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Article

Contrasting effects of genotype and root size on the fungal and bacterial communities associated with apple rootstocks

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Abstract

The endophytic microbiome of plants is believed to have a significant impact on its physiology and disease resistance, however, the role of host genotype in determining the composition of the endophytic microbiome of apple root systems remains an open question that has important implications for defining breeding objectives. In the current study, the bacterial and fungal microbiota associated with four different apple rootstocks planted in April, 2018 in the same soil environment and harvested in May, 2019 were evaluated to determine the role of genotype on the composition of both the bacterial and fungal communities. Results demonstrated a clear impact of genotype and root size on microbial composition and diversity. The fungal community was more affected by plant genotype whereas the bacterial community was shaped by root size. Fungal and bacterial abundance was equal between different-sized roots however, significantly higher microbial counts were detected in rhizosphere samples compared to root endosphere samples. This study provides information that can be used to develop a comprehensive and readily applicable understanding of the impact of genotype and environmental factors on the establishment of plant microbiome, as well as its potential function and impact on host physiology.

Introduction

Grafting together different combinations of scion and rootstock genotypes to impart specific cultural characteristics is a common horticultural practice in fruit production, especially apple [1–3]. Traits, such as growth habit, disease resistance, dormancy, cold hardiness, drought tolerance, and a range of other physiological traits can all be conveyed to the developing scion cultivar as it grows and develops into maturity. Therefore, as with scion cultivars, rootstock breeding programs are designed to develop new rootstock genotypes with specific properties and/or the ability to impart specific traits to grafted scion cultivars. The most common use of rootstocks are to control tree size and impart disease resistance (e.g. apple replant disease caused by several species of soilborne plant pathogens and resistance to fire blight caused by the bacterium, *Erwinia amylovora*), other traits have also been explored [4]. In particular, the ability of different rootstocks to differentially acquire water and various nutrients from the soil has been of significant recent interest [5].

A larger, perhaps more theoretical, question of significant practical importance centers on the degree to which the microbial community may contribute to determining the properties of a rootstock, such as disease resistance, growth, and fruit quality. In this regard, the rhizosphere microbial community of plants, and its role in plant health and stress tolerance has been widely studied and the significant positive impact of the rhizosphere and endophytic microbial community on plant physiology has been well documented [5–8]. For example, some bacterial taxa have been designated as plant growth-promoting rhizobacteria (PGPR) for their ability to foster plant growth and health in response to biotic and abiotic stress [9]. Additionally, some microbial strains have also been used as seed treatments to promote germination under adverse environmental conditions and generally improve plant health, disease resistance, and

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stress tolerance. Regarding apple, microbial application of endophytes derived from *Salix* and *Populus* to the soil of pots growing non-grafted "Honeycrisp" apple trees was reported to increase fruit size and glucose levels [10]. Van Horn *et al*. demonstrated that rootstock genotypes that expressed greater tolerance to apple replant disease (ARD) harbored a microbial community that differed from rootstocks that were more susceptible to ARD [11]. In that study, microbial communities exhibited significant differences in composition between the rhizosphere and endophytic environments in terms of species diversity, content, and abundance. The composition and function of plant microbiomes are highly dependent on the plant genome and the environment in which they reside [12, 13]. Translating this knowledge into agricultural practices, however, will require additional comprehensive studies of microbial communities and an interdisciplinary, systems-level approach to understand how these communities interact with other components of the agroecosystem.

The role of genotype in determining the composition of the rhizosphere and endophytic microbiome of plants has been a subject of several studies [14–17]. In addition to the research conducted by Van Horn *et al*. [11], Liu *et al*. reported a distinct impact of both scion and rootstock genotype on the composition of the endophytic community of "Golden Delicious", "Royal Gala", and "Honecrisp" apple trees grafted on either "M.9" or "M.M.111" rootstocks [15]. The study also indicated that pedigree was related to the composition of the endophytic microbial community, with "Golden Delicious" and "Royal Gala" being more closely-related in terms of pedigree, clustering together in a Principal Components Analysis of beta diversity based on Bray-Curtis dissimilarity indices, with "Honeycrisp" clustering completely separately.

To what degree does genotype affect the recruitment and composition of plant-associated microbiota and how this effect determines the phenotype of a plant genotype, remain open questions? Microbial communities in bulk soil are shaped largely by edaphic factors, such as soil pH and organic matter content, but the rhizosphere microbiome is shaped and supported by nutrients, exudates, secondary metabolites, mucilage, and border cells secreted by roots (i.e. rhizodeposits), all of which can differ among plant species and genotypes, and lead to host-specific rhizosphere communities that differ substantially in composition from those of bulk soil [18–22]. An even smaller and more specialized subset of these microbes are able to establish themselves in host tissues as endophytes [11, 15, 23, 24]. The present study was designed to determine: i) how rootstock genotype affects the composition and diversity of rhizosphere and endophytic fungal and bacterial communities? ii) the recruitment patterns of apple roots, i.e. from bulk soil, to rhizosphere, to the endosphere, and iii) the relationship between root size and community composition. The bacterial and fungal microbiota associated with four different apple rootstocks planted in the same soil environment were evaluated by amplicon sequencing and RT-qPCR. Samples used to characterize the microbial taxa included bulk soil, rhizosphere, and endophytes present in fine roots (≤ 2 mm), intermediate roots (2–4 mm), and large roots (4–12 mm).

Results

General summary of sequencing results

Representative photographs of the rootstock planting directly after planting and during mid-summer of the first year are presented in Fig. 1. Samples of bulk and rhizosphere soils were initially sampled followed by root samples for endophytic microbiome analysis. High throughput sequencing yielded a total count of 714 278 ITS2 reads which were assigned to 4995 fungal ASVs and 1 335 057 16S reads which were assigned to 26 381 prokaryotic ASVs (Table S1).

Effect of genotype on the rootstock microbial diversity and community composition

Analysis of the root endophytic and rhizosphere sample indicated that genotype had a significant effect on the fungal diversity of apple rootstocks (Table 1). Pairwise comparisons among genotypes, however, indicated that only "G.935" had a significantly higher Shannon diversity relative to the other genotypes (Fig. 2A). In contrast, the effect of rootstock on bacterial Shannon diversity was not significant (Table 1 and Fig. 2B). Notably, however, a strong effect of genotype was observed on the composition (beta diversity) of the fungal community, accounting for 12% of the observed variation ($P = 0.001$). Similarly, rootstock genotype also had a significant effect on the bacterial community composition, although accounting for less variation (5%) ($P = 0.001$) than fungi (Table 2).

A Principal Coordinate Analysis (PCoA) of the fungal and bacterial communities based on Bray–Curtis dissimilarity indices (Fig. 2C and D), along with a hierarchal clustering analysis (Fig. 2E and F), revealed that the effect of genotype on community composition was more evident for the fungal community than it was for the bacterial community. Notably, bacterial communities present in the bulk soil and rhizosphere clustered together, while fungal communities of these same sample types were distinctly separate. The collective root endophyte samples (small, intermediate, and large) of the fungal community exhibited a significant level of independent clustering within each genotype (Fig. 2C). In contrast, clustering of the collective root endophytic samples of the bacterial community based on genotype was less evident (Fig. 2D).

Hierarchical clustering of the microbial communities based on genotype (including all sample types), also displayed a distinct clustering for both the fungal and bacterial communities (Fig. 2E and F). Different colors in the hierarchical clustering in Figs. 2E and F represent related groupings based on similarities in their fungal and bacterial communities. In both cases, bulk soil as

Figure 1. Photographs of the rootstock planting taken after the initial planting in April, 2018 (A) and then during mid-summer (B). Trees were removed from the ground in May, 2019 and destructively sampled. The row was blocked into five sections and a replicate of each tree was randomly assigned a planting site within each of the blocks.

