Rootstock–scion combination contributes to shape diversity and composition of microbial communities associated with grapevine root system

Ramona Marasco,†*, Hend Alturkey,† Marco Fusi,† Michele Brandi,‡ Isabella Ghiglieno,§,∥ Leonardo Valentii and Daniele Daffonchio,†*,‡

1Biological and Environmental Sciences and Engineering Division (BESE), King Abdullah University of Science and Technology (KAUST), Thuwal, Kingdom of Saudi Arabia.
2Marchesi Frescobaldi Società Agricola s.p.a. Fattoria Poggio a Remole, Sieci, Italy.
3Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, University of Milan, Milan, Italy.
4Department of Civil, Environmental, Architectural Engineering and Mathematics (DICATAM), University of Brescia, Agrofood Research Hub, Brescia, Italy.

Summary

To alleviate biotic and abiotic stresses and enhance fruit yield, many crops are cultivated in the form of grafted plants, in which the shoot (scion) and root (rootstock) systems of different species are joined together. Because (i) the plant species determines the microbial recruitment from the soil to the root and (ii) both scion and rootstock impact the physiology, morphology and biochemistry of the grafted plant, it can be expected that their different combinations should affect the recruitment and assembly of plant microbiome. To test our hypothesis, we investigated at a field scale the bacterial and fungal communities associated with the root system of seven grapevine rootstock–scion combinations cultivated across 10 different vineyards. Following the soil type, which resulted in the main determinant of the grapevine root microbial community diversity, the rootstock–scion combination resulted more important than the two components taken alone. Notably, the microbiome differences among the rootstock–scion combinations were mainly dictated by the changes in the relative abundance of microbiome members rather than by their presence/absence. These results reveal that the microbiome of grafted grapevine root systems is largely influenced by the combination of rootstock and scion, which affects the microbial diversity uptaken from soil.

Introduction

The bacterial and fungal microbiota are essential component of the plant holobiont (Sánchez-Cañizares et al., 2017; Hassani et al., 2018; Ceja-Navarro et al., 2021). These microorganisms add an external second metagenome to those of the plants, supplying key ecological functions and services (Zilber-Rosenberg and Rosenberg, 2008; Turner et al., 2013; Vandenkoornhuyse et al., 2015; Rosenberg et al., 2016), including promotion of plant growth, fitness, health and adaptation to environmental challenges (Berendsen et al., 2012; Rolli et al., 2017; Mommer et al., 2018; Pugnaire et al., 2019). Understanding the dynamics and factors driving the recruitment and assembly of the plant microbiome is becoming more and more important to translate fundamental ecological knowledge into effective manipulative strategies aimed at the engineering of plant microbiomes (Lakshmanan et al., 2014; Mueller and Sachs, 2015; Dini-Andreote and Raaijmakers, 2018; Toju et al., 2018; Singh et al., 2020).

Plants actively recruit and select microorganisms from the surrounding soil by means of the rhizosphere (i.e. the transition zone between root and soil influenced by root exudates) and a subset of such microorganisms could further cross the rhizoplane barrier to become endophytes (Van Der Heijden and Schlaeppi, 2015; Santoyo et al., 2016; Zhalkina et al., 2018). Most studies have been conducted on conventional plant species (cultivated and wild), while little attention has been directed at ‘chimeric plants’; i.e. individual plants made up of two or multiple species. Examples of these chimaeras are given by grafting, a mechanism of plant–plant interaction that is widespread in nature, in which parts of two different
plants are physically joined to create a new individual (Fuentes et al., 2014; Warschefsky et al., 2016; Gaion et al., 2018). Typically, a grafted plant has two genetically different components: the root system of one plant species (rootstock) and the shoot (scion) from another plant cultivar or even species (Fuentes et al., 2014). Grafting is widely used with trees and shrubs, but recently it was also applied in vegetables (Gaion et al., 2018), to combine the rusticity and resistance of the rootstock, and the quality and productivity of the scion (Warschefsky et al., 2016; Riaz et al., 2019). Today, most flowering and fruit trees (e.g. apple, avocado, citrus fruit, grapevine, peach, and rose) and horticulture plants (e.g. up to 40% of tomatoes and eggplants, 75% of cucumbers, 5%–10% of peppers and 90% of watermelons) are grafted onto rootstocks (Koevoets et al., 2016; Warschefsky et al., 2016; Gaion et al., 2018). Of these, grapevine is an iconic and the most widespread grafted crop, covering up to 7.5 million hectares worldwide (OIV The International Organisation of Vine and Wine, 2020). Since the 19th century, in Europe Vitis vinifera (the European grape) cultivars have been grown as scions grafted onto rootstocks of other species of the genus Vitis (V. rupestris, V. berlandieri and V. riparia, and their derived hybrids) to prevent vineyard failure due to the aphid-like hemipteran Phylloxera (Battey and Simmonds, 2005; Riaz et al., 2019). To date, grafting on rootstocks is used as a general practice, because the rootstock can affect several physiological parameters of the plant and make the grapevine growing in otherwise unsuitable soils. Thus, rootstock selection and development are an important component of modern viticulture. Rootstocks have been initially developed for improving plant resistance to certain soil-borne pests and diseases (Wallis et al., 2013; Warschefsky et al., 2016), and for improving plant resistance to adverse climatic or soil conditions (Bert et al., 2013; Henderson et al., 2014; Berdeja et al., 2015; Hamrouni et al., 2015; Habran et al., 2016). Grafting also influences the reproductive performance, scion vigour, biomass accumulation and distribution, grape yield and quality, and phenology (Whiting, 2003; Wallis et al., 2013; Bonghi et al., 2016; Warschefsky et al., 2016). In grafting, plant vigour and productivity depend on the efficient integration mechanisms between the rootstock and the scion, including wound healing, tissue fusion, vascular reconnection and shoot–root signalling regulation (Pina et al., 2017; Venema et al., 2017; Melnyk et al., 2018; Gautier et al., 2019). The grafting process unequivocally results in a two-sided influence on the phenotype (physiological/morphological traits) of both components; i.e. the rootstock affects the scion and vice versa (Martínez-Ballesta et al., 2010; Hamrouni et al., 2015; Habran et al., 2016; Warschefsky et al., 2016; Ruan et al., 2020). However, to date, research has mainly been focused on how the rootstock affects the scion phenotype, with little attention paid to the reverse effect of scion on the rootstock phenotype and on the mechanisms of scion–rootstock interactions (Gautier et al., 2019).

