava*	16.67±0.10	28.92±1.50	-	52.1±3.18
ACT5	Actinobacteria	*Kocuria palustris*	-	-	-	18.3±0.71
ACT6	Actinobacteria	*Micrococcus sp.*	-	-	-	7.1±0.35
ACT7	Proteobacteria	*Methylobacterium sp.*	-	7.69±0.10	-	55.4±0.71
ACT8	-	*Uncultured*	7.35±2.48	-	-	32.9±2.47
ACT9	Actinobacteria	*Rhodococcus sp.*	-	22.46±1.00	-	46.7±3.54
ACT10	Actinobacteria	*Mycobacterium hodleri*	-	-	-	57.5±3.01
ACT11	Actinobacteria	*Nocardioides sp.*	-	-	-	21.3±3.28
ACT12	**Actinobacteria**	***Asanoa sp.***	**96.43±0.10**	**16.92±0.10**	**-**	**56.7±2.50**

ACT13	Actinobacteria	*Plantibacter sp.*	-	-	-	59.6±0.35
ACT15	Actinobacteria	*Methylobacterium sp.*	4.17±0.71	45.54±1.00	-	59.7±0.35
ACT16	Actinobacteria	*Cellulomonas sp.*	25.88±1.41	-	-	-
ACT17	Actinobacteria	*Nocardioides cavernae*	10.00±4.24	-	-	65±1.41
ACT18	Proteobacteria	*Pseudomonas sp.*	96.43±0.10	10.46±0.50	-	71.7±0.71
ACT19	Proteobacteria	*Methylobacterium adhaesivum*	-	7.69±0.10	-	44.6±0.35
ACT20	Proteobacteria	*Methylobacterium sp.*	-	3.64±0.50	-	63.3±0.71
ACT21	Proteobacteria	*Methylobacterium adhaesivum*	-	-	-	42.5±2.12
ACT22	Proteobacteria	*Methylobacterium tardum*	-	8.62±0.50	-	41,3±3.18
ACT23	Proteobacteria	*Methylorubrum extorquens*	-	1.23±0.50	-	24.6±3.50
ACT24	Actinobacteria	*Nocardioides sp.*	-	16±0,50	-	30±0.71
ACT25	**Proteobacteria**	***Rhizobium sp.***	**25.29±0.71**	**11.82±0.10**	**-**	**61.3±0.35**
ACT26	Proteobacteria	*Methylobacterium adhaesivum*	-	-	-	42.1±1.06
ACT27	Proteobacteria	*Methylobacterium sp.*	25.89±0.71	-	-	44.6±0.35
ACT28	Proteobacteria	*Methylorubrum sp*.	-	44.62±0.10	-	37.5±0.71
ACT29	Proteobacteria	*Methylobacterium sp.*	-	-	-	27.9±2.47
ACT30	Actinobacteria	*Nocardia sp.*	-	24.31±1.00	-	-
ACT31	Actinobacteria	*Nocardioides sp.*	95.29±1.41	-	51.76±3.50	85.8±0.71
ACT32	Actinobacteria	*Streptosporangium sp.*	-	-	-	-
ACT33	Proteobacteria	*Rhizobium sp.*	-	14.15±1.50	-	69.2±2.12
ACT35	**Proteobacteria**	***Methylobacterium radiotolerans***	**96.43±0.10**	**1.23±1.50**	**-**	**49.6±0.35**
ACT36	Actinobacteria	*Mycobacterium sp.*	35.71±0.10	2.15±1.50	-	77,9±1.06
ACT37	Proteobacteria	*Methylopila oligotropha*	-	-	-	24.2±3.00
ACT38	Actinobacteria	*Microbacterium sp.*	-	21.54±1.00	-	41.3±1.77
ACT39	Proteobacteria	*Agrobacterium sp.*	1.47±1.77	-	-	50±0.00
ACT40	Actinobacteria	*Microbacterium sp.*	-	-	-	11.7±0.00
ACT41	Actinobacteria	*Micrococcus yunnanensis*	2.06±1.06	-	-	25.4±3.18
AR1	Proteobacteria	*Pseudomonas psychrotolerans*	94.04±0.10	19.55±1.77	-	54.6±1.06
AR2	Proteobacteria	*Achromobacter insuavis*	96.47±0.10	15.45±1.41	-	79.6±0.71
19VE21-2	**Proteobacteria**	***Achromobacter xylosoxidans***	**94.05±0.10**	**78.64±0.50**	**31.91±0.53**	**85±0.71**
19VE21-3	**Proteobacteria**	***Achromobacter xylosoxidans***	**94.64±0.10**	**76.82±0.10**	**28.82±3.50**	**86.3±2.25**
P.Fluo	**Proteobacteria**	***Pseudomonas sp.***	**95.23±0.71**	**87.08±0.10**	**-**	**62.1±3.18**

Table 2. Evaluation of plant growth promoting (PGP) traits of selected bacteria constituting the SynCom. Symbol – indicates negative result and symbol + positive result. Positive results have been ranked with an intensity scale: +, slight activity; ++ medium activity; +++ good activity. Data for ACCdeaminase activity are expressed as ACC concentration remaining in the DF-ACC medium containing 3 mM ACC after incubation of each ACC-utilizing bacterial isolate for 24h. Data for IAA-production are expressed as IAA concentrations (µg/mL) after incubation of each isolate for 48h in Luria Bertani broth. Data for siderophore production: measurement (mm) of the yellow halo zone around the bacterial colonies on CAS agar plate. Data of N fixation: +, growth capability on Nfb agar medium. Data for P solubilization and starch hydrolysis: measurement of halo diameter (zone of clearance) around the bacterial colonies. Data related to salinity tolerance: intensity of bacterial growth on agar plate containing different NaCl concentrations. Data are the mean values of three replicates ± SD for each isolate.

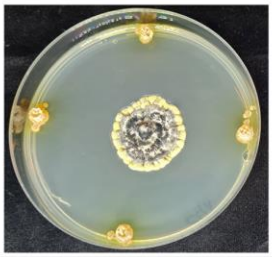
Code	ACC concentration (mmol l^{-1})	IAA Production (µg ml^{-1})	Siderophore Production	*N* Fixation	*P* Solubilization	Starch Solubilization	Salinity Tolerance
ACT2	-	-	-	+	-	++	-
ACT12	-	+ (10.06±0.32)	+	-	+	+	+
ACT25	++ (0.04±0.01)	+ (5.64±0.11)	++	-	-	+	++
ACT35	++ (0.02±0.01)	-	-	+	-	+	-
P.Fluo	-	+++ (46.92±1.31)	+	-	+	+	+
19VE21-2	+++ (0.005±0.01)	+++ (40.77±0.78)	+	+	++	+++	++
19VE21-3	++ (0.10±0.01)	++ (28.35±0.67)	++	+	+	++	++

In vitro ***evaluation of biocontrol and Plant Growth Promoting (PGP) activities of bacterial isolates***

In the biocontrol Petri dish assay, 44 strains showed different degree of pathogen containment and the most performing isolates were selected as good candidates to build a SynCom (Table 1 and Figure 1). Indeed, an antagonistic activity towards at least three pathogens and a pathogen growth inhibition rate higher than 45% facing at least one fungus was adopted as selection criteria. The seven selected bacterial strains are highlighted in bold in Table 1. Since the high efficacy in controlling pathogens and the potential production of volatile organic compounds (VOCs), the seven isolates were challenged in septate Petri dishes but none of them showed the ability to inhibit the pathogen in such experimental set-up (data not shown). Further analyses were performed to evaluate the ability of SynCom candidate members to stimulate plant growth and abiotic stress tolerance (Table 2): among the seven candidates, all of them were able to solubilize starch, with 19VE21-2 showing the largest halo zone around the colonies. Four isolates were able to solubilize phosphate, with 19VE21-2 showing the wider clear zone around the colonies. Three isolates were able to growth on NFb agar plate having thus the potential to fix nitrogen, in specific ACT2 showed the most preeminent growth. Five isolates proved to be siderophore producers, with isolates ACT25 and 19VE21-3 showing the wider yellow halo appearance around bacterial colonies in the Chrome Azurol media. Five isolates were identified as IAA producers, with isolate P.Fluo showing the most abundant production. Four strains were also able to degrade ACC, with isolates ACT35 and 19VE21-2 showing the highest consumption activities. As a last PGP-related trait we assessed the ability of the selected isolates to grow in presence of NaCl at different concentrations (0, 1,5 and 3% w/v): six out of seven candidates display a great salinity tolerance. The biocontrol activity and the PGP-traits for each of the selected

isolate is reported in Fig. 1 along with the picture taken at the end of the biocontrol assay on *G. bidwellii*. Finally, to limit reciprocal inhibition effects, the strains compatibility assay was performed, showing a good attitude of candidates to live together confirming them as promising members of a grape-specific synthetic community.

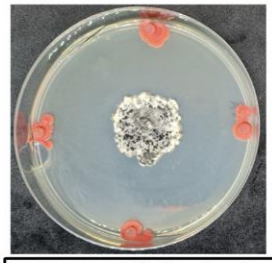
ACT2 - *Actinomadura glauciflava*



PGP traits	
ACC (mmol l^{-1})	-
IAA (µg ml^{-1})	-
Siderophore	-
N fixation	+
P solubilization	-
Starch solubiliz.	++
Salinity	-

Biological control activity			
Botrytis cinerea	*Phaeoacremonium minimum*	*Neofusicoccum parvum*	*Guignardia bidwellii*
17.86±0.10%	26.15±0.10%	-	49.60±1.77%

ACT12 - *Asanoa* sp.



PGP traits	
ACC (mmol l^{-1})	-
IAA (µg ml^{-1})	+ (10.06±0.32)
Siderophore	+
N fixation	-
P solubilization	+
Starch solubiliz.	+
Salinity	+

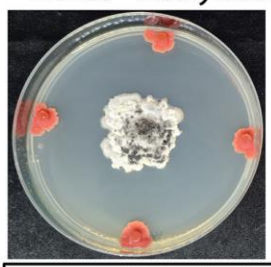
Biological control activity			
Botrytis cinerea	*Phaeoacremonium minimum*	*Neofusicoccum parvum*	*Guignardia bidwellii*
96.43±0.10%	16.92±0.10%	-	56.70±2.50%

ACT25 - *Rhizobium* sp.

PGP traits	
ACC (mmol l^{-1})	++ (0.04±0.01)
IAA (µg ml^{-1})	+ (5.64±0.11)
Siderophore	++
N fixation	-
P solubilization	-
Starch solubiliz.	+
Salinity	++

Biological control activity			
Botrytis cinerea	*Phaeoacremonium minimum*	*Neofusicoccum parvum*	*Guignardia bidwellii*
25.29±0.71%	11.18±0.10%	-	61.30±0.35%

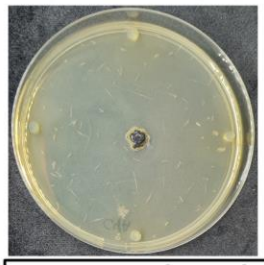
ACT35 - *Methylobacterium radiotolerans*



PGP traits	
ACC (mmol l^{-1})	++ (0.02±0.01)
IAA (µg ml^{-1})	-
Siderophore	-
N fixation	+
P solubilization	-
Starch solubiliz.	+
Salinity	-

Biological control activity			
Botrytis cinerea	*Phaeoacremonium minimum*	*Neofusicoccum parvum*	*Guignardia bidwellii*
96.43±0.10%	1.23±1.50%	-	49.60±0.35%

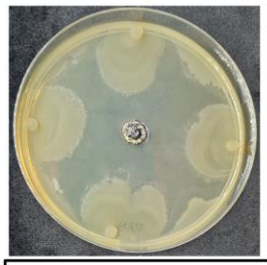
19VE21-2 - *Achromobacter xylosoxidans*



PGP traits	
ACC (mmol l^{-1})	+++ (0.005±0.01)
IAA (µg ml^{-1})	+++ (40.77±0.78)
Siderophore	+
N fixation	+
P solubilization	++
Starch solubiliz.	+++
Salinity	++

Biological control activity			
Botrytis cinerea	*Phaeoacremonium minimum*	*Neofusicoccum parvum*	*Guignardia bidwellii*
94.05±0.10%	78.64±0.50%	31.91±0.53%	85.00±0.70%

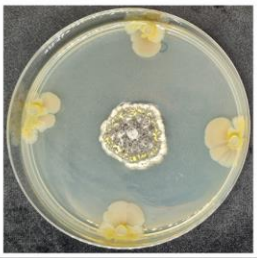
19VE21-3 - *Achromobacter xylosoxidans*



PGP traits	
ACC (mmol l^{-1})	++ (0.10±0.01)
IAA (µg ml^{-1})	++ (28.35±0.67)
Siderophore	++
N fixation	+
P solubilization	+
Starch solubiliz.	++
Salinity	++

Biological control activity			
Botrytis cinerea	*Phaeoacremonium minimum*	*Neofusicoccum parvum*	*Guignardia bidwellii*
94.64±0.10%	66.82±0.10%	28.82±3.50%	86.30±2.25%

P.Fluo - *Pseudomonas fluorescens*



PGP traits	
ACC (mmol l^{-1})	-
IAA (µg ml^{-1})	+++ (46.92±1.31)
Siderophore	+
N fixation	-
P solubilization	+
Starch solubiliz.	+
Salinity	+

Biological control activity			
Botrytis cinerea	*Phaeoacremonium minimum*	*Neofusicoccum parvum*	*Guignardia bidwellii*
95.24±0.71%	87.04±0.10%	-	62.10±3.18%

Controls - *Guignardia bidwellii*

Uninoculated

Uneffective
ACT30 - *Nocardia* sp.