Table 1. ANOVA analysis of the effects of rootstock genotype and root tissue, and their interactions on Shannon diversity of the bacterial and fungal communities on apple trees.

Shannon Anova		Df	Sum Sq	Mean Sq	F value	Pr(>F)
Fungi	**Rootstock Genotype**	**3**	**1.58**	**0.5265**	**3.979**	**0.0119**
	Root Tissue	2	0.01	0.0052	0.039	0.9615
	Genotype:Tissue	6	0.526	0.0876	0.662	0.6803
	Residuals	60	7.939	0.1323		
Bacteria	Rootstock Genotype	3	0.096	0.0321	0.323	0.80861
	Root Tissue	**2**	**1.276**	**0.6382**	**6.431**	**0.00295**
	Genotype:Tissue	6	0.955	0.1592	1.604	0.16175
	Residuals	60	5.954	0.0992		

an outlier clustered separately from rootstock genotypes. The dendrogram indicates that the fungal communities of "G.222" and "G.935" were more similar to each other than to "G.41" and "Bud.9" rootstocks. Similarly, "G.41" and "Bud.9" were more similar to each other than to the other two genotypes (Fig. 2E). The dendrogram of the overall bacterial communities (all sample types) indicates that three of the rootstock genotypes ("G41", "Bud.9", and "G.222") were more similar to each other than they were to the other rootstock genotype ("G.935").

Microbial diversity and community composition in rhizosphere and root endosphere samples

Distinct patterns in the alpha diversity (Shannon Index) were observed within each cultivar based on sample type, with fungal and bacterial diversity exhibiting contrasting trends (Fig. 3A, B). Fungal diversity was highest in rhizosphere samples and lowest in fine roots (Fig. 3A). In contrast, the highest level of alpha diversity for bacteria was observed in fine roots and the lowest in rhizosphere tissues (Fig. 3B). A general gradient in the level of fungal

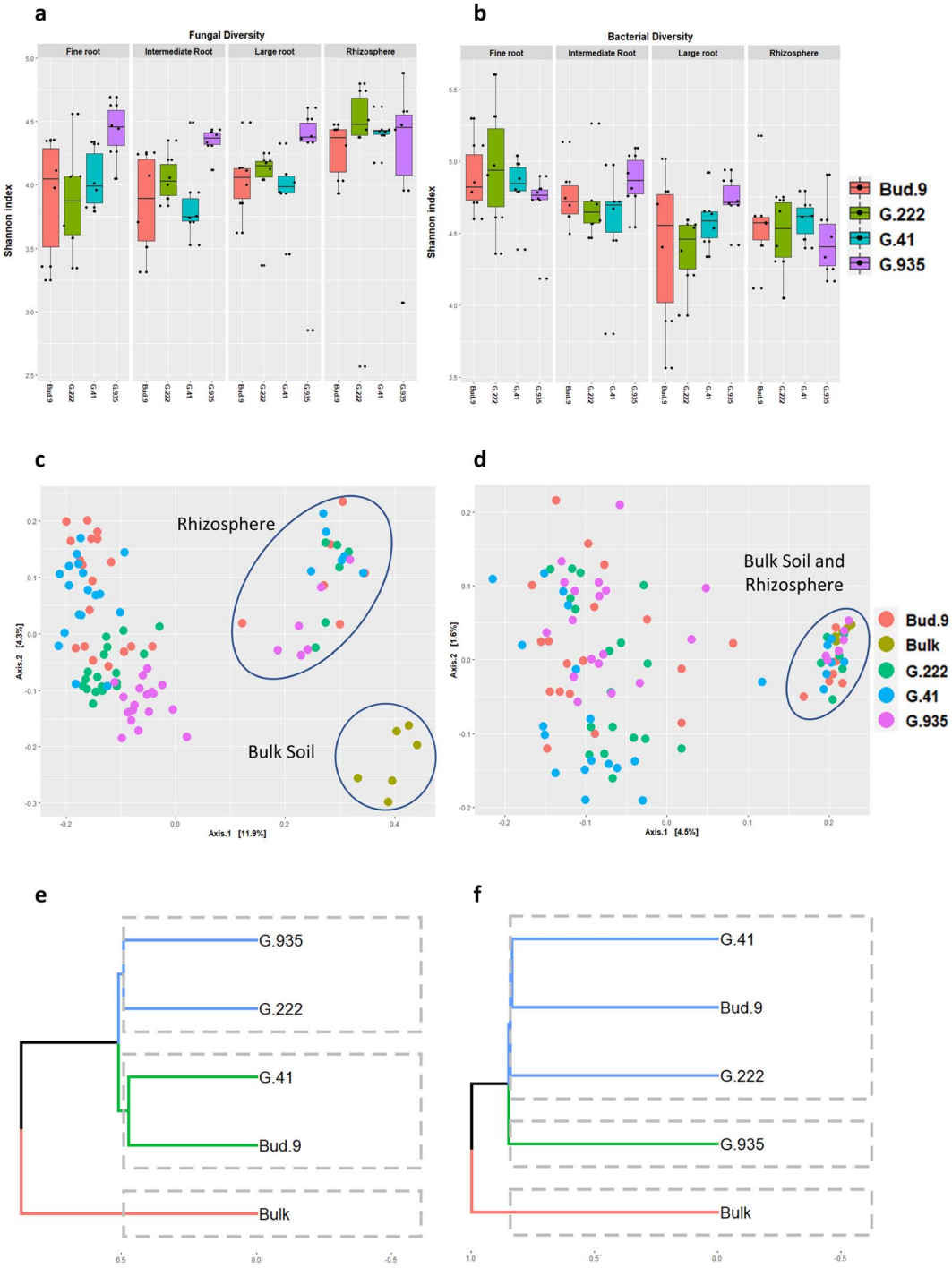

Figure 2. Effect of genotype on the plant fungal and bacterial diversity and community composition. Alpha diversity (Shannon diversity) of the fungal (A) and bacterial (B) community in the rhizosphere, fine, intermediate, and large roots of four apple rootstock genotypes ("Bud.9", "G.222", "G.41" and "G.935"). PCoA plots of beta diversity, based on Bray-Curtis, of the fungal community (C) and bacterial community (D) of the bulk soil, and four apple rootstock genotypes. Bulk soil and rhizosphere samples are circled. Samples outside of the circles represent clustering of the collective root endophyte (small, intermediate, and large) samples based on rootstock genotype. Hierarchical clustering of the fungal community (E) and bacterial community (F) of bulk soil, and four apple rootstock genotypes. Different colors in the hierarchical clustering represent related groupings based on the similarities in their fungal and bacterial communities.

and bacterial diversity was apparent within each cultivar, except for fungal diversity in the "G.935" rootstock genotype. No major differences in alpha diversity were observed between cultivars in the level of fungal and bacterial diversity in the different sample types (Fig. 3A and B).

A PCoA analysis, based on sample type (bulk soil, rhizosphere, fine, intermediate, and large roots) within a genotype, revealed a clear separation of rhizosphere samples from root endophyte samples for both the fungal and bacterial communities (Fig. 3C and D). Similar to the results on the impact of genotype on the composition of

Table 2. PERMANOVA analysis of the effects of rootstock genotype and root tissue, and their interactions on bacterial and fungal communities of apple trees.