Based on the bilateral influence of rootstock and scion, it is plausible to suppose that both and their interaction may also affect the association of microorganisms with the plant, including their recruitment, selection and assembly. Studies of spatial and temporal variations in microbial communities associated with grapevine, including the above- and below-ground parts of the plant, have revealed a myriad of factors that can influence the dynamics of grapevine microbiome assembly (Bettenfeld et al., 2021; Griggs et al., 2021). Soil heterogeneity and vineyard agricultural practices represent the main factors that affect the diversity of taxa recruited by the grapevine in the root system (Marasco et al., 2013; Campisano et al., 2014; Vega-Avila et al., 2015; Zarraonandia et al., 2015; Burns et al., 2016; Manici et al., 2017; Berlanas et al., 2019; Vitulo et al., 2019; Gamalero et al., 2020; Swift et al., 2021; Vink et al., 2021a). Furthermore, a recent body of literature has demonstrated that the rootstock genotype strongly influences the diversity of bacterial and fungal communities recruited by the root from the surrounding soil (Wallis et al., 2013; D’Amico et al., 2018; Marasco et al., 2018b; Berlanas et al., 2019; Nerva et al., 2021; Swift et al., 2021; Dries et al., 2021a; Darriaut et al., 2022). However, the effect mediated by the interaction between different rootstocks and scions (i.e. grafting combinations) on the grapevine microbiome has been poorly studied. To date, only one study has explored this in grapevine, using grafted plants obtained from the interaction between four rootstocks and four cultivar scions (Vink et al., 2021b). This study was limited to the bacterial community associated with the rhizosphere and was performed in a single soil in an experimental field. Given that the current literature has neglected the overall complexity of an extended-environmental context at ecosystem scale, in our study, we hypothesized that (i) the effect of genotype observed for the rootstock microbiome recruitment at the levels of root endosphere and rhizosphere for both bacteria and fungi is further tuned if the latest is combined with different scion cultivars, and (ii) it can be consistently detectable across heterogeneous environmental settings in a real agricultural ecosystem context. To test these hypotheses, we selected a large area devoted to grapevine cultivation, in which differential environmental and pedoclimatic conditions occur. We selected seven different rootstock–scion combinations cultivated in 10 vineyards located in two geographical areas in Tuscany, namely, Pomino and Nipozzano. These combinations are formed by four rootstocks (SO4, 420A, 110R and 3309C) and
three scions (Chardonnay, Sangiovese and Merlot). The bacterial 16S rRNA gene and fungal ITS from the root system (root and rhizosphere) and bulk soil sampled were amplified and sequenced to evaluate the effects of the rootstock, scion, and their combination, along with those of soil and geographical area, on the structure and diversity of the grapevine root system microbiome.

With this work we aim to demonstrate that the scion–rootstock combination factor is an important parameter to be considered in ecological studies of grafted plants. One requirement of the modern research in plant-associated microorganisms is to extend investigations from a confined range, e.g. microcosms or single fields (Vink et al., 2021b), to wide ecosystem scales to unravel the selective forces that influence the microbiome associated with plant across the heterogeneous conditions of real-word ecosystems (Delgado-Baquerizo, 2022; Gobbi et al., 2022). Ecosystem-scale studies can offer important contributions that can set the basis of future framework for targeted agriculture (Trivedi et al., 2021) and microbial synthetic communities applications (Delgado-Baquerizo, 2022) to support productivity and sustainability. It is important to note that the use of soil microorganisms to promote plant productivity is a promising tool also in viticulture (Rolli et al., 2017; Bettenfeld et al., 2021; Dries et al., 2021b; Darriaut et al., 2022). This study can provide insights on the effect of different rootstock–scion combinations on the microbiome assembly and recruitment that should be taken into account during the development and use of targeted-agriculture applications (e.g. biofertilizers and biopromoters), especially in the light of the predicted changes that viticulture will face soon (Morales-Castilla et al., 2020).

**Experimental procedures**

**Sample collection**

The root system (root tissue and rhizosphere soil) of 420A, SO4, 110R and 3309C rootstocks, which are originated from the breeding of the most used *Vitis riparia*, *V. berlandieri* and *V. rupestris* (Supplementary Table S1), was collected at the end of October 2016 in 10 vineyards across the Nipozzano and Pomino areas owned by Marchesi Frescobaldi Società Agricola s.p.a. (north–eastern Tuscany, Italy; Supplementary Fig. S1; Supplementary Table S2). Onto the selected rootstocks were grafted the scions of *V. vinifera* cultivars Chardonnay, Merlot and Sangiovese, for a total of seven rootstock–scion combinations (Supplementary Table S2). Each of the vineyard studied here has a unique combination of environmental conditions, strongly dictated by the soil physicochemical characteristics and geoclimatic factors (Fig. 1; Supplementary Table S3), and for this reason different rootstocks were distributed across them (Ollat et al., 2016; Bianchi et al., 2020). For instance, the SO4 rootstock (selection oppenheim, *V. berlandieri × V. riparia*) is one of the most used in Italy because it offers great adaptability to edaphic and pedo-climatic conditions, as well as good productivity. Rootstocks that push plant vigour, such as 110 Richter (110R, *V. berlandieri × V. rupestris*) are preferable in tough and dry hillside slopes, and to cope with warm climates and drought events (Bianchi et al., 2020), often experience in Italy in the last decades (Ciais et al., 2005). 420A rootstock (420A, *V. berlandieri × V. riparia*) confers low vigour to plants, limiting the yields, particularly during the first years of production, but it covers a wide range of soil type (i.e. deep, fine texture and alkaline soils), representing a possible compromise for vineyards with heterogeneous soils, such as the case of Pomino and Nipozzano estates. On the contrary, the 3309 Couderc rootstock (3309C, *V. riparia × V. rupestris*), with low to moderate tolerance to chlorosis and active limestone, and sensitivity to water stress, is not widespread in the Frescobaldi vineyards.

A total of six plants were selected in each vineyard; in the case of Rena vineyard, we have sampled two different combinations in the same field (Supplementary Table S2). The root samples (*n = 66*) were collected at a depth of 20–40 cm; this range of depth allowed us to consistently collect the active and denser portion of root system across the different vineyards and for the different rootstocks. Six samples of bulk soil (i.e. soil not influenced by the root system) from each location were also collected. All samples were collected under sterile conditions using sterile tools. The recovered samples were stored at −20°C for molecular analysis. The sampling was authorized by the vineyard owner at each location and no other specific permissions were required for this activity.

