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The Effect of Rotating Apple Rootstock Genotypes on Apple Replant Disease and Rhizosphere Microbiome

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ABSTRACT

Continuous plantation of apple trees (*Malus pumila*) at the same sites where the same or a closely related species were grown previously leads to poor establishment, reduced growth vigor, and subsequent crop losses; this phenomenon is termed apple replant disease (ARD). Management of ARD is a continual challenge due to (i) restrictions on the use of broad-spectrum soil fumigants, (ii) putative causal agents of ARD can be site-specific, and (iii) more than one causal agent can be present at a site. We conducted an experimental study at two orchard sites to investigate the succession effect of rootstock genotypes on apple tree establishment with three specific objectives: (i) whether ARD in newly planted apple trees is more severe if the same rootstock as the previous one is replanted, (ii) whether trees develop better in the aisle than in original tree stations, and (iii) the extent of association of rootstock genotypes with rhizosphere microbiome in relation to ARD. Tree growth and rhizosphere microbiome were assessed within 30 months of replanting. In one orchard, replanting trees with a rootstock genotype different from the previous one can be effective in reducing ARD development, susceptibility to ARD is likely to be genetically

controlled, and replanting trees in the previous grass aisle can reduce ARD development. However, the opposite results were obtained in the other orchard, possibly due to the lack of weed management in the grass aisle affecting tree establishment in the grass aisle. Rhizosphere microbiota associated with specific rootstock genotypes reached a stable state within 7 months of replanting. An arbuscular mycorrhizal fungi operational taxonomic unit (OTU) had reduced abundance with ARD trees; however many other OTUs associated with ARD cannot be identified to low taxonomic ranks and hence their roles cannot be easily interpreted. In conclusion, replanting trees with rootstocks that are genetically differently from the previous one can reduce ARD development. However, in adopting this strategy, the extent of genetic relationships among rootstock genotypes needs to be considered.

Keywords: apple replant disease, plant pathology, rhizosphere microbiome, rhizosphere and phyllosphere, rotating rootstocks, yield and crop productivity

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Continuous plantation of apple trees (*Malus pumila* Miller) at the same sites where the same or a closely related species were grown previously leads to poor establishment, reduced growth vigor, and subsequent crop losses. Such a phenomenon is termed apple replant disease (ARD), and is a worldwide problem affecting the production of both rootstocks and fruit (Mazzola and Manici 2012). The bearing of fruit by ARD trees can be delayed by 2 to 3 years and yields are depressed by up to ca. 60% for the duration of trees’ commercial life (Willett et al. 1994). ARD causal agents include several fungal species, e.g., *Cylindrocarpus*, *Rhizoctonia*, and *Fusarium* spp. (Braun 1995; Manici et al. 2013; Mazzola and Manici 2012; Tewoldemedhin et al. 2011), as well as several oomycetes species (Braun 1991, 1995; Mazzola et al. 2002; Tewoldemedhin et al. 2011). In addition, root lesions caused by nematodes, such as *Pratylenchus penetrans*, have been implicated in exacerbating ARD by facilitating pathogen entry into root tissues (Galkovskyi et al. 2012; Mai and Abawi 1978; Tewoldemedhin et al. 2011).

ARD can be effectively controlled by fumigating soils with broad-spectrum pesticides, such as methyl bromide and

chloropicrin; though the efficacy is often dependent on soil type, providing a variable and temporary solution. Even so, other fumigants may not be as effective as methyl bromide. Additionally, these broad-spectrum pesticides are now banned in many apple production areas because of their harmful effects on the general environment. Because of the lack of a single effective soil fumigant, the complex nature of agents causing ARD, environmental concerns and cost, chemical fumigation of soils is no longer considered a viable measure in many apple production regions. The current consensus is that ARD management should rely on implementation of several measures, especially at those sites where more than one ARD causal pathogen are likely to be present.

The relative importance of putative ARD causal agents can be site-specific (Manici et al. 2013). Furthermore, it is not yet clear how these individual ARD causal components interact with each other to affect tree growth. It is, therefore, not surprising that ARD control efficacy achieved by methods other than broad-spectrum pesticides varies considerably among sites. For instance, amending soils with *Brassica juncea* seed meal can provide effective management of ARD through generation of biologically active glucosinolate hydrolysis products and/or transformation and activity of resident soil biology (Cohen et al. 2005; Mazzola et al. 2007, 2009, 2016, 2015). However, brassica seed meal appears to be ineffective against oomycete pathogens. Thus, the seed meal needs to be combined with a post-planting mefenoxam application for effective ARD control at sites where oomycete ARD pathogens are important (Mazzola and Manici 2012). In addition, the effect of general soil compost amendment on ARD is small relative to planting positions and rootstocks (Leinfelder and Merwin 2006; Rumberger et al. 2004).

Distinctive rhizosphere microbial communities develop in association with plants of different species (Burns et al. 2015; Haichar et al. 2008). Recent studies showed that soil microbial community structure differed greatly between tree stations and grass aisle although they were only a couple of meters apart (Deakin et al. 2018b; Qin et al. 2016; St. Laurent et al. 2008). Thus, planting trees in the grass aisle instead of original tree stations could reduce ARD severity as demonstrated previously (Leinfelder and Merwin 2006; Rumberger et al. 2004). For the same principle, a “break crop” may be grown before replanting apple trees (Mazzola and Manici 2012).