		Df	Sums of Sqs	MeanSqs	F.Model	R2	Pr(>F)
Fungi	Rootstock Genotype	3	2.4551	0.81838	3.1011	0.11674	0.001
	Root Tissue	2	0.8312	0.4156	1.5748	0.03952	0.001
	Genotype:Tissue	6	1.9095	0.31825	1.2059	0.0908	0.004
	Residuals	60	15.8341	0.2639		0.75293	
Bacteria	Rootstock Genotype	3	1.4897	0.49657	1.17787	0.04917	0.001
	Rootstock Tissue	2	0.9855	0.49277	1.16885	0.03253	0.001
	Genotype:Tissue	6	2.5244	0.42074	0.99798	0.08333	0.537
	Residuals	60	25.2952	0.42159		0.83497	

The comparisons were based on Bray Curtis dissimilarity, and p-values were calculated using the adonis function in vegan and corrected using the FDR method.

the root endophytic community, within a given rootstock genotype, root-size class influenced fungal community composition to a greater extent than it did the composition of the bacterial community. Clustering of different root types within a genotype was most readily apparent in "Bud.9" and "G.41" for the fungal community. In contrast, no distinct clustering based on sample type was evident for bacterial community composition, except for fine and large roots in "G.41".

Hierarchical clustering analysis also indicated a separation in the composition of the fungal community between and within the different rootstock genotypes (Fig. 4A). As noted previously, "Bud 9" and "G.41" were more similar to each other than they were to "G.222" and "G.935" and the latter two genotypes were mores similar to each other than they were to the two former genotypes. Three of the rootstock genotypes ("Bud.9", "G.41", and "G.935") exhibited the same pattern of relatedness within the different-sized root samples. Notably, intermediate and large roots occur on the same branch of the dendrogram, while fine roots are placed on their own branch. In contrast, intermediate roots and fine roots in "G.222" were more similar to each other than to large roots, which was placed on its own branch of the dendrogram (Fig. 4A). Separation of root types between and within rootstock genotypes, however, was not evident for the bacterial community (Fig. 4B).

Effect of genotype and root size on microbial abundance

Fungal and bacterial abundance, estimated via RT-qPCR, was not affected by genotype (Fig. S1 and Table S2). Here, the rootstocks of "Bud.9", "G.222", "G.41", and "G.935" demonstrated 1.37, 8.16, 9.86 and 8.86 mean fungal copy numbers, respectively. Bacterial abundance was higher for all genotypes, giving the following respective values: 9.69×10^9, 7.9×10^9, 1.2×10^{10} and 6.9×10^9. Comparing the tissue types of all genotypes combined revealed significantly higher copy numbers in rhizosphere samples than in large root and intermediate root samples. For bacteria, rhizosphere demonstrated significantly higher copy numbers compared to all three root endosphere samples. No effect of root size (i.e. comparing endosphere samples) was observed, however, for fungal and bacterial abundance.

Comparative analysis of taxa and community network analysis

Venn diagrams were constructed to illustrate the number of shared and unique fungal and bacterial ASVs with a relative abundance >0.01% present in each rootstock genotype (Fig. 5). As illustrated in the Venn diagrams the majority of fungal and bacterial ASVs were shared between all genotypes and tissue-types. Notably, rootstock genotypes, "G.41" and "G.935" had the greatest number of unique ASVs in the majority of tissue types. This was especially true for large roots, where "G.41" exhibited eight unique bacterial ASVs and "G.935" exhibited four unique ASVs. Some uniqueness in the number of bacterial and fungal ASVs shared between specific genotypes in specific tissue-types was also evident.

There were more fungal ASVs than bacterial ASVs that were differentially abundant between the four rootstock genotypes (Fig. 6). Notably, among the differentially abundant fungal genera, the genus *Cadophora*, a known wood-decay pathogen of apples and pears, exhibited a relatively high level of abundance in "G.41". Other noteworthy differences include: a relatively low abundance of *Colletotrichum* in the "G.935" rootstock, relative to the other rootstock genotypes; A low relative abundance of *Rhizoctonia* in "G.41", relative to the other rootstock genotypes; a high relative abundance of *Lachnum* (a genus capable of producing IAA and enhancing shoot growth) in "Bud.9", compared to the other rootstock genotypes; and a significantly higher relative abundance of *Pezicula* in "G.41", relative to the other rootstock genotypes. Among the bacterial taxa, *Bradyrhizobia* (which can fix nitrogen and produce IAA) was most abundant in the "G.41, 'G.222', and 'Bud9" apple rootstock genotypes, relative to 'G.935'. Additional information on the differentially-abundant taxa are provided in Fig. S2 and Table S3.

Network analysis of the fungal and bacterial communities in the different genotypes are presented