**Physico-chemical analysis of vineyard soils**

The chemical and physical properties of the bulk soil from each vineyard were provided by the vineyard owner. Soil samples have been collected, pooled and homogenized before analysing soil texture and physicochemical parameters, including pH, cation exchange capacity (CEC), total and active limestone content (CaCO₃), organic matter, total carbon, total nitrogen, C/N ratio and available elements/nutrients (calcium, magnesium, potassium and phosphorus), base saturation and overall acidity. The results of physicochemical analyses were analysed by performing principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA) on the Euclidean distance matrix with Primer v.6.1 (Anderson et al., 2008); sample categories (two levels: Pomino and Nipozzano) were used as explanatory variables.
Total DNA extraction

The sampled roots with attached soil particles were placed in a sterile tube and 9 ml of physiological solution (9 g L\(^{-1}\) NaCl) was added. The tubes were vortexed for 5 min to detach tightly attached soil particles, then centrifuged at 4000 rpm for 10 min. The roots were removed, and the supernatant was discarded to collect the remaining soil and use it as rhizosphere soil. The soil samples (rhizosphere and bulk soil) were homogenized with sterile pestles and mortars, and 0.5–0.1 g was further used to extract total DNA using the PowerSoil DNA Kit (Qiagen), following the manufacturer’s instructions. In the case of the root tissues, the material obtained after the removal of the rhizospheric soil was further surface sterilized by soaking in 70% ethanol for 3 min, followed by sodium hypochlorite 2.5% for 5 min, 70% ethanol for 30 s, and finally by washing with sterile distilled water five times; the efficacy of the sterilization method was verified by plating pieces of the sterilized root and the water from the last washing step on plates with tryptone soy agar. The plates were examined for bacterial growth after incubation at 30°C for 3 days. The root tissues were smashed with liquid nitrogen and further homogenized before performing genomic DNA extraction (0.5–0.1 g) using the DNeasy Plant Maxi Kit (Qiagen). Eluted DNA (5 μl) was visualized on 0.8% agarose gel using electrophoresis to evaluate its quality, while quantification of DNA was performed using a Qubit 3.0 Fluorometer and dsDNA BR Assay kit. All extracted DNA samples were stored at −20°C.

Amplification, sequencing and analysis

Illumina tag screening of the V3–V4 hypervariable regions of the bacterial 16S rRNA gene was applied on
DNA, using the primers 341f and 785r (Klindworth et al., 2013) and following the protocol previously described (Mapelli et al., 2018). Briefly, PCR reactions mixture of 30 μl was performed for each sample using 1 U of Platinum Taq DNA Polymerase, High Fidelity (Invitrogen) with 1× High Fidelity Buffer, 1.5 mM of MgSO₄, 0.3 mM dNTPs mix, 0.3 μM each of forward and reverse primers, and ca. 10 ng of template DNA, along with the addition of 0.5 μM each of pPNA and mPNA clamps to reduce the amplification of host chloroplasts and mitochondria (Fitzpatrick et al., 2018; Deyett and Rolshausen, 2020). In the case of fungi, the fungal ITS2 gene was amplified using ITS3F and ITS4R primers (Marasco et al., 2018a). The sequencing libraries were constructed using a 96 Nextera XT Index kit (Illumina) and the products of amplification were cleaned using a SequaPrep™ Normalization Plate kit. All libraries were sequenced on the Illumina MiSeq platform at the Biosciences Core Lab, King Abdullah University of Science and Technology. The raw sequencing data for bacterial and fungal communities were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number PRJNA807110.

Analysis of sequences was performed using a combination of the QIME pipeline version 1.9 (Caporaso et al., 2010) and UPARSE version 8 (Edgar, 2013). After paired-end merging and quality filtering, only high-quality reads were kept for further steps in the analysis. Reads were clustered as OTUs considering a 97% sequence distance similarity and taxonomy was assigned using UCLUST against SILVA 138 and UNITE databases for bacteria and fungi respectively. All OTUs non-assigned to bacteria [mitochondria (24 626 reads, 31 OTUs), chloroplasts (24 465 reads, 63 OTUs), archaea (740 reads, 11 OTUs) and unclassified (95 530 reads, 12 951 OTUs)] and fungi [non-fungi (90 833 reads, 326 OTUs) and unclassified (1 178 884 reads, 942 OTUs)] were removed. OTUs were filtered to keep only those with a relative abundance higher than 0.001% and the rarefaction curves were generated (Supplementary Fig. S2). By using qiime feature-table rarefy plug-in without replacement (Weiss et al., 2017), the rarefaction threshold was set at 9590 and 13 400 reads per sample for bacteria and fungi respectively (Supplementary Table S3), after the removal of samples with low sampling depth (<5000 reads). In PRIMER v.6.1 (Anderson et al., 2008), Bray–Curtis distance matrices were used to perform PCOA, permutational analysis of multivariate dispersion and canonical analysis of principal coordinates (CAP). We evaluated the most important factor accounting for the beta-diversity variability between microbial communities by using the function best.r.sq() in the R package mvabund (Wang et al., 2012). Since the effect of ‘compartment’ was ranked as the most important factor (i.e. niche partitioning; Supplementary Table S4), we performed further analyses considering separately the root tissue, the rhizosphere and the bulk soil. For each compartment, we evaluated and quantified the effect of estate, field and rootstock–scion interaction factors by using a model-based approach for compositional data performed with the anova manyglm() and the best.r.sq() functions in the R package mvabund (Wang et al., 2012). Our categorical explanatory variables were ‘estate’ (two levels), ‘field’ (10 levels), ‘rootstock’ (four), ‘scion’ (three) and their interaction ‘rootstock x scion’ (seven levels). To disentangle the effect of the rootstock–scion interaction we conducted CAP, PERMANOVA and pairwise analysis using PRIMER v.6.1 (Anderson et al., 2008). Shared (i.e. core microbiome) and specific microbial OTUs across the rootstock–scion combinations within each compartment (root tissue, rhizosphere and bulk soil) were defined using Venn diagram analysis with R. We further calculated the individual contribution of each OTUs to define the diversity of bacterial and fungal communities across rootstock–scion combinations by testing univariate glm for each OTU including the parameter p.uni = ‘adjusted’ in the function anova manyglm() (Wang et al., 2012). To pinpoint those bacterial and fungal OTUs that were enriched within each sample type, an indicator species analysis combining both the abundance and occurrence of a given OTU across all rootstock–scion combinations was used. Indicator species were calculated by comparing combinations’ communities within each compartment, using the multipatt function in the R statistical package IndicSpecies (De Cáceres et al., 2020). This function creates combinations of the input clusters and compares each combination with the species in the input matrix. For each species it chooses the combination with a highest association value. Best matching patterns are tested for statistical significance of the associations. The association indices used was ‘IndVal’ that return the pattern that better matches the species observed pattern. The significance of each OTU–combination association was tested using a permutation test (n = 9999) and non-significant associations (p > 0.05) were discarded. Richness and Shannon diversity were calculated in R using the Phylloseq package (McMurdie and Holmes, 2013). We used ANOVA with Tukey’s multiple comparison tests to determine statistical significance across compartments for richness and Shannon diversity in bacteria and fungi and we used the machine learning algorithm Random Forest to rank the most important predictors of alpha diversity patterns by taking into account the mean decrease accuracy (%IncMSE) that indicates the increase of the mean squared error when a given variable is randomly permuted (Delgado-Baquerizo and Eldridge, 2019).
Results

