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Metabolic composition of apple rootstock rhizodeposits differs in a genotype-specific manner and affects growth of subsequent plantings

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ABSTRACT

Apple replant disease (ARD) negatively impacts apple tree health and reduces crop yield in new orchards established on sites previously grown to the same or related species. Use of tolerant rootstock genotypes can diminish the growth limiting effects of ARD, and while current research characterizes differential root gene expression by ARD tolerance among genotypes, the potential role of genotype-specific rhizodeposits contributing to ARD tolerance has not been intensively examined. A Q-TOF LC/MS metabolic profiling approach targeting phenolic compounds was used to characterize water-soluble phenolic rhizodeposit metabolites collected from water percolated through the rhizosphere of apple rootstocks planted in pasteurized quartz sand. Four rootstock genotypes (two with ARD field tolerance, G935 and G41, and two with ARD susceptibility, M9Nic29 and M26) differed in both rhizodeposit composition of metabolites and quantity over the course of time, with overall quantity of metabolites increasing as leaf area increased. Total metabolite quantity recovered did not differ with relative rootstock tolerance to ARD. Benzoic acid levels were consistently higher in rhizodeposits derived from G935, while rutin was higher in M26. Phloridzin and phloretin, two compounds previously examined in relation to apple root disease pathogenesis, were higher in the ARD-susceptible M9Nic29 at the inception of the experiment, but did not differentiate ARD tolerant from susceptible genotypes at later time assessments. Other untargeted compounds, identified by accurate mass, mass spectral features, and retention time, separated rootstocks according to ARD tolerance, but their chemical identity remains unconfirmed. Orchard soil treated with apple rhizodeposits had lower pH than soil collected from no-tree controls. Seedling growth in rhizodeposit treated soils differed according to rootstock genotype in a subsequent bioassay, but not according to expected replant tolerance. Differences in metabolite composition of rhizodeposits according to rootstock genotype, and temporal dynamics of their production during early stages of rootstock growth following dormancy, offer insight apple rootstock rhizodeposition, and provide the basis to further investigate their impact on soil chemistry, soil microbiology, and plant health.

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1. Introduction

Apple replant disease (ARD) is caused by a pathogen complex, which includes fungi (*Ilyonectria* spp. and *Rhizoctonia solani*), oomycetes (*Phytophthora* spp. and *Pythium* spp.), and plant parasitic nematodes (Mazzola, 1998). Field tolerance to ARD has been reported for certain apple rootstock genotypes (Fazio et al., 2006; Robinson et al., 2015), but specific genetic resistance mechanisms to individual pathogens, although recently proposed (Shin et al., 2016; Zhu et al., 2016), have not been fully elucidated. Furthermore, multitrophic interactions among multiple pathogens and rhizosphere microbial communities with as yet undefined rootstock genotypic preferences for specific environments and soil chemistries complicate full comprehension of disease etiology. Differences in rhizosphere microbial consortia recruited in a rootstock genotype-dependent manner may determine the severity of ARD development in subsequent orchard plantings (Rumberger et al., 2007; St. Laurent et al., 2010); however the specific rootstock attributes regulating composition of these rhizosphere communities have not been explored. An intriguing lead is the insight that genotype-specific root phenolic concentration in the fine distal roots may contribute to ARD tolerance (Emmett et al., 2014), but how this connects with rhizosphere microbiome derived suppression of replant organisms remains unknown.

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Compounds released by roots into the rhizosphere have been termed exudates or rhizodeposits, with exudate implying an active secretion (Weston et al., 2012). "Rhizodeposits" include all metabolites originating from the root that move into the surrounding soil, even passively, and the precise mechanism of release from the root is not strictly defined. Rhizodeposits can be released through active secretion or passive diffusion (Weston et al., 2012), or may emanate from root debris, i.e., root turnover (Leigh et al., 2002), and may include compounds sloughed off from root epidermis or actively growing root tips (McCully and Boyer, 1997). Rhizodeposits consist of a range of metabolites, from simple bicarbonate or hydrogen ions (Marschner and Romheld, 1983; Romheld et al., 1984; Shahbaz et al., 2006) to sugars, organic acids, and amino acids (Sandnes et al., 2005; Chaparro et al., 2013), as well as polymeric compounds such as tannins (Bekkara et al., 1998) and proteins (De-la-Pena and Vivanco, 2011). Metabolites produced by plant roots can have growth promoting or inhibitory effects on soil microbes (Osbourn, 1996; Broeckling et al., 2008), and estimates of photosynthetically derived rhizodeposits range from 5 to 40% of total fixed carbon (Marschner, 1995; Jones et al., 2009).

Rhizodeposition is a dynamic process that is influenced by microbial activity, which cycles carbon and nutrients back into the plant (Jones et al., 2009), in turn affecting rhizodeposition (Dessaux et al., 2016). Rhizodeposit composition and quantity may vary in a temporal manner and changes can correlate to functional gene expression by corresponding microbes (Chaparro et al., 2013). Other classes of compounds, specifically phenolics, which in Arabidopsis constitute 84% of the secondary metabolites exuded from the roots (Narasimhan et al., 2003), can inhibit microbial growth (Niro et al., 2016), and were purportedly an important determinant of the rhizobiome community composition (Chaparro et al., 2013). Specific classes of compounds, i.e., benzoxazinoids, can attract Pseudomonads to the rhizosphere (Neal et al., 2012). Benefits of both chemical changes due to rhizodeposit release as well as corresponding microbial activity to plants include nutrient sequestration and solubilization through changes in pH and H_2CO_3 (carbonic acid), with the intriguing feedback of microbe released CO_2 increase also having plant growth promotional effects (Glenn and Welker, 1997). Plant species (Hartmann et al., 2009) and genotype have demonstrated effects on the rhizosphere microbiome and may even influence genotypic composition of functional traits including antibiotic production (Mazzola et al., 2004). Apple rootstock genotype influences microbial community composition (St. Laurent et al., 2010), but the cultivar specific metabolic composition of rhizodeposits driving these differences has not been explored.

In apples, numerous research articles note that phloridzin, a dihydrochalcone glycoside, is a component of apple root exudates (Hoffman et al., 2009) and is present in high concentration within the roots (Emmett et al., 2014). Levels of phloridzin vary in response to pathogen infection of roots, although the quantity does not alter the level of pathogen damage to the root system (Hoffman et al., 2009). Hoffman et al. (2009) suggested phloridzin levels correlate positively with apple host susceptibility to pathogens in ARD, but other research indicates phloridzin's aglycone, phloretin, can suppress growth of plant pathogenic oomycetes or fungi (*Phytophthora capsici*, *Rhizocotonia solani* AG4, and others) (Shim et al., 2010). A variety of phenolic compounds have been detected in apple orchard soil (Jinshui et al., 2014), although root origin was not ascertained.

Our fundamental objective was to contrast and describe rhizodeposition in apple rootstocks with differing field tolerance to ARD using targeted and untargeted metabolic profiling approaches, and to then determine the effects of these rhizodeposits on soil chemistry (pH) and the next generation of trees grown in rhizodeposit treated soil. Targeted compounds included phenolic metabolites previously found in association with apple roots (detected in root extracts and orchard soil), and untargeted compounds included other metabolites compatible with a phenolic metabolite Q-TOF LC/MS analysis solvent system. The primary hypothesis was that the metabolic composition and quantity of rhizodeposits would differ among apple rootstock cultivars relative to ARD tolerance, and that specific metabolites potentially driving soil microbial community differences, including pathogens, could be defined. As the initial experimental results were considered, follow-up sub-hypotheses were also defined, specifically that subsequent tree growth in this soil would be affected by microbial and chemical changes induced by rhizodeposits, and that attributes of microbial populations found to be present in rhizodeposits corresponded to leaf area. A validation experiment using axenically propagated trees was also performed to affirm that rhizodeposits were of tree origin versus potential microbial origin when rhizodeposits were generated and collected in a non-sterile environment.

2. Material and methods

A primary experiment was performed twice followed by several validation experiments (Fig. 1). The main experimental goal was to determine the apple rootstock genotype specific composition and quantity of rhizodeposits and assess their impact on soil. The intent of the study was to gain insight into the relative effects of rhizodeposits from disease tolerant and susceptible rootstock genotypes on soil chemistry and biology. In response to results from the primary experiments, several sub-experiments or follow-up tests were conducted including 1) performing a bioassay assessing the impacts of rhizodeposits on growth of next-generation trees, 2) quantifying microbes in greenhouse rhizodeposits in tandem with measuring tree leaf area, and 3) validating the composition of tree-originating rhizodeposits obtained in greenhouse experiments with axenically grown micropropagated trees.

2.1. Rootstock selection

Four apple rootstocks (M9Nic29, M26, G41, G935) were selected on the basis of their relative field tolerance to ARD (Robinson et al., 2012), with M9Nic29 and M26 representing highly susceptible genotypes, while G41 and G935 exhibit superior performance in soil with a history of ARD, and specifically are less susceptible to *Pythium* spp. and *Pratylenchus* spp. in WA (Mazzola et al., 2009).

