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REGULAR ARTICLE

Rootstock genotype succession influences apple replant disease and root-zone microbial community composition in an orchard soil

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Abstract Apple replant disease (ARD) is a soil-borne disease complex that affects young apple trees in replanted orchards, resulting in stunted growth and reduced yields. Newly developed rootstock genotypes with tolerance to ARD may help to control this disease. We determined the effects of rootstock genotype rotations during orchard renovation, by investigating root-zone soil microbial consortia and the relative severity of ARD on seven rootstock genotypes (M.9, M.26, G.30, G.41, G.65, G.935, and CG.6210) planted in soil where trees on four of those same rootstocks (M.9, M.26, G.30 and CG.6210) had grown for the previous 15 years. Rootstock genotyping indicated that genetic distances among rootstocks were loosely correlated with their differential responses to ARD. Root-zone fungal and bacterial community composition, assessed by DNA fingerprinting (T-RFLP), differed between M.26 and CG.6210. Soil bacterial communities were influenced most by which rootstock had grown in the soil previously, while fungal communities were influenced more by the current replanted rootstock. In a clone library of bacteria from M.26 and CG.6210 root-zone soil, β-Proteobacteria was the most abundant phylum (25% of sequences). Sequences representing the *Burkholderia cepacia* complex were obtained only from CG.6210 soil. Rootstock genotypes that were grown in the orchard soil previously affected subsequent ARD severity, but replanting with the same or closely related rootstocks did not necessarily exacerbate this disease problem. Our results suggest that genotype-specific interactions with soil microbial consortia are linked with apple rootstock tolerance or susceptibility to ARD.

Keywords Apple replant problem · Clonal rootstocks · Soil microbial consortia · Disease suppressive soil

Abbreviations

AMMI	Additive main effects with multiplicative interaction
ANOVA	Analysis of variance
ARD	Apple replant disease
OTU	Operational taxonomic units
T-RFLP	Terminal restriction fragment length polymorphism

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Introduction

In many orchard replant sites, young apple trees (*Malus domestica* BORKH.) develop poorly, suffering necrotic lesions of fine feeder roots, nutrient and drought stress, stunted growth, and reduced yields (Mai and Abawi 1981). This soil-borne disease syndrome is known as Apple Replant Disease (ARD), and its putative causal pathogens include soil-borne fungi, bacteria, nematodes, actinobacteria, and oomycetes, in site-specific combinations (Mai et al. 1994; Mazzola 1998). These pathogens accumulate in the root-zone of previous fruit trees, often causing no visible harm to previously established trees, but seriously damaging newly planted trees on susceptible rootstocks. Even when orchards are planted in sites with no history of fruit growing, apple root pathogens can become a problem within a few years (Mazzola 1998, 1999).

Modern orchards usually consist of trees propagated by grafting buds of a scion cultivar (a cultivated genotype) onto clonally propagated rootstocks that influence important tree traits such as size (vigor), yield precocity and efficiency, and adaptability to soil physical, chemical and biological conditions (Rom and Carlson 1987). These apple rootstocks are propagated from rooted vegetative cuttings in nursery layering beds, so that all rootstocks of a given cultivar represent a single mass-produced genotype. The Cornell-Geneva (CG) rootstock breeding program has emphasized selection for genotypes with multiple disease resistance (Cummins and Aldwinckle 1974; Norelli et al. 2003; Fazio et al. 2005); and several rootstocks from the CG program are reportedly resistant or tolerant to ARD (Isutsa and Merwin 2000; Leinfelder and Merwin 2006). However, it is not known whether the ARD tolerance of these CG rootstocks, or the ARD susceptibility of most Malling rootstocks, are determined more by their genetic differences (or similarities) or by the influence of the rootstocks grown previously in a given replant site soil.

Soil microbial community composition differs between ARD-conducive soils and supposedly "healthy" soils that have not supported orchards previously (Časka et al. 1982; Benizri et al. 2005). Mazzola (1998, 1999) observed decreased *Burkholderia cepacia* and *Bacillus megaterium* abundance, and increased prevalence of pathogenic *Phytophthora*, *Pythium*, and *Rhizoctonia* species as ARD severity increased long-term in Washington orchards. Manici et al. (2003) attributed differences in ARD severity among orchards in Italy to differences in soil fungal abundance and diversity. Previous research in our program has shown that ARD-tolerant and susceptible rootstocks differed in their associated root-zone fungal and bacterial communities (Rumberger et al. 2004, 2007; Yao et al. 2006). However, there have been no previous studies on ARD-conducive or suppressive species carryover effects that may be unique to specific rootstock genotypes. The present study tested the hypothesis that rootstock-specific microbial consortia may increase or decrease ARD severity, when genetically similar or dissimilar rootstock clones are planted sequentially in the same orchard soil.

We sought to determine whether (1) replant problems are exacerbated when trees are replanted on the same rootstock genotype that was grown in the orchard previously, (2) the ARD resistance observed in certain rootstocks (e.g. CG.6210) is diminished when that same genotype is replanted successively in the same soil, (3) ARD-resistant rootstocks suppress ARD severity for otherwise susceptible rootstock genotypes subsequently replanted in the same soil, (4) characteristic microbial species or consortia are associated with the roots of ARD resistant or tolerant rootstocks.

Materials and methods

Previously planted orchard site

In 1991, an orchard was established in Ithaca, NY, to evaluate and compare the performance of 'Empire' apple grafted onto seven experimental rootstocks from the CG breeding program. The planting also included 'Indian Summer' crab-apple pollenizers on 'Malling 26' (M.26) rootstocks, and an orchard of 'Mutsu' on M.9 rootstocks was established at the same time adjacent to this planting. Apple trees had been grown at the site since 1936. The soil is a glacial lacustrine silty clay loam (mixed, mesic Udic Hapludalf), averaging 4.5% organic matter content, with a pH of 6.2 at the time of planting.

Soil collection and treatment

In March, 2006, soil was collected from beneath trees on the rootstocks G.30, CG.6210, M.26 and M.9 in this 15-year-old orchard. In total, 300 L of soil were collected to 35 cm depth beneath the drip-line of four randomly selected replicate trees of each rootstock genotype, within a weed-free tree-row strip that had been maintained by annual glyphosate herbicide applications for the past decade. These four-tree composite soil samples were handled separately for each rootstock genotype, and mixed thoroughly with an equal volume of coarse sterile perlite (Whittemore, Lawrence, MA, USA) to provide adequate drainage for growing test rootstocks in containers. Half of each soil-perlite rootstock sample mixture was placed into sterilized 10-L containers and stored at 2°C to provide an unpasteurized soil for comparison, while the other half was pasteurized with forced air and steam at 80°C for 1 h, and then placed into sterilized 10-L containers. The container pots of pasteurized or unpasteurized soil from each of the four preceding rootstock sources were then allowed to ventilate and settle in an outdoor nursery for 2 weeks prior to planting the test rootstock liners into each container.