Geoclimatic and edaphic physicochemical characterization of vineyards

Pomino and Nipozzano are estates belonging to the Frescobaldi company located in the north-eastern area of Tuscany (Italy), close to the Apennines (Supplementary Fig. S1). The two estates are characterized by different geology (PERMANOVA soil textures, $F_{1,8} = 5.99; p = 0.023$; Supplementary Fig. S3A; Supplementary Table S5); whereas in the Nipozzano estate the heterogeneity of the soil texture led to distinct vineyard clusters, in the Pomino estate the dominance of sand (range, 57%–75%) favoured the establishment of similar edaphic conditions across the vineyards (Supplementary Fig. S3B). The physicochemical characteristics of the vineyard soils (Supplementary Table S5) confirmed the differences between the two estates ($F_{1,8} = 3.03; p = 0.013$; Supplementary Fig. S3C). For instance, while all the vineyard soils were around neutral-slightly alkaline, with pH from 6.5 to 8 at Pomino and from 7.7 to 8.1 at Nipozzano, organic carbon, limestone, exchangeable ions and CEC determined the soil diversity (Supplementary Table S5; sequential test and AIC model in Supplementary Table S6). Additional details on the ecosystem and agronomic practices applied in the two estates are also provided in Supplementary Result S1. Both estates use the 110R, 3309C, SO4 and 420A rootstocks obtained by crossing of different vitis species (V. berlandieri, V. riparia and V. rupestris). These rootstocks are grafted with Chardonnay, Merlot and Sangiovese, while ungrafted plants are not present in the Frescobaldi estates.

Compartmentalization of bacterial and fungal communities associated with grapevine root system

The bacterial and fungal diversity associated with the root system (root tissue and rhizosphere) of seven rootstock–scion combinations obtained from four rootstocks and three scions, and related bulk soils not influenced by grapevine root systems have been measured (Supplementary Tables S1 and S2). PCoA revealed strong clustering of the microbial communities according to the different compartments sampled (root tissue, rhizosphere and bulk soil) for both bacteria and fungi (manyglm, bacteria: $Dev_{2,185} = 206 634, p = 0.001$ and fungi: $Dev_{2,187} = 29 238, p = 0.001$; Fig. 1A and B; Supplementary Table S4). The microbial communities of the compartments were mainly differentiated along the first axis of PCoA (Fig. 1A and B) with cross-validation of 100% for the samples within each compartment (CAP). A selection determined by the plant and a rhizosphere effect were consistently observed in bacteria and fungi: we detected a horseshoe-shaped distribution in the ordination space with the three compartments succeeding each other (Fig. 1A and B). This pattern indicates the presence of a dual step process of recruitment and selection from the soil to the root tissues (Van Der Heijden and Schlaeppi, 2015). Notably, within each compartment two distinct sub-clusters based on the geographical/environmental origin of the samples were detected (Pomino vs. Nipozzano; Supplementary Fig. S4).

Different levels of dispersion (within-beta diversity) were observed along the root system compartments (Fig. 1C and D). The highest dispersion values were detected for root microbial communities, indicating that endophytes had the largest intrinsic variability, possibly due to their limited diversity (low richness, Fig. 1E and F) and uneven distribution (low Shannon index, Fig. 1G and H). In contrast, the rhizosphere and bulk soil, which harboured more diverse and more even microbial communities (Fig. 1E–H), had less dispersion (Fig. 1C and D). It is important to note that the compartment was also identified as the most important factor determining the differences observed in the alpha-diversity indices (Supplementary Table S7).

The niche partitioning described in terms of beta-diversity and alpha-diversity was confirmed by the differential composition of the main bacterial and fungal classes across the three compartments (Supplementary Fig. S5). Bacterial communities were dominated (on average) by Proteobacteria (37.5%: 21% Alphaproteobacteria and 16.5% Gammaproteobacteria), Actinobacteria (16%), Bacilli (8%), Bacteroidia (7%), Blastocatellia (6%) and Clostridia (3%), followed by Vincamibacteria, Thermoleophilica, Verrucomicrobiae, Polyangia, Gemmatimonadetes, Acidobacteriae, Acidimicrobiia, Anaerolineae and the remaining classes with low relative abundance (<1%). Most bacterial classes were differentially distributed across the compartments, with Actinobacteria and Clostridia enriched in root tissues, Gammaproteobacteria in the rhizosphere, and Blastocatellia, Gemmatimonadetes, Acidobacteae, Verrucomicrobiae and Anaerolineae in bulk soil (Supplementary Fig. S6). In the case of fungal communities, Ascomycota was the dominant phylum mainly represented by Sordariomycetes (40%), Dothideomycetes (17%), Eurotiomycetes (8%), Leotiomycetes (5%) and Pezizomycetes (3%), and it was followed by Basidiomycota (7% Agaricomycetes and 5% Tremellomycetes), Mortierellomycetes (8%) and Mucoromycetes (1%). The remaining part was constituted by rare fungal taxa (relative abundance <1%) and unclassified fungi (2% and 4% respectively). All of them were differentially distributed across compartments except for Agaricomycetes, Leotiomycetes and the dominant Sordariomycetes. Members of Dothideomycetes were enriched in the root tissues, while...
Eurotiomycetes and Mucoromycetes were enriched in the rhizosphere, and Pezizomycetes in the bulk soil (Supplementary Fig. S7). The list of bacterial and fungal OTUs that most contributed to determine the differences across compartments is reported in Supplementary Table S8.