2.2. Greenhouse experiments

0.95 cm diameter (sold as 3/8 inch liners) dormant rootstocks (G.935, G.41, M.26, M.9Nic29 [Willow Drive Nursery, Ephrata, WA]) were planted in 30 mesh Lane Mountain Sand (Valley, WA) in D40 Deepots (25.4 cm [10 inch] long x 6.4 cm [2.5 inch] wide; Greenhouse Megastore, Danville, IL). Soil or sand was added to obtain a growth medium depth in the pot of 20.4 cm. Six trees were planted for each genotype in Experiment 1, and 8 trees for Experiment 2; further specifics to each experiment are detailed below. Sand was pasteurized at 80 °C for 8 h on two successive days prior to rootstock planting, with 12 h between each heating session. Pots were surface sterilized in a solution of 10% Clorox bleach (Oakland, CA, USA) [v/v in water] (active ingredient, 8.25% NaCLO) for 10 min and a piece of sterilized fabric mesh was inserted into the bottom of the pot to prevent sand or fine soil loss. Trees were rinsed clean of dirt and debris and roots were surface sterilized by submersion for 5 min in 10% bleach (as above) and rinsed with distilled water prior to planting. No additional nutrients were applied to the trees

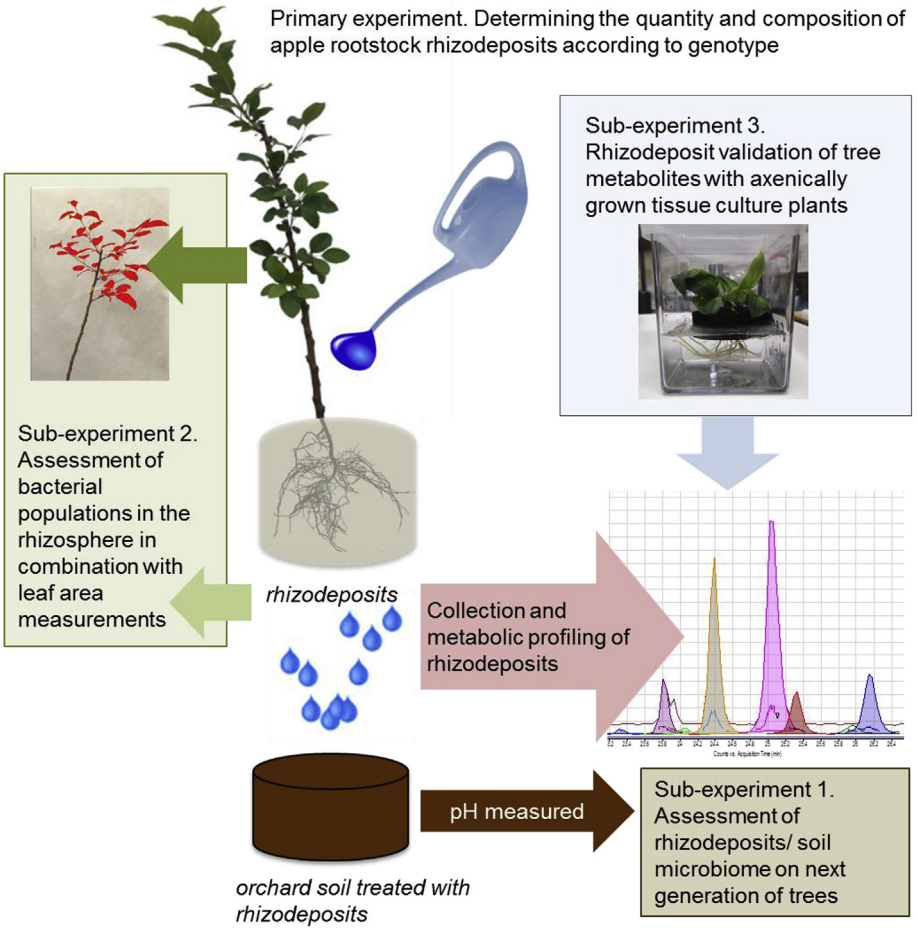

Fig. 1. Experimental diagram. Analytical and experimental systems included metabolite collection, LC-MS QTOF analysis, greenhouse and aseptic hydroponic experimentation systems, and axenic micropropagation to validate apple rootstock root system metabolites.

during the experiment. The experiment was conducted using a completely randomized design.

A second tier of pots containing soil from an orchard with a history of ARD (Sunrise Research Orchard, near Rock Island, WA [47.311551, −120,068531]) was placed beneath the pots containing rootstock liners (Fig. 1). The ARD pathogen resident to this orchard soil was previously characterized (Mazzola et al., 2015). The negative (no-disease) control consisted of sand alone (no tree) in the top tier above pasteurized Sunrise soil in the second tier. The positive (disease expected) control consisted of sand alone (no tree) above Sunrise soil in the second tier.

The purpose of the 2-tier experimental design was to allow water to percolate through the root systems of the rootstock liners, supported by quartz sand, washing rhizodeposits from the rhizosphere that then flow onto the second tier of pots containing orchard soil. Every two weeks rhizodeposits were collected by placing polypropylene specimen containers (4.5 oz [133 ml]) (VWR Inc., Randor, PA) beneath the pots in tier 1 prior to the noon watering. All of the percolated water sample was retained for sample analysis; after samples were collected, the second tier of pots was watered manually.

Upper tier pots were irrigated automatically twice a day with 75 ml of water, using pressure compensating emitters to regulate the flow volume evenly throughout the irrigation line. After the first week, where the plants were watered every 2 h for the first two days, every 4 h for the next two days, and then every 6 h for the final 3 days, the timer was set to irrigate once every 12 h, at noon and midnight.

At the end of the experiment, soil from each pot in the second tier was collected, mixed, and 30 cc allocated for pH measurement. For pH measurement, 30 ml of distilled water was added to the soil, the mixture gently agitated by hand to completely mix soil and water, and allowed to sit for 1 h prior to measuring pH (Beckman 300 pH meter [Beckman Coulter, Inc., Brea, CA] coupled with an Oakton double-junction electrode [Cole-Parmer, Vernon Hills, IL]).

2.3. Greenhouse temperature and light conditions

During winter months (December thru mid-March) the greenhouse was warmed with an overhead butane heater, and in the spring months (mid-March and beyond) cooled using an evaporative cooling system as needed to achieve target temperatures. Temperature was recorded every 30 min using a data logger (WatchDog Data Logger, Model 425; Spectrum Technologies, Inc.). The target temperature was 22 °C, although the average temperature during the recorded period was 19.5 °C, the minimum 8 °C, and the maximum 34.5 °C (supplemental material Graph of greenhouse temperature data. xlsx). Ambient light was supplemented using LED lights. Photosynthetically active radiation (PAR, 400–700 nm) at the level of the rootstock canopy was between 100 and 250 μmol m^{-1} sec^{-2} as measured with Li-COR Li-1500 light sensor logger, using a single point quantum sensor (Q53292 [LI-190SA])

(Li-COR, Lincoln, NE).

2.4. Rhizodeposit-treated soil seedling bioassays

'Gala' apple seed stratification (in order to meet chilling requirement for germination), seedling preparation and plant growth bioassays were conducted essentially as previously described (Mazzola et al., 2009) (sub-experiment 2, Fig. 1). Seeds from 'Gala' apples were germinated in pasteurized perlite (80 °C for 12 h, twice, with a 12 h interval at room temperature between the two sessions), and then an individual seedling was transplanted into 110 ± 5 g ARD soil from each second tier pot (seedling age for each experiment is indicated below). Cotton balls were inserted into perforations in the bottom of each pot to prevent soil loss. Seedlings were harvested 6 weeks after transplanting and total plant mass, root mass, and seedling height were assessed.

2.5. Specific experimental details for experiment 1

Six rootstock liners were utilized for each genotype. Rootstock rhizodeposits were allowed to percolate from the rootstock root system onto Sunrise orchard soil for 10 weeks (12/21/15-03/01/16) prior to bioassay inception. The bioassay was conducted using 18 d old apple seedlings, with 18 seedlings per treatment, and plants were harvested after 6 weeks.

2.6. Specific experimental details for experiment 2

Eight rootstock liners were utilized for each genotype. Rootstock rhizodeposits were allowed to percolate onto Sunrise orchard soil for 12 weeks (02/03/16-04/20/16) prior to bioassay inception. Procure 480SC fungicide (triflumazole; Chemtura Corporation, Middlebury, CT) was applied to the foliage of rootstocks to control powdery mildew 4 weeks after planting. Five days prior to initiation of the 12 week rhizodeposit collection, 1 mL of percolated rhizodeposit was collected and serial dilutions of the solution were plated onto $1/10^{th}$-strength tryptic soy agar (TSA; 3 g/L tryptic soy broth, 15 g/L agar) to estimate the density of culturable bacteria contained in the rhizodeposits. The bioassay employed seedlings that were 14 days old at planting and seedlings were harvested after 30 days.