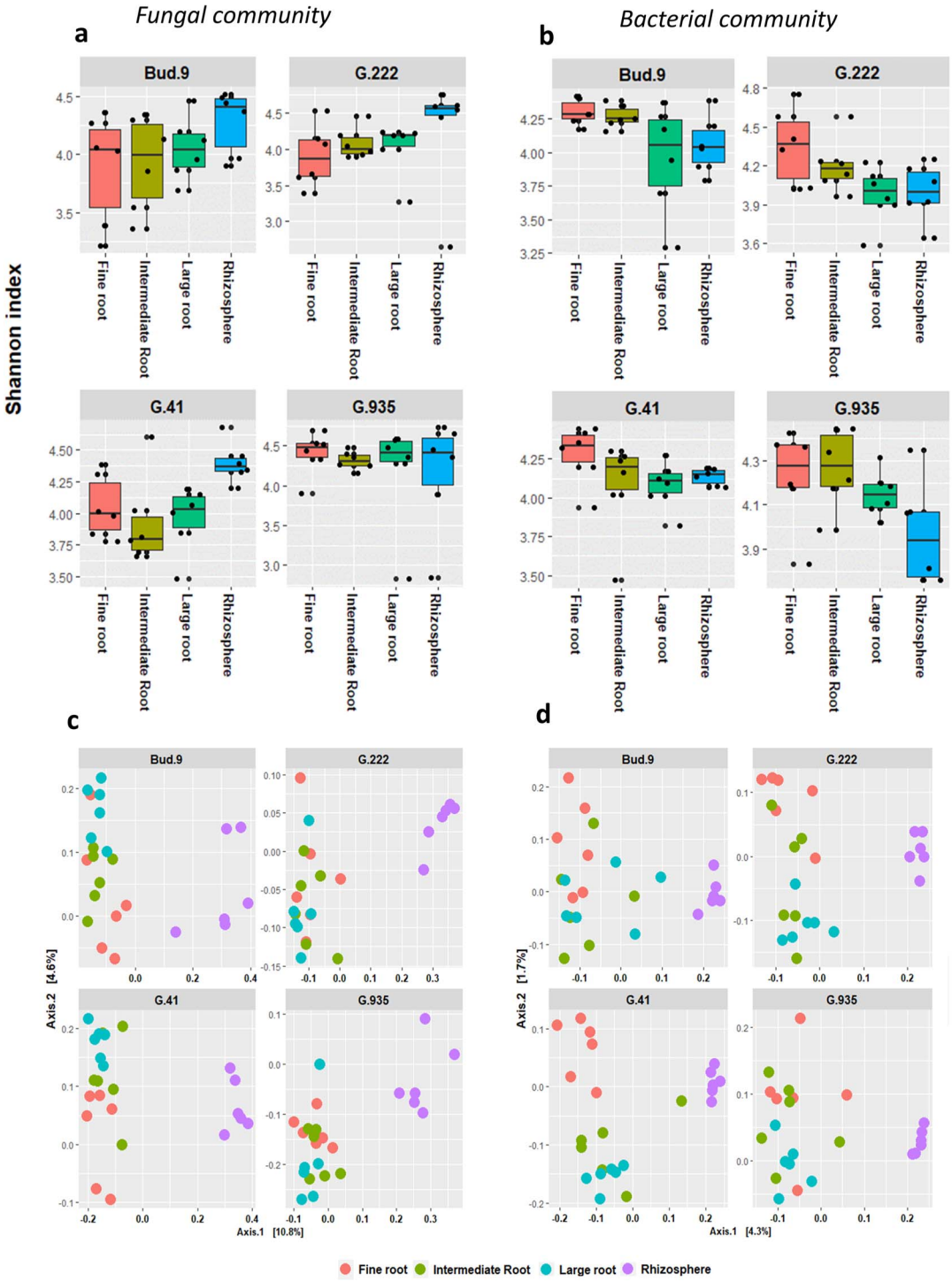

Figure 3. Alpha diversity (Shannon diversity) of the fungal community (A) and bacterial community (B) in the rhizosphere, fine, intermediate, and large roots within each of the four apple rootstock genotypes ("Bud.9", "G.222", "G.41" and "G.935"). PCoA plots of beta diversity, based on Bray-Curtis, of the fungal community (C) and bacterial community (D) of the rhizosphere, small, intermediate, and large roots within each of the four apple rootstocks.

in Figs. 7 and 8, respectively. Keystone taxa in each genotype are indicated with an arrow. Importantly, taxa presented in Figs. 7 and 8 are at the level of order while networks at the highest definable taxa level are presented in Figs. S3–S10. This was done to reduce the level of complexity of the presented data. Notably, the bacterial interactions were dominated by co-occurrence i.e. positive interactions, while the fungal network exhibits a mixture of co-occurrence and co-exclusion i.e. positive and negative interactions. Although the size of the fungal networks (avg. number of nodes = 146, avg. no, edges = 814) was larger than the bacterial networks (avg. no. nodes = 51, avg. no. edges = 814), the bacterial network density was much greater for bacteria (Average = 0.222) than fungi (Average = 0.082). This indicates that the members of the bacterial community had a much higher tendency to interact with each other than fungi.

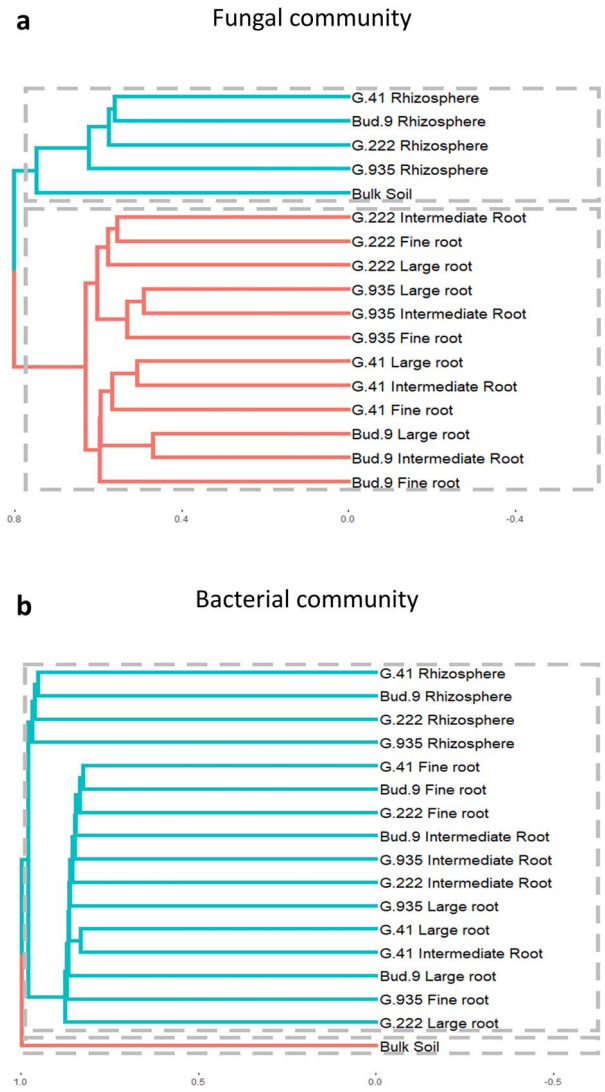

Figure 4. Hierarchical clustering of the fungal community (A) and bacterial community (B) in bulk soil, and the rhizosphere, small, intermediate, and large roots within each of the four apple rootstocks ("Bud.9", "G.222", "G.41" and 'G.935).

Discussion

Recently developed DNA sequencing technologies and the use of universal primer sets has allowed researchers to begin to explore microbial communities inhabiting the surface and interior of plants and provide a wealth of new information. Despite this new information, numerous additional studies are needed to develop a complete and readily-applicable understanding of the impact of genotype and environmental factors on the establishment of the plant microbiome, as well as its function and impact on host physiology. Plant tissues and the rhizosphere recruit complex fungal and bacterial communities, and their composition is affected by host traits such as genotype, tissue type, tissue age, and environmental conditions [25, 26]. Rootstocks are widely-used in commercial apple orchards to obtain a range of economically beneficial cultural traits [5, 26, 27]. Despite the importance of the rhizosphere and endophytic root microbial community as reservoirs of plant microorganisms, little is known about the impact of different apple rootstock genotypes on the composition of their associated microbial communities. In the present study, the diversity and composition of the fungal and bacterial communities in the rhizosphere and endosphere of different-sized roots of four apple rootstocks planted in the same soil environment was characterized to determine the effect of genotype and root size on those microbial communities. High-throughput amplicon sequencing indicated that bacterial diversity was lowest in the rhizosphere and highest in fine roots, while the highest level of diversity for fungi was observed in rhizosphere soils and lowest in fine roots. This stands in contrast to the report by Poudel *et al.*, who reported that alpha diversity of the bacterial community was higher in the rhizosphere than it was in the endosphere of tomato root systems [28]. Notably, however, Kim *et al.* analyzed the structure of the bacterial and fungal community in both the rhizosphere and endosphere of ten-year-old kiwifruit plants, and also found that the microbial community of the rhizosphere was less diverse than the root endosphere community [29]. Van Horn *et al.* reported that rhizosphere bacterial and fungal communities were more diverse than the corresponding endophytic community across multiple orchard soils and apple rootstock genotypes [11]. Whether the differences in these results can be attributed to variations in nutrient availability in the different soils and types of samples (rhizosphere vs. fine roots) or the trophic properties of the bacterial and fungal taxa present will require further study.

The statistical analysis (Tables 1 and 2), the PCoA plots (Fig. 2C and D), and hierarchical clustering (Fig. 2E and F) revealed a significant effect of rootstock genotype on the Shannon diversity of the fungal and community but not the bacterial community. Genotype did have a significant influence on the composition (beta diversity) of both the fungal and bacterial community (Table 2). Although it was more evident in the fungal community. Different root types also had a significant impact on the composition (beta diversity) of both the fungal and the bacterial community (Table 2) and a significant interaction between genotype and sample type (tissue type) was observed on the composition of the fungal community but not the bacterial community. These results are consistent with our previous report indicating a significant effect of rootstock genotype on the composition of the endophytic fungal community of roots [15], although in that study pooled roots of different sizes were analyzed. Rootstock genotype was also shown to have a significant influence on the composition of root-associated fungal and bacterial community composition [11, 30]. Notably, bacterial and fungal community profiles in general diverged between Geneva series and Malling series rootstocks. While genotypes within the Geneva or Malling rootstock series are derived from similar parentage, the two series as a whole are genetically divergent, supporting the role of genetic components in