Effect of biotic and abiotic factors on beta-diversity and alpha-diversity of microbial communities associated with the root system of grafted grapevine and bulk soil

It is well known that the micro-ecosystem of each vineyard specifically influences the diversity and composition of edaphic microorganisms available for plant recruitment (Gobbi et al., 2022). In our study the ‘field’ factor was confirmed to be the most important abiotic factor shaping the diversity of microbial communities associated with root system compartments (root and rhizosphere) and bulk soil (bacteria, Fig. 2A–C; fungi, Fig. 2D–F; manyglm in Table 1). The interaction between rootstock and scion was the second factor for importance in shaping the root system microbial diversity (Fig. 2; Table 1). It was consistently ranked as the first and most important plant-related factor, explaining a percentage of observed variability higher than that explained by the rootstock or the scion considered alone (Fig. 2). Furthermore, when testing the best combination of factors for explaining microbial variability, the rootstock–scion interaction was always included, together with the field, as a meaningful explanatory factor in the best models for both bacterial and fungal communities in all compartments (see sequential test in Fig. 2). The scion, and in most cases the rootstock, were discarded because they did not provide the model with any additional explanatory power. The role of the interaction between rootstock and scion (i.e. rootstock–scion combination) in the assembly of microbial communities was corroborated by the low percentages of misclassification from cross-validation (CAP, Fig. 3A and D) for rhizospheric (8% and 22% for bacteria and fungi respectively) and endophytic (21% and 33% for bacteria and fungi respectively) microbial communities. Notably, while the pedo-climatic conditions of the Nipozzano and Pomino estates were more important than the scion and rootstock biotic components in shaping the edaphic microbial communities of the bulk soil (Fig. 2E and F), the formers gave the lowest contribution for the endophytic microbiomes (Fig. 2A and D).

In terms of microbial alpha-diversity, the field and the rootstock–scion interaction were confirmed as the most important factors explaining richness and Shannon

Fig. 2. Results of variation partitioning models used to identify the effects of abiotic (estate) and biotic (rootstock, scion and their interaction, i.e. rootstock–scion combination) factors analysed. The analyses are reported separately for (A–C) the bacterial and (D–F) the fungal community across (A and D) root tissues, (B and E) rhizosphere and (C and F) bulk soil compartments. Results of sequential test run considering all the factors are also reported as pie chart for bacterial and fungal communities at compartment level.

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diversity (Table 2). However, in contrast to what was observed for beta-diversity, alpha-diversity was strongly influenced by the estates, which in certain circumstances represent the most important factor.

Identification of core and signature microbial discriminants across rootstock–scion combinations

For each compartment we evaluated the bacterial and fungal communities associated with different grapevine rootstock–scion combinations, independently of their field of origin. A complex picture emerged from the taxa distribution in root tissue, rhizosphere and bulk soil across the seven rootstock–scion combinations (see summary of bacterial and fungal classes in Fig. 4). Even though the microbiomes associated with the different rootstock–scion combinations were significantly different, the shared OTUs defining the core microbiome across the combinations in the rhizosphere and bulk soil were 21% and 31.5% for bacteria respectively (contributing to 79.6% and 75.6% of total bacterial relative abundance), and 11.7% and 11% for fungi respectively (contributing to 69.5% and 67.5% of total fungal relative abundance; Fig. 4B and D). The edaphic core microbiomes included both abundant (>1%)

### Table 1. Multivariate generalized linear model (manyglm) reporting bacterial and fungal communities’ variability explained by each factor (estate, field and interaction between rootstock and scion) for the three compartments (RT, root tissue; RH, rhizosphere; BS, bulk soil).

| Kingdom | Factor            | RT               | RH               | BS               |
|---------|-------------------|------------------|------------------|------------------|
| Bacteria| Estate            | Dev$_{1,55}$ = 8199, $p = 0.001$ | Dev$_{1,64}$ = 52 774, $p = 0.001$ | Dev$_{1,63}$ = 60 243, $p = 0.001$ |
|         | Field             | Dev$_{9,46}$ = 20 482, $p = 0.001$ | Dev$_{9,55}$ = 77 717, $p = 0.001$ | Dev$_{9,54}$ = 97 921, $p = 0.001$ |
|         | Rootstock × Scion | Dev$_{6,46}$ = 3861, $p = 0.001$ | Dev$_{6,55}$ = 6339, $p = 0.001$ | Dev$_{6,54}$ = 9318, $p = 0.001$ |
| Fungi   | Estate            | Dev$_{1,58}$ = 4231, $p = 0.001$ | Dev$_{1,55}$ = 13 432, $p = 0.001$ | Dev$_{1,62}$ = 12 190, $p = 0.001$ |
|         | Field             | Dev$_{9,49}$ = 17 778, $p = 0.001$ | Dev$_{9,54}$ = 28 676, $p = 0.001$ | Dev$_{9,53}$ = 29 202, $p = 0.001$ |
|         | Rootstock × Scion | Dev$_{6,49}$ = 1673, $p = 0.001$ | Dev$_{6,54}$ = 2274, $p = 0.001$ | Dev$_{6,53}$ = 2130, $p = 0.001$ |

Deviance and $p$-value are reported.
Table 2. Effect of abiotic (estate and field) and biotic (rootstock, scion and their interaction) factors on alpha-diversity (Shannon diversity and richness) in bacteria and fungi.

| Kingdom | Factor | Scion | Estate | Filed | Rootstock | Rootstock × Scion |
|---------|--------|-------|--------|-------|-----------|------------------|
| **Bacteria** | Abiotic | 13.1  | 31.3  | 28.8  | 23.1      | 35.5             |
|         | Field   | 6.1   | 22.3  | 1.2   | 8.7       | 17.7             |
|         | Rootstock |      |       |       | 11.6      | 3.5              |
|         | Scion   | 8.8   |       |       | 22.2      | 14.3             |
| **Fungi** | Abiotic | 1.3   |       |       | 21.6      | 8.2              |
|         | Field   | 6.1   |       |       | 15.1      | 1.1              |
|         | Rootstock | 8.4   |       |       | 3.2       | 6.9              |
|         | Scion   | 8.4   |       |       | 3.6       | 6.9              |
|         | Rootstock × Scion | 13.1  | 25.7  | 22.2  | 22.5      | 21.1             |