Apple rootstock genotypes differ in their responses to ARD (Fazio et al. 2012; Leinfelder and Merwin 2006; Rumberger et al. 2004). The dwarfing rootstock M.9 has been instrumental in the development of high density planting systems used in dessert apple production. Unfortunately, it is also severely affected by ARD (Auvil et al. 2011). Semidwarfing rootstocks, such as MM.106, MM.111, and more recently M.116, are typically used in cider orchards in the United Kingdom and show variable levels of susceptibility to putative ARD causal agents (Auvil et al. 2011; St. Laurent et al. 2008; Wilcox 1990). G.41 is a recently bred rootstock and shows greater tolerance toward ARD than other rootstocks, including M.9—the current commercial standard for dessert apple (Isutsa and Merwin 2000; Mazzola et al. 2009). One possible way to exploit differential responses of rootstock genotypes to ARD is to replant trees grafted with a rootstock different from the previous one, i.e., rotating rootstock genotypes with different tolerance/resistance to ARD; but this approach is currently limited in intensive orchards by the insufficient commercial uptake of other dwarfing rootstocks to alternate with M.9. Furthermore, interactions between rootstock genotypes and individual ARD causal agents are not well understood, making the prediction of rootstock rotation effect on ARD difficult. Conventionally, rotation is usually operated between different plant species to manage soilborne pathogens (Krupinsky et al. 2002; Niu et al. 2016).

In the present paper, we report results from two field trials to study the succession effect of rootstock genotypes on early apple tree establishment (hence ARD) and the differences in rhizosphere microbiome between rootstock genotypes over time. Specifically, we studied (i) whether ARD severity in newly planted apple trees is greater if the same rootstock genotype as the previous one is replanted, (ii) whether trees will develop better in the aisle than in original tree stations, and (iii) how soon different rootstock genotypes will assemble distinct rhizosphere microbiome following planting. Comparison of relative abundance of individual microbial operational taxonomic units (OTUs) was restricted to comparison between trees showing ARD symptoms and non-ARD trees. No attempts were made to compare individual microbial OTUs among pairwise rootstock genotypes as it would not only generate too many differential results to report but also was not an objective in the present study.

MATERIALS AND METHODS

Study design. Experiments were conducted at two geographically and agronomically distinct apple orchards: one cider and one dessert apple (about 240 km apart). The general characteristics of the two orchards were reported previously (Deakin et al. 2018a, b).

Two factors were studied: rootstock genotype (eight genotypes) and location within an orchard (original tree station and the corresponding aisle grass location). The experimental design and layout are given in Supplementary Figure S1. Within each orchard, a randomized block design was used with three blocks. Each block was ca. 20 m long with eight consecutive trees (locations). Within each block, there were eight pairs of locations: original tree station and the corresponding midpoint in the grass aisle (ca. 2 m away from the tree station). Each pair of the eight locations within each block was randomly assigned to two trees with one of the eight rootstock genotypes.

General characteristics of the two orchards. At the dessert site, the soil (of Wickham series) had uniform clay loam texture. At the cider site, the soil (of Whimple series) had predominantly silty clay loam texture (blocks 1 and 3, adjacent to each other) with block 2 (situated below block 1) containing a proportionately smaller silt fraction resulting in clay loam texture. Soil pH was slightly alkaline at the dessert orchard, ranging between 7.9 and 8.1, and was medium acid at the cider orchard (pH 5.4 to 5.8).

The two orchards represented two different rootstock and scion combinations typical for the production of dessert/culinary and cider apples (*Malus pumila* Miller) in the United Kingdom. The dessert orchard was planted with cultivars Bramley’s seedling and Golden Delicious on M.9 rootstock (row distance 3.75 m, and an in-row tree spacing of 1.6 m) between 1992 and the winter of 2014–15. Since 1988 the cider orchard had been planted with cider apple varieties (row spacing of 5.5 m and in-row tree spacing of 2.75 m), the most recent planting prior to the initiation of the present study was the cultivar Katy on two different rootstocks: MM.106 and MM.111. The planting of cultivar Katy was dug-up and removed after 12 years in 2014. The three blocks were located in the areas where trees with MM.106 were previously grown.

Rootstock and scion genotypes. Eight rootstocks were selected to reflect a range of vigor, genetic background (derived from various *Malus* species), and reported tolerance to ARD, including the preceding rootstock genotypes (M.9 and MM.106) used at the two orchards. These included seven commercial rootstocks—M.9 (unknown pedigree), M.26 (M.16 × M.9), M.27 (M.13 × M.9), MM.106 (Northern Spy × M1), M.116 (MM.106 × M.27), G.11 (M.26 × *M. robusta* 5), and G.41 (M.27 × *M. robusta* 5)—as well as

an advanced selection from the East Malling breeding program: EM_SEL1 (*M. robusta* 5 × Ottawa 3). G.41, M.116, and M.27 were reported to be most tolerant to ARD; M.9 and M.26 the least. M.27 is very dwarfing; M.9, G.11, G.41, and EM_SEL1 dwarfing; M.26 semidwarfing; and M.116 and MM.106 semivigorous. These rootstocks were sourced from two nurseries in slightly different sizes: M.9, M.26, MM.106, M.116, G.11, G.41, and EM_SEL1 from a French nursery, and M.27 from a U.K. nursery. The root systems of all rootstocks were washed prior to grafting with cultivars Discovery and Worcester Pearmain in April 2015 for use in the dessert and cider orchard, respectively. Grafted trees were potted up in a mix of peat and sand and grown in pots for nearly 7 months. In October 2015, prior to planting, all trees were tipped and lateral shoots removed; the most similar trees of each of the rootstock-scion combinations were paired (original tree station and the corresponding grass aisle site) for planting in each block. The cider and dessert orchard was planted on 14 and 30 October 2015, respectively, in land that had been prepared by subsoiling and rotavating only (i.e., without land inversion) to minimize soil disturbance following the standard practice.