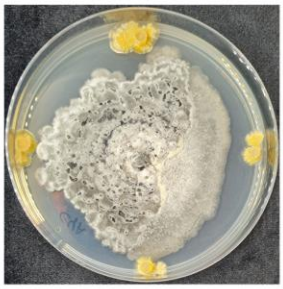


Fig. 1. Plant growth promotion and biological control traits of the selected isolates forming the SynCom. For PGP traits the symbol + represents the presence of growth/halo, while the symbol – represents negative results for the test. Data for 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity: +++, good; ++, medium; +, slight; values are the mean ± SD. Data for IAA production: +, quantity <20 µg ml-1; ++, quantity between 20 and 40 µg ml-1; +++, quantity >40 µg ml-1; values are the mean ± SD. Data for siderophore production: +, zone of yellow halo up to 2 mm; ++, zone of yellow halo bigger than 2 mm up to 5 mm. Data for *N* fixation: +, growth capability on Nfb agar; -, absence of growth. Data of *P* solubilization: +, isolates with halo diameter up to 1 mm; ++, halo diameter bigger than 1 mm up to 5 mm. Data for starch hydrolysis: + (0 cm up to 0,4 cm), ++ (0,4 cm up to 1 cm), +++ (higher than 1 cm) means low, medium and high halo diameter on the media after incubation, respectively. Data related to salinity tolerance: +, low growth; ++, medium growth; +++, good growth. For each PGP trait, at least three replicates per isolate were performed. Data for biological control activity are referred to the % of growth inhibition rate toward each selected fungal pathogen.

AM root colonization analysis

To confirm the establishment of mycorrhizal symbiosis on roots of rooted cutting inoculated with the commercial formulation (AMF+B), the percentage of arbuscules in root cortical cells was calculated on three randomly selected plants for each treatment. Microscopic observations of stained roots revealed the presence of mycorrhizal structures with different extent depending on the treatment. Roots of AMF+B treatments showed a significantly higher percentage of mycorrhization frequency (F) ranging around 100%; intensity of the mycorrhizal colonisation in the root system (M) or fragments (m) both ranging around 60%; arbuscule abundance in root fragments (a) ranging around 94% or in the root system (A) ranging around 54% (Fig. 2a). Conversely, very few fungal structures were observed in the roots from SynCom and CTRL samples (Fig. 2a). Additionally, to check the functionality of AM symbiosis, the assessment of three AM symbiosis-related genes (*i.e.*, *VvCCD7*, *VvCCD8* related to strigolactones biosynthesis and a grape phosphate transporter, *VvPT1-3*) was performed. *VvCCD7* was significantly up-regulated in AMF+B-treated plants and SynCom when compared to CTRL-treated plants (Fig. 2b). Conversely, *VvCCD8* showed no significant difference among treatments (Fig. 2c). Regarding the *VvPT1-3* gene reported as marker of functional AM symbiosis establishment in grapevine (Balestrini et al., 2017; Nerva et al., 2021a), its expression level was affected by AMF+B with a significant 185 up-regulation with respect to both the CTRL and SynCom treatments that instead did not show any significant differences between them (Fig. 2d). These results suggested that AMF+B roots are efficiently colonized, and the well-functioning of AM symbiosis is noticed. In contrast, colonization from native AMF in SynCom and CTRL plants was not relevant.

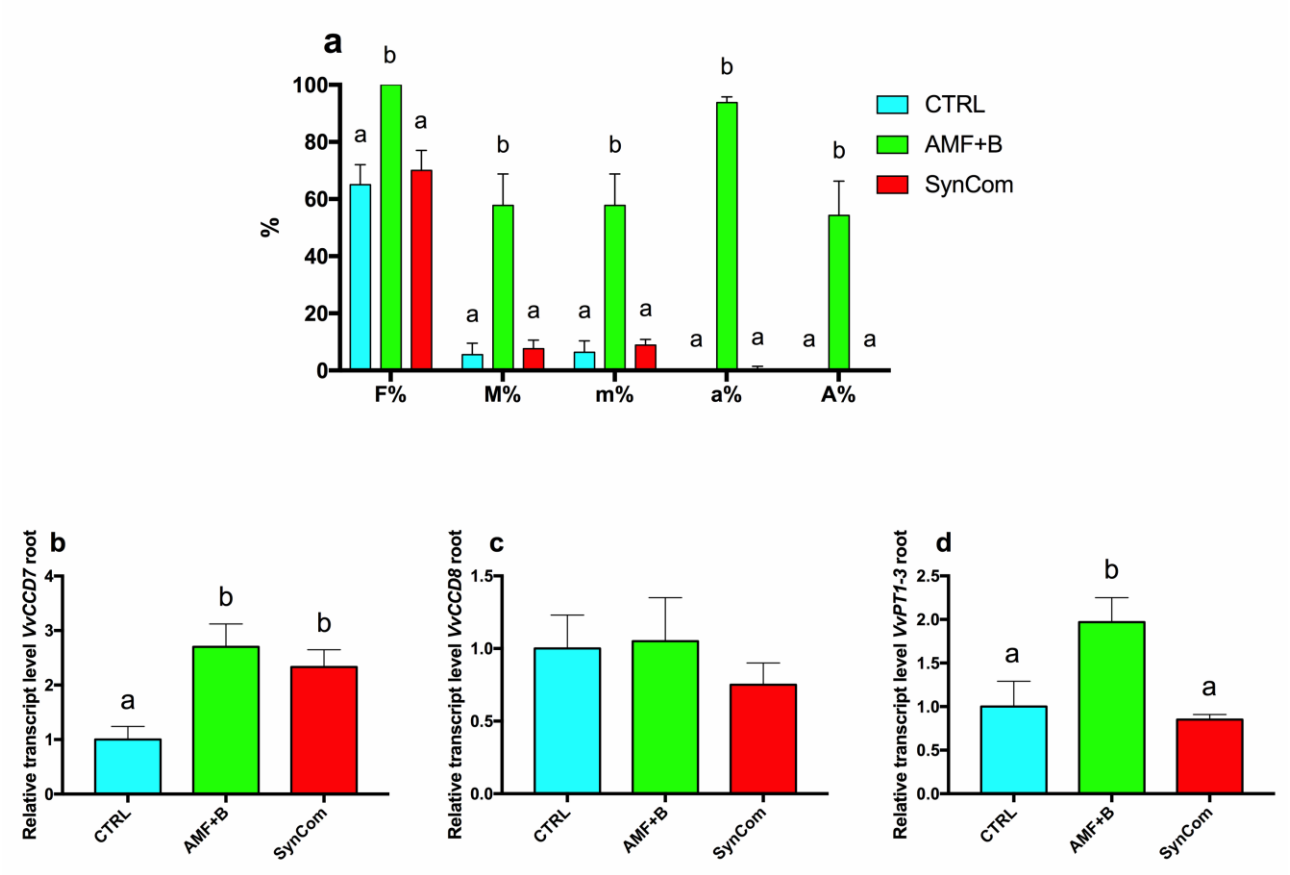


Fig. 2. AMF colonization in grapevine roots. a) Colonization rate in CTRL, AMF+B and SynCom treatments. Data are expressed as mean ± SD (n = 3). F%, Frequency of mycorrhiza in the root system; M%, Intensity of the mycorrhizal colonization in the root system; m%, Intensity of the mycorrhizal colonization in the root fragments; a%, Arbuscule abundance in mycorrhizal parts of root fragments; A%, Arbuscule abundance in the root system. b,c) Strigolactones biosynthesis related genes *VvCCD7* and *VvCCD8*, respectively. d) grapevine phosphate transporter as marker of functional symbiosis *VvPT1-3*. Lowercase letters above bars denote significant differences attested by Tukey's HSD test ($P \leq 0.05$). CTRL, control plants; AMF+B, commercial AMF + Bacteria mixed inoculum-treated plants; SynCom, Synthetic Community-treated plants.

Physiological parameters and biochemical responses in field

AMF+B as well as SynCom treatment altered leaf physiological performances of vines under field conditions. Regarding to Pn, rooted cutting inoculated with AMF+B showed significantly greater Pn values compared to CTRL and SynCom-treated plants, with the latter that showed significantly lower values when compared to both AMF+B and CTRL treatments (Fig. 3a). Gs was characterized by rising trend in AMF+B and SynCom compared to the CTRL, but they did not significantly differ among the two treatments (Fig. 3b) and only AMF+B is significantly higher with respect to the control plants. Looking at Ci values, SynCom-treated plants displayed slightly higher values compared to the CTRL and to AMF+B plants, although not significantly differences among treatments are evident (Fig. 3c). ACE (calculated as Pn Ci-1) showed significant lower level in SynCom plants with respect

to the other treatments (Fig. 3d). Similarly, iWUE (calculated as Pn gs-1 202 ratio) resulted significantly lower in SynCom, while no significant differences were found between AMF+B and CTRL plants (Fig. 3e). Overall, these findings showed a marked photosynthetic imbalance in SynCom inoculated vines. Moving to biochemical responses in both leaves and roots, we considered metabolites involved in growth (*e.g.*, IAA) and defence processes (*e.g.*, ABA, t-resveratrol and viniferin). Starting from ABA, no relevant differences were detected in leaves (Fig. 4a), although ABA level appeared to be lower in both the inoculated plants (AMF+B and SynCom) in comparison to the CTRL ones. Conversely, significantly higher concentrations were found in roots of both AMF+B and SynCom inoculated plants compared to the CTRL (Fig. 4b). IAA level in leaves did not show any difference among treatments (Fig. 4c), while in roots AMF+B showed lower levels with respect to CTRL (even if not significant) and SynCom plants, while the latter exhibited the highest values (Fig. 4d). As regard to stilbenes, *t*-resveratrol in leaves showed significant higher concentration in AMF+B and SynCom plants, with the latter having the higher concentration with respect to AMF+B and CTRL (Fig. 4e). Similarly, accumulation was strongly elicited in roots in SynCom as well as AMF+B-treated plants, with significant higher concentration when compared to CTRL (Fig. 4f). Viniferin showed a similar trend with significantly higher values in SynCom and AMF+B-treated plants compared to CTRL ones in both leaf (Fig. 4g) and root (Fig. 4h) tissues.

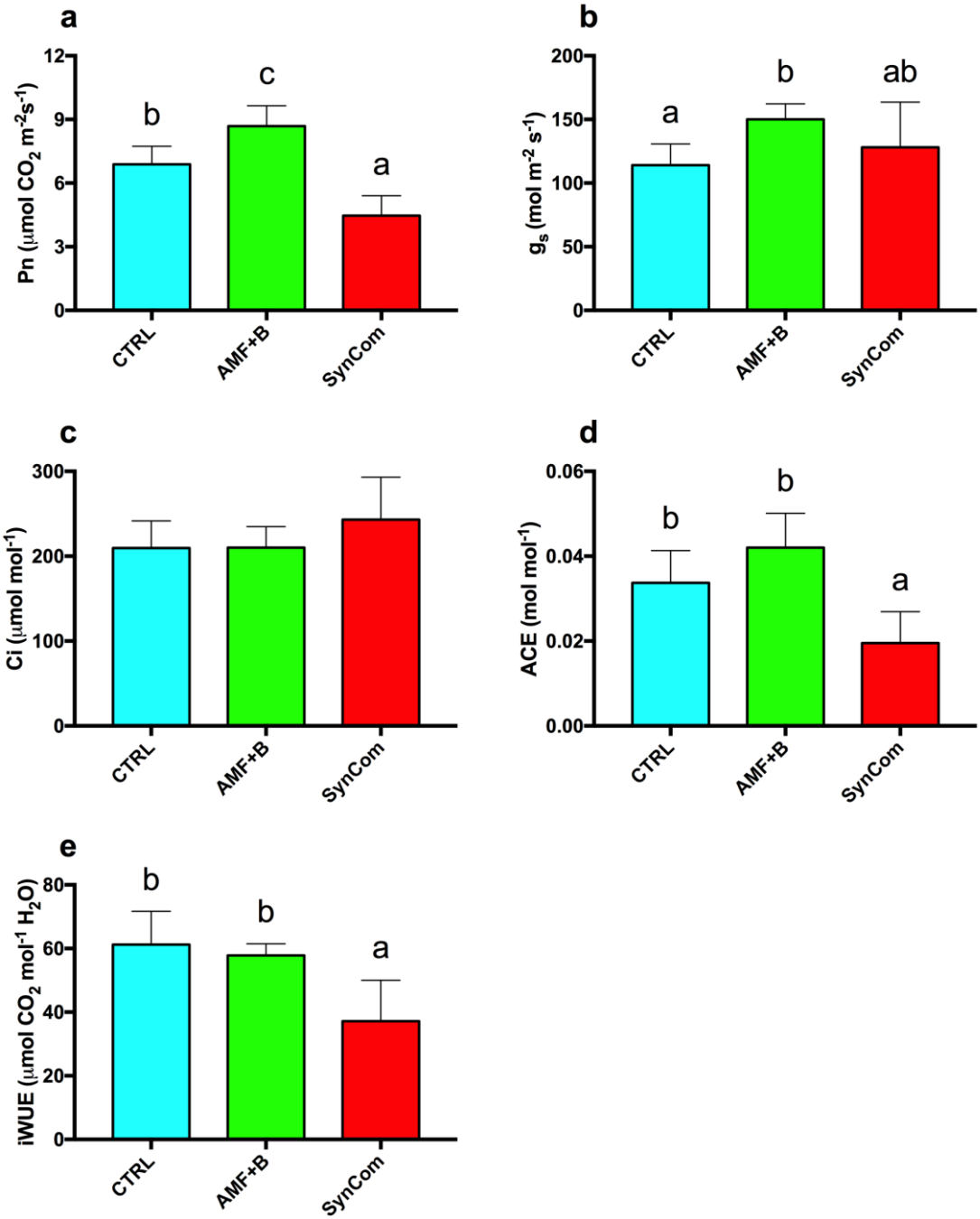


Figure 3. Instantaneous leaf gas exchange measurements. Performances records of: **a)** net photosynthesis (Pn); **b)** stomatal conductance (gs)**; c)** intercellular CO2 concentration (Ci); **d)** apparent carboxylation efficiency (ACE); **e)** intrinsic water use efficiency (iWUE). Data are expressed as mean ± SD (n = 18). Different lowercase letters above bars indicate significant differences according to Tukey's HSD test ($P < 0.05$). CTRL, control plants; AMF+B, commercial AMF + Bacteria mixed inoculum-treated plants; SynCom, Synthetic Community-treated plants.