Rootstock material and reciprocal plantings

In mid April of 2006, rootstock liners of M.26, M.9, G.30, G.41, G.65, G.935, and CG.6210 were planted into the 10-L containers of pasteurized or unpasteurized orchard soil in which either the same rootstock genotype, or different rootstocks of M.26, M.9, G.30 and CG.6210, had been growing during the previous 15 years. The M.26 rootstock liners for replant container tests were obtained from Willamette Nurseries (Canby, OR, USA). All other replant rootstock liners were obtained from the CG apple rootstock breeding program of the USDA Plant Genetic Resources Unit (PGRU) in Geneva, NY, USA. The parentages of these CG and Malling rootstocks are shown in Table 1, and their genetic similarity based upon SSR marker analysis is given in Fig. 1.

The bioassay rootstocks were grown as ungrafted liners bearing their own shoot and root system. Before planting, the fresh weight and caliper of each rootstock were measured, and the number of lateral roots per rootstock shank was graded on a scale from 0 (no roots) to 10 (abundant roots). Because there were substantial differences in the quality of our initial rootstock materials, each group of rootstocks was ranked by its initial biomass and lateral root abundance, and assigned to the preplant soil treatments (the preceding rootstocks in the old orchard site, and ± soil pasteurization) in a stratified random order so that rootstocks of comparable quality were planted into each preplant soil treatment. There were five replicates per combination of seven replant rootstock genotypes, four soil sources (preceding rootstock soil from the old orchard), and two (±) soil pasteurization treatments.

Growing conditions in the nursery

The rootstock liners were grown for 5 months (May through Sept. of 2006) in containers randomly arranged in an outdoor nursery in Ithaca, NY. Rootstock growth was measured as caliper width at 10 cm above the soil line at monthly intervals, and uniform drip irrigation was provided to each container as needed daily to weekly. When shoot elongation had commenced on all viable rootstock liners (about 40 days after planting), 200 ml of a 13% (w/v) solution of N-P-K fertilizer (15:5:15; equivalent to 200 ppm Nitrate-N) with micronutrients (Miracle Gro Excel, Scotts Company, Marysville, OH) was provided to each container, every other week. The containers were hand-weeded, and arthropod or fungal pests on leaves were controlled as needed with non-systemic, non-soil-active pesticides.

Rootstock bioassay harvest

The rootstock liners were harvested in late Sept. 2006 after 5 months of growth, when they had set terminal buds and ceased visible shoot growth. The adherent soil was washed gently off the roots with running tap water. The roots were blotted dry with paper towels, and their fresh weight (wt) and lateral root abundance were rated. Each plant was then divided into above- and below-ground parts, weighed, and held at 2°C until further processing. Shoot and root biomass not sub-sampled for microbial analyses were dried at 70°C for 5 days and then weighed to determine total plant dry weight in each treatment combination.

Table 1 Parental pedigrees of the rootstocks used in this study. Plant Introduction (PI) numbers indicate accessions that are maintained by the USDA National Plant Germplasm System repository (www.ars-grin.gov) in Geneva, NY, USA

Rootstock	Parentage or origin
Cornell Geneva 6210 (CG.6210)	Ottawa 3 × Robusta 5
Geneva® 30 (G.30)	Robusta 5 × Malling 9
Geneva® 41 (G.41)	Malling 27 × Robusta 5
Geneva® 65 (G.65)	Malling 27 × 'Beauty Crab'
Geneva® 935 (G.935)	Ottawa 3 × Robusta 5
Malling 26 (M.26)	Malling 16 × Malling 9
Malling 9 (M.9)	*Malus X domestica* open pollinated selection
Ottawa 3 (PI 588881)	Malling 9 × *Malus prunifolia*
Robusta 5 (PI 588825)	*Malus X robusta* (*M. baccata*, *M. prunifolia*) open pollinated selection
Malling 27	Malling 9 × Malling 13
'Beauty Crab' (PI 589235)	*Malus X robusta* open pollinated selection
Malling 13	*Malus X domestica* open pollinated selection

Analysis of soil fungal and bacterial communities

Our previous research indicated that M.26 was susceptible and CG.6210 was tolerant to the ARD complex in the orchard sampled (Leinfelder and Merwin 2006; Rumberger et al. 2004; Yao et al. 2006). Thus, with limited resources available for cloning and sequencing, we selected root-zone microbial DNA from the four reciprocal combinations of replant and preplant M.26 and CG.6210 rootstocks, to characterize the microbial communities in each soil treatment. When the replant rootstock liners were harvested in Sept. of 2006, five replicate soil samples were taken from each container of unpasteurized soil

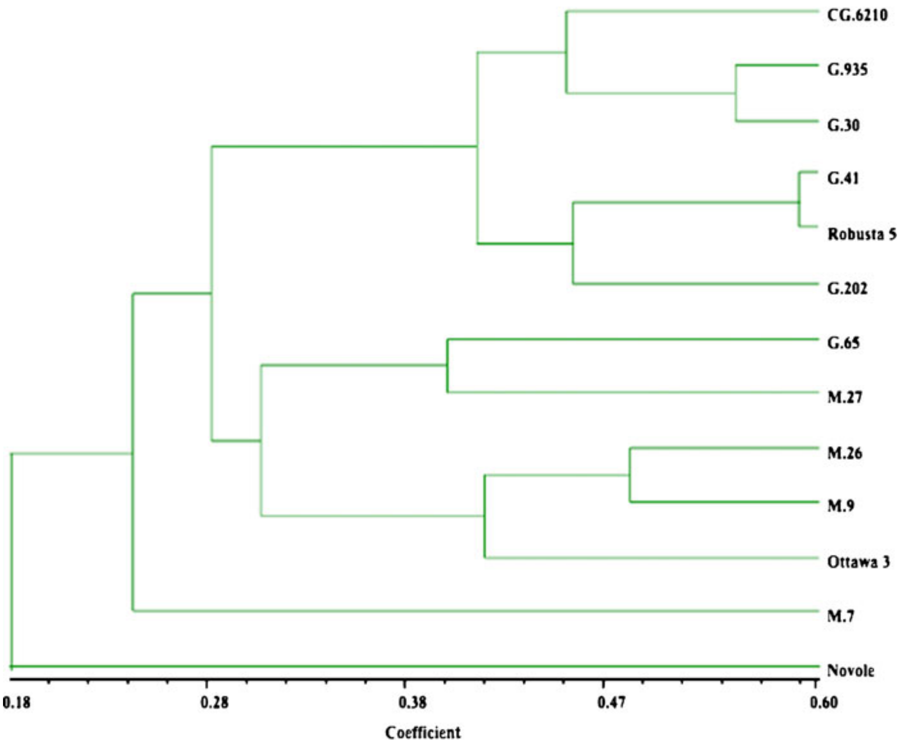

Fig. 1 Dendrogram representing genotypic similarity based upon Jaccard's coefficient matrix of Single Strand Repeats (SSRs), for the apple rootstocks included in this study and some of their parents. 'Novole' (*Malus prunifolia*) and 'Malling 7' rootstocks were included as out-groups for reference purposes

in which M.26 and CG.6210 replant rootstocks had grown, and all samples were frozen at −20°C until further processing as follows. Soil DNA was extracted with a FastDNA®Spin for Soil kit (MP Biomedicals, Solon, OH), using 1.5 g soil per extraction (the manufacturer's recommended quantity of 0.5 g did not yield sufficient DNA for subsequent analyses). The DNA extracts were purified using QIAquick® PCR Purification Kit (Qiagen Inc., Valencia, CA, USA), and then diluted tenfold for PCR-amplification.