Orchard management, assessment, and sampling soils. Throughout the experimental period, trees were managed with commercial practice except that herbicides were not used. In the winter 2017 (January) and 2018 (February), each tree was assessed for girth (measured at 5 cm above the graft union), height (from the ground level), and pruning weight (two-thirds of a branch were removed for all branches).

Soils were sampled three times: Year 0 (before planting: 1 May 2015 [dessert] and 3 June [cider] 2015), Year 1 (3 June [dessert] and 27 June [cider] 2016), and Year 2 (24 May [dessert] and 1 July [cider] 2017). In 2015, three separate cores (depth 10 cm) of bulk soils per location (tree station or the corresponding grass aisle) were taken; in addition, six samples of soils were taken from the grass area near the orchard boundary hedge, in each orchard. The same sampling strategy was used in 2016 and 2017, except three cores of rhizosphere (not bulk) soils at the depth of 10 to 20 cm were taken per tree (location). Fine roots (i.e., <2 mm diameter, nonwoody, feeder roots) were taken at ca. 10 to 20 cm depth. Excised roots were shaken free of loosely attached soil and the remaining adhering soil was considered to be “rhizosphere” soil, which was removed with the aid of a sterile spatula (Bramwell et al. 1995) and collected. At each sampling time, 150 samples were taken in each orchard: three samples per tree (or location for time 0 bulk soil) × eight rootstocks × three blocks × two locations (tree station and grass aisle), in addition to six control samples (Supplementary Table S1).

Each bulk or rhizosphere soil sample was placed in a separate labeled, sealable polythene bag. Bags were placed on dry ice in the field and immediately transferred to a –80°C freezer when returned to the lab for storage prior to DNA extraction. Microbial community structure in relation to vegetation type (grass aisle and tree station) over all locations for the samples taken in Year 0 (2015) had already been reported (Deakin et al. 2018a, b).

DNA extraction and sequencing. The diversity and composition of bacterial and fungal microbiota in the bulk or rhizosphere soil was determined by amplicon sequencing; the detailed methodology was reported previously (Deakin et al. 2018b). Briefly, genomic DNA was extracted from bulk or rhizosphere soils using the DNeasy PowerSoil Kit (Qiagen, Carlsbad, CA) in conjunction with a bead beating benchtop homogenizer (Fastprep FP120, Qbiogene, Carlsbad, CA). The marker regions V4 16S rRNA and internal transcribed spacer (ITS)1 + ITS2 of the 18S rRNA gene for bacteria and fungi, respectively, were amplified by PCR using the following primer pairs: Bakt_341F/Bakt_805R (Herlemann et al.

2011) and EkITS1F/Ek28R(= 3126T) (Gardes and Bruns 1993; Sequerra et al. 1997), to which Nextera transposase adapters (Illumina, San Diego, CA) had been appended. All PCR reactions were performed in 25-μl reaction mixtures comprising 1× PCR buffer containing 2 mM MgCl₂ and 1 U of Platinum *Taq* DNA Polymerase (Invitrogen, Life Technologies, Carlsbad, CA), 0.2 mM dNTP (Fisher Bioreagents, Thermo Fisher Scientific, Pittsburgh, PA), 0.2 mM forward and reverse primers each (Integrated DNA Technologies, Coralville, IA), 8 ng of template DNA, and molecular biology reagent water (Sigma, St. Louis, MO). Amplicons for each marker region were cleaned using solid phase reversible immobilization beads (Agencourt XP Ampure beads; Beckman Coulter, Brea, CA) and pooled by individual sample. A Nextera XT DNA Library Preparation Kit (Illumina) was used to prepare and barcode amplicon libraries for sequencing with a MiSeq sequencer (Illumina) using 2 × 300 bp v3 reagent kits (Illumina).

Bioinformatic analysis of sequence reads. All sequence data were submitted to the NCBI database (Project PRJEB32801). Sequence processing followed previously reported methods/pipelines (Deakin et al. 2018b). FASTQ reads were demultiplexed into bacterial (16S) and fungal (ITS) datasets based on their primer sequences. Reads with any ambiguous positions in the primer region, or nonmatching forward and reverse primers were discarded, and 16S forward and reverse reads were merged with a maximum difference in overlap of 5%.

The pipeline was then carried out in two separate steps to (i) generate representative OTUs and (ii) produce an OTU frequency table. All analyses were carried out with the USEARCH 10.0 pipeline (Edgar 2013) unless specified otherwise.

Generating OTUs. All 16S forward and reverse primers were removed. Merged reads containing adapter contamination or fewer than 300 nucleotides were excluded from further analysis. Remaining merged reads were then filtered for quality with a maximum expected error threshold of 0.5 per sequence (Edgar and Flyvbjerg 2015).

The expected distance between fungal ITS primers was larger than twice the MiSeq read length. Consequently, forward and reverse reads could not be merged. For this experiment, only the forward reads were used and treated as unpaired reads. Reads shorter than 200 nucleotides were discarded. Forward primers were removed before quality filtering with an expected error threshold set to 1 per sequence.