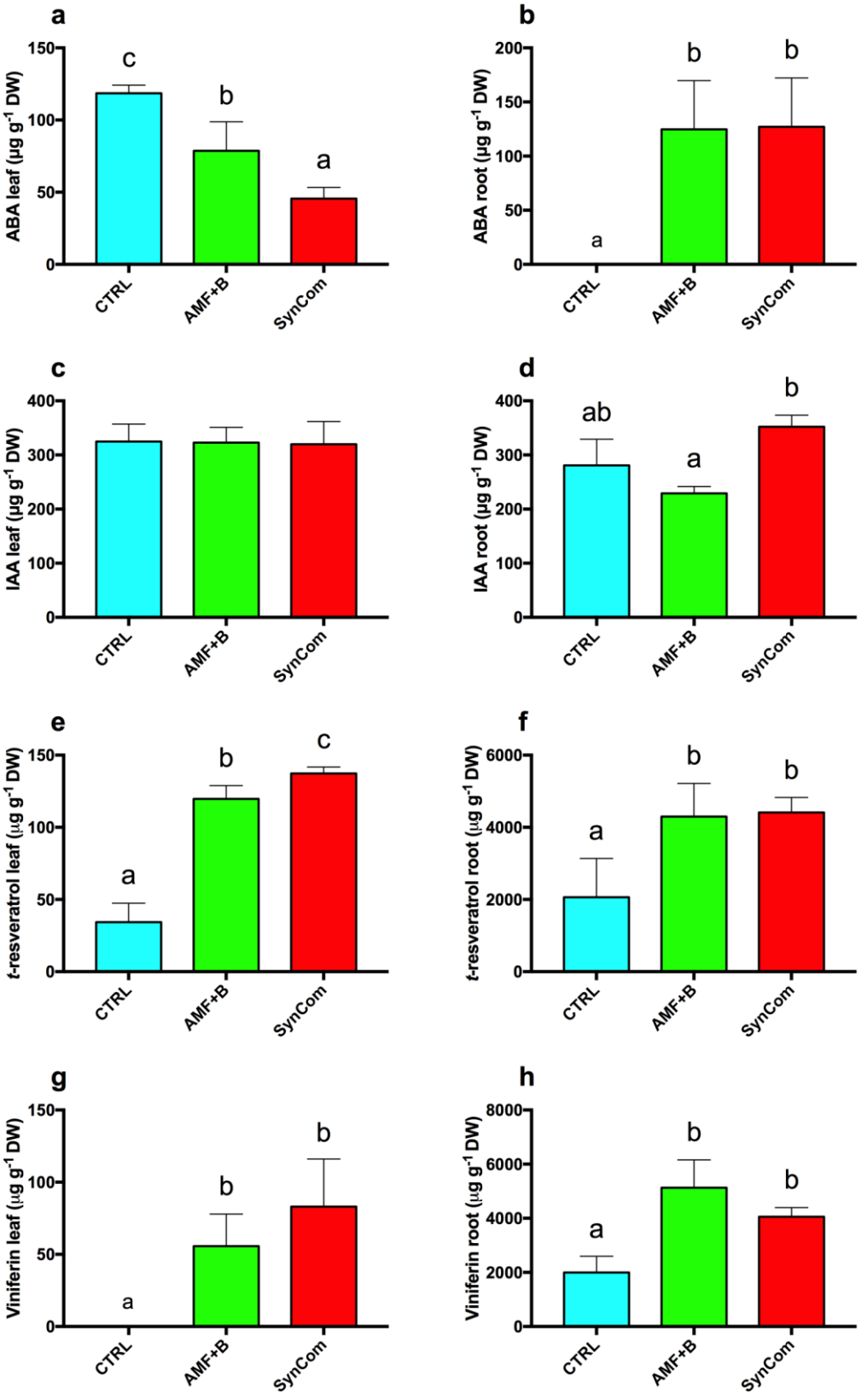

a
ABA leaf (µg g-1 DW)
150
100
50
0
c
b
a
CTRL
AMF+B
SynCom
b
ABA root (µg g-1 DW)
200
150
100
50
0
a
b
b
CTRL
AMF+B
SynCom
c
IAA leaf (µg g-1 DW)
400
300
200
100
0
CTRL
AMF+B
SynCom
d
IAA root (µg g-1 DW)
400
300
200
100
0
ab
a
b
CTRL
AMF+B
SynCom
e
t-resveratrol leaf (µg g-1 DW)
150
100
50
0
a
b
c
CTRL
AMF+B
SynCom
f
t-resveratrol root (µg g-1 DW)
6000
4000
2000
0
a
b
b
CTRL
AMF+B
SynCom
g
Viniferin leaf (µg g-1 DW)
150
100
50
0
a
b
b
CTRL
AMF+B
SynCom
h
Viniferin root (µg g-1 DW)
8000
6000
4000
2000
0
a
b
b
CTRL
AMF+B
SynCom

Figure 4. Target metabolites concentration in leaf and root tissues. a, b) Abscisic acid (ABA) quantification in leaf and root tissues, respectively. **c, d)** Indolacetic acid (IAA) quantification in leaf and root tissues, respectively. **e, f)** *trans*-resveratrol (*t*-resveratrol) quantification in leaf and root tissues, respectively. **g , h)** Viniferin quantification in leaf and root tissues, respectively. Data are expressed as mean ± SD (n = 3). Different lowercase letters above bars indicate significant differences according to Tukey's HSD test ($P < 0.05$). CTRL, control plants; AMF+B, commercial AMF + Bacteria mixed inoculum-treated plants; SynCom, Synthetic Community-treated plants.

Gene expression analysis

Transcripts levels were analysed in leaf tissues by means of RT-qPCR on target genes involved in growth or defence pathways. In detail, *VvPAL* (a gene encoding for a phenylalanine amino lyase) showed significantly higher expression level in AMF+B and SynCom treatments with respect to CTRL both in leaves and roots. Particularly, *VvPAL* showed higher expression level in leaves of SynCom-treated plants, even if not significant, when compared to AMF+B plants (Fig. 5a). In roots, expression level was higher in both AMF+B and SynCom plants with respect to CTRL (Fig. 5b). Regarding to stilbenes synthase gene, *VvSTS1* was significantly affect by all treatments compared to the CTRL but with an opposite trend in roots and leaves: it was strongly up-regulated in leaves of SynCom- and AMF+B-treated plants (Fig. 5c), while it was significantly down regulated in roots in both treatments (Fig. 5d). Looking at key players of the immunity system, *VvLOX* (coding for a lipoxygenase) and *VvNPR1* (coding for NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1) are known as target genes involved in the onset of ISR and SAR, respectively. *VvLOX* was influenced by both AMF+B and SynCom treatments showing an opposite trend with a significant up-regulation in SynCom plants and a slightly down-regulation trend in AMF+B treatment both in leaves and roots (Fig. 5e-f). About *VvNPR1,* no significant differences among the treatments are present in leaves (Fig. 5g), while a significant up-regulation in roots of both AMF+B and SynCom-treated plants with respect to CTRL ones (Fig. 5h) was observed. It is worth noting that among treatments, SynCom showed a stronger influence on *VvNPR1* expression level although not significantly different from AMF+B (Fig. 5h). We also looked at some genes involved in physiological responses to highlight differences occurring among treatments. The expression analysis of *VvCHL* (a chlorophyllase gene) showed significantly higher values in leaves of SynCom-treated plants when compared to the AMF+B and CTRL treatments among which no significance difference was detected (Fig. S1a). Looking at the expression of the ABA synthesis gene *VvNCED3*, transcript level was significantly higher in roots of AMF+B and SynCom treatments (Fig. S1b), while in leaves expression level was lower for both treatments with respect to CTRL samples (Fig. S1c). Finally, *VvYUC3*, a key gene involved in the auxin synthesis pathway, showed a different trend in root and in leaf tissues. In leaves, the expression level was not affected by AMF+B treatment with respect to

CTRL, while in SynCom treated plants expression level was significantly higher if compared to both CTRL and AMF+B treatments (Fig. S1d). Conversely, in roots, it was significantly downregulated in AMF+B and SynCom treatments with respect to the CTRL (Fig. S1e).

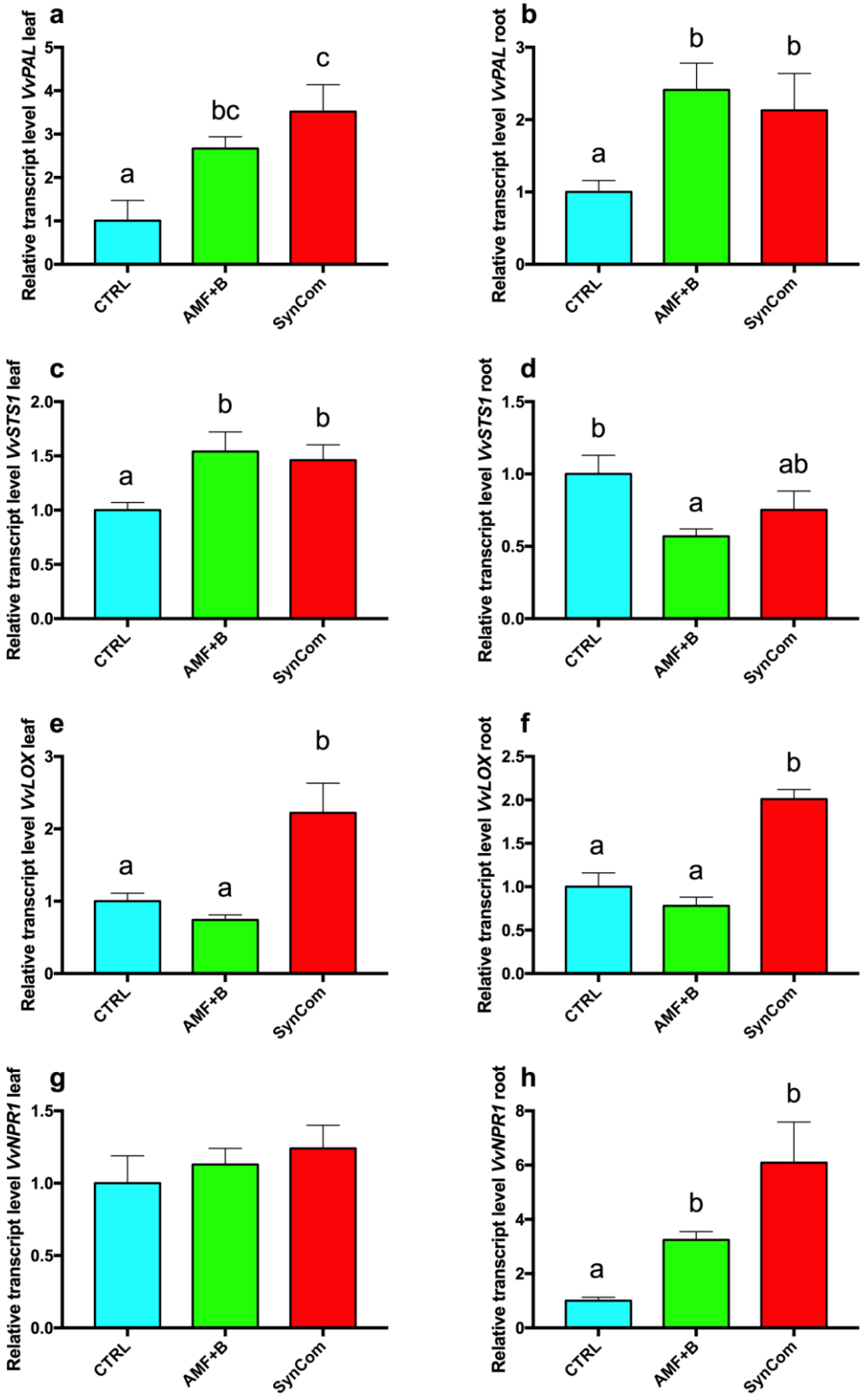


Figure 5. Expression changes of defence-related genes. a,b) Relative expression level of *VvPAL* in leaf and root tissues, respectively. **c, d)** Relative expression level of *VvSTS1* in leaf and root tissues, respectively. **e, f)** Relative expression level of *VvLOX* in leaf and root tissues, respectively. **g, h)** Relative expression level of *VvNPR1* in leaf and root tissues, respectively. Data are expressed 917 as mean ± SD (n = 3). Different lowercase letters above bars indicate significant differences according to Tukey's HSD test ($P < 0.05$). CTRL, control plants; AMF+B, commercial AMF + Bacteria mixed inoculum-treated plants; SynCom, Synthetic Community-treated plants.

Discussion

To date, increasingly attention has been placed on the functions played by plant-associated microbes in response to environmental stresses (Marulanda et al., 2006; Sandrini et al., 2022b). From long time scientists reported beneficial plant effects conferred by associated bacteria or fungi as a single strain or mixed to form consortia. Thanks to the –omics advances, during the last decade methods for microbial consortia development evolved in SynCom formulations, improving our knowledge on the still debated mechanisms activated by the microbe-host relationships (Liu et al., 2019; Dastogeer et al., 2020). Attention have been primarily posed on the use of soil microorganisms to improve plant response to drought tolerance (Tufail et al., 2022). By contrast, tailored SynComs to trigger plant immunity pathways against pathogens is an approach still poorly explored (Li et al., 2021). In this fashion, here a customized bacterial SynCom formed by strains showing a marked *in vitro* biocontrol activity against *Vitis vinifera* fungal pathogens has been formulated. It is worth noting that isolates employed in this study were already described as grapevine endophytes and therefore well-adapted to live in association with its tissues as well as able to easily colonize them, thus facilitating the induction of priming responses induction (Berendsen et al., 2018). The customized SynCom was inoculated in young grape cuttings and a multidisciplinary approach was used to investigate its effect on plant physiological and defence responses in open field, comparing results with a commercial SynCom and an uninoculated control.