Soil bacterial and fungal communities were analysed using terminal restriction fragment length polymorphism (T-RFLP) analysis. For each 50-µL PCR reaction, 4 µL of the 1:10 diluted DNA extracts was added to a reaction mix containing 1.25 U Go-Taq DNA polymerase, 160 µM L^{-1} dNTPs, 1× PCR-buffer, 3 mM L^{-1} MgCl$_2$ and 0.5 µM L^{-1} of each primer in ultra pure water. All PCR-master-mix components were obtained from Promega (Madison, WI, USA). Amplification conditions and primers used are given in Table 2. Successful PCR was verified by electrophoresis on a 1.5% agarose gel of PCR amplicons stained with SYBR®-Green (Sigma, St. Louis, MO, USA) mixed into the loading dye at a ratio of 1 µL mL^{-1}.

Two replicate 50-µL PCR reactions per sample were pooled and quantified against a calf thymus DNA standard curve in an ethidium bromide (EtBr) solution using a BioRad Fluor-S™ MultiImager and Quantity One™ software (BioRad, Hercules, CA, USA). The restriction enzymes Hha1 and Sau96*I* (New England Biolabs, Ipswich, MA, USA) were used to digest amplified sample DNA. A 30-µL restriction-enzyme digest was prepared per sample, containing 1 U restriction enzyme, 1× reaction buffer (New England Biolabs), 0.3 g L^{-1} bovine serum albumin (New England Biolabs), and 300 ng of amplified sample DNA in nuclease-free water. Restriction digests were carried out in a PTC100 thermal cycler (MJ-Research, Waltham, MA, USA) held at 37°C for 4.5 h with a final step of 80°C for 20 min to stop the reaction. Complete digestion of the

Table 2 Primer targets, primer names, reaction conditions and post-PCR analysis methods used to characterize bacterial and fungal communities as applied in this study

Target	Primer	Method	PCR conditions	Reference
Bacteria, 16S rDNA	27f [6FAM] 1378r	T-RFLP	95°C 5 min $\left.\begin{array}{l}95^{\circ}\mathrm{C}30\mathrm{s}\\47^{\circ}\mathrm{C}45\mathrm{s}\\72^{\circ}\mathrm{C}45\mathrm{s}\end{array}\right\}27\times$ 72°C 10 min 4°C ∞	Marchesi et al. 1998 Moeseneder et al. 1999
Bacteria, 16S rDNA	F985 1492r	Cloning	95°C 5 min $\left.\begin{array}{l}95^{\circ}\mathrm{C}30\mathrm{s}\\50^{\circ}\mathrm{C}45\mathrm{s}\\72^{\circ}\mathrm{C}45\mathrm{s}\end{array}\right\}35\times$ 72°C 10 min 4°C ∞	Moeseneder et al. 1999
Fungi, ITS	ITS1Ff [6FAM]* ITS4r	T-RFLP	95°C 5 min $\left.\begin{array}{l}95^{\circ}\mathrm{C}30\mathrm{s}\\59^{\circ}\mathrm{C}45\mathrm{s}\\72^{\circ}\mathrm{C}60\mathrm{s}\end{array}\right\}35\times$ 72°C 15 min 4°C ∞	Bruns et al. 1991
Plasmid	T7 SP6	Cloning	95°C 5 min $\left.\begin{array}{l}95^{\circ}\mathrm{C}30\mathrm{s}\\48^{\circ}\mathrm{C}45\mathrm{s}\\72^{\circ}\mathrm{C}45\mathrm{s}\end{array}\right\}35\times$ 72°C 7 min 4°C ∞	Wallace et al. 1981

DNA was verified by inspecting digested PCR products run on a 1.5% agarose gel, stained with EtBr and visualized using a Fluor-S™ MultiImager (Bio-Rad).

Digested DNA was purified using a PERFORMA® DTR Edge Plate (Edge BioSystems, Gaithersburg, MD, USA) and then lyophilized. DNA was resuspended in a 10 µL mix containing 9.85 µL of formamide and 0.15 µL of Liz 500 size standard (Applied Biosystems, Foster City, CA, USA). Terminal fragment size analysis was performed using a 3730 X1 ABI electrophoretic capillary sequencer (Applied Biosystems) in conjunction with the Genemapper Software (Applied Biosystems) at Cornell University's Biotechnology Resource Center (Ithaca, NY, USA).

Soil bacterial DNA was amplified from the root-zone soils sampled from M.26 replant liners grown in M.26 preplant soil, and all CG.6210 liners grown in CG.6210 soil, as summarized in Table 2. All PCR-products of the five replicate DNA-extracts per treatment were pooled, cleaned and concentrated using QIAquick® PCR Purification Kit (Qiagen Inc.), according to manufacturer's protocol. Three microliters of cleaned PCR product were used for the ligase reaction, using a pGEM® T-Easy Kit System II (Promega Co.) according to manufacturer's protocol. After heat-shock transformation, cells were spread on S-Gal^{TM}/LB-Agar Blend (Sigma Co.) amended with 100 µg L^{-1} sodium ampicillin (Sigma Co.) and incubated at 37°C overnight. White colonies were picked and grown overnight in liquid LB-medium amended with 100 µg L^{-1} sodium ampicillin. Length of the insert was checked by amplifying 1 µL of each bacterial culture as a template in a 50 µL reaction (Table 2) and subsequent electrophoresis of the PCR-product on a 1.5% agarose gel. Six microliters of each PCR-product with the right insert length was cleaned for 15 min at 37°C with 2 µL ExoSAP-IT® (USB Corp., Cleveland, OH, USA), followed by 15 min at 80°C for enzyme deactivation. The resulting 507 bp of cleaned PCR-product was sequenced at the Cornell Biotechnology Resource Center, Ithaca, NY.