Filtered sequences were then dereplicated and unique sequences with fewer than four reads were discarded. These unique sequence reads were then clustered into OTUs at the level of 97% similarity and a representative sequence for each OTU was generated. The clustering algorithm also removed chimeras during the clustering process. The UCLUST algorithm (http://drive5.com/usearch/manual/tax_conf.html) then assigned each OTU representative sequence to taxonomic ranks by alignment with the gene sequences in the reference databases ‘Unite V7’ (fungal ITS) (Koljalg et al. 2013) and ‘RDP training set 15’ (16S) (Cole et al. 2014).

Generating OTU tables. Finally, all (unfiltered) sequences were aligned with the OTU representative sequences at the level of 97% similarity to generate an OTU frequency table.

Statistical analysis. All statistical analyses were carried out in R 3.4.0 (R Core Development Team 2008).

Analysis of morphological characters. In the present study, ARD is indicated when there is substantial differential tree growth between the original tree station and its corresponding grass aisle for the same rootstock genotype. Because of the inherent differences in tree vigor among rootstock genotypes, the extent of ARD associated with different rootstocks cannot be based on simply comparing the overall tree growth among rootstock genotypes.

Furthermore, because of the inherent differences in tree sizes at planting between rootstock genotypes, it would be also inappropriate to compare tree growth directly among different rootstock genotypes. Thus, a relative rate of increase (RRI) was calculated for tree girth and height as

$$RRI = \frac{(x_2 - x_1)}{x_1}$$

where x_1 and x_2 are the values at time points 1 and 2, respectively. It is the difference in the RRI values between the aisle and tree station for the same genotype that was statistically analyzed by analysis of variance (ANOVA). ANOVA was applied to individual orchards separately since it is expected that ARD severity of each rootstock genotype depends on specific orchards because of the difference in the previous rootstocks used. In addition to the differences in the RRIs, the ratio of RRIs between the tree station and aisle was also analyzed. As the ratio gave similar results as the difference, only results based on the difference are presented. Pruning weight was transformed onto a logarithmic scale before ANOVA to achieve variance homogeneity.

Comparative analysis of rhizosphere microbiomes. OTU counts were normalized for library size using the median-of-ratios method implemented in DESeq2 (Anders and Huber 2010; Love et al. 2014), and where appropriate transformed using the DESeq2 variance stabilization transformation (VST). Reads for fungal OTUs assigned to the same species and with 95% UTAX confidence at the species level were combined. Samples that either failed sequencing or had very few total reads relative to other samples were excluded. Before analysis, the three core samples taken from each sampling point were pooled; for sampling points with less than three valid replicates (due to sequencing failures or with very few reads), pooling the available replicates was adjusted accordingly. OTUs with less than five reads across all samples were excluded from statistical analysis (except for alpha diversity measures). All analyses were carried out separately for fungal and bacterial data.

Alpha (α) diversity (Chao1, Shannon, and Simpson) indices were calculated using the R vegan 2.3-1 package (Dixon 2003). The rank of (α) diversity indices were subjected to ANOVA to assess the overall contribution of study factors to the observed differences in microbial community structures via a permutation test for significance. The present study factors include (i) orchards, (ii) sampling times, (iii) vegetation type (tree station or grass aisle), and (iv) rootstock genotypes. Principal components (PCs) were calculated from VST transformed OTU counts using prcomp (R stats package) with default settings. ANOVA was carried out on the first four PCs to estimate the contribution of study factors to the observed variance in each of the four PCs (separately for each orchard). Similar ANOVA was also carried out for each individual sampling time point and/or orchards.

Finally, DESeq2 was used to compare the relative abundance of individual OTUs to identify association of specific microbes with

ARD. Whether a specific rootstock genotype displays ARD was determined from the analysis of morphological characters. Thus, if G1 and G2 genotypes showed ARD and non-ARD, respectively, the following two specific comparisons were applied in DESeq2 at the same sampling points after planting: (i) (G1:Tree_station – G1:Aisle); and (ii) (G1:Tree_station – G1:Aisle) – (G2:Tree_station – G2:Aisle). The second comparison will identify OTUs that are significantly different between G1:Tree and G1:Aisle but not significantly different between G2:Tree and G2:Aisle, i.e., OTUs uniquely different in the G1 (ARD) comparison. Significance was taken at the 0.05 level according to Benjamini-Hochberg adjusted (Benjamini and Hochberg 1995).

RESULTS

Tree growth. Trees established reasonably well in the dessert orchard. Similar establishment was observed at the cider orchard but trees appeared to have less branching. In addition, all six trees with G.11 rootstocks were dead in Year 1 at the cider orchard. The reason for the death of these trees was not established but may have been due to delayed incompatibility with the Worcester Pearmain scion, or latent viruses in the scion (Robinson et al. 2003).

Average tree height and girth measured 1 year after planting were nearly identical between trees planted in the grass aisle and original tree stations within each orchard (Table 1). However, there were contrasting differences in the tree growth between the two orchards in relation to the tree location (original tree station or grass aisle) when assessed 2 years after planting. In the cider orchard, the tree growth rate (girth and height) was generally greater in the grass aisle than in the original tree location; the opposite was true for the dessert orchard (Figs. 1 and 2). The average RRI for girth was 0.18 and 0.59 for the aisle and tree station in the dessert orchard, respectively, compared with the corresponding values of 0.29 and 0.21 in the cider orchard. Similarly, the RRI for height in the aisle (RRI 0.28) was less than in the tree station (RRI 0.40) in the dessert orchard, but the opposite was true (RRI 0.35 versus RRI 0.27) in the cider orchard. Such orchard differences in the effect of grass aisle and original tree stations on tree growth, i.e., interaction between orchard and planting locations, accounted for 20 and 27% of the total variability ($P < 0.001$) in the relative growth rate in the respective tree height and girth. Of the three variables, height was most significantly ($P < 0.01$) affected by rootstocks, accounting for ca. 18% of the total variability.