2.7. Assessment of bacterial populations in rhizodeposits

In a follow-up experiment (sub-experiment 2, Fig. 1), ten rootstocks of each of G935 and M26 were planted in pasteurized sand as previously described and placed in a controlled environment growth chamber. Trees were watered twice daily for 2 min with deionized water at 9:30 a.m. and 9:30 p.m. using a pressure compensating drip emitter system; emitters were ½ gallon per hour (1.9 L per hour) size. Growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) conditions were 25 °C during 12 h light, and 18 °C for 12 h dark. Photosynthetically active radiation (PAR; 400–700 nm) was approximately 250 μmol s^{-1} m^{-2} at the tree canopy height. Twelve hours after planting and weekly thereafter for 4 weeks, water percolating through the root system following the 9:30 a.m. watering was collected in sterile specimen cups. After 15 s of gentle agitation, 1 mL was removed and serial dilutions of the solution were plated onto $1/10^{th}$-strength TSA to estimate culturable bacteria populations.

2.8. Micropropagation of trees and rhizodeposit collection for metabolite validation

Micropropagated rootstock trees (genotypes MM.106, M.26, and G.935) were grown axenically for metabolite validation (sub-experiment 3, Fig. 1) with methods and media similar to Dobránszki and Teixeira da Silva (2010) with further details in Zhu et al. (2016). Briefly, shoots were grown in Magenta boxes in a growth chamber (12 h light 25 °C/12 h dark 20 °C) in shoot multiplication media to a height of ~4 cm, and then sterilely excised from the callus and transferred to rooting media. Shoots were maintained in rooting media for 1 week in the dark prior to transferring to root elongation media (REM). Once transferred to elongation media, roots proceeded to emerge and allowed to extend until encircling the base of the Magenta box (~2 months). Trees were removed from REM, roots were rinsed in sterile distilled water and the tree was placed in a 2" neoprene float (Ehydroponics.com, Santa Cruz, CA) which had been sterilized by soaking in 95% ethanol for 24 h, and dried on a sterile Petri plate in a laminar flow hood. The plantlet was then transferred to a sterile Magenta box containing 85 mL sterile distilled water to allow remaining callus tissue to slough off. After 24 h, the tree was again transferred to a new Magenta box containing 85 mL sterile distilled water. After 5 days, a 100 μl aliquot of solution from the Magenta box was plated onto $1/10^{th}$-strength TSA to test for microbial contamination, and a 5 mL aliquot was removed for pH measurement. The remaining root dip water was filtered through glass wool inserted in a 60 mL syringe and frozen to −80 °C immediately. After 3 days of incubation at 25 °C, any samples that exhibited bacterial growth on TSA were removed from the freezer and discarded. Root water samples were processed for rhizodeposits in the same manner as described above for rhizodeposit samples from greenhouse Experiments 1 and 2. Control samples of 1 cm^2 REM dissolved in 85 mL water as well as neoprene floats alone were processed in order to enable subtraction of any metabolites originating from these sources. Rhizodeposits were frozen and stored at −80 °C, and lyophilized until water removal was complete, approximately 5 days (Unitrap II, Virtis, Gardiner, NY). After lyophilization, rhizodeposits were resuspended in 3 ml methanol to enable complete washing down the sides of the specimen cup. Rhizodeposits were dried under a stream of nitrogen gas and resuspended in 200 μl of 10% acetonitrile and filtered (PVDF membrane, 0.22 μm pore size, 4 mm diameter, Millex, EMD Millipore, Billerica, MA) immediately prior to sample analysis.

2.9. Metabolic profiling

Concentrated rhizodeposit samples were analyzed for metabolite composition using an Agilent 1260 HPLC equipped with a 6520 Accurate-Mass Q-TOF LC/MS (Agilent Technologies, Inc., Santa Clara, CA). 10 μl of sample was injected. Solvent flow rate was 0.350 ml/min through a 1.8 μm pore size "Extend" C18 column, with a 2.1 mm internal diameter and 50 mm length (Agilent Technologies, Inc.). The solvent temperature during resolution maintained at 30 °C. Solvent A consisted of 0.2% acetic acid (Fisher Scientific, Fairhaven, NJ) in HPLC grade filtered water, and solvent B consisted of 0.2% acetic acid in acetonitrile (HPLC grade, Sigma-Aldrich, St. Louis, MO). The solvent system was 0–5 min, 90% A; 6–18 min, 70%–10% gradient; 18–23 min, 10% B, and 24–38 min 90% A for column re-equilibration. The ion source used was ESI in negative ion polarity mode, and data was stored in the MS mode, with mass ranges between 70 and 1200 m/z, at an acquisition rate of 1 spectra/s and 1000 ms/spectrum. The drying gas temperature was 350 °C at a flow rate of 11 L/min. The nebulizer gas pressure was 35 psi. The fragmentor voltage was 125 V, the skimmer 65 V, and the voltage cap was 3500 V. The instrument was calibrated before each run with ESI-Low Concentration Tuning Mix (Agilent Technologies, Inc.), and during each run the reference masses 119.0360 and 980.0163 (G 1969-85001 ES-TOF Reference Mass Solution Kit,

Agilent Technologies Inc.) were used, prepared per protocol in Agilent 6500 Q-TOF LC/MS maintenance guide. The auto recalibration reference mass parameters were 100 ppm with a minimum of 1000 counts.

2.10. Mass spectra processing

Mass spectral data files were processed with MZmine (Pluskal et al., 2010). Three peak detection libraries were created, one containing metabolites identified by co-elution with authentic standards ("Standard library"), one containing metabolites from micropropagated trees root water collection ("Tree specific library"), and one from greenhouse root system percolate ("All library"). The greenhouse rhizodeposit samples were then analyzed using each of these libraries. Settings for library generation and analysis are in supplemental material MZmine settings. xlsx. The targeted peak detection library ("Standard Library") containing compounds identified by co-elution, accurate mass agreement, and mass spectral correspondence with authentic standards (Sigma-Aldrich, St. Louis, MO) is indicated in Table 1.

2.11. Method validation

Control samples (water percolated through sand with no planted tree) and solvent blanks (no analyte) were analyzed to check for impurities present in water/experimental system. Data from these analyses for the greenhouse metabolite library ("All library") were retained for contrast of rhizodeposit samples to these controls. The presence of microbes in the greenhouse rhizodeposit samples was confirmed in Experiments 2 and 3. Since the presence of bacteria and their metabolites could confound the tree-origin of root zone metabolites, a separate analysis of the greenhouse experiment data was completed using a MZmine library generated from micropropagated trees ("Tree specific library"). For the "Tree specific library", any ions found solely in the control samples (neoprene floats, REM media, and no-tree percolate) were removed. Internal standards, including 2-(4-(2-Methylpropyl)phenyl)propanoic acid (ibuprofen) and salicin, were added prior to lyophilization to account for potential sample losses during the extraction process. Ibuprofen (Sigma-Aldrich, St. Louis, MO) was utilized in sample normalization in subsequent analyses in MetaboAnalyst 3.0 (Xia et al., 2015).

Table 1
Phenolic compounds whose identity was confirmed through accurate mass agreement and corresponding retention time to authentic standards.

m/z [M-H]-	Retention time	Standard name
353.0950	1.12	chlorogenic acid
289.0734	1.19	catechin
137.0255	1.31	4-hydroxybenzoic acid
179.0365	1.59	caffeic acid
289.0734	2.00	epicatechin
163.0256	3.12	*p*-coumaric acid
193.0514	4.24	ferulic acid
121.0307	5.30	benzoic acid
609.1476	8.40	rutin (quercetin-3-*O*-rutinoside)
147.0449	9.12	cinnamic acid
435.1322	9.96	phloridzin
301.0374	10.78	quercetin
273.0796	11.21	phloretin
285.0424	11.35	kaempferol
205.1179	14.60	ibuprofen (internal standard)
455.3551	19.26	ursolic acid

2.12. Plant growth assessment and data normalization

A photograph was taken each week and used to determine an estimate of leaf area in ImageJ (Lobet et al., 2011), and at the end of the experiment, leaves were collected and area measured with a leaf area analyzer (LI-3100 Area Meter, Li-COR Inc.) in order to validate photography area estimates. At the end of the experiment, fresh and dry weights for leaves, stems, and roots were determined to enable normalization of metabolite data to plant vigor.

2.13. Statistics

Leaf area, root dry weight, and soil pH data were analyzed using SAS software (SAS Inc, Cary, NC). Where F-stat was significant at $p < 0.05$ in type III sums of squares, *post-hoc* means comparisons and letter groupings were assigned according to Fisher's least significant difference test. SAS proc corr was used for correlations among leaf area as measured by digital photography at the final measurement photograph date and the leaf area measured for detached leaves.

Metabolite peak areas were analyzed in MetaboAnalyst 3.0 (Xia et al., 2015). Peak areas were normalized to peak area of the reference standard in each sample (ibuprofen) and then autoscaled (means centered and divided by the square root of the standard deviation within each metabolite). Data were then analyzed using ANOVA, ANOVA-simultaneous component analysis (ASCA) and partial least squares discriminant analysis (PLSDA). ASCA assesses univariate experimental factors in metabolic profiling data; the experimental factors assessed by ASCA were rootstock genotype and time. PLSDA is a multivariate technique which summarizes latent trends in data according to subjective factors (in this case, rootstock genotype in each experiment separately, and the rhizodeposit levels for each genotype in the first experiment versus the second experiment).