The most important factor that explains the pattern of alpha-diversity in root tissues (RT), rhizosphere (RH) and bulk soil (BS) was determined by Random Forest analysis. The mean decrease accuracy (%IncMSE) was reported for each tested factor.

and rare (<1%) OTUs, and they were dominated by bacteria belonging to Alphaproteobacteria, Bacteroidetes, Gammaproteobacteria, Actinobacteria and Bacteroidia, and fungi belonging to Sordariomycetes, Dothideomycetes, Mortierellomycetes and Tremellomycetes. The total of OTUs specific for each rootstock–scion combination (i.e., present only in a single combination) were only 5% and 4.8% in the bacterial communities of the rhizosphere and bulk soil respectively, while they were up to 14.4% and 19.8% in those of fungi (Fig. 4B and D). However, these OTUs specific of each combination covered less than 3% of relative abundance, underlining the importance of rare species in community assembly processes and plant–microbes interactions (Zhang et al., 2022). In contrast, the root microbiomes shared only 1% of bacterial OTUs and 3.4% of fungal OTUs, corresponding to 24% (13% Alphaproteobacteria and 6% Bacilli) and 46.4% (24% Sordariomycetes and 17.5% Dothideomycetes) of total abundance, along with a higher percentage of rootstock–scion combination-specific OTUs (34% (3% of relative abundance) and 48% (5.3% of relative abundance) respectively; Fig. 4B and D). These combination-specific bacterial OTUs mainly belong to Rhizobium (Alphaproteobacteria) and Alloprevotella (Bacteroidia) in SO4–Chardonnay, Nonlabens (Flavobacteria) and Arcticibacter (Sphingobacteria) in 420A–Sangiovese, Candidatus Phytoplasma (Bacilli) in 420A–Chardonnay, Ezakiiella (Closiortia) and Cetibacterium (Actinobacteria) in 110R–Chardonnay, Turicula (Actinobacteria) in 110R–Sangiovese, Flavobacterium (Flavobacteria) and Chitinophaga (Chitinophagia) in 3309C–Chardonnay, and Corynebacterium (Actinobacteria) and Steroidobacter (Gammaproteobacteria) in 420A–Merlot. In the case of fungi, we mainly detected members of Psathyrella (Agaricomycetes) and Rhizophyllum (Chytridiomycetes) in 110R–Chardonnay, Parasola (Agaricomycetes) and the arbuscular mycorrhiza Glomus (Glomeromycetes) in 110R–Sangiovese, Catapyrenium (Eurotiomycetes) and Hymenoscyphus (Ascomycetes) in 3309C–Chardonnay, Fusarium (Sordariomycetes) and Mortierella (Mucoromycota) in 420A–Chardonnay, Saccharomyces (Saccharomycetes) and Donadinia (Pezizomycetes) in 420A–Merlot, the symbiotic Claroideoglosum (Glomeromycetes) in 420A–Sangiovese and Thelebolus (Leotiomycetes) in SO4–Chardonnay. The remaining OTUs were shared across the rootstock–scion combinations (minimum two, maximum six; see ‘Multi combo’ in Fig. 5B and D).

To distinguish the OTUs that could discriminate the microbial communities across the rootstock–scion combinations – not limited to their presence/absence – we analysed the deviance of OTUs abundance. Ranked by their importance values (i.e., highest deviance), the top 20 bacterial and fungal OTUs were reported (Fig. 5A–F; full list in Supplementary Table S9). In the rhizosphere, the discriminant bacterial OTUs mainly belonged to Gammaproteobacteria (11 OTUs), Actinobacteria (3) and Alphaproteobacteria (2), while in bulk soil they belonged to Acidobacteria (3), Bacteroidia (2), Bacteroidetes (2), Thermoleophilia (2) and Gammaproteobacteria (2). Notably, in root tissue Actinobacteria (15) were the most important bacterial discriminants, along with Candidatus Phytoplasma OTUs (2) that were detected only in the 420A–Chardonnay bacterial communities (Fig. 5A). In the case of fungal communities, the rootstock–scion combination-discriminant OTUs were Sordariomycetes (five and six in the rhizosphere and bulk soil respectively), Eurotiomycetes (three and six) and Dothideomycetes (three and five); Leotiomycetes (1) Mortierellomycetes (1) Mucoromycetes (1) and Tremellomycetes (2) were rootstock–scion combination signatures only in the rhizosphere (Fig. 5D–F). Overall, the 20 OTUs distinguishing rootstock–scion combinations had a low level of abundance in the rhizosphere.

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Fig. 4. Legend on next page.
and bulk soil bacterial communities (range, <1%–11%), while they reached up to 26% of relative abundance in those of fungi. The majority of these OTUs belong to the core microbiome (i.e. present across all rootstock–scion combinations; see stars in Fig. 5), suggesting that their distribution-relative abundance rather than their presence/absence make them microbial discriminants. In contrast, in root tissues the relative abundance of influential OTUs was consistently greater compared to that of edaphic compartments, reaching up to 50% and 53% in bacterial and fungal communities respectively. However, in the heterogeneous, highly dispersed and uneven endophytic communities, only a few of these OTUs made up part of the core microbiome (see stars in Fig. 5).