Biocontrol-based SynCom formulation

According to the protocol reported by Nerva et al., (2022a), all bacterial strains were isolated directly from the inner woody tissue of grape main trunk (*Vitis vinifera* cv Glera) building a collection of grapevine endophytes and ensuring in this way that these bacteria can live in strictly association with *Vitis vinifera* tissues. In fact, looking at the literature, the output obtained from field applications of experimental SynComs has been often contrasting (Ownley et al., 2003) and one of the main causes is that species-dependent effects are not taken in consideration, often obtaining non-specific microbial cocktails with only milden effects on their plant hosts (Sandrini et al., 2022a). Nowadays, the importance of Actinobacteria and the possibility to exploit them as sustainable tool in agriculture has been repeatedly discussed and confirmed (Viaene et al., 2016). For instance, Mahesh et al., have recently demonstrated that drought-tolerant endophytic actinobacteria are able promote growth of wheat (*Triticum aestivum*) under water stress conditions (Yandigeri et al., 2012). However, just few species have been already used as biological control agents (Bressan, 2003) and for this reason an Actinobacteria-specific isolation protocol (Dhanasekaran and Jiang, 2016) has been adopted for this study, obtaining 22 out of 44 isolates belonged to Actinobacteria phylum. Although a specific

protocol with semi-selective media has been used, just the 50% of strains collection has resulted to be composed by Actinobacteria and only 2 out 7 have been selected for the SynCom formulation. Since the isolation of Actinobacteria using classical bacterial isolation protocols is in fact often disappointing in terms of the rates obtained, the selected protocol allowed us to successfully enrich the Actinobacteria members being able to have 50% of the isolates belonging to this phylum. The number of isolates forming SynComs greatly varied among the studies, from 3-4 to hundreds as reviewed in Sandrini et al., (2022a). Here, a great number of isolates were shown to be effective to limit the growth of different fungal pathogens, at least *in vitro.* Among these isolates, the seven most performing isolates (two Actinobacteria and five Proteobacteria) were selected to build the grape customized SynCom. The use of such simplified SynCom is because plants excrete in soil by roots a considerable amount of root exudates to attract microbes (Massalha et al., 2017) and the more of them are applied, the greater would be the competition among themselves (Li et al., 2021). To limit such effect, in our study a simplified SynCom was formulated using isolates that successfully confirmed their compatibility after mutual exclusion test. To underline the potential of bacterial endophytes as biocontrol agents in agriculture, about 50% of tested isolates exhibited a pathogen growth inhibition rate higher than 45%, towards at least one of the four pathogens. This inhibition is likely due to diffusible compounds since no VOCs activity has been detected by means of septate-petri dish assay (Wan et al., 2008; Olivera et al., 2021). As cited before, the main goal of our study was the development of a customized SynCom formed by biocontrol agents able to trigger direct and indirect defence responses against pathogens in vines. However, given the well-known capacity of several bacteria to promote plant growth and stress resilience (Guerrieri et al., 2020), the seven candidates to form the synthetic community were also screened for some of the main PGP traits such as nitrogen fixation, phosphate and starch solubilization or siderophore production, IAA production and ACC-deaminase activity. Additionally, they were tested also for tolerance to an abiotic stress, *i.e.*, the salt stress, since in viticulture soil salinity is increasingly, becoming a current problem due to both high fertilization rates and the increasing incidence of drought events (Corwin, 2021). Among the seven PGP traits evaluated *in vitro*, each candidate was found to be positive for at least two of them, highlighting the potential to improve plant growth and abiotic stress resilience in field. However, such effects may not be observed in field due to the occurrence of complex environmental interactions (Li et al., 2021).

Plants inoculated with the customized SynCom showed growth-defence imbalance responses in field with respect to AMF+B ones

Although there are some works in literature regarding the study and the formulation of SynComs, only a few of them concern their application in glasshouse or experimental fields (Armanhi et al., 2021), and, at the best of our knowledge, no one of them were focused on grapevine. The goal of our study was to determine whether the application of synthetic communities (both customized or commercialized: SynCom or AMF+B, respectively) is a feasible approach in viticulture and whether this last can benefit, increasing its sustainability. Customized SynCom has been inoculated on rooted cuttings before fielding to foster the interaction establishment during the early developmental stages of rooted cuttings. Indeed, Carlström et al. (2019), have recently demonstrated that community assembly is historically contingent and subjected to priority effects, so the early timing of microbiome inoculation is essential to obtain a stable SynCom in planta. Additionally, adult plants are characterized by rich and complex microbiome that remain largely unaffected by latecomers (Toju et al., 2018). Their results indicate also that individual strains of both Proteobacteria and Actinobacteria (both constituting our SynCom), have the greatest potential to affect community structure as keystone species (Carlström et al., 2019). The influence of plant-associated microorganism on plant fitness has been already well demonstrated (Yu et al., 2019). However, new knowledges regarding the effect about microbial inoculants on plant wellness and physiology are needed to better understand plant-microorganism interactions. To achieve this goal, different plant parameters deciphering the effects of SynComs on plant performances have been evaluated. The main finding emerged was a diverse influence on growth-defence trade-off in both SynCom and AMF+B treatments compared to the CTRL. AMF+B-treated plants showed better photosynthesis performances indicating a positive effect on plants fitness, probably thanks to the synergy of AMF and rhizobacteria in accordance with several works in literature (Nerva et al., 2021a). For instance, Gou et al., (2020) provided a theoretical and practical basis for large scale development of integrated biofertilizers using beneficial rhizobacteria confirming their ability in plant growth promotion. Moving to AMF, these fungi improve nutrient uptake in mycorrhizal plants, where the nutritional exchange are thought to occur across the symbiotic interface between the plant and the fungus inside the roots, ameliorating plant growth and physiological performances. Conversely, a negative impact on photosynthesis rates, ACE and iWUE has been revealed by SynCom-treated plants compared to both AMF+B and CTRL ones. These finding was further confirmed by *VvCHL* expression level (gene encoding a chlorophyllase) which appeared higher in SynCom respect to CTRL- and AMF+B treatments, suggesting a growth imbalance likely due to the plant responses triggered by the customized SynCom. Among phytohormones, ABA is well known to play key roles in plants by improving stress tolerance and

adaptation strategies to stressful factors (Egamberdieva et al., 2017). In this study, increased endogenous levels of ABA were observed in roots of both SynCom- and AM-inoculated plants, suggesting an impact on plant response toward abiotic stress and thus making colonized plants more resilient to the incoming climate change scenario (Sharp and LeNoble, 2002). Additionally, IAA hormone concentration, commonly used as growth marker since its involvement in growth and development pathways of plants (Teale et al., 2006), was almost not affected among treatments in leaves while in roots diverse levels were observed in the different treatments. This finding further suggests a diverse growth modulation pathway among SynCom and AMF+B treatments as discussed in the section below (see section 4.3). Bacterial community have been also already reported to activate plants immunity to prime them against pathogen infections. Stilbenes are the main defence-related metabolites synthesized in grapevine with well documented antioxidant and antifungal properties which are modulated by several factors, including the associated microbiota (Verhagen et al., 2010). Here, *t*-resveratrol and viniferin concentrations were measured to understand whether the influence on plant physiology was also accompanied by a sway on the defence metabolites production. The analysis showed that both AMF+B and SynCom treatments led to an increase in stilbenes accumulation both in roots and leaves clearly highlighting a stimulating effect mediated by the associated microbes from both SynCom and AMF+B treatments (Li et al., 2021; Nerva et al., 2021a). In summary, SynCom-treated plants displayed simultaneously better defence responses and worst photosynthetic performance compared to the other treatments suggesting a shift in photosynthates allocation towards defence. Conversely, AMF+B treatment showed potentiated defence responses even if to a less extent with respect to SynCom, without inhibiting photosynthetic rates. Although in the commercial consortium (AMF+B) the bacterial strains are not described, AM-symbiosis positive effects on leaf gas exchanges and biotic or abiotic stress resilience have been reported for several crops, including grapevine, suggesting a major role in priming the inoculated plants (Augé et al., 2016; Chitarra et al., 2016; Goddard et al., 2021; Nerva et al., 2021a; Nerva et al., 2023).

Linking molecular markers with functional ecophysiological and biochemical traits

Plants are known to finely tune the immune system during the interaction with beneficial microorganisms regulating gene expression related to different defence pathways (Alagna et al., 2020). Based on this, to better understand the observed physiological and metabolic responses, selected target genes were analysed by means of RT-qPCR. Firstly, *VvSTS1* (a stilbene synthase gene) showed an opposite trend in leaves and roots with higher values in SynCom and AMF+B leaves. *Vice versa,* likely due to the higher concentration of both stilbene compounds in roots, the gene expression of *VvSTS1* was downregulated. This finding further confirmed an organ-dependent immunity system

elicitation in plants, leading them in a priming status against biotic stresses (Nerva et al., 2021a). Additionally, it has also been demonstrated a role for stilbenes in controlling accommodation of beneficial microorganisms, maintaining a homeostasis in the whole plant associated microbial community as reported by Liu et al. (2020). At molecular level, the presence of a systemic resistance response triggered by both treatments (AMF+B and SynCom) was also evaluated, looking at the expression level of target ISR and SAR defence-related marker genes (*VvLOX* and *VvNPR1*, respectively). Results showed a different trend depending on the treatments. Both SynCom and AMF+B plants displayed signatures of a defence priming status in accordance with previous reports (Goddard et al., 2021; Nerva et al., 2021a). Although in AMF+B the ISR marker gene was not affected, it has been already demonstrated by Cameron et al., (2013) that AM plants can develop an enhanced defensive capacity against pathogens through the so-called 'mycorrhiza-induced resistance' (MIR) that shares some characteristics with SAR and ISR systems (Bruisson et al., 2016; Goddard et al., 2021). Svenningsen et al. (2018) reported that AMF ecosystem services might be negatively affected by some rhizosphere bacterial groups and this finding could explain the non-modulation of *VvLOX* (*i.e.*, ISR marker gene) in AM-colonized plants. In fact, AMF+B treatment, as mentioned before, is a commercial SynCom composed by AMF plus some unknown bacterial strains, due to the absence of information on the label, that could influence AMF-host interaction, in accordance with previously reported by Goddard et al. (2021) in grapevines inoculated with *Rhizophagus irregularis*. Considering the plant immune system elicitation in response to the application of synthetic communities as well as the relationship between the production of defence metabolites and the secondary metabolism pathway, the activation of *VvPAL,* which catalyse the phenylpropanoid pathway, was evaluated. It displayed an up-regulation in both AMF+B and SynCom treatments, confirming the activation of secondary metabolism pathways (*e.g.*, producing phenolics and inducing lignification to prevent pathogen invasion) and leading inoculated plants in a priming status also against environmental stresses in accordance with previous works (Van Huylenbroeck et al., 1998; Oh et al., 2009; Giudice et al., 2022). To verify this point, the expression of the ABA biosynthetic gene (*VvNCED3)* was analysed both in leaves and roots since this phytohormone is involved in responses to different abiotic stresses (*e.g.*, drought) and it can also play a role in the interactions with phytopathogens (Ton et al., 2009; Egamberdieva et al., 2017). Here, ABA endogenous concentration and *VvNCED3* transcripts were strongly 423 higher in roots of both AMF+B and SynCom treatments proving the fact that ABA biosynthesis is positively influenced during the interaction with root-associated beneficial microbes (Chitarra et al., 2016). Similarly, Martín- Rodríguez et al. (2016), reported that ABA biosynthesis is finely tuned in AM-colonized tomato roots and overall data obtained from this study confirmed this trend. About the SynCom inoculated plants, it is interesting

to underlay that, to best of our knowledge, the effects of SynCom inoculated plants on the ABA concentration was never reported before. Indeed, several studies just reported the ability of single isolate to reduce plant susceptibility to drought by lowering the ABA concentration (Curá et al., 2017) or the ability of some specific bacterial strain to produce small amount of ABA (Ullah et al., 2019) that could help upon stress arrival. Here, we report the significant enhancement of ABA concentration in SynCom inoculated roots and the consequent upregulation of *VvNCED3* in root tissues. Such result represents a novel topic that deserve to be deepened by further studies on drought resilience. Since five out seven isolates constituting the SynCom were found to be IAA producer, the plant auxin balance has been evaluated to verify the impact of the IAA produced by the inoculated bacteria. Looking at the expression level of the selected IAA biosynthesis gene (*VvYUC3*), a down regulation was detected in roots of both SynCom and AMF+B with respect to the CTRL. This modulation might suggest that plant is using the IAA produced by the SynCom saving energy to invest in other biochemical or physiological processes. In fact, Bacterial IAA Producers (BIPs), by input the plants auxin pool, showed a positive effect on root system elongation and development, thus helping water and nutrient uptake (Guerrieri et al., 2020). A crosstalk between IAA and induction of SA biosynthesis has been noticed in IAA-primed wheat seeds (Iqbal and Ashraf, 2007). This finding could explain the higher expression level of the target SAR gene (*VvNPR1*) together with the higher IAA concentration in SynCom roots with respect to AMF+B and CTRL plants. Conversely, in AMF+B plants IAA concentration in roots was lower with respect to SynCom and CTRL ones. This is not surprisingly, since AMF are not reported as IAA producers but, as previously demonstrated, mycorrhizal roots showed higher cell hydraulic conductivity (Lpc) and water permeability in maize AM root cells with consequent higher gs and photosynthetic capacity with respect to the non-colonized ones (Quiroga et al., 2019). This is in accordance with gas exchange performances observed in AMF+B-treated plants further highlighting the diverse AMF-mediated growth and defence priming that are IAA independent. In summary, since the new developed SynCom is formed by antagonistic bacteria with strong antibiosis activities against some fungal pathogens, they may, directly or indirectly, boost the plant immune system and the related defence responses. Such responses may hide their PGP effects observed *in vitro*, thus resulting in a shift of the gr 457 growth-defence tradeoffs toward defence responses, limiting photosynthesis performances and water use efficiency conversely to what observed in AMF+B-treated plants.

Concluding remarks

The exploitation of synthetic community selected for biocontrol activity could be considered an interesting tool to manage the unbalanced growth-defence tradeoffs of modern genotypes mostly

shifted towards growth and higher yields. As underlined in the recent literature, modern genotypes are often characterized by low defence and high growth performances leading to a large use of chemical input for field management (Nerva et al., 2022b). In this study, a simplified SynCom formed by seven grape bacterial isolates (firstly identified in a previous metatranscriptomics study, Nerva et al., 2022a) has been formulated and inoculated in young cuttings. In addition to an uninoculated controls, results have been compared with those obtained using a commercial SynCom (formed by AMF and unknown bacteria). The SynCom assembly was performed using isolates with marked antagonistic activity to boost biotic stress resilience in the host plants, leading them in a priming status against pathogens and potentially enabling a reduction in phytochemicals application.