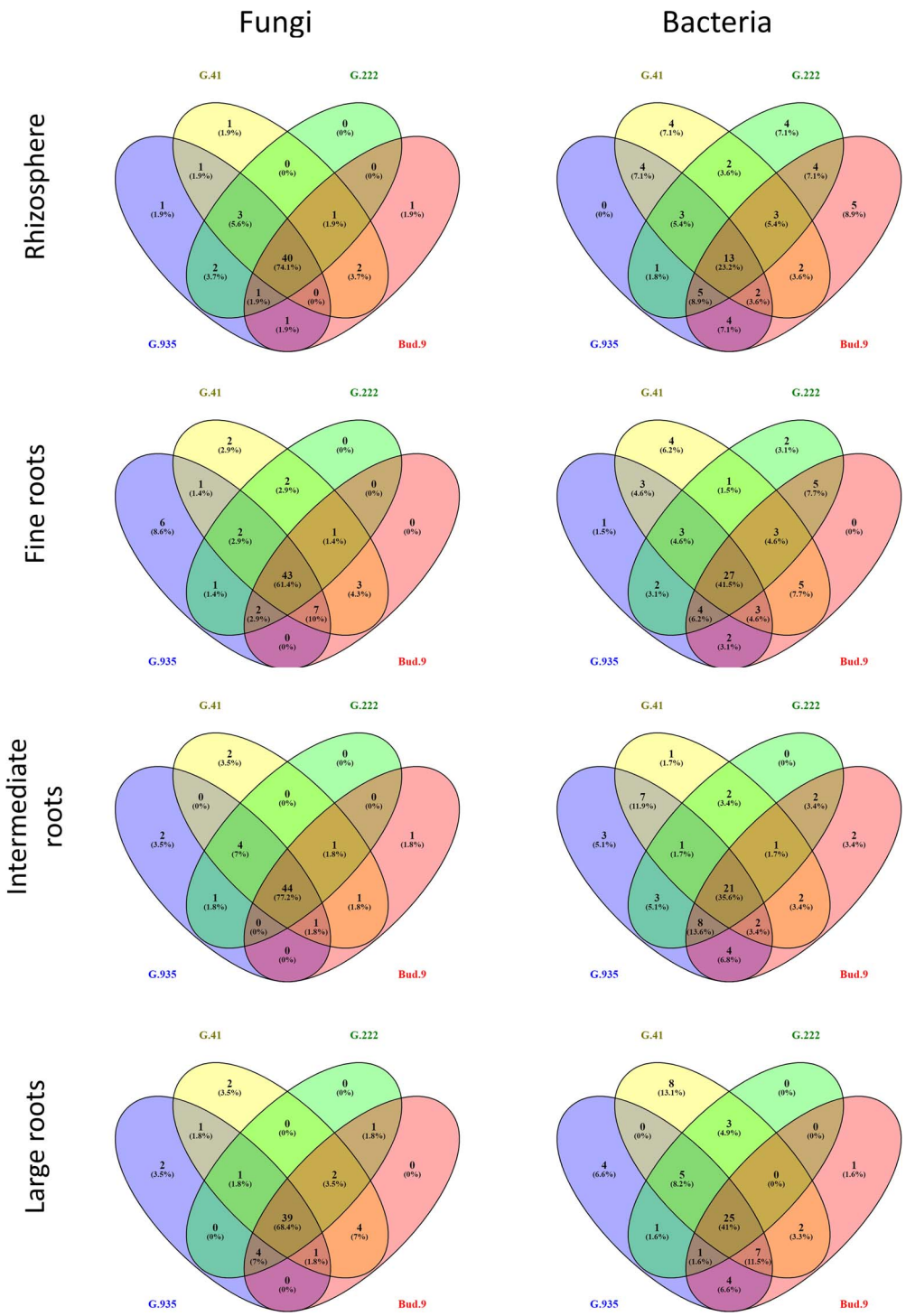

Figure 5. Venn diagram indicating the shared and unique fungal and bacterial ASVs among apple rootstock genotypes in different roots tissues. Taxa were filtered to include the most abundant ASVs, with at least 0.01% relative abundance.

shaping microbiome composition. A preliminary study by Thompson, *et al*. also reported an effect of apple rootstock genotype on the root-zone microbial community [5]. A study examining the effect of cover crop on the microbial community in apple orchards also reported a significant effect of apple rootstock genotype [31]. When cultivated in Brassicaceae seed-meal-amended orchard replant soil, rootstock genotype was shown to significantly influence the composition of the apple rhizosphere microbiome. Disease control attained in response to the seed meal soil amendment was dependent upon the activity of an altered rhizosphere microbiome which produced altered expression in genes related to plant defense and hormone signaling [32]. Thus, rootstock genotype may have a significant effect on multiple disease control or fertility management practices that rely upon the activity of the root-associated microbiome. Similarly, Marasco, *et al*. reported a significant effect of grapevine rootstock