Rootstock genotyping

Leaf and apical meristem tissue for DNA extraction was harvested from bud-wood and rootstock liners of the Cornell-Geneva repository of rootstock genotypes. Twenty-one published apple Single-Strand Repeat (SSR) primers (Hokanson et al. 1998; Liebhard et al. 2002) spanning 12 linkage groups were used as the source for microsatellite data. All PCR primers were labeled with 6-FAM-blue, HEX-green, or NED-yellow [corresponding to filter set D of the ABI-PRISM 3130 DNA fragment analyzer (AME Bioscience, Toroed, Norway)], and used in multiplex PCR reactions to amplify SSR loci. The PCR reactions for SSR markers were performed in 15 µL volumes of a uniform reaction mixture (3 mM MgCl, 0.2 mM dNTPs, 15–20 ng of DNA, 0.4 µM of each primer, Taq polymerase, and commercial buffer (Promega Co.) incorporating 10 µL of light weight mineral oil overlay (Fisher Scientific, Pittsburg, PA). The PCR reactions were resolved on a 36 cm capillary on the ABI-PRISM 3130 DNA fragment analysis system. Electropherograms were analyzed with the Genescan and Genotyper software (ABI-PRISM) and allele size information was manually checked for accuracy and entered into a database for analysis. Alleles were coded as individual characters, tracking presence or absence. Genetic similarity was investigated with NTSYS 2.12 software for phylogenetic analysis (Exeter Software, Setauket, NY). A similarity matrix was generated for the dataset. Jaccard dendrograms were constructed using the SAHN clustering UPGMA method (Unweighted Pair-Group Method, Arithmetic average) within the NTSYS 2.12 software package.

Statistical analyses

Significant treatment effects and differences were inferred at $p=0.05$ for main effects, and $p=0.10$ for interactions. Results of rootstock biomass measurements were analyzed using the Mixed Model procedure (SAS, v. 9.1, SAS Institute Inc., Cary, N.C.), for least-square means (LSmeans). Rootstock liners that died at the outset of the replant study were excluded from subsequent statistical analyses.

For T-RFLP analyses, each fragment size was treated as an operational taxonomic unit (OTU). The resulting fingerprints were analyzed with the Additive Main Effects Multiplicative Interaction (AMMI) model (Gauch and Furnas 1991) using the MATMODEL™ software (Microcomputer Power, Ithaca, NY) The AMMI model (also known as a doubly centered PCA) combines the additive elements of ANOVA with the multiplicative elements of PCA (Culman et al. 2008), combining analysis of variance to partition the species main effects, environment main effects, and species-by-environment interaction effects—followed by principal component analysis to partition the species-by-environment interaction into several components. The main purposes of AMMI analysis are: (1) to understand complex species-by-environment interactions, including delineating environmental effects, and (2) to gain accuracy by separating a signal-rich parsimonious AMMI model from a discarded noise-rich residual (Gauch 2007). Sequences were aligned with Clustal-W alignment using the Mega 4 program (www.megasoftware.net), and identified with BLAST-searches and phylogenetic trees containing known species. Bacterial sequences were considered to be the same "species" at 98% sequence similarity, and all sequences were deposited in GenBank under accession numbers EU051656-EU051822.

Results

Rootstock genotyping

Two major branches (a Cornell-Geneva group and a Malling group) were evident in the dendrogram of phylogenetic similarity developed for the rootstocks and their parents in this experiment (Fig. 1). Within the Malling group, two sub-clusters (M.9, M.26, and Ottawa 3) were aligned in accordance to their familial relationship. Full sibling relationships were noticeable with G.41 and G.202 (both M.27 X Robusta 5 progeny), and with CG.6210 and G.935 (both Ottawa 3 X Robusta 5 progeny; Table 1; Fig. 1). It was noteworthy that except for G.65, the Cornell-Geneva series of rootstocks aligned more closely with Robusta 5 than with their other parents (M.27, M.9, and Ottawa 3). This may be due to the intense selection pressure for disease resistance placed on progeny during selection of the CG rootstock series, with repeated massive disease inoculations that eliminated most progeny during the early screening of seedlings, and likely favored resistance genes derived from Robusta 5.

Rootstock quality and viability at planting

Liners of the seven replanted rootstocks differed substantially in their initial biomass, lateral root abundance, and viability at the outset of this experiment, due to genotype differences and field conditions in the stool-beds where they were obtained (Table 3). The M.9 and M.26 rootstocks were larger and had more lateral roots than the CG rootstocks at planting time. The liners of G.30 were of particularly poor quality and low viability when obtained for this study, and 35% of them failed to initiate growth after they were planted. Among all of the CG rootstocks, 17 liners (mostly G.30) had necrotic lesions on the main stem at planting time, and most of these rootstocks failed to initiate growth and died shortly after planting. Another 23 liners died during the first 30 days of the experiment from undetermined causes; all 40 of these dead or defective liners were excluded from further analyses.

Table 3 Rootstock biomass (g fresh weight), root abundance rating at planting (0 = none, to 10 = abundant), and the percentage of liners of each rootstock that did not initiate growth in container soil bioassays. Data are means±standard deviation of the mean

Rootstock cultivar	Biomass at planting (g)	Root density score (0 to 10 scale)	Failed to initiate growth (%)
M.9	42±18 a*	6.0±2.3 a	0
G.30	23±11 b	2.3±1.4 c	35
CG.6210	21±9 b	2.2±1.1 c	2.5
M.26	20±4 b	6.3±1.1 a	0
G.65	12±3 c	2.3±0.9 bc	2.5
G.41	10±4 c	2.5±1.2 bc	2.5
G.935	10±3 c	2.9±1.1 b	0

*Values followed by different letters within the same column are significantly different from each other at $p=0.05$, based on Tukey's HSD

Replant rootstock growth in the nursery

No stem caliper increase was observed during the first month of rootstock growth in the containers. The M.9 rootstocks initiated visible shoot growth before the other rootstocks, but measurable radial stem expansion was first observed for M.26 and G.935. The last rootstocks to begin visible growth were G.30 and CG.6210. These differences were attributed to different intrinsic chilling and heat unit requirements to terminate dormancy for these rootstocks (Rom and Carlson 1987)

After 5 months growth, there were substantial differences in replant rootstock biomass increase (final rootstock biomass minus biomass at planting) among the two (+/-) soil pasteurization treatments and between the preceding rootstock soils from the orchard (Table 4). Soil pasteurization increased subsequent replant growth somewhat for all seven replant rootstocks, averaged across the four previous rootstock soils from the old orchard (Fig. 2a). However, the replant biomass difference between pasteurized vs. unpasteurized soil was statistically significant for only three of the replant rootstocks—M.9, G.30, and G.41 (Fig. 2a). Lateral root abundance scores at harvest were highest for G.935 and G.6210, with an average score of 9, and lowest for M.9 and M.26, with an average score of 7 (data not shown).

