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ORIGINAL ARTICLE

Fine-root system development and susceptibility to pathogen colonization

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Abstract Root development may exert control on plant–pathogen interactions with soil-borne pathogens by shaping the spatial and temporal availability of susceptible tissues and in turn the impact of pathogen colonization on root function. To evaluate the relationship between root development and resistance to apple replant disease (ARD) pathogens, pathogen abundance was compared across root branching orders in a bioassay with two rootstock genotypes, M.26 (highly susceptible) and CG.210 (less susceptible). Root growth, anatomical development and secondary metabolite production were evaluated as tissue resistance mechanisms. ARD pathogens primarily colonized first and second order roots, which corresponded with cortical tissue senescence and loss in second and third order roots. Defense compounds were differentially allocated across root branching orders, while defense induction or stress response was only detected in first order and pioneer roots. Our results suggest disease development is based largely on fine-root tip attrition. In accordance, the less susceptible rootstock supported lower ARD pathogen abundance and altered defense compound production in first order and pioneer roots and maintained higher rates of root growth in both the ARD soil and pasteurized control compared to the more susceptible. Thus, this rootstock's ability to maintain shoot growth in replant soil may be attributable to relative replant pathogen resistance in distal root branches as well as tolerance of infection based on rates of root growth.

Keywords Apple replant disease · *Cylindrocarpon* · Fine roots · Oomycetes · Root anatomy · Root architecture · Root branching order · Soil-borne pathogens

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Introduction

Diseases of the fine-root system of perennial plants are a major constraint on the establishment, growth and longevity of trees in horticultural (Mai and Abawi 1981) and forest ecosystems (Jönsson 2006; Packer and Clay 2003; Reinhart and Clay 2009). These diseases present a challenge to study and manage because they often manifest symptoms irregularly (Jönsson 2006) and may be attributed to multiple causal organisms (Braun 1995; Mazzola 1998). Woody plants are a particular challenge because the maturation of the finest roots leads to tissues of different structure and function (Hishi 2007). This developmental and structural differentiation may affect the susceptibility of tissues to pathogen infection and colonization (Watt et al. 2006), as well as the impact of such colonization on the plant. Thus, our ability to understand root disease causation, as well as the nature of susceptibility and resistance, would be improved with the explicit recognition of how root development affects specific plant–pathogen interactions.

Root branches of different structure and function result from maturation of tissues with age and distance from the

growing tip. Regions near the root tips are in a state of primary development. These roots have a high percent area of metabolically active cortex and are the principal site of resource uptake. With increasing distance from the tip, the formation of the endo- and exo-dermis limits resource uptake by regulating solute flow, while also regulating biotic interactions (Enstone et al. 2002). Further from the tip, roots undergo secondary development including vascular differentiation, formation of the woody periderm, and loss of the cortex. These changes mark a transition in function from resource uptake to transport (Esau 1965). These structural and chemical changes may (1) constrain the quantity and quality of available pathogen habitat, or (2) shift allocation of plant defenses against pathogen infection and colonization. Hierarchical branching order provides a classification of fine roots that has been repeatedly shown to correlate with these structural changes better than classification by root diameter (Guo et al. 2008; Pregitzer et al. 2002). This classification may be useful in the study of plant–pathogen interactions if it provides a framework for predicting where pathogen infection and colonization are likely to occur and the tissue types and functions that may be disrupted by pathogen colonization.

In addition to the differences in structure and function that arise from longitudinal development, differences in root susceptibility to colonization may result from the distribution of plant defenses within the root system. Plant optimal defense theory predicts that defenses will be allocated based on the importance of a tissue for plant performance and the likelihood of pathogen attack of that tissue (McCall and Fordyce 2010; McKey 1974). Here again, a hierarchical classification of root classes is useful, for higher orders may be preferentially defended because entire downstream branches depend on their function (Wells and Eissenstat 2002), while growing root tips are most likely to encounter pathogen propagules (Watt et al. 2006). Additionally, roots within the same branching order may receive greater defense allocation if they are on a growth trajectory that leads to longer lifespan or function as a higher order root. Previous studies have distinguished short-lived first order fine-feeder roots from larger, faster growing root tips (pioneer roots) that expand the root system and typically become higher order roots (Wilcox 1968; Zadworny and Eissenstat 2011). In a study of multiple tree species, pioneer roots had more layers of hypodermis with fewer passage cells than first order fine-feeder roots and were less likely to be colonized by mycorrhizal and non-mycorrhizal fungi (Zadworny and Eissenstat 2011). In addition to structural defenses, plant roots contain a diversity of secondary metabolites that defend against pathogen and herbivore attack (Bennett and Wallsgrove 1994). These compounds may be constitutively produced or may increase rapidly following pathogen attack (Bednarek and Osbourn 2009). To our knowledge, constitutive or induced chemical defenses have not been compared among fine-root classes of different branching orders or growth trajectories. However, differences in chemical defense allocation among root tissues of different stages and functions are expected within the plant optimal defense framework and should be correlated with tissue-specific differences in anatomical defenses.