3. Results

3.1. Rootstock vigor, metabolic libraries, and general metabolic profiling trends

The four rootstock genotypes exhibited measures of relative tree vigor (defined by leaf area and root mass) consistent with previous observations (Fazio et al., 2006; Robinson et al., 2007; Autio et al., 2009) (Fig. 2), with G.935 and M.26 exhibiting greater growth than G.41 and M9. Nic29.

All rhizodeposit samples were analyzed on Q-TOF LC/MS from Experiment 1 (2, 4, 6, 8, and 10 weeks), but rhizodeposit data from Experiment 2 was analyzed only at 12 weeks. Samples from both experiments were pooled to create a metabolite library ("All library") with which to analyze the Q-TOF LC/MS data files in MZmine.

Levels of "Tree specific" metabolites in the greenhouse experiments (the "Tree specific" metabolites are the overlap indicated in Venn diagram) (Fig. 3) corresponded to rootstock vigor, but when corrected according to dry root weight, total metabolite concentration did not differ significantly among the rootstocks. The relationship between metabolite quantity and leaf area (peak area/cm^2) was also assessed using ASCA (Supplemental material – major metabolic trends. doc), as root mass could not be assessed until the end of the experiment. Overall quantity of metabolites increased with the duration of the greenhouse experiments, but metabolite levels did not increase proportionately with the increase of leaf area (Supplemental material – major metabolic trends. doc).

Rootstock genotype-specific differences in the root zone

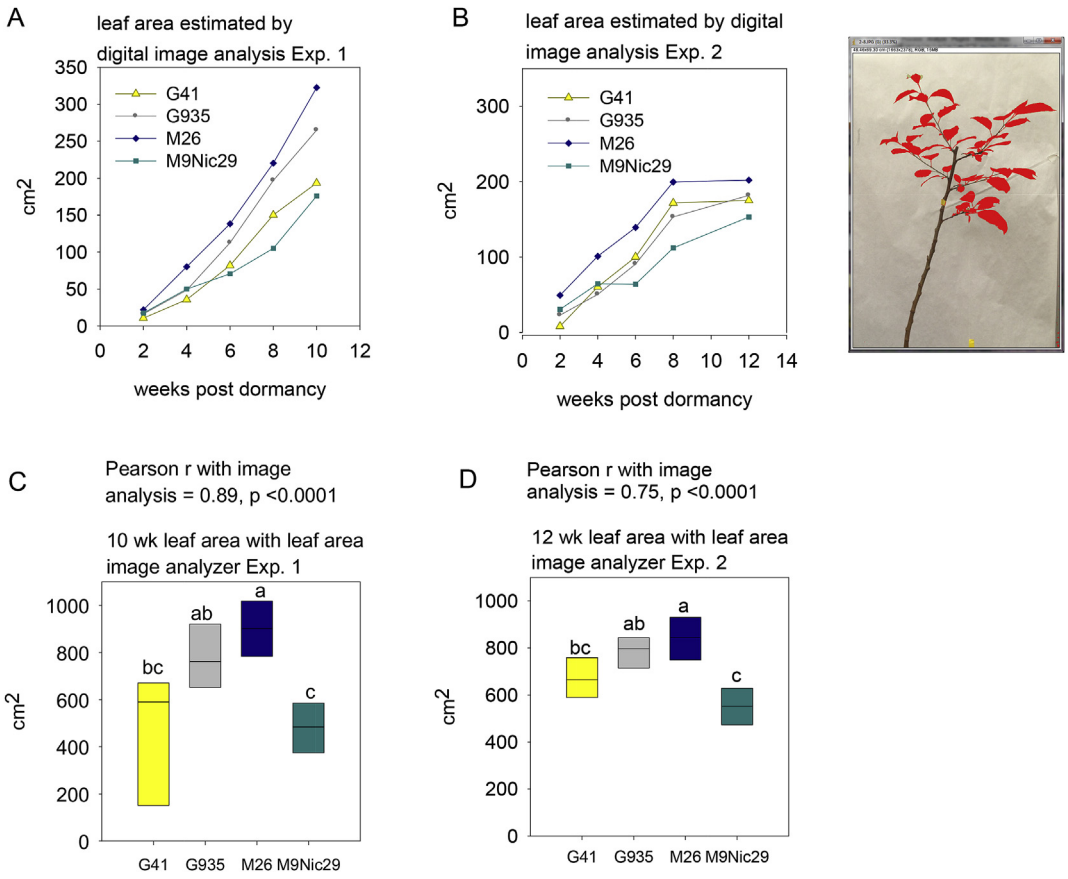

Fig. 2. Relative vigor of rootstocks as measured by leaf area in Experiment 1 and Experiment 2. Leaf area was estimated utilizing digital photography image analysis (A and B), and at the termination of the experiment, leaf area was measured with a flatbed leaf area analyzer (C and D). Although image analysis underestimates actual leaf area, data from digital photography correlates well with actual leaf area (C and D). Leaf area was used for metabolite level normalization (peak area/cm^2) prior to some data analyses.

percolate metabolic profile were confirmed in the analysis of the combined data sets from two experiments (Fig. 4). Results indicate consistent rhizodeposit similarities within a rootstock genotype, irrespective of experiment (Fig. 4A), but also that trial-specific factors differentially influenced the rhizodeposit metabolome resulting in some differences within a genotype between experiments (Fig. 4B).

3.2. Differences among the rootstocks for identified phenolic compounds

Identify of compounds confirmed using authentic standards included benzoic acid, *trans*-cinnamic acid, 4-hydroxybenzoic acid, phloridzin, phloretin, kaempferol, and rutin. Peak area data from the final time point (10 weeks and 12 weeks, in Experiments 1 and 2, respectively) were adjusted for the corresponding root dry weight (peak area/root DW [g]) prior to analysis. In both experiments, benzoic acid was significantly higher in G.935 rhizodeposits (ARD tolerant) relative to M26 and M9Nic29 (ARD-intolerant) (Fig. 5). 4-hyrdroxybenzoic acid was higher for G935 than M26 and M9Nic29 in the first experiment, but was not different in the second experiment. Kaempferol levels were higher in the rhizodeposits of M26 compared to G41 and M9Nic29 in the first experiment, and higher in M26 than all the genotypes tested in the second experiment. Rutin levels were higher in rhizodeposits from the second experiment for M26.

Both phloridzin and phloretin content (not adjusted for root mass or leaf area) decreased with time in experiment 1 (Fig. 6A) (full time course data from experiment 2 was not generated). Phloridzin levels at the termination of the experiment (adjusted for dry root mass) differed among genotypes although not consistently (Fig. 6B). Phloretin levels adjusted for dry root mass did not differ among genotypes in either experiment (Fig. 6B).

3.3. Root associated bacteria

Bacteria were detected in rhizodeposits collected from each rootstock (documented first in Experiment 2, and confirmed in a larger study in Experiment 3) through infiltration of water through the root zone; very low levels were also detected in no-tree controls (Fig. 7). Total bacterial densities corresponded with plant vigor in Experiment 2 (corresponding to leaf area) (supplemental material: Exp2 12 wks CFU counts. xlsx). A follow-up experiment (Experiment 3) was performed which confirmed that bacterial populations increased with leaf area (Fig. 7).

3.4. Rhizodeposit effects on soil pH and subsequent gala seedling growth

The two tiered pot experiment (see Fig. 1) was designed to allow rhizodeposits to percolate from the root system of rootstocks planted in pasteurized sand and onto orchard soil with a history of ARD and a well characterized pathogen complex (Mazzola et al., 2015). Controls included sand where no tree was planted (no