There were significant differences in replant rootstock biomass increase, depending upon the preceding rootstocks that had grown in the unpasteurized orchard soil (Fig. 2b). The rootstocks M.9, M.26, and G.65 grew similarly and relatively weakly following all four of the rootstocks (M.9, M.26, G.30, and CG.6210) that had grown in the old orchard soil previously. Growth of the G.30 rootstocks was greater following M.9 than following the other rootstocks. Growth of the G.41 rootstocks was greater when replanted following M. 26 than following M.9. The G.935 rootstocks grew more strongly in soil where CG.6210 had grown previously, compared with soil where M.26 or G.30 had grown. The growth of CG.6210 rootstocks was greatest in soil where CG.6210 itself had been grown previously (Fig. 2b). In general, both G.935 and CG.6210 grew more vigorously than the other five replanted rootstocks, regardless of which rootstock genotype had grown in the soil previously.

Table 4 ANOVA table for main effects and significant interactions, and main effect means (± SD) for seven replant rootstocks, four preceding rootstocks, and soil pasteurization effects on replant rootstock growth. There were no significant interactions ($p>0.1$) among preceding rootstock * replant rootstock * pasteurization, or for preceding rootstock * pasteurization

Main effects	**df**	**F-value**	***P*-value**
Replant rootstocks	6	21.46	<0.001
Preceding rootstocks	3	4.32	0.006
Pasteurization	1	21.24	<0.001
Repl. rootstock * Pasteurization	6	1.81	0.098
Repl. rootstock * Preced. rootstock	18	1.97	0.013
Replant rootstock effect		**Mean replant fresh wt increase±one SD** (g)	
M.9		83±9	
M.26		85±5	
G.30		117±16	
G.41		93±10	
G.65		86±5	
G.935		144±8	
CG.6210		187±13	
Previous rootstock effect			
M.9		103±7	
M.26		107±8	
G.30		108±7	
CG.6210		133±11	
Pasteurization effect			
Pasteurized		126±6	
Non-pasteurized		99±5	

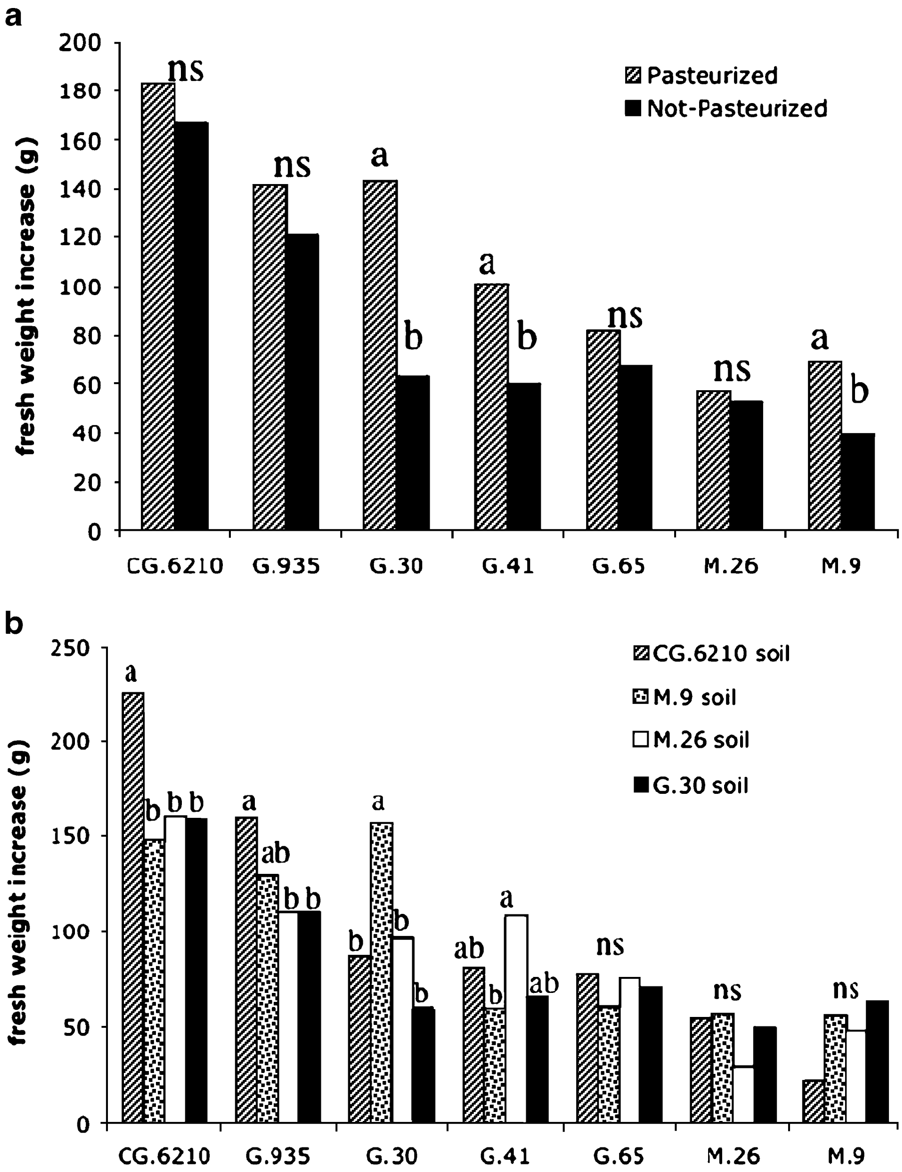

Fig. 2 **a** Increase in fresh weight (g) for seven rootstock cultivars (M.9, M.26, G.30, G.41, G.65, G.935, and CG.6210) grown as replant liners for 5 months in pasteurized or non-pasteurized soil from the previous orchard (*n*=7). **b** Increase in fresh weight (g) of the same seven replant rootstock cultivars, grown for 5 months in containers of unpasteurized soil sampled from locations where four apple rootstocks (M.9, M.26, G.30, and CG.6210) had grown for the preceding 15 years in a NY orchard (*n*=18). Columns beneath different letters were significantly different; **ns** denotes no significant differences at *p*=0.05

Soil microbial communities on rootstocks

Bacterial and fungal DNA amplified from the root-zone soils and digested with Hha1 and Sau961 restriction enzymes produced very similar T-RFLP results in the AMMI analyses; therefore, only the results for the Hha1 restriction digests are presented (Fig. 3a, b; Table 5). Since the data obtained for soil fungi were very noisy, we opted for AMMI analysis rather than PCA alone. Even after 5 months of replant rootstock growth in the nursery containers, soil bacterial community composition was influenced most strongly by the rootstocks grown previously in the orchard soil, as indicated by the separation of treatment values along the horizontal axis in the AMMI analysis (Fig. 3a). Bacterial communities were similar in soil where G.30 or CG.6210 had grown previously (grouping closely in the lower left quadrant of Fig. 3a), while they separated widely along the horizontal axis in soils previously planted to M.26 vs. M.9. Along the vertical axis in the AMMI plots, bacterial communities separated mostly according to the replant rootstocks in soil where M.26, CG.6210, and M.9 had grown in the old orchard (Fig. 3a). However, where CG.30 had grown for the preceding 15 years, the replant rootstocks did not influence soil