To test if explicit recognition of root development and fine-root classes can inform investigations of pathogen–plant interactions, we used apple replant disease (ARD) as a model system. ARD is characterized by the poor growth and yield of trees in replanted apple (*Malus domestica* Borkh.) orchards and is attributed to the buildup of a core group of common fungal and oomycete pathogens including *Cylindrocarpon* spp., *Rhizoctonia solani* Kuhn, *Pythium*, *Phytophthora*, and a nematode pathogen (*Pratylenchus penetrans* (Cobb) Filip. and Schur.-Stekh.) in the rhizosphere of established trees (Jaffee et al. 1982a, b; Mazzola 1998; Tewoldemedhin et al. 2011b). Not all pathogens are necessarily present and active at a given site and some may act synergistically (Braun 1995; Tewoldemedhin et al. 2011b).

Rootstocks, the predominantly clonal genotypes on which desirable apple varieties are grafted, vary in their performance in replant soils. Hypotheses to explain the mechanisms of improved performance include elevated tolerance based on root vigor (Yao et al. 2006) as well as resistance to key replant pathogens (Isutsa and Merwin 2000; Mazzola et al. 2009). To evaluate alternate hypotheses requires identifying specific tissues that are colonized by pathogens and determining differential rates of colonization between different rootstocks.

In this paper, we test the hypothesis that root development leads to tissue-level resistance to replant pathogens. We predict that root branching orders vary in resistance to colonization by replant pathogens and we explore mechanisms of anatomical development and chemical defense to explain observed patterns. We further address the hypothesis that plants invest differentially in root classes based on their importance for plant performance and predict that pioneer roots are more resistant to pathogen infection, compared to first order fine-feeder roots, as a result of altered allocation of chemical defenses. Finally, we test the hypothesis that improved rootstock performance is a result of resistance to colonization by replant pathogens and explore chemical defense as a potential mechanism of rootstock resistance.

Materials and methods

Soil collection, plant material, and plant growth conditions

Soil was collected from the Cornell Orchards in Ithaca, NY, from a known replant site (Leinfelder and Merwin 2006)

in November 2011. Soil from this site is a Hudson silty clay loam. Soil was sampled at a depth of 0–30 cm under trees growing on Malling (M).26, M.7 and M.9 rootstocks, which are susceptible to ARD and are widely planted in commercial orchards. Soil was mixed with perlite 2:1 (vol/vol) in a cement mixer. Half of the soil mixture was steam pasteurized at 80 °C for 2 h and the remaining field soil (FS) returned to a cooler at 4 °C. The pasteurized soil (PS) was allowed to ventilate, open to the air, at room temperature for 1 week prior to planting.

Clonal plantlets of ARD-susceptible (M.26) and resistant (CG.210) rootstocks were propagated in tissue culture on Murashige and Skoog media (PhytoTechnology Laboratories, Shawnee Mission, KS) with supplemental vitamins. Plants were rooted in vitro and planted in a soilless medium following root initiation (Cornell Mix, Ithaca, NY). Plants were acclimated for 3 weeks in a fog tunnel and 1 week on a greenhouse bench at 25–28 °C before final planting in 1.5-L plastic pots containing bioassay soil. Thirty plantlets of each genotype were ranked by size and distributed evenly among each soil treatment and each of three harvest dates resulting in five biological replicates. Three plantlets of M.26 died following transplanting, leaving four replicates of M.26 in pasteurized soil for each harvest date. Plants were grown on a greenhouse bench from December 2011 to February 2012 with a 14 h photoperiod of supplemental light (≈100 $\mu M/m^2/s$). Day and night temperatures in the greenhouse were 22 and 15 °C, respectively. Plants were watered daily; irrigation water was supplemented with 150 ppm nutrient solution (21-5-20 with micronutrients) (Scotts, Marysville, OH) three times per week.