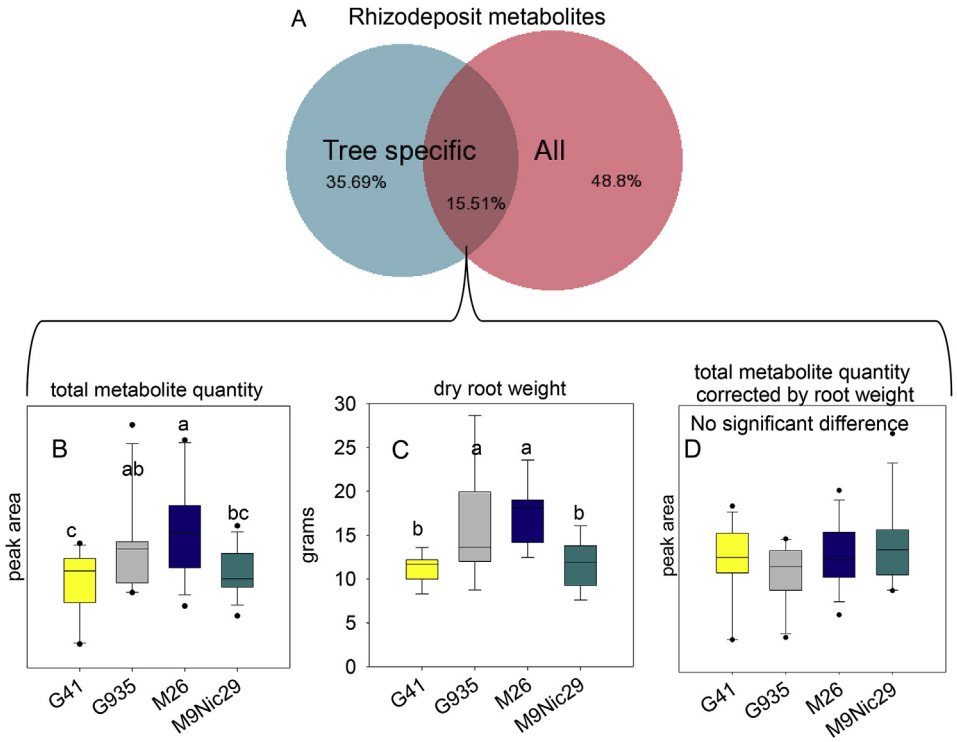

Fig. 3. Untargeted metabolic libraries were developed from tissue culture propagated rootstocks ("Tree specific") and from root zone percolated rhizodeposits ("All), but a portion of metabolites from both libraries were disparate (A). "Tree specific" metabolites in the greenhouse experiments (overlap indicated in Venn diagram) (B) corresponded to rootstock vigor (C), but when corrected for dry root weight (D), metabolite quantity (at 10 wks [Experiment 1]) and 12 weeks [Experiment 2]) did not differ significantly among the rootstocks. Data in B and D are simple peak area sum from LC-MS analysis of percolated exudate samples collected at the final metabolite collection point for two greenhouse experiments, and as uncalibrated compounds, are represented as relative quantity. Pearsons' correlation between total metabolite quantity (B) and dry root weight (C) is r = 0.40, $p = 0.0029$.

rhizodeposit source) and pasteurized or non-pasteurized ARD orchard soil in the second tier. Percolate from rootstock planted containers reduced the soil pH irrespective of rootstock genotype compared to both "no-tree" controls in the first experiment. In the second experiment these same eluates reduced soil pH relative to that of the pasteurized ARD soil (Fig. 8).

The effects of the rootstock rhizodeposits on the subsequent generation of trees was assessed using a 'Gala' seedling bioassay conducted in orchard soil from the second tier of pots. Growth of 'Gala' seedlings in the ARD intolerant M26 eluate treated soil and the pasteurized control were equivalent in the first experiment, and seedling root weight in the M26 treated soil was greater than the controls in the second (Table 2). Seedling height was also higher in soil treated with the M26 eluate relative to other genotypes and the ARD control in both experiments.

3.5. Untargeted and unidentified metabolites differing among rootstock genotypes over time

As noted above, significant differences existed among rootstock genotypes in presence or abundance of metabolites that had been confirmed using authentic standards. However, none of the metabolites with confirmed identity differed in presence or abundance among the rootstock genotypes according to ANOVA simultaneous component analysis ("significant features") when both time and genotype were considered. Examples of unidentified metabolites differing both according to time and genotype are indicated in Fig. 9. Some metabolites could be confirmed in the "Tree Specific Library", whereas others were present only in the "All Library", indicating potentially microbial origin.

4. Discussion

This study offers new insight into the comparative effects of apple rootstock genotypic rhizodeposits on the surrounding soil. Modes of rhizodeposition include active secretion, passive diffusion, and root turnover or root debris (Leigh et al., 2002; McCully and Boyer, 1997; Weston et al., 2012); this project collected rhizodeposits from all of these sources indiscriminately. Previous studies regarding apple rootstock genotype interaction with soil systems have tended toward molecular approaches (i.e. DNA or RNA sequencing) of the plant material itself; there are few previous studies that approach the rootstock-soil interface via broader metabolic profiling of the rhizodeposits.

4.1. Experimental impacts on rhizodeposits

Environmental conditions (natural temperature and light variation) contributing to rhizodeposit differences between experiments, as well as intra-genotypic variability, and the detection of increasing bacterial populations in rhizodeposits despite efforts to minimize their presence, highlight the dynamic nature of the tree, environment, and microbiome interaction. Essentially, while seeking to identify apple rootstock genotype-specific rhizodeposit characteristics, we also assessed rhizodeposits of apple tree holobionts (trees plus their microbial consortia) (Bordenstein and Theis, 2015) and variability there-in. None-the-less, this study describes apple rootstock genotype-specific rhizodeposits (confirmed

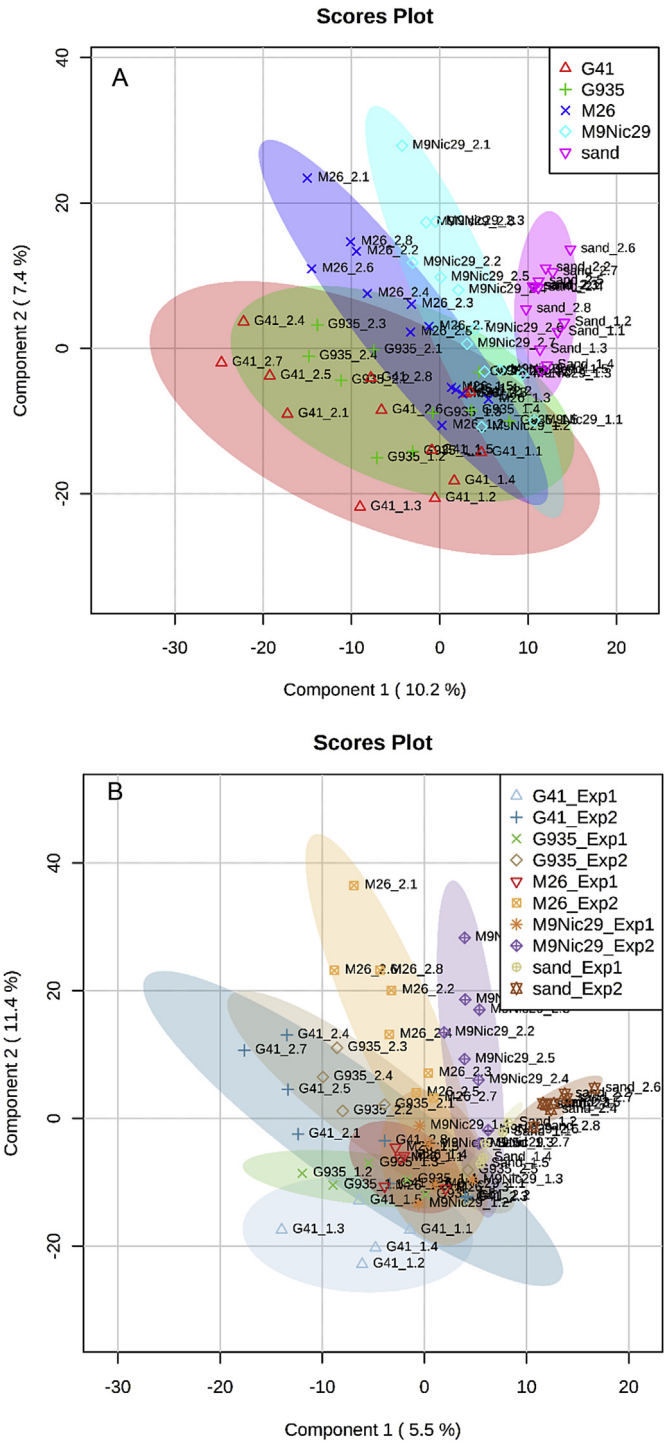

Fig. 4. Partial least squares discriminant analysis (PLS-DA) scores plots greenhouse-grown apple rootstock combining percolated root exudate metabolic profiles from the end of each experiment. Profiles were built utilizing an aseptically propagated apple rootstock exudate metabolite library. In plot A, data from Experiment 1 and Experiment 2 were compiled and analyzed only according to rootstock, and results indicate similarities within each rootstock genotype, irrespective of experiment. In plot B, data from Experiment 1 and Experiment 2 were separated, and results indicate differences among experiments as well.