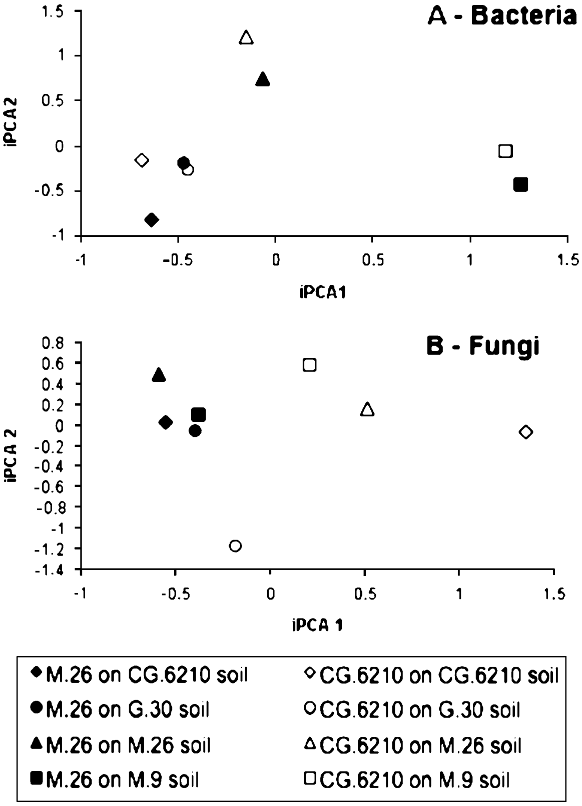

Fig. 3 AMMI plots of the bacterial (**a**) and fungal (**b**) community composition assessed using T-RFLP analysis with the restriction enzyme Hha1, in soil where apple trees had grown for the preceding 15 years on the rootstocks M.9, M.26, G.30, and CG.6210, that was subsequently replanted for 5 months to M.26 or CG.6210 rootstock liners. Values are the mean of five replicates for each treatment combination

bacterial community composition as strongly. ANOVA for the components in the AMMI model indicated that treatments (rootstocks) and both IPCA axes were significant effects in the model (Table 5).

For root-zone soil fungal community composition the replant rootstocks appeared to have a greater effect on soil fungi than the preceding rootstock genotype that had grown in the old orchard. The CG and Malling rootstocks separated clearly along the horizontal axis in the AMMI analyses (Fig. 3b). Root-zone fungal communities grouped closely together in one quadrant of the AMMI graph for M.26 replant rootstocks, regardless of the rootstock genotype grown in the orchard soil previously. In contrast, the fungal consortia associated with CG.6210 were scattered across three quadrants of the AMMI plot, reflecting the strong interaction of both pre-plant and replant rootstock genotypes on root-zone fungi.

Identification of root-zone bacteria

A total of 167 different bacterial sequences, representing 80 genotypes at the 98% sequence similarity level, were obtained from soil where M.26 and CG.6210 had been both the preceding and the replant rootstocks (Table 6). Of these 80 putative genotypes, 53 (32%) matched with GenBank entries at a minimum of 95% sequence coverage and 98% sequence similarity. Very similar results were obtained for sequence matches with the GreenGene database (data not shown). The two rootstock genotypes differed substantially in their associated bacterial species composition. The most frequently represented phylum was β-Proteobacteria, comprising 25% of all observed sequences. Four sequences obtained from the CG.6210 root-zone represented the *Burkholderia cepacia* complex, while no sequences from *Burkholderiaceae* were obtained from M.26 soil. All γ-Proteobacteria clones obtained in this study belonged to the family Xanthomonadaceae, representing the genera *Xanthomonas*, *Frateuria,*

Table 5 ANOVA tables for AMMI analyses of bacteria and fungi using Hha1 digestions, based on TRFLP presence or absence in DNA fragments from soil samples

Source	df	SS	MS	*P* values
Bacteria				
Total	8549	2099.58444	0.24559	
TRT	1799	1362.03444	0.75711	0.001
Band	224	1073.93913	4.79437	0.001
Environ 7	1.82066	0.26009	0.020	
B x E	1568	286.27465	0.18257	0.001
IPCA 1	230	93.23075	0.40535	0.001
IPCA 2	228	45.22978	0.19838	0.001
Residual	1110	147.81413	0.13317	0.001
Error	6750	737.55000	0.10927	
Fungi				
Total	8189	2086.93285	0.25485	
TRT	1679	1544.88285	0.92012	0.001
Band	209	1411.30629	6.75266	0.001
Environ 7	3.69589	0.52798	0.001	
B x E	1463	129.88068	0.08878	0.155
IPCA 1	215	48.46515	0.22542	0.001
IPCA 2	213	19.93985	0.09361	0.128
Residual	1035	61.47568	0.05940	0.001
Error	6510	542.05000	0.08326	

Fulvimonas, Rhodanobacter, and *Dokdonella*. Considerably more γ-Proteobacteria clones were obtained from CG.6210 than from M.26 soil (25% versus 12%). In contrast, 27% of the M.26 bacterial sequences vs. only 7% of those obtained from CG.6210 represented Acidobacteria. Other potentially important groups observed were α-Proteobacteria and Verrucomicrobia, with 15% of sequences representing each group.

Only 11% of the observed bacterial sequences represented Gram-positive organisms. Five closely related clones obtained from CG.6210 soil could not be assigned to any phylum using BLAST searches. However, one of these five clones matched at the "species" level with the sequence of GenBank accession number EF516654, an uncultured soil bacterium. Another sequence that could not be attributed to any phylum formed an out-group root of the comparative dendrogram, and its closest match (80% coverage, 95% sequence similarity) in the GenBank was EF516653—another uncultured soil bacterium. Most of the observed bacterial phyla formed distinct groups in the comparative phylogenetic tree, but sequences identified as δ-Proteobacteria or Bacteriodetes did not cluster closely together.

Discussion

Previously grown rootstock genotypes differentially affect the severity of ARD

Results presented here show that the rootstock genotypes grown previously in an old orchard can influence quite specifically the growth of trees replanted on similar or different rootstocks at that orchard site. Preceding rootstock genotype effects on subsequent growth and microbial consortia of replant rootstocks varied among the genotypes we tested (Fig. 2). There was no clear correlation between the genetic relationships among the rootstocks (Fig. 1) and their growth effects when replanted in an ARD soil (Fig. 2). For example, CG.6210 grew most vigorously in soil where it had grown previously, but so did its relatively distant sibling G.935. Moreover, G.30 grew better following M.9 (one of its parents), than in soil where G.30 itself had grown previously. While the rootstock grown previously could affect subsequent ARD severity for some rootstock genotypes, our observations do not support the hypothesis that replanting orchards with the same or closely related rootstock cultivars will necessarily exacerbate ARD problems. This is consistent with earlier reports that ARD sometimes occurs even when orchards of other tree-fruits (e.g. *Prunus* sp.) have preceded the apple planting (Mai et al. 1994).