**Microbial taxa as indicators of rootstock–scion combinations in grafted grapevine root system**

Indicator species analysis was performed to test the strength and statistical significance of the relationship between microbial OTUs and the different rootstock–scion combinations (Table 3), and thus to establish which...
OTUs are characteristic for individual or multiple (from two to six) combinations. The analysis detected 35% and 27.3% of bacterial and fungal OTUs respectively, as specifically associated with the rhizosphere of one or more rootstock–scion combinations, while in the case of endophytic communities we detected 12.7% and 44.4% of indicator bacterial and fungal OTUs respectively (Table 3). As a general trend, indicator species tended to be associated with only one rootstock–scion combination, with 81% and 86% bacterial and fungal indicator OTUs in the root tissues respectively, and 50% and 68% in the rhizosphere (see the group with one combination in Table 3). Considering the group containing two rootstock–scion combinations, we observed a decline in microbial indicator OTUs for root tissues (18% and 11% in bacteria and fungi respectively) and the rhizosphere (29% and 23% respectively). Bacterial endophytic indicators were dominated by members of *Rhizobiaceae* (Alphaproteobacteria), *Micromonosporaceae* and *Streptomycetaeae* (Actinobacteria), *Comamonadaceae* and *Steroidobacteraceae* (Gammaproteobacteria) and *Microscillaceae* (Bacteriodia), except for 420A–Chardonnay that presented *Candidatus Phytoplasma (Bacilli)* as indicator OTU (Supplementary Table S10A). The taxonomic diversity of indicator OTUs increased in the rhizosphere, with the presence of numerous families, including *Micromonosporaceae*, *Nocardioidaceae* and *Pseudonocardiaeae* within *Actinobacteria*, *Beijerinckiaceae*, *Rhizobiaceae*, *Sphingomonadaceae* and *Xanthobacteraeae* within *Alphaproteobacteria*, *Comamonadaceae*, *Nitrosomonadaceae*, *Pseudomonadaceae* and *Steroidobacteraceae* within *Gammaproteobacteria*, and several members of *Bacteroidia*, *Gemmatimonadetes*, *Plantomycetes*, *Polyangia*, *Thermoleophila*, *Vencomycicrobaceae* and *Vicinamibacteria* (Supplementary Table S10A). In the case of fungi, the endophytic and rhizosphere indicator OTUs of rootstock–scion combinations were mainly composed of *Nectriaceae*, *Lasiosphaeraceae*, *Chaetomiaceae* (Sordariomycetes) and unclassified of the same class, unclassified Dothideomycetes, unclassified Agaricomycetes, *Herpotrichiellaceae* and unclassified within the *Eurotiales* class, *Rhizasmataceae* and *Helotiales* (Leotiomycetes), *Glomeraceae* (Glomeromycetes) along with members of the *Mortierellomycetes*, *Pezizomycetes*, *Orbiliomycetes* and *Tremellomycetes* classes (Supplementary Table S10B).

### Discussion

Environmental factors, such as physicochemical characteristics of soil, along with plant-related factors (among others, plant genotype) have been reported as the main factors regulating grapevine performance, as well as recruitment and selection of its associated microbiome (Costa et al., 2006; Pancher et al., 2012; Marasco et al., 2013; Zarraonaindia et al., 2015). However, in grafted plants the physical/morphological/chemical mechanisms modulating the plant–soil feedback (Hu et al., 2018; Olanrewaju et al., 2019) are influenced by the interaction...
of, at least, two genotypes, *i.e.* the rootstock and the scion (Gautier *et al*., 2019).

Belowground, the root metabolites, which act as growth substrates for suitable microbial partners, attract the surrounding edaphic microorganisms that form the rhizosphere microbiome (van Dam and Bouwmeester, 2016; Massalha *et al*., 2017; Sasse *et al*., 2018; Vives-Peris *et al*., 2020). This ‘selective pressure’ imposed by the plant on its immediate surrounding results in a sharp decrease in microbial diversity with proximity to the plant (*i.e.* bulk soil > rhizosphere > root tissue; Fig 1), consequently shaping the composition and relative abundance of microbial species associated with the grapevine compartments (Figs 3 and 4) (Bulgarelli *et al*., 2013; Van Der Heijden and Schlappi, 2015; Zarraonaindia *et al*., 2015; Saleem *et al*., 2018). The root exudation pattern is genetically controlled by different factors innate to the plant, including the genotype, with inevitable consequences on the establishment of mutualistic relations with the surrounding edaphic microorganisms (Vives-Peris *et al*., 2020; Chai *et al*., 2022). Recent studies have shown that within a vineyard grapevine rootstock genotypes grafted with the same scion are associated with different rhizospheric and endophytic microbiomes (D’Amico *et al*., 2018; Marasco *et al*., 2018b; Berlanas *et al*., 2019). However, here, we have shown that at ecosystem level across the heterogeneous conditions of different vineyards, when the interaction between rootstock and scion is considered, this is more important than the rootstock or scion considered alone (Fig. 2). This reinforces, at ecosystem scale, what was recently observed for young grapevines cultivated in an experimental field (Vink *et al*., 2021b) and for apple trees from a single orchard (Chai *et al*., 2022). In addition, our study provides evidence that this process is extended to the endophytic root communities, and to the fungal components of the grape microbiome.

The aboveground component of the plant (the scion) is involved in communication with the rootstock belowground for nutrient exchange, growth signalling and many other aspects of plant physiology (Tandonnet *et al*., 2009; Aloni *et al*., 2010; Martínez-Ballesta *et al*., 2010; Bonghi *et al*., 2016; Gautier *et al*., 2019). Among them, we find the production and distribution of phytohormones. Auxins, which are produced in the shoot apices, are translocated to the roots where they regulate many aspects of plant vegetative and reproductive development, such as root system growth, architecture and functioning (Persello-Cartieaux *et al*., 2001; Aloni *et al*., 2010; Overvoorde *et al*., 2010; Egamberdieva *et al*., 2017). Although the mechanism was not fully elucidated, it has been shown that in young grapevines the scion genotype confers different root vigour possibly through signal molecules and hormones rather than by carbon supply from the shoot (Tandonnet *et al*., 2009). Since roots play a direct role in the establishment and maintenance of the interaction between host plants and soil microorganisms, any modification in their architecture (*i.e.* overall length, density, branching and biomass) and root morphology (*i.e.* diameter, surface area and root hairs) can affect the plant-associated microbial communities and the recruitment of beneficial microorganisms (Herms *et al*., 2022). For instance, thinner root diameter recruits a more diverse rhizosphere community in different plants, including arboreal and herbaceous species, which can often result in a positive impact on plant health and productivity (Bardgett and van der Putten, 2014; Saleem *et al*., 2019; Herms *et al*., 2022).