As there were large and significant interactions between orchard and planting location, the relative growth rates were analyzed for each orchard separately. For the dessert apple, rootstock genotypes did not differ in the differences in their relative development rates between the aisle and tree stations (Figs. 1 and 2). On the other hand, the differences in the relative increase rate in the girth and height between the aisle and tree stations differed ($P < 0.01$)

TABLE 1 Average tree development for those planted in the grass aisle and original tree stations at two locations (dessert and cider orchards) ^a							
Orchard	Location	Girth (cm)		Height (cm)		Pruning weight (g)	
		2017	2018	2017	2018	2017	2018
Dessert	Aisle	3.8 ± 0.10	4.3 ± 0.10	86 ± 3.65	103 ± 3.86	7.4 ± 1.04	5.8 ± 0.65
	Tree station	3.8 ± 0.10	6.0 ± 0.17	89 ± 3.75	119 ± 4.35	6.6 ± 0.96	18.9 ± 2.24
Cider	Aisle	3.3 ± 0.09	4.3 ± 0.16*	109 ± 6.47	146 ± 6.54*	1.5 ± 0.41	4.7 ± 1.19
	Tree station	3.3 ± 0.05	4.0 ± 0.11*	107 ± 5.42	135 ± 7.21*	1.5 ± 0.87	2.7 ± 0.64

^a Trees were planted in October 2015. Number after ± is the standard error. * Indicates significant interaction between rootstock genotype and location.

among genotypes for the cider apple. For girth, M.116 had the largest difference (0.35) between the aisle and original tree stations, which was significantly greater than the other rootstocks, ranging from 0.01 for G.41 to 0.11 for EM_SEL1 (Fig. 1). For height, M.116 had greater ($P < 0.05$) differences (RRI 0.51) than MM.106 (RRI 0.21) and M.27 (RRI 0.16), which in turn, were greater ($P < 0.05$) than the other four rootstocks, with RRI ranging from 0.0 (EM_SEL1) to -0.09 (M.9) (Fig. 2).

Pruning weight from trees planted in the original stations was significantly greater ($P < 0.01$) than trees in the aisle for the dessert

orchard in 2018 (Fig. 3A, Table 1), accounting for 51% of the total variability on the logarithmic scale. The effect of rootstock genotypes on pruning weight was close to statistical significance ($P = 0.056$). There were no significant interactions between rootstock genotypes and planting position. For the cider orchard, only the effect of rootstock genotypes was close to statistical significance ($P = 0.059$), accounting for 26% of the total variability on the logarithmic scale. Although there were no significant interactions between genotypes and planting position, pruning weight was greater in the aisle than in the tree stations for M.116, MM.106,

Fig. 1. Relative increase rate in tree girth measured 5 cm above the graft union in relation to rootstock genotypes and planting locations (aisle or original tree station). Tree was measured in January 2017 and January/February 2018 after planting in October 2015. For the dessert orchard, the previous rootstock was M.9, whereas for the cider orchard, it was MM.106.

M.9, and EM_SEL1; the opposite was true for G.41, M.26, and M.27 (Fig. 3B).

Microbiome data. There were 13 and 16 (sub)samples, for fungi and bacteria, respectively, which either failed sequencing or had less than 1,000 total sequence reads. These samples were excluded from analyses. There was a total 3.38×10^7 and 4.04×10^7 fungal and bacterial reads, respectively, evenly distributed between the two sites (Supplementary Table S2). There were on average 104,213 fungal and 124,732 bacterial reads per sequence sample, ranging from over 9,000 to over 300,000 for fungi, and 9,000 to over

400,000 for bacteria. Rarefaction curves of the samples with the lowest read count indicated that depth of sequencing was adequate (Supplementary Fig. S2). In addition, all samples associated with G.11 (trees or locations) in the cider orchard were omitted from microbiome analysis as all six trees with G.11 rootstocks were dead in Year 1 at the cider orchard.

Alpha diversity and population structure at the phylum level. Several alpha diversity metrics were calculated across the three time points (bulk for the first time point and rhizosphere soils for the other two times) and are shown in Supplementary Figure S3. There

Fig. 2. Relative increase rate in tree height in relation to rootstock genotypes and planting locations (aisle or original tree station). Tree was measured in January 2017 and January/February 2018 after planting in October 2015. For the dessert orchard, the previous rootstock was M.9, whereas for the cider orchard, it was MM.106.

was a significant effect of time on all calculated alpha diversity metrics at the cider orchard for both bacteria and fungi (Supplementary Table S3). At the dessert orchard, the Chao1 metric for fungi and Shannon and Simpson metrics for bacteria were significant. Restricting the analysis to the later two time points (i.e., excluding bulk soil samples), there was still an effect of time for all the diversity measures in the bacterial community at the cider orchard, and in the Chao1 metric for the fungal communities (Table 2).

Figures 4 and 5 present OTU abundances aggregated at the phylum taxonomic rank for each combination of planting location,

time points and orchards for fungi and bacteria, respectively. Fungi at the cider orchard were dominated by species in the Ascomycota and Basidiomycota phyla, whereas the dessert orchard contained a much larger proportion of Ascomycota. For bacteria, the cider orchard consisted of mostly *Proteobacteria*, *Verrucomicrobia*, and *Acidobacteria*. In contrast, the dessert orchard had much lower levels of *Verrucomicrobia*. The relative abundances at this high rank level did not differ much between planting locations or over time.