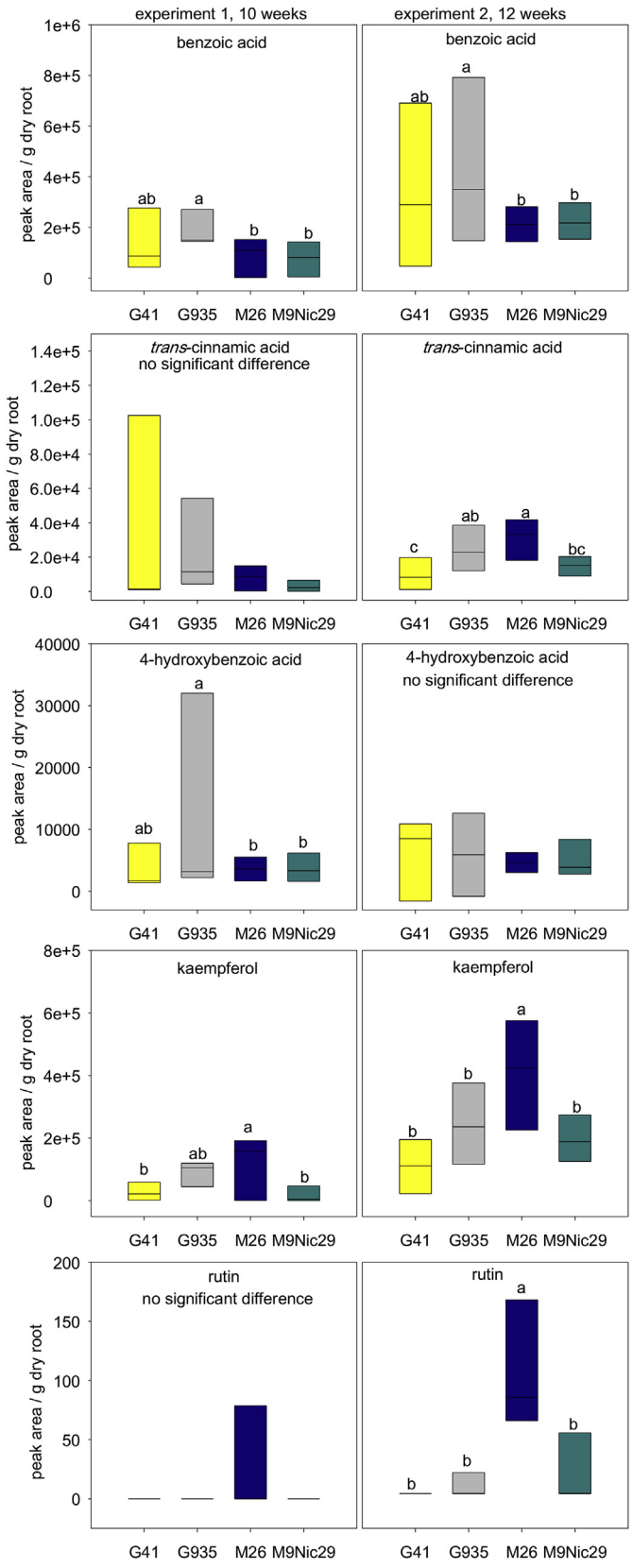

Fig. 5. Peak area of identified root exudate metabolites, adjusted for root dry weight (DW), and their differences among the rootstock genotypes at 10 weeks in Experiment 1 and 12 weeks, in Experiment 2. Data were analyzed with Proc GLM in SAS, and where type III sums of squares were significant at $p < 0.05$, *post hoc* letter groupings were assigned with Fisher's least significant difference (means with the same letter grouping are not significantly different).

by axenically micropropagated trees) and outlines time-course dynamics of rhizodeposits in relation to plant growth and development. The unexpected impacts of these rhizodeposits on a subsequent generation of trees when applied to orchard soil indicates

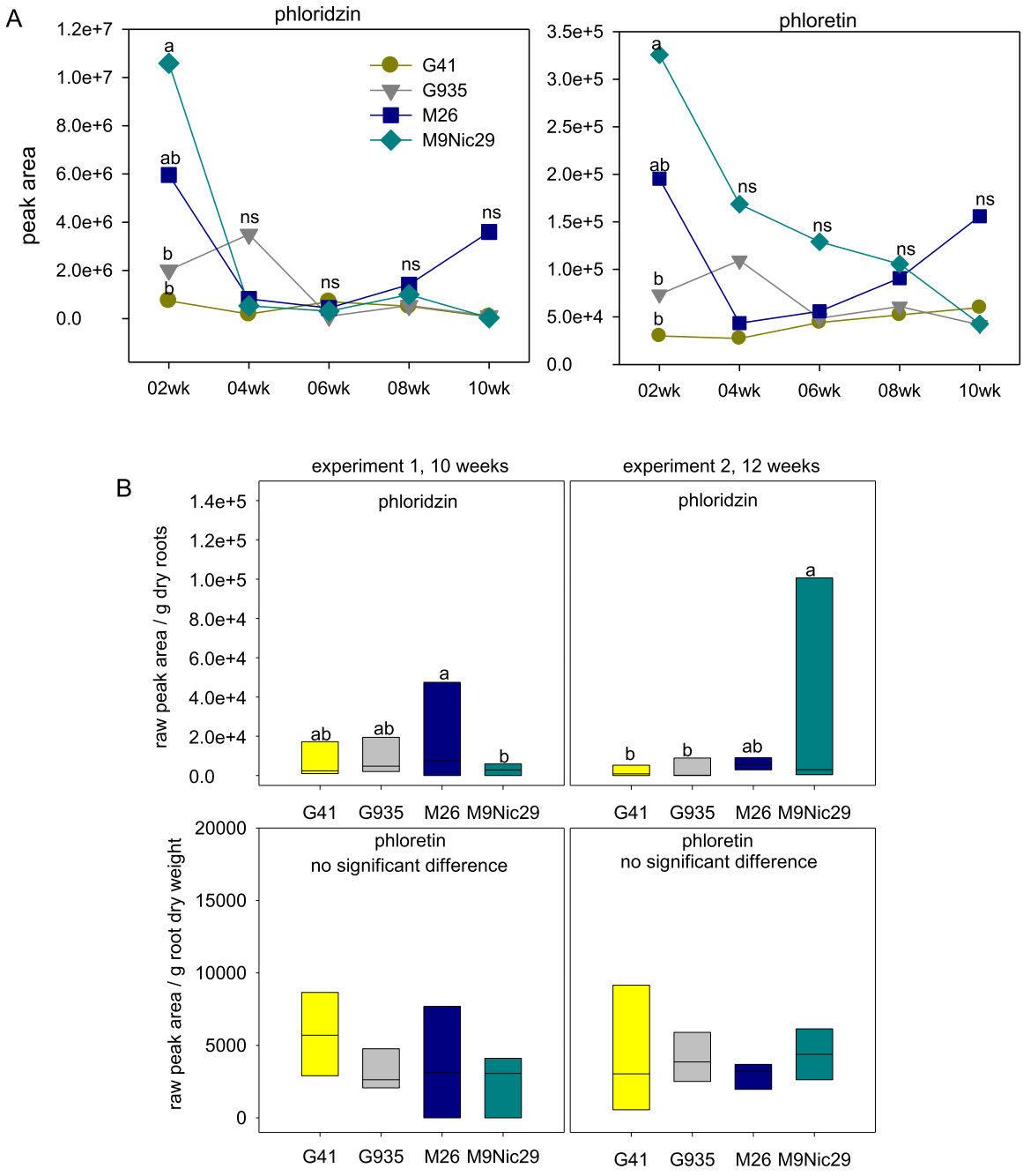

Fig. 6. Total phloridzin and phloretin peak area (not adjusted for root mass) generally decreased with time (Experiment 1) (A). When the final percolated rhizodeposit was adjusted for the dry weight of roots, phloridzin levels varied among rootstocks and among experiments, while phloretin did not (B). Statistics in (A) were performed separately at each time point.

the complexity of the rhizodeposit and soil interaction.

In the second greenhouse experiment, trees were treated with a foliar fungicide to control powdery mildew, a leaf disease caused by the fungus *Podosphaera leucotricha*. The pathogen infects new leaves as well as other parts of the tree, reducing their functionality and vigor. The incidence of initial "strikes" (new infections) on the leaves required that the fungicide Procure 480SC was applied to control the infection. Although the effects of a foliar fungicide application on rhizodeposition is unknown, the application was performed immediately subsequent to the 4 week rhizodeposit collection, and no anomalies were observed in the 6 week rhizodeposit collection.

4.2. Total metabolite time-course recovery in the rhizosphere corresponded to tree growth

The defining time-course trend of total metabolites collected in tree root percolate was an increase in the total quantity of detected metabolites with increase in plant biomass, which is similar to findings from previous work involving *Arabidopsis* (Chaparro et al.,

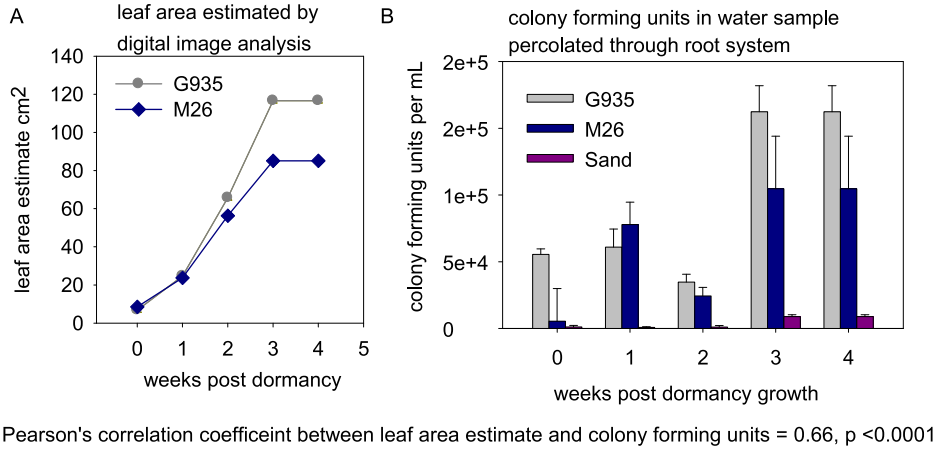

Fig. 7. A follow-up experiment confirmed the presence of bacteria living in the root systems of rootstock liners (B), whose numbers generally increased with increasing leaf area (A) ($r = 0.66$, $p < 0.0001$).