Use of ARD tolerant rootstocks may help develop suppressive soils

Isutsa and Merwin (2000) compared the growth of 17 clonal rootstocks and 40 seedling lots representing 941 different genotypes, in pasteurized vs. unpasteurized soil from five NY orchards with documented replant problems. They concluded that the rootstocks G.30 and CG.6210 were relatively tolerant to the ARD complex in NY orchards. Leinfelder and Merwin (2006) also found that G.30 and CG.6210 were tolerant of the ARD-complex, in field experiments conducted nearby the present study site on the same soil type. In contrast, M.26 and G.65 were rated as susceptible to ARD in the study by Isutsa and Merwin (2000), and M.26 also performed poorly in the ARD study by Leinfelder and Merwin (2006).

In the present study, M.9 and G.30 replant rootstock growth increased twofold in response to soil pasteurization (Fig. 2a), suggesting that growth of M.9 and G.30 was restricted by soil organisms that were reduced or eliminated by pasteurization, whereas CG.6210, M.26, G.935 and G.65 grew similarly in pasteurized and unpasteurized soils (Fig. 2a). Weak lateral root development and low viability of the G.30 rootstocks obtained for this study might explain why it was less tolerant of ARD than it had been in our previous studies. Low grafting success rates and poor stool-bed viability of G.30 rootstocks have also been reported by several commercial nurseries (T. Robinson, personal communication). Soil bacterial and fungal consortia differed substantially in the root-zone soil of M.26 and CG.6210 (Fig. 3; Table 6). This was consistent with previous orchard-based observations that different rootstocks were associated with distinct rhizosphere microbial communities (Rumberger et al. 2004; Yao et al. 2006).

Our recent observations suggest that soil bacterial communities in orchards are influenced most by the rootstock that had grown in old orchard soil previously, while soil fungal communities are influenced

Table 6 Percentage of clones and number of Operational Taxonomic Units (OTUs) belonging to a phylum in DNA isolated from soil planted to M.26 and CG.6210 rootstocks. Phyla were identified via BLAST searches. Genotypes were differentiated at 98% sequence similarity. A total of 167 clones were sequenced. 'Unknown' signifies groups that could not identified as belonging to any known phylum via BLAST searches

Phylum	Percentage of clones		Number of OTUs		Number of OTUs common to both rootstocks
	M.26	CG.6210	M.26	CG.6210	
β-Proteobacteria	26	25	5	6	2
γ-Proteobacteria	12	25	5	7	4
Acidobacteria	27	7	16	6	2
Verrocomicrobia	10	8	3	4	1
α-Proteobacteria	9	9	6	8	2
δ-Proteobacteria	6	2	5	2	0
Actinobacteria	2	6	2	4	0
Bacteriodetes	4	3	3	1	0
Chloroflexi	2	3	2	3	0
Unknown	0	6	0	1	0
Gemmamonadetes	0	4	0	2	0
Division TM7	2	1	2	1	1
Unknown	0	1	0	1	0

more by the current, replanted rootstock (Fig. 3). In our studies of rhizosphere microbial community development over several years at a nearby ARD site (Rumberger et al. 2007), the persistent effects of replant location (in previous tree rows vs. previous grass drive lanes) were observed in soil bacterial communities for 3 years after replanting the orchard site, but the replant rootstock fungal communities in these two planting locations converged after just 1 year

In previous studies, we observed that bacterial root-zone communities on CG.6210 and G.30 were similar (Rumberger et al. 2004, 2007; Yao et al. 2006). However, in the present study there were substantial differences in replant growth and carry-over ARD effects of CG.6210 vs. G.30. This discrepancy may indicate that the microbial fingerprinting techniques (T-RFLP and Denaturing Gradient Gel Electrophoresis) used in our prior studies were not discriminating enough to reveal differences between the soil microbial consortia of these two rootstocks. Neither of these molecular fingerprinting methods provides quantitative information about active populations of potential beneficial or pathogenic microorganisms on rootstocks at ARD sites, so we cannot make definitive inferences about the potential ARD-suppressive roles of bacterial and fungal consortia associated with the roots of CG.6210 in these studies.

The frequency of various bacterial phyla differed between the clone libraries obtained for CG.6210 and M.26 soils (Table 6). A higher percentage of sequences representing γ-Proteobacteria—all belonging to the family Xanthomonadaceae—were obtained from CG.6210 soil than from M.26 soil. One genus in this family (*Frateuria*) reportedly produces antifungal compounds in-vitro (Matsushita 1990) and might have a role in ARD suppression by CG.6210. Unfortunately, little is known about potential disease antagonists in the genera *Fulvimonas, Rhodanobacter,* and *Dokdonella*, which have all been isolated from soil and rhizosphere environments and named within the last decade (De Clercq et al. 2006; Mergaert et al. 2002; Nalin et al. 1999; Yoon et al. 2006).

Acidobacteria were the dominant phylum observed in M.26 soil in this study. Chan et al. (2006) observed that a subgroup of Acidobacteria appeared to thrive in humus rich soil layers around tree roots; but to date only a few Acidobacteria have been cultured, and little is known about their potential interactions with plants and other soil microorganisms.

Four sequences representing members of the *Burkholderia cepacia* complex were obtained from CG.6210 soil, while none were observed in M.26 soil (Table 6). Various strains of *Burkholderia cepacia* are reportedly suppressive to fungal and oomycete root pathogens, including some previously implicated in

ARD (Bevivino et al. 1998; Hebbar et al. 1998; Mazzola 1998). These findings suggest that a greater abundance of antagonistic rhizosphere bacteria might be contributing to the ARD tolerance of CG.6210 itself, and to the increased growth of G.935 rootstocks replanted in soil where CG.6210 had grown.

In prior studies, we also observed that M.9 and M.26 supported more culturable bacteria and fungi in their rhizospheres, compared with CG.6210 (Rumberger et al. 2007). It is possible that CG.6210 supports a smaller rhizosphere community, in which strong competition for carbon substrates may reduce the signal strength of inducer molecules needed for fungal spore germination. That hypothesis is worth testing in future experiments.

Relatively few (11%) of the sequences obtained were classified as Gram-positive bacteria. Our previous ARD study using orchard soil without perlite added yielded more than twice as many sequences representing Gram-positive bacteria (St. Laurent et al. 2008). Mixing perlite with soil samples to improve drainage in the container pots also reduced the DNA yields from soil, compared to DNA extracted from very similar soils in our previous studies. At the bead-beating step in soil sample pulverization, perlite fragments may interfere with the lysis of comparatively thick-walled Gram-positive bacteria; this observation may be useful to future researchers extracting DNA from soils amended with perlite.