Microbial community structure over time. For both orchards, a large proportion of the variability in community structure was due

Fig. 3. Average pruning weight (grams) in January/February 2018 in relation to rootstock genotypes and planting locations (aisle or original tree station). Trees were planted in October 2015. For the dessert orchard, the previous rootstock was M.9, whereas for the cider orchard, it was MM.106.

to the differences between planting positions within an orchard (Supplementary Table S4 and S5). For instance, in the fungal community at the cider orchard, the planting position accounted for 44 and 33% of the variability in the first two PC scores; the corresponding values were 42 and 29% for the time factor. The interactions between tree location and sampling time were very small. Similar trends were observed for the bacterial community. The differences in fungal community between aisle and tree stations were generally more pronounced than in the bacterial community.

Overall, for both bacteria and fungi at both orchards, nearly all the differences in the first two PC scores among the three time points were due to the differences between the first time and the latter two times, i.e., between bulk and rhizosphere soils. These temporal differences are illustrated in Figure 6. At the dessert orchard, the large differences in the bacterial community between the first sampling time and the other two were strikingly clear, when compared with the cider orchard (Fig. 6C and D).

Effects of genotypes on microbiome alpha diversity. There were no significant interaction effects on the alpha diversity between rootstock genotypes and time points 1 and 2 (Table 2; Supplementary Figs. S4, S5, S6, and S7), i.e., the microbiomes associated with each genotype did not vary differently over time. There was a significant effect of rootstock genotypes at the cider orchard for bacteria when measured by Shannon and Simpson metrics.

Effects of genotypes on microbiome community structure. The overall difference in the rhizosphere fungal and bacterial community between the grass aisle and tree stations was reduced by ca. 50 to 75% 6 to 7 months after planting (Table 3) compared with the bulk soil microbiomes before planting. For instance, at the dessert orchard, this positional effect accounted for 12.4% of the

variability in fungal community structure before tree planting but only 3.0% 7 months after planting. Overall, the reduction in the positional effect was less pronounced for bacteria than for fungi (Table 3). There were also significant interactions between the position and rootstock genotypes for both rhizosphere fungal and bacterial community structure, accounting for 14 to 15% of the total variability for both the latter sampling times (Table 3). The overall magnitude of positional and genotypic effects and their interaction changed very little 12 months after the second sampling time (Table 3).

There were no clear differences in the rhizosphere microbiomes between rootstock genotypes over the latter two time points (Fig. 7), except that M.9 did not cluster closely together with the other genotypes (especially for the tree station samples). Redundancy analysis constrained for genotype was able to display clusters for the rootstocks, especially for the fungal microbiome (Supplementary Fig. S8). It confirmed a trend for M.9 rootstock to cluster away from the other rootstocks; there is a lack of greater separation of samples for the same genotypes between the two sampling points.

Differences in rhizosphere microbiome between M.116 and other genotypes. Many OTUs were filtered out by the DESeq2 default criteria before statistical comparison (Supplementary Table S6). For instance, only about a quarter of the fungal OTUs were retained for the comparison between M.116 and the other four rootstock genotypes that showed significant differences in relative tree growth from M.116.

Irrespective of the comparisons and time points, only a very small proportion of fungal OTUs (ca. 2 to 5%) differed ($P < 0.05$) in their relative abundance in the following two comparisons: (i) M.116_tree_station versus M.116_aisle, and (ii) M.116 (tree_station versus

TABLE 2 Percentage of variation (% var) in each of the alpha diversity metrics accounted for by the differences between the later two sampling time points, together with their <i>P</i> values for bacterial/fungal rhizosphere microbiomes in two orchards						
Orchard	Chao1		Shannon		Simpson	
	% Var	<i>P</i>	% Var	<i>P</i>	% Var	<i>P</i>
Fungi						
Cider orchard						
Time	13.30	0.001	1.21	0.204	1.02	0.182
Genotype	9.74	0.085	1.41	1.000	1.34	0.980
Interaction	9.08	0.125	6.54	0.318	7.34	0.374
Dessert orchard						
Time	7.10	0.008	0.68	0.882	1.43	0.282
Genotype	9.22	0.199	5.79	0.655	5.96	0.702
Interaction	11.78	0.082	3.00	0.926	3.78	0.737
Bacteria						
Cider orchard						
Time	13.49	0.002	7.08	0.010	9.57	0.002
Genotype	7.23	0.422	13.16	0.029	15.35	0.021
Interaction	3.10	1.000	4.45	0.700	3.00	0.802
Dessert orchard						
Time	0.07	0.922	4.37	0.024	0.12	0.603
Genotype	4.73	0.792	7.58	0.387	6.46	0.853
Interaction	3.58	0.971	2.69	0.905	4.41	0.653

Fig. 4. Normalized count abundances aggregated at the fungal phylum taxonomic rank for **A**, cider orchard and **B**, dessert orchard. The figure has been faceted into time points. Operational taxonomic units (OTUs) were assigned to the phylum at 0.9 UTAX confidence. OTUs not assigned at this confidence are aggregate under Fungi(k). Phyla with less than 1% of total abundance have been pooled under others.

aisle) versus M.26, M.9, G.4, and EM_SEL1 (tree_station versus aisle), i.e., the list of differential OTUs in M.116 excluding any differential OTUs in any of the other comparisons. Of those OTUs with significant differential abundance, about 60% of OTUs had lower abundance in the samples associated with M.116 ARD trees. Similarly, for bacteria only a small proportion of OTUs (ca. 2 to 9%) differed ($P < 0.05$) in their relative abundance for the two specific comparisons.