Results suggested that the customized SynCom successfully triggered vines priming activating the defense responses while lowering photosynthetic performances, including carboxylation efficiency and water use efficiency as growth-defense imbalance (Karasov et al., 2017). The used approach takes into consideration physiological, biochemical and molecular responses, giving back a clearer picture of the responses occurring between the plant and its inhabiting microbiome, confirming that a holistic vision can make lights on the "dark side-effects" of SynCom application (Fig. 6). Our results showed that the use of a similar *ad hoc* SynCom could be useful for viticultural areas with high diseases pressure, although negative effects on the photosynthetic performances with potential drawback on plant yields could be also present. However, results obtained with the AMF+B inoculum suggest that the priming for defence due to SynCom inoculation could be potentiated with the contemporaneous application of AMF isolates, leading to improved ecophysiological performances and defence reactions against pathogens in both aerial and underground parts. Lastly, the development of tailored SynComs represents a very useful approach for research purposes aimed to dissect the microbiome-host interaction mechanisms that are still poorly explored in field environment. Additionally, the SynCom approach represents also a promising weapon that could be exploited as future sustainable alternative in agriculture by building tailored SynComs (using both beneficial bacteria and fungi) for specific environmental settings, thus reducing the requirement of agrochemical and/or water inputs.

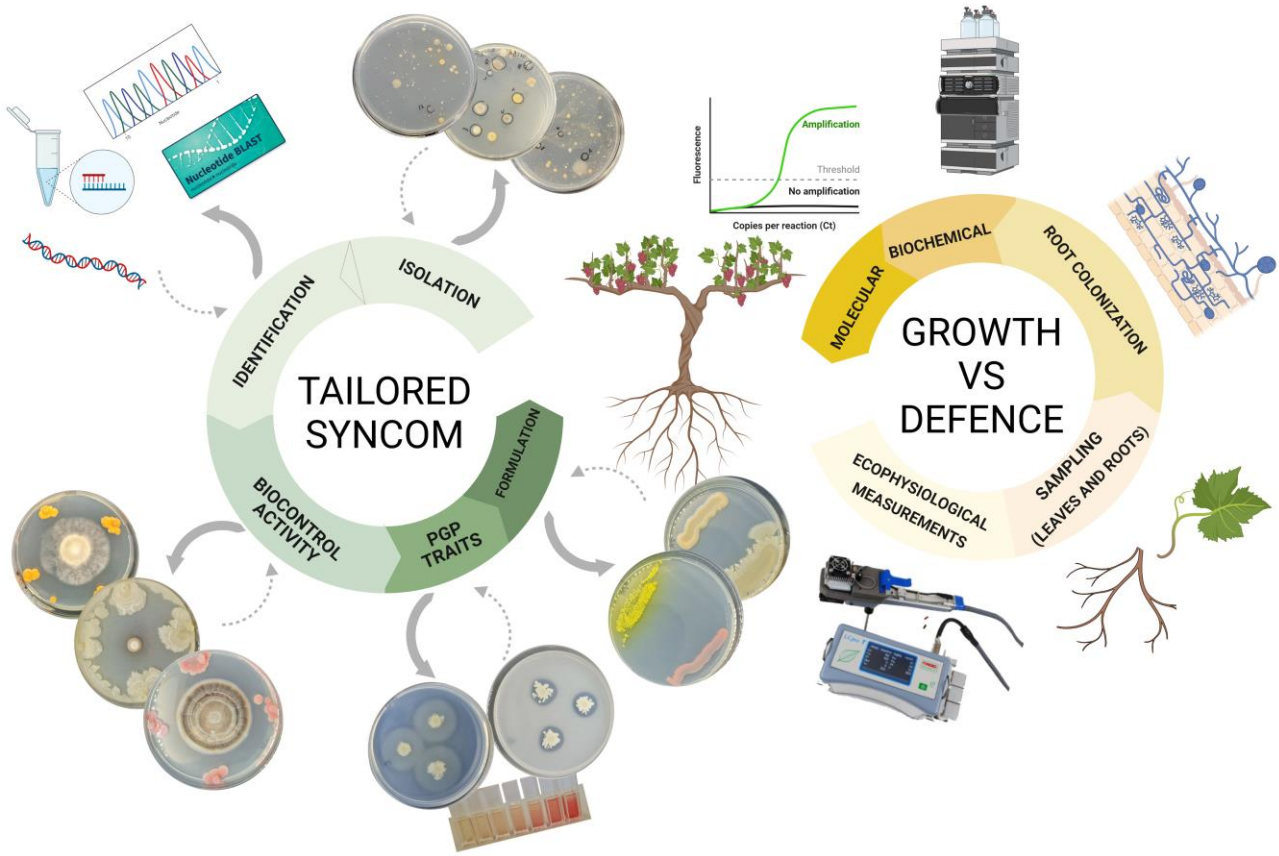


Figure 6. Overview of the holistic approach. To study the SynCom developed in this work we took in consideration several specific features of each isolate (*e.g.*, PGP traits, biocontrol activity, formulation characteristics, etc…) and once formulated we analysed the holobiont responses to decipher the impact on the plant trade-off features. With this approach we were able to observe physiological, biochemical and biochemical rearrangements induced by the SynCom application.

Materials and methods

Culture-dependent identification of bacteria isolates

To ensure the capability to stably colonize grape tissues, cultivable pure bacteria were previously isolated from the inner woody tissue of field-grown *Vitis vinifera* cv Glera plants as reported by Nerva et al., (2022a) and stored in the CREA – Research Centre for Viticulture and Enology microbial bank (https://www.revine-prima2020.org/vimed) both as lyophilized and glycerol stocks (40% v/v) at -80 °C. Forty-four isolates were identified, after total genomic DNA extractions, using the 16S sequence amplification and sequenced by Sanger method at BioFab Research srl (Italy). A search for similar sequences was conducted with the BLAST tool on the GeneBank database as reported in Table 1.

In vitro *antagonism screening against grape fungal pathogens and volatile effect*

The ability of bacterial isolates to control *Botrytis cinerea* (bunch gray mold causal agent), *Phaeoacremonium minimun*, *Neofusicoccum parvum* (the latter two are key players in the esca syndrome) and *Guignardia bidwellii* (black rot causal agent) was evaluated *in vitro*. To perform biocontrol activity tests, each isolate was grown on solid media (CYA) for at least 5 days and then inoculated four times at the boundary of a 90mm Petri dish containing CYA media. The specific pathogen was inoculated 48 hours later as mycelial plug or as conidial suspension according to specific pathogen characteristics and then monitored for growth. Each combination of bacterial isolate and fungal pathogen was made in triplicate and the colony diameter measured twice for each biological replicate and each time point. For *B. cinerea* and *N. parvum* (both considered as fast growing) the biocontrol assay lasted 10 days, for *P. minimum* and *G. bidwellii* (both considered slow growing) the assay lasted for 20 days. Additionally, when inhibition was observed, the potential antifungal activity of volatile organic compounds (VOCs) was evaluated according to (Oukala et al., 2021). In detail, the two room-plate method was used against the fungal pathogens reported above using at least three replicates for each bacterial isolate. After incubation at 28 °C, the percentage of mycelial growth inhibition was recorded and compared with their respective controls (Table 1). The 7 best performing isolates were selected for the evaluation of PGP traits (see below) and for SynCom formulation.

In vitro *evaluation of plant growth promoting traits and compatibility test*

In vitro compatibility test was performed between the 7 selected isolates (Table 1, bold highlighted isolates) on CYA plates in triplicate. Each bacterial strain, after overnight culture (500 µL, CYA media), was streaked in solid media plates and co-cultured with each of the others. After ten days at 28°C, the growth was compared with a control plate where the isolate was cultured alone and, if present, inhibition effects were reported. The selected isolates were also screened and evaluated for different PGP traits such as production of indole acetic acid (IAA) (Guerrieri et al., 2020), ACC-deaminase activity (Li et al., 2011), siderophore production (Louden et al., 2011), N fixation (Geetha et al., 2014) using Jensen's Nitrogen Free bacteria (JNFb) medium, phosphorus solubilization (Singh et al., 2020), starch hydrolyzation (Kokare et al., 2004) and salt stress resilience at diverse % of NaCl (0%, 1,5%, 3% w/v) (Gopalakrishnan et al., 2014) (Table 2). To evaluate IAA-production and ACC-deaminase activity, quantitative methods were adopted, whereas for siderophore production, phosphorus solubilization, starch solubilization and salinity resistance semi-quantitative methods were used. Finally, N fixation activity was assessed by qualitative method (presence or no presence of growth). In detail, regarding to phosphorus solubilization, bacterial ability was analyzed measuring

the halo zone diameter around the bacterial colonies: isolate with a halo bigger than 0 mm up to 1 mm was considered as slight activity (+), a halo bigger than 1 mm up to 5 mm as medium activity (++) and a halo bigger than 5 mm as high activity (+++). Data for siderophore production was instead classified as: zone of yellow halo appearance bigger than 0 mm up to 2 mm was evaluated as slight siderophore production, zone of yellow halo appearance higher than 2 mm up to 5 mm as medium siderophore production and a zone of yellow bigger than 5 mm as good siderophore production. Moving to starch solubilization, data were classified as: a halo bigger than 0 cm up to 0,4 cm was considered as slight solubilization (+), a halo bigger than 0,4 cm up to 1 cm as medium solubilization (++) and a halo bigger than 1 cm as high solubilization (+++). Finally, for salinity resistance three different classes were set based on bacterial growth intensity: low growth (+), medium growth (++), good growth (+++).

Plant inoculation and field experiments

Two hundred and thirty cutting vines of 'Pinot gris' cultivar grafted onto Kober 5BB and certified as 'virus free' were purchased from an Italian nursery (Vivai Cooperativi Rauscedo, Italy; http://www.vivairauscedo.com). Cuttings were treated as previously reported in (Nerva et al., 2021a) prior to plantation. Three treatments were compared in this study: i) non-treated control plants, CTRL; ii) SynCom-inoculated plants, SynCom; iii) SynCom-inoculated plants with a commercial consortium formed by different AMF species and rhizosphere bacterial strains, AMF+B. The experiments were repeated twice, two independent rounds of cuttings inoculation and transplanting were performed for both SynComs (50+50 plants for each) while as CTRLs, 15 and 15 uninoculated plants were used for the first and second round of experiments. As cited before, in this study we designed a 7-strain SynCom with isolates showing strong antagonistic activities previously isolated from grapevine woody tissues (see above) (Nerva et al., 2022a). The seven selected and compatible strains were mixed to form the SynCom and inoculated in roots of one-year 'Pinot gris' cuttings using equal volume of each strain (~ 10^8 cells mL^{-1}). In detail, prior to field planting, cuttings were maintained for 30 days in a plastic container filled with sterilized substrate (80% sand and 20% peat) supplemented with the formulated SynCom to a final concentration of 10^6 cells mL^{-1} of substrate. For AMF+B treatment, grapevine cuttings were inoculated with a soluble powder-based commercial SynCom (MycoApply DR, Sumitomo Chemical Agro Europe SAS) formed by AMF mixed inoculum (*Rhizophagus irregularis*, *Claroideoglomus luteum*, *Claroideoglomus etunicatum*, *Claroideoglomus claroideum* corresponding to 1% of the total inoculum as reported in the label) with rhizosphere bacteria (2,180,000 UFC g^{-1}) following manufacturer instruction. As for the 7-strain SynCom, AMF+B inoculated cuttings were maintained for 30 days in containers with steam sterilized substrate

amended with the commercial inoculum. For CTRL plants, cuttings were prepared similarly and maintained in the same substrate for 30 days but without any microbiological inocula. Trials were carried out in a semi-controlled experimental field, specifically dedicated to this experiment, located at Cantine Rauscedo, Rauscedo, Italy (GPS coordinates: 46.054978N, 12.816345E). The about 3000 m^2 of vineyard available for this study was composed of sandy loam soil (pH 7.3; available P 8.4 mg kg^{-1}; organic matter 1.70%; cation exchange capacity 22.11 mew 100 g^{-1}) that was not cultivated for three years prior to our experiments. After 60 days from field planting, leaf ecophysiological measurements and sampling of leaf and root tissues for molecular and biochemical analyses were performed. The collected samples were freeze-dried and stored at -80 °C until use. A part of the root apparatus was used to estimate the level of mycorrhiza colonization (*i.e.*, arbuscule abundance in the root system by morphological observation of thin roots fragments as previously described (Chitarra et al., 2016; Nerva et al., 2021a). Data obtained from the two independent experiments were collected and biological replicates (at least 4 for each replicate) were mediated and analyzed (see below).

Leaf gas exchange measurements

Instantaneous measurements of net photosynthesis (Pn), stomatal conductance (gs), intercellular CO_2 concentration (Ci), apparent carboxylation efficiency (ACE, calculated as ratio between Pn and Ci) and intrinsic water use efficiency (iWUE, obtained as the ratio between Pn and gs) were carried out on 6 randomly selected vines from each treatment. For each plant three fully developed non senescent leaves at the same physiological age (4^{th} to 5^{th} leaf from the shoot apex) were measured using a portable infrared gas analyzer (ADC-LCi T system; Analytical Development Company, BioScientific Ltd., UK) as previously reported (Belfiore et al., 2021). During measurements, ambient parameters such as light intensity ranged from 1.600 to 1.700 μmol photons m^{-2} s^{-1}, temperature ranged from 25 to 28 °C and the concentration of the CO_2 in the air ranged from 420 to 440 ppm.

Targeted metabolite analyses

Leaf and root collected samples were used to determine abscisic acid (ABA), indole-acetic acid (IAA), *t*-resveratrol and viniferin concentration using a high-performance liquid chromatographer (HPLC) (Nerva et al., 2021a; Nerva et al., 2021b). Briefly, for each time, treatments and biological replicate, 100 mg of freeze-dried samples were aliquoted with 1 mL of extraction buffer (80% methanol-H2O, 8:2 v/v, with 0.1% v/v of acetic acid). The mixture was then sonicated in an ultrasonic bath for 1h at maximum intensity. After sonication, samples were centrifuged at maximum speed for 10 min at 4°C and filtrated using a 0.20 μm PTFE membrane filter (Chromafil® Xtra PTFE-20/13, Macherey Nagel). The supernatant was analyzed by an HPLC apparatus, Agilent 1200 Infinity LC

system model G4290B (Agilent, Waldbronn, Germany) equipped with gradient pump, auto-sampler and column oven set at 30°C. A C18 column (4.6 mm x 150 mm, 5 µm, XTerra®RP18) was used for the chromatographic separations. Original standards of ABA, IAA, *t*-resveratrol and *t*-viniferin (purity ≥ 98.5% and ≥99% for the latter two respectively, Merck KGaA, Darmstadt, Germany) were used to prepare the calibration curves and for the identification by comparing retention time and UV spectra. Analysis was run in reverse phase with an elution gradient method: eluent A was 0.1% formic acid in water and eluent B was acetonitrile; flow rate was fixed at 500 µL min^{-1}. From 10% to 35% of B in 20 min, from 35% to 100% of B for 5 min, from 100% to 10% in 1 min and conditioning for 10 min. Twenty microliters were injected for each sample and at least three biological replicates were run for each treatment.