Five replicate plants of each genotype and soil combination were destructively harvested 3, 6 and 9 weeks after planting. Roots were washed free of adhering soil under running tap water. Whole root systems, or intact subsamples, were scanned on a modified flatbed scanner (Epson Expression 10000XL, Nagon, Japan) and dissected by root branching order (Fitter 1982). A Strahler-branching order definition was used, except that first order roots of larger diameter (approximately ≥0.5 mm) were designated as pioneer roots and assigned their own category. Dissected roots were frozen at −80 °C, lyophilized in a Labconco Freeze Dry System (Kansas City, MO, USA), weighed and then stored at −20 °C for subsequent analyses.

Root system measurements

Root system scans were converted to black and white through color thresholding in ImageJ (Abramoff et al. 2004) and analyzed in WinRhizo Pro version 2007d (Regent Instruments, Victoria, Canada) for root length. Where subsamples of root systems were scanned, a correction factor was applied to estimate total root length (root $length_{total} = root\ length_{scanned} \times (biomass_{total}/biomass_{scanned})$. Quality of scaled estimates were crosschecked for correlation with plant biomass, one point was an extreme outlier (studentized residual > 3) and was removed from root length analysis.

DNA extraction and quantitative PCR

DNA was extracted from each root order and growth trajectory (pioneer roots) of five replicate plants of each genotype growing in field soil at each harvest date. Depending on available sample quantity, between 1 and 15 mg of lyophilized root tissue was ground in a MiniBeadBeater (BioSpec Products, Bartlesville, OK) for two 45-s cycles in a screw cap microfuge tube with four steel 2-mm beads (MoBio, Carlsbad, CA, USA). Following tissue homogenization, DNA was extracted with the Qiagen DNeasy Plant Mini Kit (Valencia, CA, USA) following the manufacturer's protocol. DNA was then purified and concentrated by an ethanol precipitation (Sambrook et al. 1989) and re-eluted in 50 µl of TE buffer (Promega, Madison, WI, USA). DNA concentration was determined fluorometrically with a Quant-iT PicoGreen dsDNA probe (Invitrogen, Grand Island, NY, USA) and diluted to 10 ng/µl. Samples with less than 10 ng/µl were kept at original concentrations.

Pathogen abundance was estimated with a qPCR assay using species-specific primer sets (Tewoldemedhin et al. 2011b) (Online Resource A1) to measure pathogen DNA concentration in root DNA samples. All reactions were carried out using the SyberGreen-based Qiagen Quantifast chemistry on 96-well plates in a BioRad iQ5 real-time PCR detection system (Hercules, CA). Each assay consisted of four plates including multiple reactions of negative control root DNA from plants grown in pasteurized soil. Each plate included duplicate standard curves of tenfold dilutions of pure culture target DNA, non-template controls (water), negative controls (non-target pathogen DNA) and root samples. To generate a standard curve for each target, DNA was extracted from pure cultures of *Pythium sylvaticum* Campbell and Hendrix, *Phytophthora cactorum* Lebert and Cohn, *Pythium irregulare* Buisman, *Pythium ultimum* Trow, and *Rhizoctonia solani* Kühn anastomosis group 5 (AG-5). DNA extracts were quantified with the Quant-iT PicoGreen kit and serially diluted. Standard curves generated by the YT2F/CYL-R primer pair targeting *Cylindrocarpon* spp. have been reported to vary between species (Tewoldemedhin et al. 2011c). Our standard was generated using an isolate of *Ilyonectria robusta* A.A. Hiddelbrand (Booth *Cylindrocarpon* group 3) (Chaverri et al. 2011) obtained from the study site, and we refrain from comparing absolute estimates between *Cylindrocarpon* and other organisms in this study.

Reactions for all target species except *P. sylvaticum* were carried out in 20-µl volumes consisting of 10 µl of

Quantifast 2×, forward and reverse primers at 1 μM final concentration and ≤20 ng template DNA. *P. sylvaticum* reactions contained forward and reverse primers at 0.6 μM to balance amplification efficiency and limit primer-dimer formation. Cycling parameters followed the manufacturer's instructions for a two-step protocol: initial denaturing 95 °C for 5 min, and 40 cycles of denaturing at 95 °C for 10 s and combined annealing extension for 30 s.