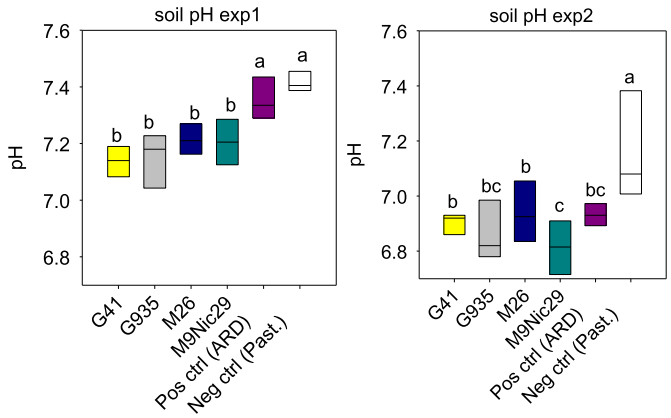

Fig. 8. Rhizodeposit-treated soil pH in two experiments after 10 weeks (Experiment 1) and 12 weeks (Experiment 2). Columns with the same letter groupings are not statistically different according *post hoc* Fisher's least significant difference. The positive control (Pos. ctrl (ARD)) = "no tree" as source of rhizodeposits, and orchard soil was not pasteurized prior to experimental inception; Negative control (neg. ctrl (Past.)) = "no tree" as source of rhizodeposits, and orchard soil was pasteurized prior to experimental inception.

2013). However, when metabolite quantities, whether "Tree Specific Library" or "All Library" metabolites, including putative microbial metabolites, were corrected according to leaf area, the trend reversed itself indicating a relative decrease in quantity of these metabolites with increasing leaf area. In other words, rhizodeposit metabolite quantity increased overall during the experiment, but did not correspond to leaf development. This may be due to the fact that metabolites monitored in the present study were primarily phenolics, and did not include the carbohydrates and primary metabolites that would be more immediately connected with carbon fixation by the leaves. Newly fixed carbon is detectable in rhizodeposits after 7 days in *Populus tremuloides* Michx. (Norris et al., 2012), but the rate of incorporation of newly fixed C into rhizodeposits in apple roots, and specifically the putative secondary phenolic metabolites assessed here, is unknown. It could be expected that in more vigorous genotypes that greater carbon allocation to rhizodeposits occurs – whether through exudation directly connected to photosynthesis or through greater turnover due to greater root volume. This is supported in the present work, where the more vigorous genotypes, M26 and G935, had greater total rhizodeposit peak area than the less vigorous rootstocks, M9Nic29 and G41.

4.3. Quantity of rhizodeposits differed among rootstocks

The total quantity of phenolic metabolites assessed using the present method correlated well with root mass. M26 was the most vigorous among the rootstocks utilized in this study, and both root mass and quantity of rhizodeposits were higher than other genotypes – until quantities of rhizodeposits were adjusted for the total root mass. The present result of "no relative difference" in total quantity of rhizodeposits (as determined by simply summing the peak area for each sample, and dividing by the dry root mass) does

Table 2
Bioassay results Gala growth measurements as proxy for impacts of rhizodeposits on soil properties and soil microbiome after growing in rhizodeposit-fed soil for 6 weeks (experiment 1) or 4 weeks (experiment 2). n = 18 seedlings (pots) per treatment.

Rhizodeposit source	Soil	Seedling height (cm)	Experiment 1			Experiment 2	
			Total seedling weight (g)	Root weight (g)	Seedling height (cm)	Total seedling weight (g)	Root weight (g)
Sand	pasteurized Sunrise soil[a]	4.95 ab	1.11 a	0.54 a	4.96 ab	0.84 ab	0.28 b
G935	Sunrise orchard soil	4.51 b	0.88 bc	0.43 bc	5.42 a	0.87 a	0.39 a
G41	Sunrise orchard soil	4.51 b	0.84 c	0.38 c	5.26 ab	0.79 abc	0.33 ab
M9 Nic 29	Sunrise orchard soil	4.23 b	0.90 bc	0.43 bc	4.81 b	0.85 ab	0.38 a
M26	Sunrise orchard soil	5.18 a	0.98 ab	0.63 a	5.42 a	0.78 bc	0.36 a
Sand	Sunrise orchard soil[b]	4.47 b	0.83 c	0.41 c	4.77 b	0.73 c	0.28 b

[a] "No-disease control".
[b] "Disease-expected control".

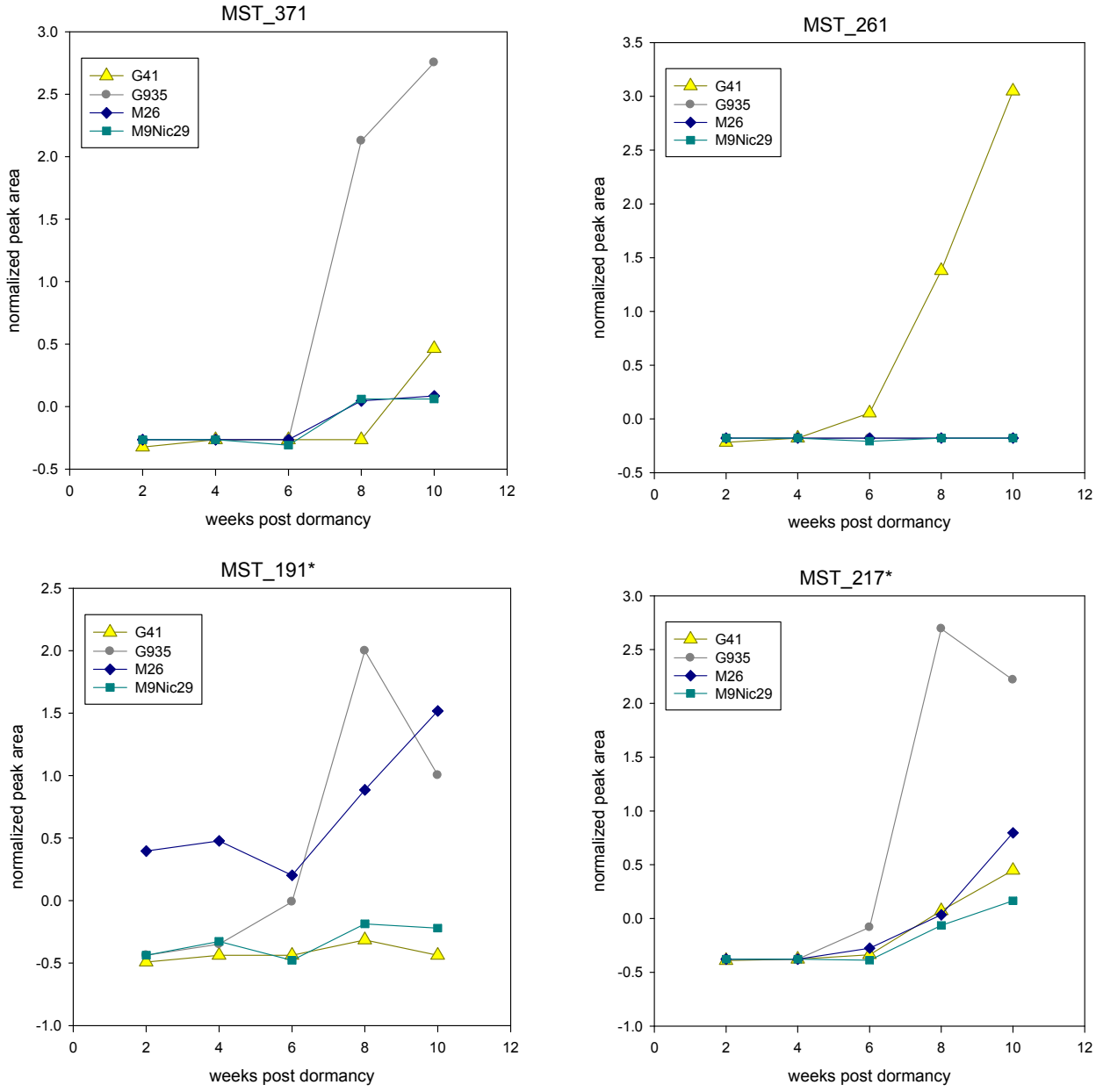

Fig. 9. Examples of mass spectral tags (unidentified compounds) differing among the rootstock genotypes per ANOVA-simultaneous component analysis (ASCA) in Experiment 1. Data were not normalized for leaf area nor root mass, but were autoscaled for the analysis (means centered and divided by the square root of the standard deviation) to put the metabolites on a similar scale. **metabolites not found in the "tree specific" library, implying that they could be microbial in origin.*

not fully disprove the possibility that metabolite quantity relative to root or leaves differs among genotypes there are certainly unmeasured metabolites (for example, proteins, sugars, organic acids, amino acids, and other classes of compounds) in the rhizodeposits. However, this finding may support the hypothesis that rhizodeposit composition is more critical in shaping the holobiont than sheer quantity of rhizodeposit.