RNA isolation and RT-qPCR

Root and leaf samples were processed to isolate RNA starting from at least 50 mg of lyophilized tissues using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) following manufacturer's instructions (Nerva et al., 2021b). RNA concentrations were checked using a Nanodrop™ (Thermo Fisher Scientific) apparatus. Then, RNA samples were treated with DNase I (Thermo Fisher Scientific) following manufacturer's instructions. The absence of DNA contamination was checked prior cDNA synthesis by quantitative real-time PCR (qPCR) using *VvCOX* grapevine specific primer (Table S1). After DNAse treatment, samples were subjected to cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific), starting from 500 µg of total RNA. qPCR runs were performed in a final volume of 10 µL using SYBR® green chemistry (Bio-Rad Laboratories Inc.) and 1:5 diluted cDNA as template. Reactions were performed in a Bio-Rad CFX96 instrument (Bio-Rad Laboratories Inc.) using the following conditions: denaturation phase at 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. Each amplification was followed by a melting curve analysis (65-95 °C) with a heating rate of 0.5 °C every 5 s. All reactions were performed with at least two technical replicates. Relative expression level was calculated by the comparative cycle method using plant reference genes (elongation factor, cytochrome oxidase and ubiquitin, *i.e.*, *VvEF*, *VvCOX* and *VvUBI* for root, and *VvCOX* and *VvEF* for leaf tissues) for gene expression normalization. Oligonucleotide sequences are listed in Table S1. Gene expression data were calculated as expression ratio (relative quantity, RQ) to CTRL plants.

Statistics

Data were analyzed by analysis of variance (ANOVA). When ANOVA was significant, mean separation was performed according to Tukey's HSD test at a probability level of $P \leq 0.05$. Standard deviation (SD) or error (SE) of all means was calculated. The SPSS statistical software package (version 22) was used to run statistical analyses.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression changes of photosynthesis and hormones-related genes. **a)** Relative expression level of *VvChl* in leaf. **b,c)** Relative expression level of *VvNCED3* in root and leaf tissues, respectively. **d,e)** Relative expression level of *VvYUC3* in leaf and root tissues, respectively. Data are expressed as mean ± SD (n = 3). Different lowercase letters above bars indicate significant differences according to Tukey's HSD test ($P < 0.05$). CTRL, control plants; AMF+B, commercial AMF + Bacteria mixed inoculum-treated plants; SynCom, Synthetic Community-treated plants.

Supplemental Table S1. Oligonucleotides used in this study. Acknowledgments The authors are grateful to Dr. Giancarlo Babbo formerly employed in Sumitomo Chem Italia for kindly providing MycoApply product used in this study.

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Conflict of interest statement. There are no conflicts to declare.

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CHAPTER 6 | BREEDING TOWARD IMPROVED ECOLOGICAL PLANT MICROBIOME INTERACTIONS

Trends in Plant Science

Opinion

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Abstract

Domestication processes, amplified by breeding programs, have allowed the selection of more productive genotypes and more suitable crop lines capable of coping with the changing climate. Notwithstanding these advancements, the impact of plant breeding on the ecology of plant–microbiome interactions has not been adequately considered yet. This includes the possible exploitation of beneficial plant–microbe interactions to develop crops with improved performance and better adaptability to any environmental scenario. Here we discuss the exploitation of customized synthetic microbial communities in agricultural systems to develop more sustainable breeding strategies based on the implementation of multiple interactions between plants and their beneficial associated microorganisms.

Keywords

Domestication Syndrome, Holo-omics Approaches, Holistic Breeding, Mycorrhizal Symbiosis, Plant Microbiome

Domestication syndrome and plant-microbiomes

Plant domestication (see Glossary) is the outcome of a selection process that leads to increased adaptation of plants to cultivation and utilization by humans [1]. This process is based on the implementation of breeding programs via the selection and modification of wild plant species and aims to select for useful plant traits for human requirements. Increasing crop yield has consistently been one of the most important goals during the domestication process to provide humans with a continuous and constant food supply [2]. However, plants do not have unlimited quantity of energy and the allocation of limited carbon sources is consequently influenced by a growth-defence trade-off [3]. This phenomenon is based on the concept that the limited carbon sources produced by photosynthesis are allocated toward growth or defence processes to maximize the plant's adaptation strategies and fitness costs in diverse environments [4]. If plants focus their energy mainly on growing, they automatically have less ability to deal with different kind of stresses such as pathogen infections or harsh environmental conditions. Furthermore, domesticated plants are much more nurtured than their wild parents, through fertilization, irrigation, and other protective measures, so they are characterized by less ability to interact with or adapt to the surrounding environment.

The ability of plants to interact with thousands of microorganisms that are surrounding and supporting them in dealing with both biotic and abiotic stresses, is one of the most important traits that should be reinforced. Several studies have already demonstrated that plant-associated microorganisms are essential to improve plants' wellness and sustainability of agricultural systems [5,6]. Modern agriculture is entering into a second green revolution and the exploitation of beneficial soil microorganisms is playing a significant role with several microorganism-based products currently coming to the market [7]. However, there is evidence that domestication processes have profoundly altered the interactions between plant hosts and associated microorganisms [8,9]. Recent studies have found significant differences between the microbiome of commercial genotypes with that of their relative wild types. It has been shown that wild ancestors and primitive landraces of wheat (*Triticum aestivum*), breadfruit (*Artocarpus altilis*), and maize (*Zea mays*) can benefit more from mycorrhizal symbiosis [10,11] compared to selected cultivars, suggesting that the modified microbiome due to domestication is not beneficial to the plant. Studies on other plants species, including arabidopsis (*Arabidopsis thaliana*) [12], sugar beet (*Beta vulgaris*) [13], barley (*Hordeum vulgare*) [14], and lettuce (*Lactuca sativa*) [15], also suggested that human-centered breeding led to compositional changes in root-associated microbiomes. How this negative trend can be mitigated, and the plant-microbiome equilibrium restored are still open questions. Although the tight interactions of indigenous crops with the associated microbiota reinforce the ability and flexibility of crops to deal

with diverse environmental stresses, a difficulty for exploiting their potential is correlated with the low ability of separating the targeted functional microbes [16]. We also still need to understand which plant traits maybe involved in the interactions with beneficial microorganisms and how we can identify them and select for them in future breeding programs (see **Outstanding questions**).

The neglected shortcomings of traditional and new plant breeding techniques.

Intersection of anthropocentric breeding and plant–microbiome interactions

The incessant selection of genomic plant traits, and the considerable number of inputs needed to sustain the selected genotypes, negatively influence the interactions among plants and beneficial microorganisms [17,18]. Breeding processes reduced in fact the microbial biodiversity and functioning associated with plants in agricultural systems, hampering the essential interactions that make wild species more resilient to biotic and abiotic stresses (Figure 1) [18]. This effect is most probably due to the fact that the selected crop cultivars might have lost some of the genetic traits needed to recruit host-specific microbiota as compared with their wild relatives. It was shown that long-term nitrogen fertilization resulted in the recruitment of less and less-functional rhizobacteria in leguminous species, providing so fewer benefits to the host [19]. Similarly, Kiers et al. [20] demonstrated that older soybean cultivars had a higher ability to reach their full symbiotic potential in the presence of a rhizobia-strain cocktail, with different symbiotic effectiveness compared with newer soybean cultivars. Furthermore, Chaluvadi and Bennetzen [21] have demonstrated that there are species-specific differences in the belowground microbiome associated with wild and domesticated Setaria, highlighting how crop domestication plays an important role in selecting prokaryotes present in the rhizosphere. There is also evidence of the impact of plant breeding on the assembly of rhizosphere fungal communities that seem to be strongly influenced by host genotype [22]. Recent studies have shown that root exudates are essential for plants to assemble a functional microbiome and changes in plant genetics derived from breeding programs result in different root exudate composition, undermining microbiome assembling and functioning [18,23] and playing a role also in symbiotic relationships [24,25]. Martín- Robles et al. stated that colonization by arbuscular mycorrhizal fungi is lower, whereas the infection rate by nematodes is higher, in the roots of plants that grow in soils previously cultivated by domesticated plants in comparison with wild progenitors [26]. Conversely, rhizosphere microbial communities induced systemic changes in tomato root exudates, suggesting the presence of a long-distance signaling [27]. Despite the improvement in understanding root exudate composition, little information is available so far about exudate spatial distribution and regulation in root, considering that homogenized samples are

generally used [28]. Döll et al. dissected the root in the three fractions analyzed for tissue-specific metabolic profiles, correlating those profiles with protein abundances involved in biosynthetic pathways resolved spatially and showing the presence of differentially abundant compounds in diverse root tissues and exudate of asparagus roots [28]. Volatile organic compounds (VOCs) also have a relevant role in the communications and interactions with rhizosphere-inhabiting microorganisms, and they are signal molecules with a potential for application in agriculture [29]. VOCs emitted belowground can stimulate the migration of distant soil bacteria, attracting them toward the roots. Additionally, changes in the blend of root VOCs, due to a stress situation such as a fungal soilborne pathogen infection, lead to the recruitment of specific beneficial bacteria from outside the rhizosphere that might have a role in helping plants to cope with the stress condition [30]. However, rhizosphere and root-associated microbiota, in addition to supporting plant growth, also offer a further level of genetic variability that was little considered by breeders until now [31]. Future breeding strategies should treat root features, including traits related to the microbial recruitment (Box 1), and strategies for root phenotyping should be implemented, also considering the interactions with rhizosphere microbiota. A deeper investigation of the relationships between roots and associated microbiota is particularly relevant to breeding highly efficient root systems and the consequent selection of climate change–resilient genotypes [32].

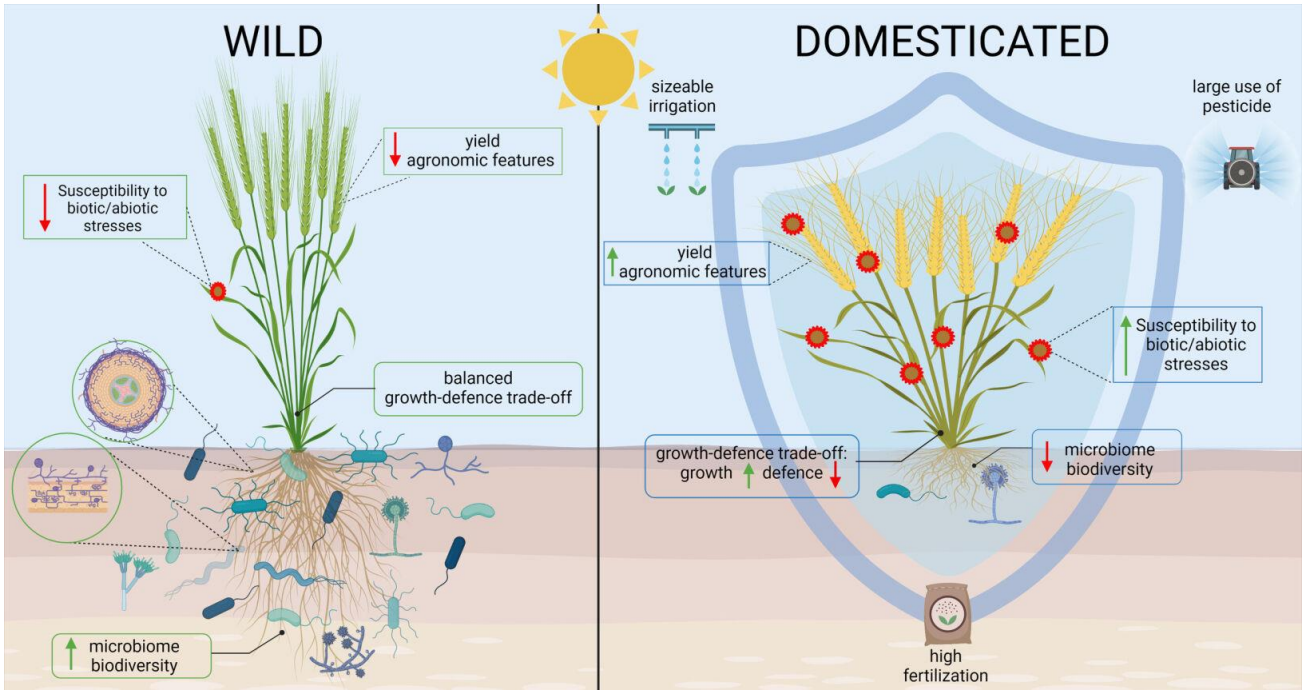

Figure 1. Comparison between wild and domesticated plant species. (A) Wild relative displays a better adaptation to environmental stresses (both biotic and abiotic), a balanced growth–defense trade-off, and a rich associated microbiome, but a low yield. (B) Domesticated plant shows an improved productivity but a reduction of both stress resilience and ability to recruit plant-associated microbes. To cope with the increased susceptibility, human practices such as irrigation, fertilization, and pesticide application are needed.

Despite breeding programs that aim to produce cultivars resilient to diseases and environmental stresses, many traits are regulated by several genes (i.e., polygenic resistance) and, for this reason, are hardly transmissible to the progeny in a single crossing [33]. In this scenario, the monogenic resistance, exploited by conventional breeding programs, is highly effective in moving single gene traits, but it is easily suppressed by the pathogen over time [34,35]. Regarding woody plants, breeding programs encounter several further limitations, such as linkage drag, long and laborious times needed for backcrossing, and high heterozygosity degrees, hampering the development of resilient genotypes and raising the costs [36–38]. The obtained resilient/resistant genotypes are often associated with modifications that could be detrimental from the commercial point of view, such as an altered phenotype and/or biochemical profile (related to texture, taste, and/or organoleptic profiles) [39], often less accepted by consumers [40,41].