Genera	FDR corrected P	Bulk	G.935	G.41	G.222	Bud.9
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__	0.000439	0.00%	6.98%	11.90%	14.71%	10.34%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae;g__	0.025821	0.00%	5.61%	5.53%	7.70%	10.40%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Rhodoplanes	0.042023	0.00%	2.71%	7.99%	10.62%	8.18%
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__	0.044007	0.00%	5.15%	2.52%	3.14%	6.52%
k__Fungi;p__Ascomycota;c__unidentified;o__unidentified;f__unidentified;g__unidentified	0.000001	0.00%	0.57%	3.17%	0.52%	2.85%
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Agaricales;f__Hygrophoraceae;g__Hygrocybe	0.000001	6.68%	2.11%	0.20%	0.02%	0.18%
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Sordariales;f__Chaetomiaceae;g__Trichocladium	0.000027	0.00%	0.18%	0.63%	0.33%	2.09%
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Polyporales;f__Xenasmataceae;g__Phlebiella	0.000027	0.00%	0.00%	0.00%	0.00%	0.90%
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Glomerellales;f__Glomerellaceae;g__Colletotrichum	0.000027	0.00%	0.36%	1.98%	0.76%	1.38%
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Cantharellales;f__Ceratobasidiaceae;g__Rhizoctonia	0.000047	0.00%	5.82%	0.01%	5.83%	0.10%
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Helotiales;f__Helotiales_fam_Incertae_sedis;g__Cadophora	0.000069	0.11%	7.90%	20.82%	13.84%	13.44%
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Corynesporascaceae;g__Corynespora	0.000098	0.00%	0.89%	0.30%	0.31%	0.00%
k__Fungi;p__Rozellomycota;c__Rozellomycotina_cls_Incertae_sedis;o__GS10;f__unidentified;g__unidentified	0.000372	20.80%	21.58%	8.43%	11.95%	13.35%
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Chaetothyriales;f__unidentified;g__unidentified	0.000894	0.00%	2.17%	1.53%	2.92%	1.03%
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Helotiales;f__Helotiaceae;g__Infundichalara	0.000894	0.00%	0.00%	1.37%	0.00%	0.12%
k__Fungi;p__Mortierellomycota;c__Mortierellomycetes;o__Mortierellales;f__Mortierellaceae;g__Mortierella	0.001224	33.30%	8.32%	9.28%	11.73%	7.90%
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Dothideales;f__Aureobasidiaceae;g__Kabatiella	0.001518	0.00%	0.00%	1.41%	0.01%	0.01%
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Trechisporales;f__Hydnodontaceae;g__Trechispora	0.001518	0.00%	2.30%	3.03%	5.66%	4.58%
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Ascochyta	0.002204	0.79%	2.70%	2.03%	0.96%	2.37%
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Didymella	0.002204	0.22%	0.86%	0.32%	0.13%	0.67%
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Dothideales;f__Aureobasidiaceae;g__Aureobasidium	0.003153	0.00%	1.31%	1.42%	2.79%	1.26%
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Agaricales;f__unidentified;g__unidentified	0.003546	11.82%	2.01%	1.79%	2.11%	1.58%
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Helotiales;f__Hyaloscyphaceae;g__unidentified	0.003809	0.00%	1.60%	2.70%	0.47%	0.38%
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Conioscyphales;f__Conioscyphaceae;g__Conioscypha	0.004304	2.25%	0.30%	0.09%	0.01%	0.08%
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Helotiales;f__Hyaloscyphaceae;g__Lachnum	0.004763	0.00%	2.69%	4.74%	4.52%	9.06%
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__unidentified;f__unidentified;g__unidentified	0.005307	0.46%	1.53%	0.88%	2.40%	0.81%
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Cantharellales;f__Ceratobasidiaceae;g__Thanatephorus	0.007876	4.79%	0.00%	0.00%	0.00%	0.00%
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Cystofilobasidiales;f__Cystofilobasidiaceae;g__Cystofilobasidium	0.008416	0.83%	0.60%	0.19%	0.31%	0.04%
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__unidentified;g__unidentified	0.009497	0.32%	0.49%	0.28%	0.12%	0.95%
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Helotiales;f__Leotiaceae;g__Gorgomyces	0.010117	0.00%	5.52%	5.43%	6.17%	5.87%
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Mycosphaerellaceae;g__Mycosphaerella	0.010117	0.00%	1.91%	1.27%	0.44%	1.38%
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Chaetosphaeriales;f__Chaetosphaeriaceae;g__unidentified	0.022482	0.00%	0.02%	0.09%	0.02%	0.56%
k__Fungi;p__Chytridiomycota;c__Rhizophydiomycetes;o__Rhizophydiales;f__unidentified;g__unidentified	0.023225	3.08%	0.34%	0.35%	0.32%	0.46%
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Helotiales;f__Helotiaceae;g__Hymenoscyphus	0.023225	0.00%	0.43%	2.29%	0.59%	0.72%
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Tremellales;f__Bulleribasidiaceae;g__Vishniacozyma	0.030105	0.65%	0.49%	0.10%	0.02%	0.12%
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Helotiales;f__unidentified;g__unidentified	0.033560	0.20%	5.76%	3.63%	4.14%	4.18%
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Chaetothyriales;f__Chaetothyriaceae;g__unidentified	0.033560	0.00%	0.35%	0.18%	0.36%	0.08%
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Clavicipitaceae;g__Metarhizium	0.035285	0.52%	2.85%	4.18%	5.28%	7.38%
k__Fungi;p__Glomeromycota;c__Archaeosporomycetes;o__Archaeosporales;f__unidentified;g__unidentified	0.036988	1.63%	0.27%	0.00%	0.35%	0.11%
k__Fungi;p__Chytridiomycota;c__unidentified;o__unidentified;f__unidentified;g__unidentified	0.039951	1.64%	0.46%	0.24%	0.29%	0.84%
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Helotiales;f__Dermateaceae;g__Pezicula	0.047725	0.00%	0.32%	0.72%	0.12%	0.00%

Figure 6. Fungal and bacterial taxa exhibiting significant differences in relative abundance in different apple rootstock genotypes ("G935", "G.41", "G.222", and "Bud.9". Shades of red and green color indicate higher (red) and lower (relative abundance).

genotype on the microbial community of the root system [33]. Interestingly, the composition of the fungal microbiota of "G.41" and "G.222", which have a similar pedigree, exhibited a degree of overlap, suggesting that the pedigree of a rootstock genotype may have a direct impact on determining the composition of at least the endophytic fungal microbiota of roots. Notably, different size classes (fine roots, intermediate roots and large roots) exhibit significant differences in the composition of root fungal endophytes of "Bud.9" and "G.41", but not the bacterial community. The relative abundance of certain fungal endophytes known to function as root pathogens of apple were also reported to differ across different root size classes [34]. Interestingly, the clustering of "G.935" and "G.222" corresponds with the higher levels of boron and calcium, and low levels of potassium in these rootstock cultivars [35]. No such correlations were identified, however, for the endophytic bacterial community in different type of roots among rootstocks. Overall, a significantly greater number of fungal taxa were identified as endophytes than bacterial taxa. The number of fungal saprophytes identified as endophytes in root samples is interesting as they appear to be resident but may lie dormant ready to take over when the root dies. Additionally, their presence may influence root physiology through hormone and metabolite production, and nutritional interactions, as occurs with plant-growth-promoting rhizobacteria (PGPR) and other bacterial endophytes.

Based on the findings of the current study, it appears that root-associated fungal communities were more strongly influenced by host genetic factors than bacterial communities. The composition of the fungal community was different in bulk, rhizosphere, and endophyte samples of different apple rootstocks. Our results are in general agreement with previous reports on the relationship between rootstock genotype and the composition of their associated microbiota [11, 15, 28, 36–38]. Arrigoni *et al.* and Thompson *et al.* reported that apple scion and rootstock genotype influence the composition of their associated microbiota [5, 25], which was also reported in our previous study [15]. Apple rootstock genotype was also found to influence the relative abundance of vesicular arbuscular mycorrhizal fungi detected in the endophytic fungal community [11]. Pérez-Izquierdo *et al.* stated that genotype was pivotal in structuring the fungal communities of Mediterranean pine (*Pinus* sp.) [39]. The soil fungal community of mulberry (*Morus* sp.) was also reported to be associated with its growth status [40]. Host genotype was also reported to influence the composition of foliar fungal communities in balsam poplar (*Populus balsamifera*) [41], and the composition of the rhizosphere microbiome was reported to be influenced by rootstock genotype in grapes [42].