None of the fungal and bacterial OTUs with differential abundance for the two specific comparisons is a known apple pathogen or parasite. If we restricted the comparison with the time point 2 involving M.116 and the other four rootstock genotypes (since this comparison is expected to be more reliable in identifying microbial organisms associated with ARD), there was only one fungal OTU with an obvious biological interpretation. This OTU is from Glomeromycota (mycorrhizal fungi) that had lower abundance on the rhizosphere of M.116 trees planted in the original tree station in the cider orchard. However, it should be noted most of the OTUs with significant differences in their relative abundance cannot be assigned with confidence to a taxonomic rank lower than family.

DISCUSSION

Due to withdrawal of broad-spectrum chemical fumigants and the complex nature of ARD, it is generally accepted that effective management of ARD in commercial nursery and orchard fruit production will more likely result from an integration of several alternative measures (Mazzola and Manici 2012). Replanting trees with a rootstock genotype differing in its response to ARD from the previous one is one of such measures since previous research demonstrated differential responses of rootstocks at replanting sites (Auvil et al. 2011; Fazio et al. 2012). Another measure is to replant trees in the grass aisle instead of the original tree stations because of differential microbial community structure associated with the vegetation types (Deakin et al. 2018b). The present study has focused on testing these two hypotheses/expectations: rotating rootstocks and planting in the grass aisle.

The two experimental orchards yielded different results in relation to the effects of planting trees in the grass aisle on tree development. At the cider orchard, overall tree growth two years after planting was significantly greater for those planted in the aisle than in the original tree stations, agreeing with previous studies (Leinfelder and Merwin 2006; Rumberger et al. 2004). However, the opposite was true for the dessert orchard. One possible explanation for this contrasting outcome is the difference in the intensities of weed competitions between the two orchards. The dessert orchard aisle was covered with denser grass and no specific weed management was applied during the experimental period; in contrast, the original tree stations were generally free of grass or other weeds because of weed management applied in the previous plantation. At the cider orchard, although weeds were present there was much less grass cover. Following the standard commercial practice, deep soil plowing was not applied to all the orchard land before planting. Thus, relative to the original tree stations, trees in the grass aisle may have been facing less competition from weeds at the cider orchard than at the dessert orchard. Differential soil compactness may also influence tree development; however, we did not notice any apparent differences in soil compactness between the two orchards. The difference in root systems of previous trees may partially be responsible for ARD occurrences in the two orchards. MM.106 and MM.111 are likely to have more widely dispersed but less dense root systems than M.9; therefore, more M.9 roots could have been removed when previous trees were removed, reducing the chance of new roots meeting old roots. The difference in the pH

between the two orchards may also affect ARD development as pH is one of the key drivers in shaping rhizosphere microbiomes (Fierer 2017; Fierer and Jackson 2006). However, differences in the root system and pH cannot easily explain why tree growth is generally less in the grass aisle in the dessert orchard.

As the relative growth rate did not differ significantly among rootstock genotypes in the dessert orchard, only the cider orchard was used to assess whether rotating rootstocks could contribute to ARD suppression. Due to the inherent differences in tree vigor among rootstock genotypes, we can only assess ARD severity associated with each rootstock genotype by comparing tree growth at the original tree station and the corresponding aisle site. The greater the tree growth in the aisle relative to the tree station, the more severe the ARD is. Present results demonstrated that M.116 experienced severest ARD among the seven rootstocks and that MM.106 and M.27 also experienced noticeable ARD. Given that the previous rootstock in the orchard was MM.106 and that M.116 was derived from the cross MM.106 $\times$ M.27, the present study suggests that rotating rootstock genotypes can be an effective measure to reduce ARD development. Similarly, we can also infer that the tolerance/resistance to ARD is likely to be partially controlled genetically. Such genetic control could be directly mediated by resistance/tolerance against ARD causal agents or indirectly through rhizosphere microbiota mediated through root exudates.

Although M.116 has been believed to be among the most tolerant to ARD, the present result suggests otherwise. The previous consensus was based from comparisons in tree vigor rather than from controlled comparisons in the relative tree growth in the replant (e.g., original tree stations) and non-replant site (e.g., the aisle) among genotypes. Since M.116 was semivigorous and hence used in cider apple production, tree growth vigor on this

Fig. 5. Normalized count abundances aggregated at the bacterial phylum taxonomic rank for **A**, cider orchard and **B**, dessert orchard. The figure has been faceted into time points. Operational taxonomic unit (OTUs) were assigned to the phylum at 0.9 UTX confidence. OTUs not assigned at this confidence are aggregate under Bacteria(k). Phyla with less than 1% of total abundance have been pooled under others.

rootstock even under replanting conditions can thus be still higher relative to other rootstocks. For instance, the average height increase and girth expansion rates for M.116 at the original tree stations were among the best rootstocks in the cider orchard. Thus, without comparing the growth rates at the aisle and original tree stations, we may have mistakenly concluded that M.116 is one of the genotypes with the best tolerance to ARD. Furthermore, it should be noted that most previous ARD studies compared tree growth in replant soil with fumigated soil whereas here we are comparing aisle versus planting row.