Application of biotechnological approaches in breeding processes has recently led to development of new plant breeding techniques (NPBTs), able to modify specific target DNA sequences without altering other regions and, if applied with the DNA-free or marker-free approaches, without the need of long backcrossing stages [42–44]. These techniques are very promising to overcome the limits imposed by traditional breeding in terms of both time and costs. Although NPBTderived

products are accepted in several countries, many restrictions remain, especially in Europe [45,46], and traits related to the ability in recruiting beneficial microbiota are not so far sufficiently considered. Roots in fact share their habitat with many microorganisms, such as bacteria and fungi, that can have a positive role on plant productivity and tolerance/resilience to environmental stresses [47–51]. The exploitation of these microorganisms to improve plant traits may overcome the limits associated with both conventional breeding and NPBTs, leading to a reduced impact on the marketable characteristics of the final products, preserving the original genotype and therefore without requiring specific safety assessments on the products (e.g., those for genetically modified organisms) and leading to a less expensive and less time-consuming application than breeding programs, especially for woody plants (Figure 2).

A comprehensive understanding of mechanisms governing the selection of microbial community by the plant will provide useful information to improve future agriculture. The application of novel approaches, such as (i) the selection and characterization of specific microorganisms to restore growth–defense trade-off balance in commercial genotype, (ii) the exploitation of complementary omics tools, and (iii) the synthetic microbial communities (SynComs) application seems to be a good way to reach this goal.

Box 1. The importance of root traits

Efficient root systems are essential to enhance crop productivity; indeed, studies on plant genotypes better adapted to stresses are now focusing on root traits [89,90]. The use of sophisticated systems and sensors enabled researchers to follow the development of the root system and to evaluate the uptake of water and nutrients at the root level. Plants can adapt root apparatus to optimize the availability in water and nutrients, thus having an impact in plant resilience and productivity [91]. However, they do not live alone in the soil, and root-associated microorganisms are known to play a fundamental role in plant adaptation to adverse environmental conditions. One of the main questions regarding plant–microbe interactions concern the identification of functional mechanisms that plants exploit to shape their microbiome. It is worth noting that if, on the one hand, root traits are able to influence the composition of the root-associated microbes, on the other hand, microbes are able to interact with the plant-modifying root traits [92]. Among root features, architectural traits (root depth/angle, length, density) and morphological traits (root hairs, root diameter, aerenchyma/cell size, root cap properties) are the physical properties playing important roles in the two-way interaction by specifically altering the plant–microbe interaction interface [88,93,94]. In parallel, the unique biochemical fingerprint profile of a plant deeply contributes to the recruitment, colonization ability, and function outline of specific microbiomes [95]. The root exudates, and the cell wall composition, vary considerably according to the age, developmental stage, and species also between different genotypes and varieties of the same species and constitute a primary feed source for the rhizosphere-associated microorganisms, making the soil surrounding roots more suitable for bacterial and fungal proliferation [96]. The influence of root architecture and exudates on rhizosphere and root environments, and consequently on the microbial recruitment, nowadays represents a hotspot in research aimed at studying root interactions and is always attracting more attention [97–99]. Agricultural sustainability can be potentially boosted by implementing breeding programs able to consider the abovementioned root properties, enhancing the possibility to recruit microbiomes with beneficial traits. Linking root phenome to specific microbiomes (or related functional traits) may enhance our understanding of the interactions occurring in the rhizosphere and the role that these interactions play in generating climate-resilient agroecosystems [100]. Important aftermath of such approaches will impact significant soil- and root-related functional aspects such as N fixation and cycling, P dynamics, C dynamics, and plant water availability. Recently, Brisson et al. [99] suggested that root exudation in response to phosphate stress was conserved during the maize domestication (teosinte versus maize), shifting the exudation levels of specific metabolites and the microbial communities, although selective recruitment of phosphate solubilizers in response to phosphate availability has not been observed [99].

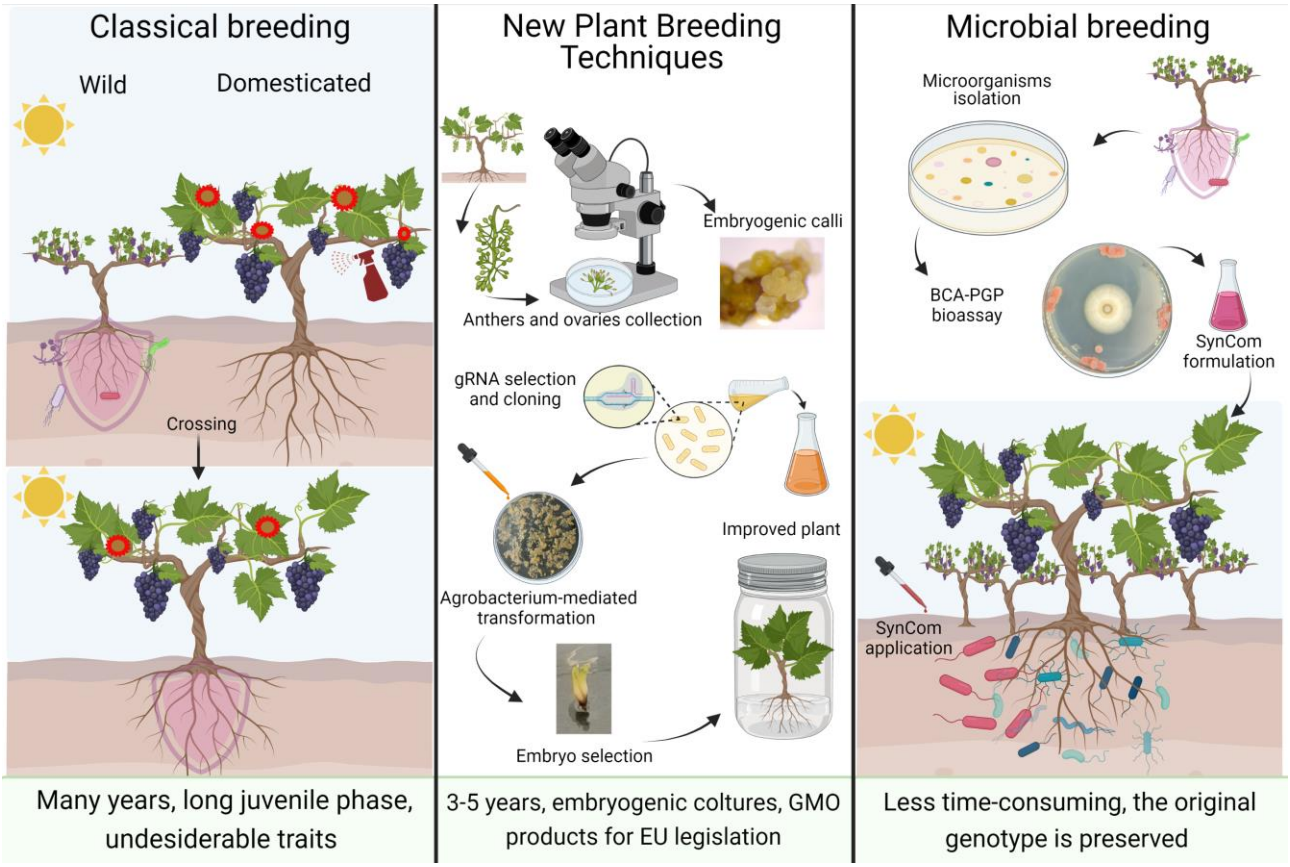

Figure 2. Overview of the classical breeding and new plant breeding techniques and the microbial breeding approaches for woody plants. (A) Classical breeding relies on the possibility to transfer traits (e.g., related to biotic or abiotic stress resilience) from wild sexually compatible species to domesticated species of high economic importance through crossing. The main limitation in such an approach, especially for woody species, is the presence of juvenile stages, which increase the time for backcrossing and the transmission of undesired traits (linkage drag). (B) New plant breeding techniques allow one to reduce the time needed for conventional breeding, but they still present limitations such as the need for specific tissues and/or cellular types (e.g., embryogenic calli) and several limitations related to genetically modified organism regulations. (C) Microbial breeding approach that can overcome the limitation of the classical breeding techniques, preserving the original genotype and reducing development times and costs of a synthetic community (SynCom).

Microbiome-mediated dynamism in the allocation of plant resources

Allocation of resources to priority biological processes, restricting others, can give rise to trade-offs between different processes/responses, mainly in natural environments where plants generally cope with limiting resources [52]. Commercial genotypes are often characterized by an unbalanced allocation of energy resources, and microbiomes represent a promising tool to restore the growth–defense trade-off balance in these plants, achieving an agricultural system able to survive with limited external inputs. Analyzing plant microbiomes and trying to select and identify genomes of microorganisms able to improve plant growth or resilience to several stresses could be the right way forward [52–54]. Recently, an involvement of arbuscular mycorrhizal fungi (AMF) in balancing growth–defense trade-off has been suggested in grapevine (Vitis vinifera), where rootstock genotypes

and AMF inoculation have a relevant role in shaping the root-associated microbes and stimulating growth and defense pathways [54]. Cultivars selected for quality and/or quantity yield are usually more sensitive to biotic and abiotic stresses: Using microorganisms able to place the plants in the so-called priming state allows them to restore the natural trade-off equilibrium, obtaining more resilient cultivars [54–58]. Priming (or acclimatization) is a complex phenomenon that consists in preconditioning the plant biotic and abiotic defense mechanisms so that responses to stress result quicker, stronger, and more effectively [59]. The simultaneous employment of biological as well as chemical agents toward further improved plant performance is gaining interest among researchers with the aim to maximize the primed status [60]. Additionally, to enable a state of priming, several rhizosphere-associated microorganisms can perform a direct antagonism toward several pathogens acting as plant allies [61,62]. However, a genotype characterized by high defense performance and low yield can be made more productive through the exploitation of plant growth–promoting (PGP) microorganisms instead of modifying their own microbiome with long breeding programs. Furthermore, because most of the resistant cultivars obtained by conventional breeding showed less crop quality and yield capacity, the possibility to exploit PGP microorganisms could partially restore the original features, avoiding plant genetic manipulation [54,63]. 'Resistant' cultivars are characterized by specific resistances toward target pathogens, but they do not make provisions for the rest of biotic and abiotic stresses. By contrast, an enrichment of certain taxa in microbiome composition guarantees a broad-spectrum tolerance toward several pathogens contributing to plant phenotypic plasticity and adaptability to the changing environment [64,65]. It has been shown that specific microbiota members can modulate plant immunity processes through bidirectional microbiota–root–shoot mechanisms relevant for plant health [66,67]. A functional and rich microbiome can stimulate plant immunity and modulate the allocation of carbon plant resources, shifting them from growth to defense processes [68,69] (see Outstanding questions). Regarding grafted plants, the choice of the rootstock genotype plays an important role in shaping microbes inhabiting the rhizosphere and the allocation of carbon resources. Indeed, rootstocks differ from each other by specific growth–defense trade-off features and for the ability to recruit different microbial consortia [69]. Modulating the interactions between rootstocks and their own associated microbe, growth and defense features could be managed through the bidirectional root-to-shoot mechanism, making grafted plants more suitable to sustainable practices.

SynComs to enhance holobiont functionality

Unearthing the functional relationships between plants and their microbial partners is the next step for improving plant fitness and for adopting breeding programs focused on the holobiont [70]. Natural

microbial communities and the interactions between them and their host plants are known to be very complex and variable and are not fully understood yet. This complexity derives from the large number of microbes inhabiting the environment coupled to the often-unknown functions for most of them and from the uncharacterized interactions occurring among one another [71]. The SynCom assembly seems to be a promising approach to exclude confounding environmental effects and to reduce the complexity of natural systems [72,73]. The establishment of a SynCom is grounded on collected knowledge of the overall composition of the root-associated microbiome needed to formulate a 'core microbiota' [72]. Carlström et al. [74] showed how community assembly is subject to priority effects (i.e., the imprint of arrival order on community structure), and, additionally, they indicated that specific strains have the greatest potential to affect community structure as keystone species. Once the keystone strain collection and the molecular identification is achieved, these microorganisms have to be tested for their antagonistic activity toward pathogens and PGP traits and for keeping out the possibility that they can have a reciprocal inhibition effect. microorganisms showing traits of interest can be used to establish a SynCom being tested before in controlled environments and subsequently in a natural environment [75–77]. Additionally, through the SynComexploitation, it is possible to highlight the role of the plant immune system in the assemblage of a protective microbiome [78]. Recent studies have demonstrated that plants can recruit beneficial bacteria upon pathogen infections, specifically disease resistance–inducing and growth-promoting ones [66,75]. Although the effectiveness of the SynCom application in agriculture has been proved to be often inconsistent due to low efficiency in establishment and survival of the selected taxa [79–82], the SynCom approach seems to be pivotal for capitalizing on associated microorganisms, increasing the agroecosystem resilience, and finally driving new breeding programs. Themain reasons for failures are related to the plant-associated microbes' ability to exert their beneficial effects. These can be reduced for several reasons that are closely linked to the host plant genotype, the microbial species compatibility with the growth environment, the spatial competition with other soil microorganisms, and the persistence in soil. The ecological interactions occurring with the naturally occurring microbial population is one of the most important aspects that must be considered when applying SynComs in real environments. The importance of ecologically based community assembly rules has been demonstrated by the survival of SynComs developed using Pseudomonas spp.: Survival rate was directly and positively correlated to the diversity of the developed consortia [83]. Additionally, the community growth and development are affected by several factors, including growth substrates and the presence of other chemical compounds [84]. The still limited knowledge on the mechanisms underlying plant ability to control its associated microbial communities and how members of microbial consortia interact with one another also strongly limit their exploitation in agriculture [79,85,86]. Interestingly, arbuscular

mycorrhizal responsiveness seems to be subjected to 'genotype × environment interaction' that refers to the phenomenon in which diverse genotypes respond to different environments in different manners [87].