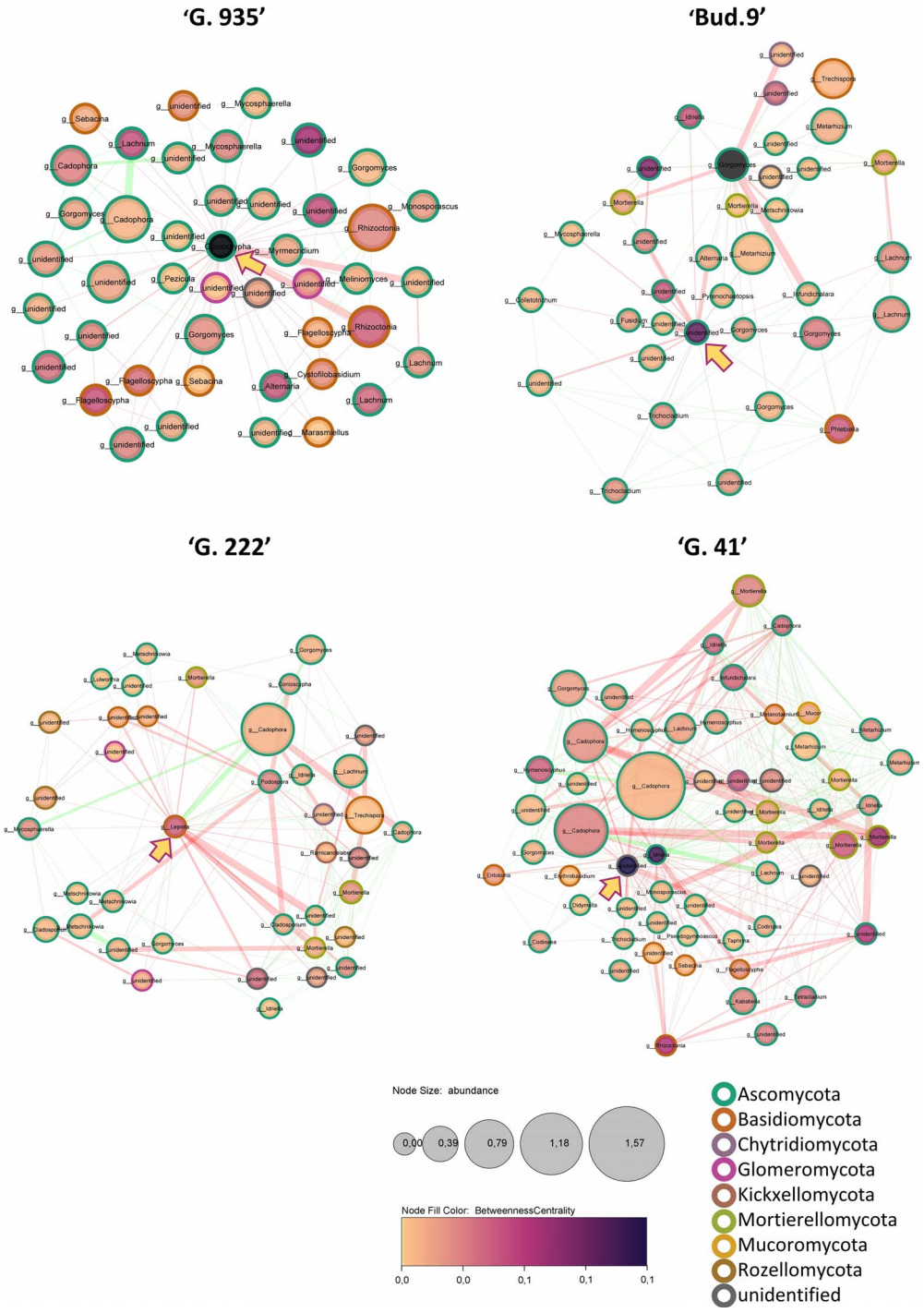

Figure 7. Fungal association networks created using Co-occurrence Network inference CoNet and visualized in Cytoscape 3.8.2 using relative entropy optimization layout. Node color correspond to with the calculated betweenness centrality, and the node border color correspond with the microbial phyla. The node size and width of the edges are correlated to the abundance of the taxa. Red and green edges represent co-exclusion and co-occurrence, respectively.

Collectively, results of the present study indicate that the endophytic fungal communities in the root system of apple rootstocks are significantly influenced by host genotype and that the composition of the endophytic community is dependent on root size in some apple rootstock genotypes. Apple rootstock genotype appears to significantly influence the selection and recruitment of fungal microbiota that may successively colonize larger roots and perhaps even aboveground plant organs [15], and may influence fruit quality by modifying water, nutrient and hormone fluxes in the scion [35, 43]. It would also be interesting to determine if a level of selection occurs as roots age and/or if there is a succession in the microbiota that occurs as the microbiome community matures. Further studies will be required to determine the mechanisms by which genotypes selectively influence the composition of their endophytic microbiome and the potential and/or actual impact that

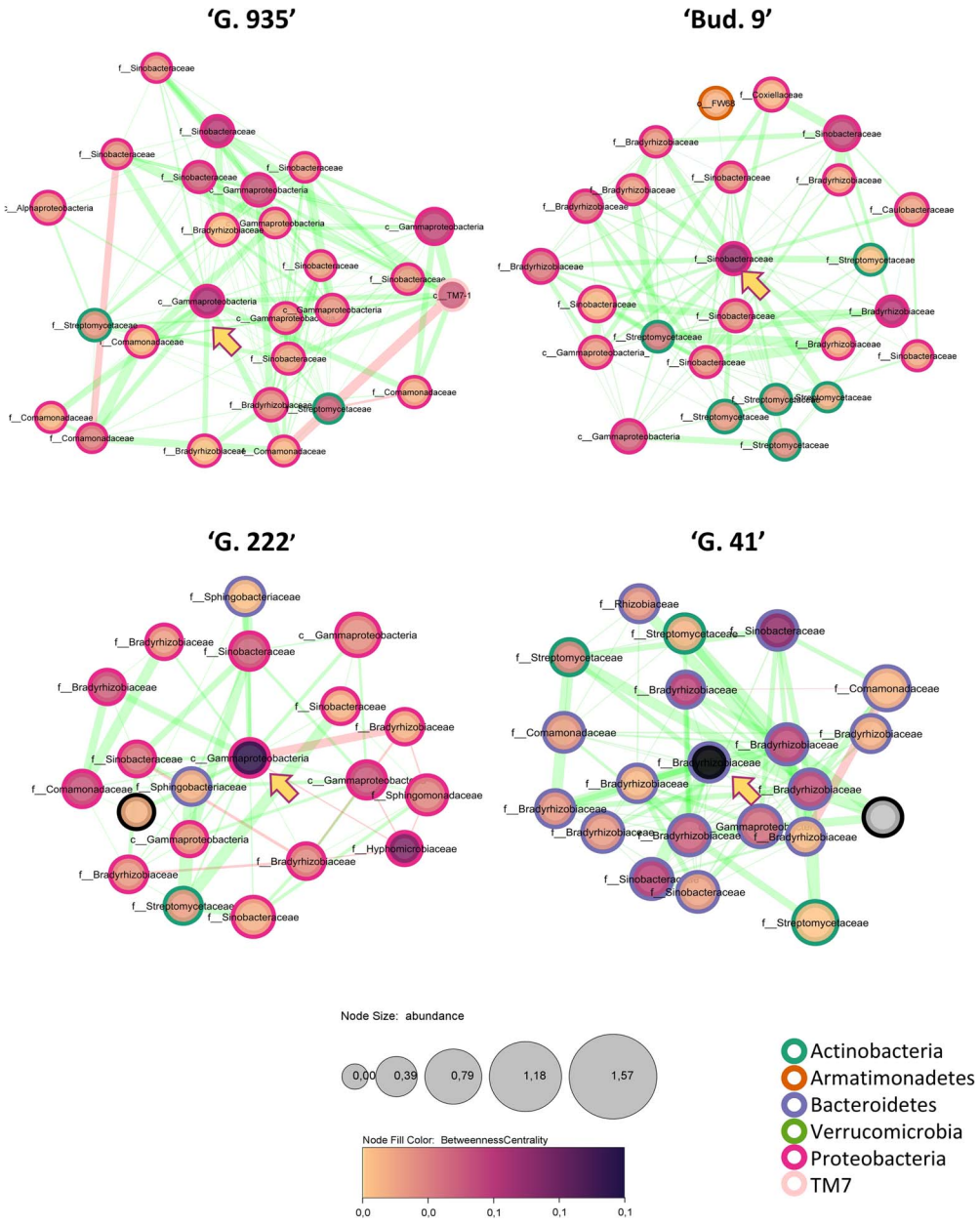

Figure 8. Bacterial association networks created using Co-occurrence Network inference CoNet and visualized in Cytoscape 3.8.2 using relative entropy optimization layout. Node color correspond to with the calculated betweenness centrality, and the node border color correspond with the microbial phyla. The node size and width of the edges are correlated to the abundance of the taxa. Red and green edges represent co-exclusion and co-occurrence, respectively.

may have on host physiology and productivity. In this regard, PGPR and mycorrhizae have been documented to impact host vigor, disease resistance, and stress tolerance through the secretion of various metabolites, including antibiotics and plant hormones [44–47]. A deeper understanding of the interaction between host genotype and the structure of its microbiome, as well as the potential influence on host physiology, will provide novel approaches to influence and perhaps select for a wide range of economically important cultural traits as Rho *et al.* was able to demonstrate by the introduction of specific endophytes into the rhizosphere of "Honeycrisp" apples [10].