Temperature gradients were conducted for each primer pair to optimize annealing temperature and maximize separation of target and non-target signals (Online Resource A1). Sensitivity of the assay was determined by the lowest dilution of standard curve with a cycle threshold (C_t) below negative controls. Melting curves were generated following all reactions to confirm target specificity. To test for the presence of PCR inhibitors, random samples of root DNA were serially diluted to test for a linear relationship between input DNA and cycle threshold. Amplification data were analyzed with the BioRad iQ5 optical software v2.0. To control for unequal extraction efficiency, pathogen DNA concentrations were normalized to root extracted DNA (fg target DNA/ng root DNA). The reliable detection threshold was set at the C_t value of either the lowest detectable standard, or a cycle below the C_t of negative control (non-target) DNA.

Root anatomical assessment

At each harvest, root anatomical development across branching orders and growth trajectory was investigated for three replicate plants of each rootstock grown in either ARD soil or the steam-pasteurized control. At 6 and 9 weeks after planting, sampling was stratified by sampling root segments representative of healthy or symptomatic roots (as assessed by necrosis or abnormal browning). Root segments were sampled from each branching order and preserved in FAA (5 ml formaldehyde, 5 ml acetic acid and 90 ml of 70 % ethanol). Root segments were dehydrated in a *tert*-butanol series and embedded in paraplast plus (Leica, Wetzler, Germany). Sections (14 μm) were cut on a Thermo Scientific Microm HM355S rotary microtome (Waltham, MA), stained in safranine-fast green and imaged under a Zeiss Axioskop II (Jena, Germany). Cross-section images were measured in ImageJ (Abramoff et al. 2004) for diameter, cortex thickness and state of senescence, stele diameter, and presence or absence of a periderm. We characterized the first four root orders, because very few plants had developed a fifth order by the final harvest. While higher root orders appeared later in the assay, their classification is dependent on distal branching, therefore higher order roots do not appear, but are reclassified as such (Fitter 1982).

Phenolic extraction and quantification

We evaluated specific secondary metabolite production across root orders and growth trajectories to investigate mechanisms of tissue resistance. Tissue phenolics were extracted from lyophilized tissue of each root order of five replicate plants from each rootstock, soil, and harvest date combination through methanol extraction. Depending on the amount of available tissue, between 1 and 15 mg of dry tissue was combined with 500 μl of extraction buffer—MeOH with 1 % 2.6-di-*tert*-butyl-4-methylphenol (BHT) (Sigma-Aldrich, USA). The sample was homogenized in a FastPrep-24 instrument (MP Biomedicals, Solon, OH, USA) set on high (6.5 M/s) for two 45-s cycles. The lysate was then centrifuged for 15 min at 14,000 rpm at 4 °C and the supernatant filtered through a 0.45-μm nylon membrane filter (Millipore, Tullagreen, Ireland).

Phenolic compounds were measured on an Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) with a Phenomenex Gemini-NX column (150 × 4.6 mm, 3 μm) (Torrance, CA, USA) and a diode array detector. Elution solvents were A: acetonitrile and B: 0.25 % phosphoric acid. 15 μl of extract was separated using the following protocol: 0–4 min 5 % acetonitrile, 4–24 min ramping to 60 % acetonitrile, 24–34 min ramping to 95 % acetonitrile and held at 95 % A for 1 min. Phloridzin (phloretin 2′-*O*-glucoside) was quantified at 320 nm; phloretin and other minor peaks were quantified at 280 nm. The spectra of compounds were also recorded between 200 and 400 nm.

Peak area was integrated using HP ChemStation software. Quantifiable peaks that had signal intensity over 100 and were present in a majority of samples were expressed in terms of peak area/mg root tissue and, when possible, assigned to compound classes based on characteristic UV spectra. Phloridzin concentration in root tissue was calculated as ng/mg based on a standard curve that was generated using a dilution series of authentic phloridzin standards to relate peak area to mass of phloridzin dihydrate (Sigma-Aldrich, USA).