Regarding the impact of human-focused breeding on microbiome assembly and functionality, additional insights into microbiomes of wild plants and native habitats could contribute to reinstating or enriching current genotypes of microorganisms with beneficial effects on plant growth, development, and health [81,88]. Thanks to a combined approach (e.g., highthroughput plant phenotyping, identification of the core microbiota strictly linked with a specific genotype, inoculation of personalized SynCom, and analysis of plant responses at molecular level), it is workable to find some microorganisms, strictly linked with a wild genotype, that can be used to restore traits lost during breeding programs.

Box 2. Holo-omics studies: Detailed information on host–microbiota interactions

To develop protocols for a new kind of breeding linked to the holobiont concept, a more detailed knowledge of the mechanisms governing plant–microbiome interactions is needed [101]. Holo-omics (i.e., the incorporation of data across multiple omic levels from both host and microbiota domains) may have the potential to resolve the functionality of a plant microbiome ecosystem by generating an image of what is being expressed, translated, and produced during plant microbiome interactions [101,102]. The combination between host and microbial datasets provides in fact a powerful approach for the development of hypotheses and advancement in the topic of plant interactions [101]. Pairing host centered omics tools, such as transcriptomics, epigenomics, and proteomics, in combination with the more commonly used microbe-focused techniques such as amplicon sequencing, shotgun metagenomics, metatranscriptomics, and exometabolomics, seems to be a very promising approach to achieve a more integrated knowledge of plant microbiome functions [101–103]. A great number of works grounded on this current approach are nowadays present in the literature [104,105]. Recently, Castrillo et al. [106] have explored the relationship between phosphate starvation response (PSR) and microbiome composition and functionality in Arabidopsis [106]. Through a holo-omics design (16S profiling and RNA sequencing), they have demonstrated that the plant root microbiome directly connects phosphate stress response and plant immune system and that gene controlling PSR contributes to the root microbiome assembly. Microbial communities of PSR mutant plants were distinct from those of the wild type, and inoculation of a specific SynCom enhanced the activity of a master regulator of PSR (PHR1) under limited phosphate conditions, confirming that PHR1 directly regulates a functionally relevant set of plant-microbe recognition genes. Moreover, Stringlis et al.

[107] demonstrated, using metagenomic and metabolomic approaches, that beneficial rhizobacteria induced excretion of the metabolite scopoletin that stimulates iron uptake and suppresses soil-borne pathogens in Arabidopsis. A combined use of omics approaches led to information on the so-called soil memory. Particularly, Li et al. [108] applied metagenomics to characterize the peanut (Arachis hypogaea) rhizosphere microbiome and root metatranscriptomics on peanut plants grown in a soil with different management histories, such as monocropping and crop rotation. These authors found that the past planting record had a significant impact on the assembly of the microbial community in the peanut rhizosphere, indicating a soil memory effect affecting crop rhizosphere microbiomes and plant physiology. Thanks to multiomics and bioinformatics technologies, it is now possible to identify core interactions between plants and native microbiomes pointing out useful traits for breeding purposes.

Concluding remarks

Domestication, focused exclusively on the selection and improvement of specific plant traits, is by its very nature an anthropocentric breeding program and has negatively affected the recruitment and/or the functionality of beneficial holobiont-associated microbes in agricultural systems. Plant-associated microorganisms are known to improve plant growth and wellness, and thus the plant's ability to interact and cooperate with microorganisms should be considered as a fundamental trait in modern breeding programs. Applying a holistic vision of plant breeding and including the exploitation of 'holo-omics' techniques (Box 2) will lead to a deeper understanding of the hidden world of plant–microbiome interactions. This will open a path to try to manage these interactions as a sustainable tool to restore plant resilience against stressful factors.

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Declaration of interests

The authors have no interests to declare.

Highlights

Domestication process and breeding programs, focused exclusively on the selection and improvement of specific plant traits, have negatively affected the recruitment and functionality of beneficial associations in agricultural systems. Rhizosphere and root-associated microorganisms play important roles in plant growth and resilience. Breeding programs aiming to rationally manipulate root traits in order to recruit beneficial microorganisms or to deter pathogens need to be developed. The development and application of synthetic communities ('SynComs') represent a sustainable way to improve plant growth and resilience.

Glossary

Arbuscular mycorrhizal: symbiosis of beneficial associations between the roots of most terrestrial plants, including several relevant crops, and the arbuscular mycorrhizal fungi, which are obligate symbiotic fungi belonging to the Glomeromycotina sub-phylum.

Core microbiota: the set of microorganisms/microbial genes that are systematically associated with a given host plant and form webs of interactions that can be exploited to optimize microbial functions at individual plant and ecosystem levels.

Holobiont: assemblage of a host and the many other species living in or around it, which together form a discrete ecological unit. Linkage drag: refers to the usually undesirable effect of moving unwanted genes in the progeny that are linked to the gene(s) or quantitative trait locus of interest.

New plant breeding techniques: methods allowing the development of new plant varieties with desired traits by modifying specific DNA sequences.

Plant domestication: coevolutionary process in which humans select wild plants based on their adaptation to cultivation and human-derived purposes.

Priming: the state that can be reached following treatments with various inducers such as chemical compounds or beneficial microbes. The primed plants are able to display faster and stronger defense responses.

Root exudates: range of compounds released by the roots into the surrounding soil with key role in supporting plant development and interactions with rhizosphere microbes, facilitating root associations with beneficial microbes and suppressing pathogens.

Synthetic microbial communities (SynComs): comprehensible system of reduced complexity. Wild microbial communities are composed of mixed microbes with several unknown functions, SynComs allow the generation of a defined system with known taxonomic as well as functional profiles.

Traditional breeding: genetic enhancement of adaptation traits, disease and pest resistance, abiotic stress tolerance, and nutritional and water use to improve crop yield and product quality

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CHAPTER 7 | CONCLUSION

Viticulture is known to be one of the most environmentally impactful agricultural system since to meet high-quality standard of wine and fruit production multiple fertilizer and pesticide applications are required across the growing season. Furthermore, grapevine modern cultivars, are much more nurtured than their wild parents through fertilization, irrigation, and other protective measures, leading over time to plants characterized by less ability to interact with or adapt to the surrounding environment. Additionally, increasing crop yield has consistently been one of the most important goals of modern agriculture to provide humans with a continuous and constant food supply. However, plants do not have an unlimited quantity of energy, and the allocation of limited carbon sources is consequently influenced by a growth–defence trade-off. This means that the modern grapevine cultivars lost their balance becoming more productive but less resilient as well year by year and the development of new strategies to mitigate this negative trend have become more relevant. The above-described anthropocentric agricultural approach and the neglect of environmental or ecosystems health and associated loss of biodiversity are a critical issue, worsening the agricultural systems sustainability. Therefore, the development of new eco-friendly practices able to restore part of the loss biodiversity and the ability of the plants to interact with the surrounding environment is globally acknowledged as a priority. An essential step to face this challenge should be to consider the roles played by beneficial associated microbes and their effect on the plant health and growth performance. However, there is evidence that domestication process, amplified by breeding programs, has profoundly altered the interactions between plant hosts and associated microorganisms and the ability of plant hosts to recruit beneficial ones. In the light of what discussed, we focus our work on better understanding the interactions between plants and their associated microorganisms and how we can improve them to restore a lost plant-microbiome balance.

We showed the involvement of AMF in the growth-defence trade-off balancing on young grapevine cuttings grafted onto two different rootstocks (1103P and SO4) and their positive impact on immunity system, suggesting that AM symbiosis triggers a mycorrhiza-induced resistance (MIR) also in a model woody plant such as grapevine. This result provided important insights which could contribute to open new perspectives, enabling the application of AMF in viticulture to achieve more sustainable and resilient agricultural system. To underlie the importance of "omic" tools for unearthing the hidden word of plant-microbiome interactions, we proposed a metatranscriptomic study to explore whether the taxonomic composition and the behaviour of microbial communities associated with grapevine plants are influenced by the health status of the host. Once identified several bacterial taxa in healthy samples, we also isolated and characterized them for biological control activities against a wood-

degrading fungal taxon obtaining very promising result. We believe that following this or similar methodologies will make the formulation of microbial synthetic communities to improve the resilience of less-performing phenotypes achievable. Finally, we isolated and characterized (e.g. biological control or PGP-Traits) several endophytes from grapevine woody tissues, developing a new database (ViMed-biomebank) constituted by more than 1000 microorganisms (fungi and bacteria) with the aim to develop synthetic communities adapted to specific agro-environmental conditions. The formulation and the application of this synthetic communities in semi-natural environment has been subsequently conducted and our results showed their ability to influence the growth-defence trade-off balancing and stimulate defences responses of inoculated plants. This could be the beginning of a regenerative path that will not consider the plant as a stand-alone entity but as a complex organism composed also by the associated microbiota (*i.e.* the holobiont). The final goal of this work is indeed to underline the importance of microbes' exploitation in viticulture and to propose a new kind of breeding based on the improvement of the whole holobiont instead of just specific plants traits. We believe that nowadays the plant's ability to interact and cooperate with microorganisms should be definitely considered as a fundamental trait in modern breeding programs and that this will be a starting point to try to manage these interactions as innovative tool to increase viticulture sustainability and biodiversity.

PUBBLICATIONS, CONFERENCES, TRAINING AND PROJECTS

PUBBLICATIONS:

- Nerva, L., Giudice, G., Quiroga, G., Belfiore, N., Lovat, L., Perria, R., Volpe, M., Moffa, L., **Sandrini, M**., Gaiotti, F., Balestrini, R. & Chitarra, W. (2022). Mycorrhizal symbiosis balances rootstock-mediated growth-defence tradeoffs. *Biology and Fertility of Soils*, *58*(1), 17-34.
- **Sandrini, M**., Nerva, L., Sillo, F., Balestrini, R., Chitarra, W., & Zampieri, E. (2022). Abiotic stress and belowground microbiome: The potential of omics approaches. *International Journal of Molecular Sciences*, *23*(3), 1091.
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- Giudice, G., Moffa, L., Niero, M., Duso, C., **Sandrini, M**., Vazzoler, L. F., ... & Nerva, L. (2022). Novel sustainable strategies to control *Plasmopara viticola* in grapevine unveil new insights on priming responses and arthropods ecology. *Pest Management Science*, *78*(6), 2342-2356.
- **Sandrini, M.,** Moffa, L., Velasco, R., Balestrini, R., Walter, C., & Luca, N. (2022). Microbe-assisted crop improvement: a sustainable weapon to restore holobiont functionality and resilience. *Horticulture Research*, *9*, uhac160.
- Nerva, L.[*], **Sandrini, M**.[*], Moffa, L.[*], Velasco, R., Balestrini, R., & Chitarra, W. (2022). Breeding toward improved ecological plant–microbiome interactions. *Trends in Plant Science*.

  [*]These authors contributed equally to this work as first authors.

SCIENTIFIC CONGRESS COMUNICATIONS – ORAL PRESENTATIONS:

- **Sandrini, M**., Nerva, L., Gambino, G., & Chitarra, W. "Double strand RNAs as sustainable alternative against botrytis cinerea in vineyard: efficiency of different application method in semi-controlled environment". Proceedings of the SIGA-SEI-SIBV-SIPAV Web Workshop "Young Scientists for Plant Health", 16 December 2020
- **Sandrini, M.,** Nerva, L., Moffa, L. & Chitarra W. "Il microbioma delle piante: un alleato per la viticoltura sostenibile". CONAVI 2022 - IX Convegno Nazionale di Viticoltura, 13-15 giugno 2022 - Conegliano (TV).

SCIENTIFIC CONGRESS COMUNICATIONS – POSTER PRESENTATIONS:

- Giudice, G., **Sandrini, M**., Chitarra W & Nerva, L. "Toward a more sustainable viticulture through the application of resistance inducers and phosphite alternatives". Proceedings of the SIGA-SEI-SIBV-SIPAV Web Workshop "Young Scientists for Plant Health", 16 December 2020.

- **Sandrini, M.,** Nerva, L., Moffa L., Giudice, G., & Chitarra, W. "Actinomycetes come to rescue of viticulture sustainability". Convegno AISSA#under40 Sassari, Dipartimento di Agraria - Università di Sassari, Sassari, 1-2 luglio 2021.
- **Sandrini, M.,** Nerva, L., Moffa L., Giudice, G., & Chitarra, W. "Actinomycetes come to rescue of viticulture sustainability". SIMBA project - Regulation, legislation& safety of biostimulants and biofertilizers, including nanoformulates, Venice, Italy, 29 Sep –2 Oct2021.
- **Sandrini, M**., Nerva, L., Moffa L., Balestrini, R., Giudice, G., & Chitarra, W. "Synthetic Communities: promising allies to sustain green transition in viticulture". PLANT BIOLOGY 22, Portland, Oregon, July 9-13-2022
- Nerva, L., **Sandrini, M**., Spada, A., Cometto, A., Bevilacqua, I., Paradiso, G., Moffa, L., Balestrini, R. & Chitarra W. "Unlocking the potential of the hologenome: development of microbial breeding approaches to face the domestication syndrome and improve resilience against climate change". 66th annual congress Italian society of agricultural genetics, Bari, 5-8 September 2023.

SCIENTIFIC TRAINING INHERENT TO PhD COURSE:

- Innovation for food resilience, UK Agri-tech centres, a virtual conference, 19th November 2020.
- Agricultural chemistry winter school - Interactions between biogeochemical cycles of elements in plant-soil-microbe systems, Italian Society of Agricultural Chemistry, University of Torino, Torino, Italy, 8-11 February 2021.
- SIMBA Training Course on "Regulation, legislation and safety of biostimulants and biofertilisers, including nanoformulates", Venice, Italy, 29 Sep –2 Oct2021.

PARTECIPATION TO RESEARCH PROJECTS:

- MICROBIO: coordinated by CREA – Research Centre of Viticulture and Enology and supported by Cariverona foundation, with the participation of ITA Trentin school, Collis Veneto Wine Group, Lallemand e SIVE – Italian Society of Viticulture and Enology.
- GRAPE4VINE: coordinated by University of Milano and supported by Cariplo foundation, with the participation of CREA – Research Centre of Viticulture and Enology as partner.
- MICRO4LIFE: coordinated by CNR – Institute for Sustainable Protection of Plants with the participation of CREA – Research Centre of Viticulture and Enology and supported by Ager foundation.
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