4.4. Metabolite composition differed among apple rootstock genotypes

Multivariate analysis of total metabolites compiled from both greenhouse experiments indicated a genotype-specific role in determining metabolite composition of rhizodeposits (Fig. 5A). These results were built from a "tree specific" metabolite library created from a 5-day root dip of axenically grown tissue culture trees and so were designed to only include metabolites of non-microbial origin. In the first two principal components, M9Nic29 and M26 rhizodeposit metabolic profiles were more dissimilar than G935 and G41, even though previous genetic analysis indicates M9Nic29 and M26 are more similar (St. Laurent et al., 2010). This result suggests that genotypes may be susceptible to ARD pathogens for different reasons (i.e. the presence or absence of certain rhizodeposits, or intrinsic root development and architecture characteristics) and potentially that ARD tolerant cultivars (G41 and G935) share similar rhizodeposit components that function to limit plant susceptibility to the causal pathogens.

Composition of rhizodeposits also differed among experiments even though assessments were conducted at a similar sampling time point. The inability to regulate specific experimental factors,

such as time of the year, may have influenced these results. This is an intriguing glimpse into the complexity and dynamic nature of rhizodeposition, and to the effects of the environment on innate and undefined attributes of a holobiont.

Analysis of untargeted and targeted rhizodeposits together indicated that the metabolites most consistently different among genotypes over time were untargeted/unidentified metabolites – some of tree origin and others not of tree origin. This indicates there are many more rhizodeposit metabolites compatible with the solvent system utilized in this study with possible functional importance to identify and characterize.

4.5. Differences in identified phenolic compounds

Metabolite levels varied from tree to tree, which affected the statistical significance with regard to differences among rootstock genotype. Tenets of the holobiont concept (Bordenstein and Theis, 2015) that may have contributed to this variability are that each hologenome or individual plant and its microbiome are individual, and subject to their own gene expression differences, both on the micro and macro level, and that both rhizodeposition and rhizosphere microbial population dynamics can have unique features. In greenhouse experiments, individual plant variation in rhizodeposition and microbial population increase connected with plant growth could have led to greater or less production of phenolic metabolites – as well as increased or reduced microbial consumption of phenolic metabolites. Regarding the latter point, follow-up studies affirmed the presence of microbes in greenhouse rhizodeposits, which could have varying functional attributes – whether acting solely as rhizodeposit consumers or otherwise modifying the rhizosphere. In a field setting or in response to pathogen infection, phenolic compounds or genes related to phenolic production can be further increased (Hoffman et al., 2009; Shin et al., 2014; Weiβ et al., 2017). This study provides elements of genotype specific rhizodeposition that are robust to individual variation and environmental conditions, as detailed below.

4.6. Phloridzin and phloretin

As one of the predominant phenolic compounds in apple fruit and tissue, phloridzin and its aglycone, phloretin, have been the topic of numerous research studies (Gosch et al., 2010). Although reported as the most concentrated phenolic compound in apple roots (Emmett et al., 2014) and rhizodeposits (Hoffman et al., 2009), phloridzin levels detected in the present study were not high relative to other compounds when adjusted for root dry weight, nor were levels different among rootstock genotypes. Several points must be considered to effectively interpret these results. First, rhizosphere inhabiting micro-organisms were detected in the experimental system, some of which may consume phloridzin (i.e. Jayasankar et al., 1969), thereby decreasing the concentration observed in the samples. Secondly, Hoffman et al. (2009) reported phloridzin levels that were significantly higher in ARD-conducive soil immediately after planting but not beyond the initial evaluation. Based on this observation, the authors proposed two functions for phloridzin exudation including function as a component of a plant defense that fails to control disease and as a host signal compound utilized by various microorganisms. In the present study, total phloridzin levels were highest in the beginning of the experiment. There was deliberate effort to minimize microorganisms in the rhizosphere at the inception of the experiment and no pathogens were intentionally introduced into the system, so it may be less likely that high initial phloridzin production was a response to biotic or abiotic stress, and more likely that rhizosphere microorganisms ultimately altered phloridzin detection by way of consumption. Furthermore, the root exudate/rhizodeposit collection systems differed: in Hoffman et al. (2009), rhizodeposits were collected by uprooting and dipping trees into a solution, and in the present study, rhizodeposits were obtained by collecting the eluate resulting from percolation of water through the root zone, which enables repeated sampling without root system disturbance. In summary, results from the present study could lend credence to the hypothesis that phloridzin production can be stimulated by pathogen infection or other environmental stresses. However, the mechanism of phloridzin deposition in the rhizosphere remains to be elucidated. In the present study, the knowledge that roots contain high levels of phloridzin (Emmett et al., 2014) combined with the fact that dry root weight normalization negated statistical differences in phloridzin concentration leads to speculation that root turnover (i.e., degradation of root debris) contributes to phloridzin accumulation in the rhizosphere.

4.7. Additional identified metabolites and potential functions

Beyond phlordizin, metabolites identified in this study could have a variety of functions in the rhizosphere. 4-Hydroxybenzoic acid and benzoic acid, which were higher in the ARD tolerant genotype G935 tested here, are both preferential substrates for *Burkholderia cepacia,* a bacterium known to provide biological control of certain soil-borne plant pathogens (Pumphrey and Madsen, 2008). In a previous study (St. Laurent et al., 2010), *B. cepacia* was present at high levels in rhizosphere soil from a genotype with tolerance to ARD (CG6210) in relation to a less tolerant rootstock (M26), leading the authors to conclude that more tolerant rootstocks support an antagonistic or suppressive consortia of microorganisms. Similarly, *Burkholderia* spp. were detected at significantly higher densities in apple rhizosphere soils that were resistant to invasion by ARD pathogens including *Pythium* spp. and *Pratylenchus penetrans* (Mazzola et al., 2015).

Rutin was higher in rhizodeposits of M26 than for any of the other genotypes tested. Previous work suggests that rutin can function as a phytohormone, i.e., stimulating rooting of micropropagated Giant Sequoia (*Sequoiadendron giganteum*) shoots (Berthon et al., 1987). M26 was the most vigorous rootstock in the present study, and, as previously reported, perhaps the most susceptible to ARD (Autio et al., 2009). In relation to microbial growth, rutin has demonstrated antimicrobial activity (Orhan et al., 2010), although impact on specific functional microbial groups in the rhizosphere are unknown.

4.8. pH and bioassay

Previous work has sought to determine how soil pH impacts nutrient uptake (Fazio et al., 2012), but this study demonstrates that apple rootstock rhizodeposits can alter soil pH. In the present study, rhizodeposits consistently lowered soil pH relative to the pasteurized orchard soil where water but no rhizodeposits were applied. In one experiment, there were differences among the rootstock genotypes with respect to effects on soil pH, which could have consequences for both the rhizosphere microbiome (Rousk et al., 2010) and relative nutrient acquisition (Armstrong et al., 1970; Shahbaz et al., 2006). Modes by which plants alter soil pH include root exudation (rhizodeposition) and respiration. Release of CO_2 through respiration can be then dissolved in soil water as carbonic acid [H_2CO_3], and release of H^+ or OH^- to balance cation or anion uptake at the soil-root interface. In addition to the direct effects of rhizodeposition on soil pH, rhizodeposits present a food source to microbes, which can also increase (Fen et al., 1996) or decrease soil pH (Hinsinger et al., 2003).

Apple seedling growth in soils treated with rootstock

rhizodeposits differed significantly, at times in a genotype-specific fashion. The expectation was that the rhizodeposits collected during the 10–12 weeks of the experiment would modify the orchard soil microbiome in a manner consistent with the relative tolerance/susceptibility of a rootstock to ARD, and that these community changes would be reflected by seedling growth in the subsequent bioassay (viz., expected reduced seedling growth in soil treated with M26 and M9Nic29, and superior growth in soil treated with rhizodeposits from G935 and G41). However, seedling growth in response to rootstock rhizodeposits demonstrated inconsistency across the two experiments. In experiment 1, seedlings planted in M26 soil were taller and had higher root mass, but not total seedling mass, than seedlings planted in soil treated with rhizodeposits from other genotypes. Seedlings grown in M9Nic29 rhizodeposit treated soil had the lowest seedling height in both experiments but possessed total seedling weight that was no different from that for the M26 treatment. Differences among the rootstock rhizodeposits in the present study that could have influenced these results include greater total metabolite quantity from M26, and lower from both G41 and M9Nic29, which essentially represents organic matter input into the soil. It is also possible that compounds such as rutin – which has been demonstrated to increase root growth in other species (Berthon et al., 1987) – also impacted growth of seedlings planted in these soils. Other considerations include the degradation of rhizodeposits by rhizosphere-inhabiting microbes in the top-tier pot, as well as differential accumulation and degradation of phenolic compounds in the orchard soil in the second tier.

5. Conclusion

This study provides novel results in relation to apple root rhizodeposition and serves as the foundation for further work into specific aspects of rhizodeposition as it relates to a variety of root functions and biology, including root disease and tree growth. Rhizodeposits differed among apple rootstock genotypes, and, during the growing season concentration of rhizodeposits corresponded to both leaf area and microbial populations in root zone. Both tree specific as well as potentially microbially derived rhizodeposits differed among genotypes. The concentration and composition of rhizodeposits was found to vary with time/plant development, but their application to replant soil were not connected with expected replant phenomenon in the subsequent planting of trees. Several phenolic compounds warrant further exploration in regards to their differences among rootstock genotypes, specifically benzoic acid and rutin. Rhizodeposit quantity correlated with the quantity of culturable rhizosphere bacteria and affected subsequent tree growth in soil treated by rootstocks.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.06.011.

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