Statistical analysis

The effect of soil pasteurization on rootstock growth was tested with ANOVA independently for each rootstock, with harvest date and height at planting included as fixed effects. This analysis avoided interactions between shoot height at planting and rootstock associated with inherent growth differences. To compare the response of the two rootstocks to each other, growth inhibition of plants growing in the field soil was calculated as the residual of observed and predicted values (derived from pasteurized soil estimates). To correct for differences in plant size, the residual was

expressed relative to the predicted value (FS residual/predicted value). Differences between the rootstocks in growth inhibition at the final harvest were tested with Welch's ANOVA.

Pathogen abundance in root branching orders was analyzed in a two-step process. A binomial logistic analysis was conducted in R (R Development Core Team 2012) using the geeglm function in the geepack package (Højsgaard et al. 2006) with plant id as a random (clustering) effect to test the main effects of order, rootstock and harvest date on the probability of detecting each pathogen. Zeros were then removed for the analysis of natural log transformed abundance.

A multivariate analysis of 11 quantifiable chromatogram peaks was conducted to test for main effects and interactions on root phenolic profiles. Peak area/mg was z-transformed to a mean of 0 and standard deviation of 1 to allow peaks measured at different wavelengths and with different variance to be analyzed together. The z-transform is sensitive to outliers, so 8 data points (of 215) were removed prior to transformation. The effects of root order, soil treatment, harvest date and rootstock were tested in a full-factorial model using the permutational multiple analysis of variance (ADONIS) function and a Euclidean distance matrix in the vegan package (Oksanen et al. 2012) in R. A factor analysis was used to group highly correlated chromatogram peaks (Noyer et al. 2011). Factors were extracted using principle component analysis with varimax rotation. The varimax rotation maximizes high loading values and minimizes low loadings, which improves the interpretability of the resulting factors (McGarigal et al. 2000). The number of factors was chosen to limit factors with single compounds yet allow compounds with different peak absorbance to separate and vary independently. This resulted in five synthetic factors, which explained 86 % of the variation in the 11 chromatogram peaks.

Differences in pathogen abundance, anatomy, and phenolic factors across branching orders and trajectory were tested with a full-factorial mixed effect ANOVA, with plant replicate included as a random effect. To evaluate differences between rootstocks in the dependent variables, the model was run separately for each root order without plant replicate as an effect.

Pairwise correlations were calculated to explore the relationship between pathogen abundance in each root order and whole plant growth inhibition and between pathogen abundance and phenolic production in first order and pioneer roots. Analysis of variance with rootstock, root order and pathogen abundance included as fixed effects and plant ID included as a random effect was used to further explore correlations between phenolic production and pathogen abundance.

Except where noted, all analyses were conducted in JMP pro v9.0 (SAS Institute Inc., Cary, NC). Fourth order roots did not appear on M.26 until later in the assay and were dropped from the factorial analysis. In all models the order variable contains first order pioneer roots and fine-feeder roots as separate levels (e.g., pioneer, first order, second order and third order).

Results

Rootstock growth in ARD soil

Following plant acclimation for 4 weeks, shoot height at planting ranged from 1 to 3 cm for CG.210 and 1–6 cm for M.26. Height at planting had a significant effect on all plant growth metrics ($P < 0.05$; Online Resource B1). However, interactions between height at planting and treatment effects were not significant (data not shown).

Steam pasteurization of soil improved growth of both rootstocks in terms of total biomass (Fig. 1a, d). Pasteurization improved root biomass accumulation for CG.210 ($P = 0.03$), but not for M.26 ($P = 0.21$; Fig. 1b, e). Root length was positively impacted by soil pasteurization for both M.26 and CG.210 ($P = 0.005$ and $P = 0.0008$, respectively; Fig. 1c, f; Online Resource B1). Whereas the effect of soil treatment on biomass accumulation in the overall model was significant for both rootstocks, plants growing in pasteurized soil were only significantly larger than those in growing in field soil for M.26 at the final harvest (Fig. 1a; Online Resource D1). By the final harvest, there was no statistically significant difference in relative growth inhibition in field soil between the two rootstocks for either biomass or root length ($P = 0.20$ and $P = 0.13$, respectively; Online Resource B2).