Phytohormones, such as auxin and salicylic acid, are also key players in mediating the crosstalk between plants and beneficial bacteria, acting as reciprocal signalling molecules in plant–microbe interactions (Lebeis *et al*., 2015; Khan *et al*., 2020). Thus, modifications in the hormones’ distribution mediated by the plant genotypes (and their interactions) could affect the composition of the plant microbiome. In our work, we found predominant amounts of Proteobacteria and Actinobacteria among bacteria, and Ascomycota and Basidiomycota among fungi in all the rhizosphere and root samples, but with differential distribution across the grafting combinations (Fig. 4). Microbial discriminants and indicator species were in part constituted by OTUs shared across all the combinations but with differential enrichment in terms of relative abundance (Fig. 5; Table 3). Such variability in the relative abundance possibly occurs because such OTUs are differentially supported by specific combination of scion/rootstock genotypes. As suggested by Semchenko *et al*., 2022, the identification of the factors that promote the plant association with microbial specialists and/or generalists will advance our understanding of the mechanisms regulating plant–soil feedback and of the ways plant–microbiome contributes to plant performances.

The compatibility of the plant genotype with the growth/association of with beneficial microorganisms is a *sine qua non* requisite for the plant to benefit from the services provided by such microorganisms and stochastic encounters with PGP microorganisms are not sufficient to explain the health benefits mediated by the plant microbiome (Haney *et al*., 2015). For instance, during bacteria-based treatments conducted in the greenhouse with Barbera plantlets grafted onto 420A, SO4 and KSBB rootstocks, it has been observed that the water deficit-tolerance mediated by PGP bacteria was a rootstock dependent trait; the three rootstocks showed different physiological responses and phenotypes (*i.e.* biomass, photosynthetic activity, evapotranspiration and stomatal conductance) in response to the treatment with a given PGP bacterium (Roli *et al*., 2012, 2015). Similarly, by exploiting the availability of 196 naturally occurring...
accessions of Arabidopsis thaliana, Haney et al. (2015) demonstrated that the genotype was an essential factor in determining whether a microbial community provides benefit or harm to its host. The authors showed that different plant genotypes within a single species (A. thaliana) have different rhizosphere microbiomes, which mainly differ in terms of the presence of beneficial Pseudomonas bacteria, with consequences for plant health. Extending this finding to our work, it is plausible to suppose that any change in the distribution of microorganisms driven by the interaction between the rootstock and the scion can have further influence on the ecological services carried to the host and to the ecosystem. One interesting example in our study is provided by Candidatus Phytoplasma, detected by different analyses exclusively as an indicator of 420A.

This suggests that this specific combination is more susceptible to colonization of the plant root by this intracellular parasite and the related insect vectors (Aryan et al., 2014). Notably, in the root tissues of 420A grafted with different scions, including Merlot and Sangiovese (both from this work) and Barbera (Marasco et al., 2018b), OTUs assigned to Candidatus Phytoplasma were not detected.

Since microbes interacting with plants are involved in sustaining plant fitness, growth promotion and disease control (Delgado-Baquerizo, 2022), the results reported here indicate that it is important to consider the rootstock–scion combination rather than only the rootstock to evaluate the process of recruitment and selection of edaphic microorganisms mediated by the plant. Such considerations are also valid when we consider the plant microbiome and its engineering for sustainable agriculture (Kaul et al., 2021): the link between the agronomic features, grafting combinations and microbiome is instrumental to understand the performance reports, often contradictory, of grafted grapevines in different areas (Ibacache et al., 2020). In the case of viticulture, the genotype-mediated selection carried out by the root system becomes a more important factor because it can also affect the microbiome associated with other plant organs, such as the fruit, potentially impacting the final product (Bokulich et al., 2016; Griggs et al., 2021).

Conclusion

Considering the importance of the microbiome for several aspects of grapevine physiology, stress tolerance and pathogen protection, it is important to elucidate the role of plant components (i.e. rootstock, scion, and their combination) in ecological processes that shape plant–microbe interactions. Which genotype, which rootstock, which scion or which of their combinations, has the greatest effect on the selection of microorganisms? The data provided here underline the importance of considering the interaction between the genotypes that compose the overall host, namely, rootstock and scion, as a factor that drives the recruitment and selection of the surrounding edaphic microbiome by the root system. In our study, the interaction of rootstock and scion resulted to be more important in shaping the root system microbiome than the rootstock and scion considered separately. Thus, selection of a given rootstock–scion combination is not only crucial for physiological adaptation of the grapevine to a certain vineyard/ecosystem but has also important repercussions on the overall metaorganism (plant and microbiome) and its fitness. Although the relative weight of each genotype in determining the composition of a microbial community has yet to be elucidated, the results obtained contribute towards creating a new perspective for research, targeted at understanding and explaining the role of rootstock–scion combination management in the ecology of the grapevine microbiome. This could also lay the foundations for including the microbiome in grapevine breeding programmes of scions and rootstocks and for improving grapevine physiology through microorganism/microbiome-based treatments.

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Availability of Data and Materials

The datasets analysed in this current study are available in the NCBI SRA repository under the BioProject PRJNA807110. The codes used to perform the analyses are available at https://github.com/MarcoFusi1980/Tuscan_Vineyard_microbial_ecology.

Authors’ Contributions

R.M., M.F. and D.D.: Conceived and designed the study and experiments. R.M., M.F. and D.D.: Sample collection. R.M. and H.A.: Performed the experiments. R.M. and M.F.: Analysed the data. D.D.: Contributed reagents/materials/analysis tools. R.M., H.A. and M.F.: Wrote the paper. D.D., I.G. and L.V.: Critical revision of the manuscript. All the authors critically revised the manuscript.
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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information.

**Supplementary Table S3.** Number of sequences and OTUs detected after quality trimming, exclusion of non-target amplicons and unclassified reads for bacterial and fungal communities associated with bulk soil, rhizosphere and root tissue. Refer to Marasco et al 2021_Supplementary Table S3.

**Supplementary Table S8.** List of OTUs and related variance detected by multivariate generalized linear model for discriminating bacterial and fungal communities across compartments. The assigned taxonomy of each OTU is displayed at the phylum and class level. Refer to Marasco et al 2021_Supplementary Table S8.

**Supplementary Table S9.** List of OTUs and related variance detected by multivariate generalized linear model for discriminating bacterial and fungal communities across rootstock–scion combinations in each compartment. The assigned taxonomy of each OTU is displayed at the class level. Refer to Marasco et al 2021_Supplementary Table S9.

**Supplementary Table S10.** List of bacterial and fungal indicator OTUs of rootstock–scion combinations in root tissues and rhizosphere compartments. Refer to Marasco et al 2021_Supplementary Table S10.