We conclude that the rootstock genotype on which apple trees were grown in a previous orchard planting can influence the severity of ARD in subsequent replant trees growing on the same or different rootstocks. The ARD tolerance of CG.6210 may involve enhanced populations of putative beneficial soil organisms such as *Burkholderia cepacia*, direct suppression of soil-borne root pathogens, or failure to induce germination of pathogen resting spores in orchard soils. Any of these mechanisms could improve the growth of apple trees replanted on other rootstocks into orchard sites where CG.6210 had been grown previously. It does not appear that replanting orchards with the same or closely related rootstocks will necessarily exacerbate ARD problems, and rootstock genotypes considered tolerant of ARD can remain tolerant to this disease complex, even when the same genotype is replanted sequentially. A salient factor in all these observations is the apparent tendency of some rootstock genotypes to promote or select distinct root-zone bacterial or fungal communities that may be associated with tolerant or susceptible responses to ARD.

References

Benizri E, Piutti S, Verger S, Pagès L, Vercambre G, Poessel JL, Michelot P (2005) Replant diseases: bacterial community structure and diversity in peach rhizosphere as determined by metabolic and genetic fingerprinting. Soil Biol Biochem 37:1738–1746

Bevivino A, Dalmastri S, Tabacchioni S, Chiarini L (1998) Efficacy of *Burkholderia cepacia*: effects of seed treatment on disease suppression and growth promotion of maize. FEMS Microbiol Ecol 27:225–237

Bruns T, White TJ, Taylor JW (1991) Fungal molecular systematics. In: Johnston RF (ed) Annual review of ecology and systematics. Annual Reviews Inc., Palo Alto, pp 525–564

Časka V, Vančura V, Hudská G, Přikryl Z (1982) Rhizosphere micro-organisms in relation to apple replant problem. Plant Soil 69:187–197

Chan C, Yang X, Fu Y, Feng Z, Sha L, Casper P, Zou X (2006) 16S RNA gene analyses of bacterial community structures in the soils of evergreen broadleaved forests in southwest China. FEMS Microbiol Ecol 58:247–259

Culman SW, Gauch HG, Blackwood CB, Thies JE (2008) Analysis of T-RFLP data using AOV and ordination methods: a comparative study. J Microbiol Meth 75:55–63

Cummins JN, Aldwinckle HS (1974) Breeding apple rootstocks. HortScience 9:367–372

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

Fazio G, Robinson TL, Aldwinckle HS, Mazzola M, Leinfelder M, Parra R (2005) Traits of the next wave of Geneva apple rootstocks. Compact Fruit Tree 38(3):7–11

Gauch HG Jr (2007) MATMODEL Version 3.0: Open source software for AMMI and related analyses. Cornell University, Ithaca, NY, 14853. Accessible at: http://www.css.cornell.edu/staff/gauch/matmodel.html

Gauch HG Jr, Furnas RE (1991) Statistical analysis of yield trials with MATMODEL. Agron J 83:916–920

Hebbar KP, Martel MH, Heulin T (1998) Suppression of pre- and postemergence damping-off in corn by *Burkholderia cepacia*. Eur J Plant Pathol 104:29–36

Hokanson SC, Szewc McFadden AK, Lamboy WF, McFerson JR (1998) Microsatellite (SSR) markers reveal genetic identities, genetic diversity and relationships in a *Malus X domestica* Borkh. core subset collection. Theoretical and Applied Genetics 97:671–683

Isutsa DK, Merwin IA (2000) *Malus* germplasm varies in resistance or tolerance to apple replant disease in a mixture of New York orchard soils. HortScience 35:262–268

Leinfelder M, Merwin IA (2006) Rootstock selection, pre-plant soil treatments, and tree planting positions as factors in managing apple replant disease. HortScience 41:394–401

Liebhard R, Gianfranceschi L, Koller B, Ryder CD, Tarchini R, Van De Weg E, Gessler C (2002) Development and characterization of 140 new microsatellites in apple (*Malus* x *domestica* Borkh.). Mol Breed 10:217–241

Mai WF, Abawi GS (1981) Controlling replant diseases of pome and stone fruits in Northeastern United-States by preplant fumigation. Plant Dis 65:859–864

Mai WF, Merwin IA, Abawi GS (1994) Diagnosis, etiology, and management of replant problems in New York cherry and apple orchards. Acta Hort 363:33–41

Manici LM, Ciavatta C, Kelderer M, Ershmaumer G (2003) Replant problems in South Tyrol: role of fungal pathogens and microbial population in conventional and organic apple orchards. Plant Soil 256:315–324

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Enivron Microbiol 64:795–799

Matsushita A (1990) F-2702 a new antifungal antibiotic produced by *Gluconobacter oxidans* F2702 I. Taxonomy and production. Jikeikai Med J 37:325–334

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

Mazzola M (1999) Transformation of soil microbial community structure and *Rhizoctonia*-suppressive potential in response to apple roots. Phytopathology 89:920–927

Mergaert J, Cnockaert MC, Swings J (2002) *Fulvimonas soil* gen. nov., sp. nov., a gamma-proteobacterium isolates from soil after enrichment on acetylated starch plastic. Int J Syst Evol Micr 52:1285–1289

Moeseneder M-M, Arrieta J-M, Muyzer G, Winter C, Herndl G-J (1999) Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl Environ Microbiol 65:3518–3525

Nalin R, Simonet P, Vogel TM, Normand P (1999) *Rhodanobacter lindaniclasticus* gen. nov., sp. nov., a lindane-degrading bacterium. Int J Syst Bact 49:19–23

Norelli JL, Holleran HT, Johnson WC, Robinson TL, Aldwinckle HS (2003) Resistance of Geneva and other apple rootstocks to *Erwinia amylovora*. Plant Dis 87:26–32

Rom RC, Carlson RF (1987) Rootstocks for fruit crops. Wiley, NY, p 494

Rumberger A, Yao S, Merwin IA, Nelson EB, Thies JE (2004) Rootstock genotype and orchard replant position rather than soil fumigation or compost amendment determine tree growth and rhizosphere bacterial community composition in an apple replant soil. Plant Soil 264:247–260

Rumberger A, Merwin IA, Thies JE (2007) Microbial community development in the rhizosphere of apple trees at a replant site. Soil Biol Biochem 39:1645–1654

St. Laurent A, Merwin IA, Thies JE (2008) Long-term orchard groundcover management systems affect soil microbial communities and apple replant disease severity. Plant Soil 304:209–225

Wallace RB, Johnson MJ, Suggs SV, Ken-ichi M, Bhatt R, Keiichi I (1981) A set of synthetic oligodeoxyribonucleotide primers for DNA sequencing in the plasmid vector pBR322. Gene 16:21–26

Yao S, Merwin IA, Abawi GS, Thies JE (2006) Soil fumigation and compost amendment alter soil microbial community composition but do not improve tree growth or yield in an apple replant site. Soil Biol Biochem 38:587–599

Yoon JH, Kang SJ, Oh TK (2006) *Dokdonella koreensis* gen. nov, sp. nov., isolated from soil. Int J Syst Evol Micr 56:145–150