Tree growth was nearly identical irrespective of planting positions within an orchard 12 months after planting; ARD symptoms only became apparent 2 years after planting. This is consistent with our unpublished results from a potted experiment (X.-M. Xu, G. Deakin, E. L. Tilston, J. Bennett, T. Passey, N. Harrison, and F. Fernández-Fernández, *unpublished data*) where we demonstrated that ARD was mainly restricted to root development within the first 15 months and the above-ground traits were mainly affected by rootstock genotypes during this period.

Fig. 6. Principal component (PC) plots of bacterial abundances for **A**, cider orchard fungi, **B**, dessert orchard fungi, **C**, cider orchard bacteria, and **D**, dessert orchard bacteria. Each point represents the centroid of individual genotypes (three samples per genotype). Control was the field edge.

TABLE 3 Percentage of variation of microbiome data accounted for by rootstock genotypes and within-orchard location (aisle or tree stations), and their interactions, as determined by analysis of variance of all principal component scores							
Organisms	Terms	Cider			Dessert		
		T0	T1	T2	T0	T1	T2
Fungi	Tree stations versus grass [1]	9.35	2.82	2.99	12.43	3.00	3.40
	Genotypes [2]		14.43	14.65		14.71	14.75
	[1] × [2]		14.38	14.33		14.64	14.85
Bacteria	Tree stations versus grass [1]	5.64	2.72	2.80	7.71	2.60	3.08
	Genotypes [2]		14.39	14.77		14.31	14.44
	[1] × [2]		14.41	13.96		14.61	14.94

When interpreting microbiome data in relation to tree locations, rootstock genotypes and sampling times, we need to assume that microbial recruitment by rootstock genotypes takes place after planting. Thus, differences in the microbiome between grass aisle and tree station samples, particularly between the bulk and rhizosphere samples, are expected to decrease over time since a nonrandom set of microbes (as implied by recruitment) of bulk soil is recruited in the rhizosphere and every rootstock genotype is present in the pair-wise location (tree station versus grass aisle). Similarly, the differences between rootstock genotypes are expected to increase over time if there is continuous microbial recruitment by genotypes. Furthermore, there may be significant interactions in the rhizosphere microbiome between genotypes and grass/aisle location because genotypes may differ in their ability of recruiting specific microbes but the abundance of these specific microbes vary between tree stations or grass aisle.

A large proportion of variability in the microbial community structure was due to the difference between the samples taken

before planting and those taken one or two years after planting. Samples taken before planting reflected the microbial community structure in the bulk soil (albeit shaped by previous trees and vegetation cover in the aisle), whereas later samples reflected microbiota in the rhizosphere, a subset of total microbiota in the soil. Rhizosphere microbiota can be considerably shaped/influenced by host genotypes (Burns et al. 2015; Haichar et al. 2008; Qin et al. 2016; St. Laurent et al. 2008), primarily through root exudates as shown for apple (Leisso et al. 2018, 2017). The present results support differential microbial recruitment in rhizosphere by rootstock genotypes. Firstly, there are significant differences in the rhizosphere microbial community structure between different rootstocks in both orchards. Secondly, the overall differences in the microbiome between the aisle and tree stations significantly decreased from first time point (before planting – bulk soil) to the later ones (rhizosphere), namely recruitment of similar microbes from bulk soil onto rhizosphere. Thirdly, there were significant interactions in the rhizosphere microbial community between

Fig. 7. Principal component (PC) plots showing the effect of genotype over time points 1 and 2 for **A**, cider orchard fungi, **B**, dessert fungi, **C**, cider bacteria, and **D**, dessert bacteria. Points show the centroids of the three biological replicates for each genotype.

rootstock genotypes and planting positions. The interactions indicate that microbial recruitment by hosts is conditioned on those microbes that are present in the specific location. For instance, a specific root exudate may be utilized by a number of microbes; however, the relative abundance of these microbes may differ considerably between the aisle and tree stations, resulting in large differences in the compositions of these microbes in the rhizosphere that can use this specific substrate between the aisle and tree station. This variable abundance of microbes may be further compounded by their differential competitiveness in rhizosphere. The exact exudates are likely to vary considerably between genotypes. These two sources of variability (microbes between aisle and tree stations; and exudates between genotypes) may result in the observed interactions between genotypes and planting positions.

Present results suggested that rhizosphere microbiota associated with specific rootstock genotypes reached a stable structure within 7 months of planting as indicated by the similar magnitude of genotypic effects between two sampling points after planting. Of course, the relative abundance of individual microbial components could still change as indicated by the differences between the samples at these two points for the third or fourth PCs. Nevertheless, the effects of rhizosphere microbiome on tree growth may take much longer to become apparent. Comparison of individual OTUs between ARD and non-ARD rhizosphere samples did not shed much light on possible microbes contributing to the observed differences in tree growth, except one mycorrhizal OTU with lower abundance in ARD trees. However, oomycetes are known to be an important potential causal agent of ARD (Mazzola and Manici 2012); but we did not specifically profile oomycetes associated with rhizosphere in this study. In addition, nearly all OTUs with differential abundance associated with ARD cannot be assigned to taxonomic ranks below the class with confidence. Thus, future studies may need to consider using other more informative primers for taxonomic assignment but may sacrifice microbial coverage.

In summary, replanting trees with a rootstock genotype different from the previous one can be effective in reducing ARD development. When adopting this strategy, the extent of genetic relationships among rootstock genotypes needs to be considered. Further evaluation is necessary to assess ARD development in relation to rotation combinations of popular commercial rootstocks. In addition, the present study also indicates that empirical evidence on the performance of rootstock genotypes in relation to ARD needs critical evaluation.

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