Materials and methods

Plant material and experimental design

Four different apple rootstocks were used in the study, "G.41", "G.222", "G.935", and "B.9". One-year-old own-rooted trees were planted in an orchard site located on the grounds of the USDA-ARS, Appalachian Fruit Research Station, Kearneysville, WV. The site was planted in grass for at least five years prior to planting the rootstock trees used in this study. An herbicide strip was applied in September, 2017 and the ground was plowed for planting in November, 2017. The trees were planted in a single row, approximately 35 m in length

with approximately 1.6–1.8 m between trees on April 23, 2018. The row was blocked into five sections and a replicate of each tree was randomly assigned a planting site within each of the blocks providing a total of five replicates ($n = 5$) for each rootstock genotype. A photograph of the tree planting is provided in Fig. 1A. The soil present in orchard is classified as a Funkstown silt loam. General characteristics of the site are: Mean annual precipitation: 83.8 to 116.8 cm; Mean annual air temperature: 4.4 to 17.8°C; Frost-free period: 141 to 168 days; and classified as prime farmland. All trees became readily established and grew well (Fig. 1B). Only a minimum level of management (application of pesticides) was conducted during the experimental period. An application of streptomycin was applied to protect against fire blight (*Erwinia amylvora*) in the spring of 2018 and 2019. The trees also received several fungicide sprays in 2018 and 2019. No other pesticides were applied.

Collection and processing of samples

Trees were harvested (i.e. removed from the soil) on May 20, 2019. Samples of bulk and rhizosphere soils were initially sampled followed by root samples for endophytic microbiome analysis. A bulk soil sample was obtained from each of the replicated blocks by sampling soil approximately 0.6–1.0 m feet away from a planted rootstock but still within the grass-free planting strip. The replicated bulk soil samples were used collectively as a reference for the subsequent comparison of rootstock genotypes and root tissue types. For The following procedure was used to collect rhizosphere samples. Excised roots were gently shaken to remove loose soil and the remaining adhering soil and treated as rhizosphere soil, which was then removed and collected with the help of a sterile spatula which was dipped in 95% ethanol between samples. Roots were gently rinsed with sterile, distilled water to remove adhering soil and then dipped in a mild solution of bleach (200 ppm) for 60 seconds, and then rinsed in sterile, distilled water several times. The roots from each tree were then divided into different class sizes: fine roots (≤ 2 mm in diameter), intermediate roots (2–4 mm in diameter), and large roots (4–12 mm in diameter). All samples (root and soil) were immediately frozen in liquid nitrogen and stored at −80°C until further processing.

At the time of DNA extraction, all individual samples within each sample-type were ground together in liquid nitrogen and then thoroughly mixed to prepare a composite sample. As previously indicated, there were five independent, biological replicates for each sample type from each genotype. Approximately 200 mg from each sample was used for DNA extraction. DNA was extracted using a DNAeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction in a Qiagen QiaCube (Qiagen, Germantown, MS, USA), using the processing routine recommended by the manufacturer for the PowerSoil kit. DNA quality and integrity were assessed using a NanoDrop UV-spectrophotometer (ThermoFisher Inc., Grand island, NY, USA) and gel electrophoresis.

Library preparation and sequencing

Sample preparation for sequencing was done as previously described [48]. Briefly, the 16S ribosomal rDNA were amplified using the 515F and 806R primers together with peptide nucleic acids (PNAs) to limit co-amplification of apples' ribosomal and mitochondrial DNA. Whereas the Internal Transcribed Spacer (ITS) regions were amplified using ITS3/KYO2 and ITS4 primers with a custom primer designed to prevent apple DNA [15, 48]. PCR reactions for 16S amplification included 12.5 μL of KAPA HiFi HotStart ReadyMix (Kapa Biosystems), 1.0 μL (10 μM) of 515F and 806R primers forward and reverse primer, 2.5 uL (5 uM) of plastid PNA, 2.5 uL (5 uM) of mitochondrial PNA, 2.5 μL of DNA template, and 3 μL nuclease-free water in a final volume of 25 μL. Sequencing of the generated amplicons was done on an Illumina MiSeq (Illumina) sequencer using V3 600-cycle chemistry.

Reverse transcription – Quantitative PCR (RT-qPCR)

For determining bacterial and fungal abundance RT-qPCR was performed using the primer pairs 515f–927r for bacteria (10 μM each) [49], and ITS1–ITS2 for fungi (10 μM each) [50]. All reaction mixes contained 5 μl KAPA SYBR Green, 0.5 μl of each primer, 1 μl template DNA, adjusted with PCR-grade water to a final volume of 10 μl. Fluorescence intensities were measured in a Rotor-Gene 6000 real-time rotary analyzer (Corbett Research, Sydney, Australia) with the following settings for bacteria: 95°C for 3 min, 30 cycles of 95°C for 5 s, 54°C for 20 s, 72°C for 5 s, and a final melt curve of 72 to 96°C; and for fungi: 95°C for 3 min, 40 cycles of 95°C for 5 s, 58°C for 35 s, 72°C for 5 s with a final melt at 72°C for 10 min and a final melt curve of 72 to 96°C. Each sample replicate was measured in individual triplicates and numbers of gene copies detected in negative controls were subtracted from the samples of the same run. Statistically significant difference in microbial abundance between the groups were calculated using non-parametric Kruskal-Wallis test and Bonferroni multiple tests correction.

Bioinformatic and statistical analyses

Demultiplexing, and quality trimming of low-quality reads, as well as creating Amplicon Sequence Variant (ASV) were done using the default parameters in DADA2 algorithm as integrated in QIIME 2 [51, 52]. Taxonomic assignment of the created ASVs was done using BLAST algorithm against the GreenGenes and UNITE databases for 16S and ITS reads respectively [53, 54]. MetagenomeSeq's Cumulative Sum Scaling (CSS) [55] was used to account for uneven sequencing depth and then used for downstream analyses including Bray–Curtis dissimilarity metrics [56], hierarchical clustering and PERMANOVA analyses.

To assess the effects of rootstock genotype and roots tissue type on the fungal and bacterial diversity, ANOVA models and Pairwise comparisons using Wilcoxon rank sum exact test with Shannon diversity and adonis (~PERMANOVA) for community composition were used. Separate analyses were conducted for bacteria and fungi. All analyses were conducted using R packages: vegan, lme4, multcomp, phyloseq, R version 3.6.2 in RStudio version 1.1.453 [57–61]. The most prevalent taxa, which had a relative abundance >0.1% across all samples, were used to evaluate differences in relative abundance of the detected taxa between tissue type and treatments using Kruskal-Wallis method [62]. Venn diagrams were constructed using an online resource (https://bioinfogp.cnb.csic.es/tools/venny/) [63]. Microbial association networks created using Co-occurrence Network inference CoNet [64] and visualized in Cytoscape 3.8.2 using relative entropy optimization layout [65].

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Author contributions

MW and XW conceived and designed the experiments. JL, AA, BW, and SD performed the experiments. JL, AA, GF, and MM analyzed the date. JL, AA, MW and XW were the major contributors in writing the manuscript. All authors read and approved the final manuscript.

Data availability

The datasets generated during the current study were deposited and are available at the National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA), under the accession number PRJNA702262 (http://www.ncbi.nlm.nih.gov/bioproject/715902). Other data generated or analyzed during this study are included in this article and its additional files.

Conflict of interests

The authors declare that they have no competing interests.

Supplementary data

Supplementary data is available at *Horticulture Research Journal* online.

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