Although growth of both rootstocks was inhibited in the field soil, growth strategies of the two rootstocks differed considerably during this assay. CG.210 accumulated most new biomass below ground (Fig. 1e), resulting in a higher root-to-shoot ratio than M.26 ($P < 0.001$; Online Resource B3). For both rootstocks, the root-to-shoot ratio increased from the first to the third harvest [CG.210: 0.36–1.38 (R/S); and M.26: 0.13–0.54]. M.26 initiated above ground growth early in the assay, but this growth ceased in the field soil and a terminal bud was set. CG.210 did not initiate measurable shoot extension until the sixth week of the assay, but at week 9, this rootstock was actively growing in both soil treatments.

Pathogen abundance in heterogeneous tissues

None of the test pathogens were detected from negative controls (root DNA from pasteurized soil) at cycle thresholds lower than the detection limit. Amplification efficiency ranged from 94 to 102 % and R^2 values for the standard curves ranged from 0.982 to 0.998 for all plates used in the

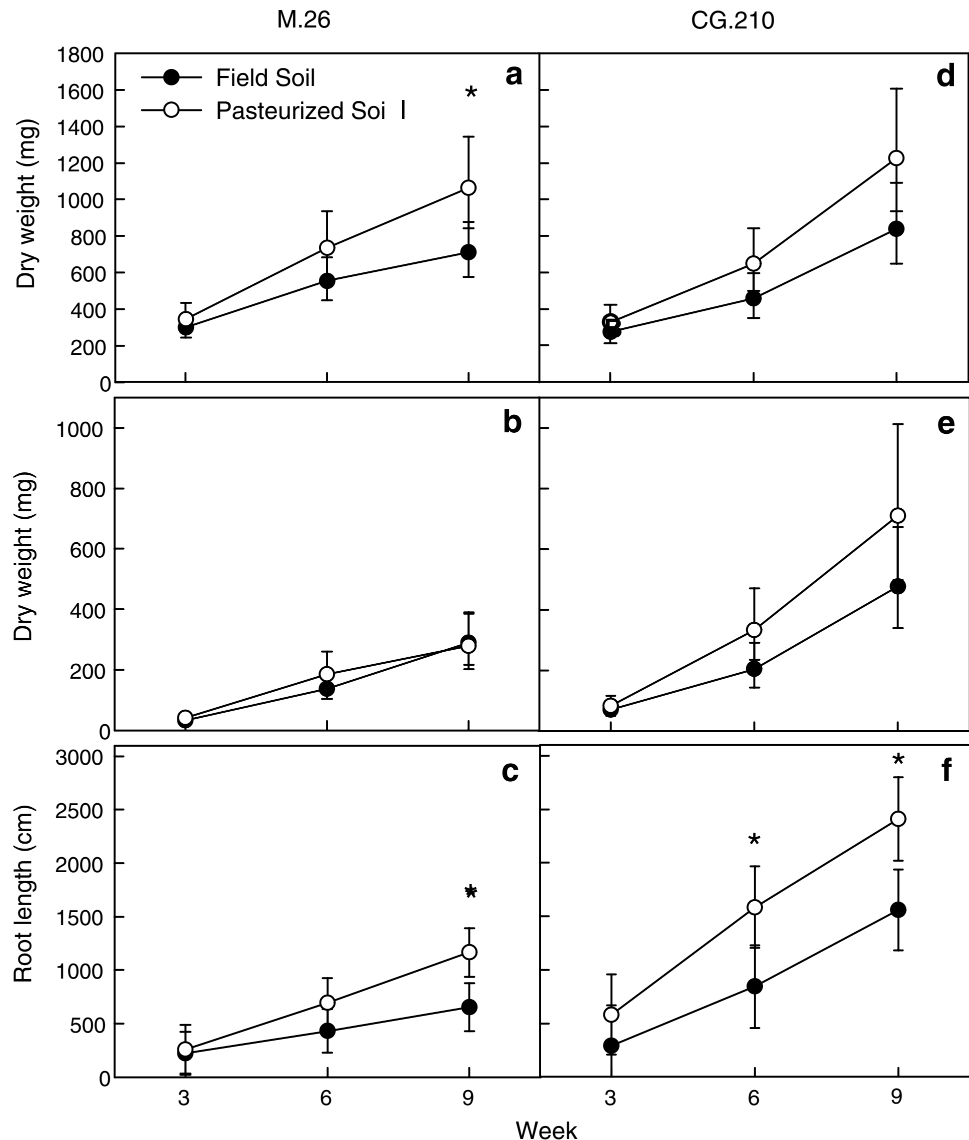
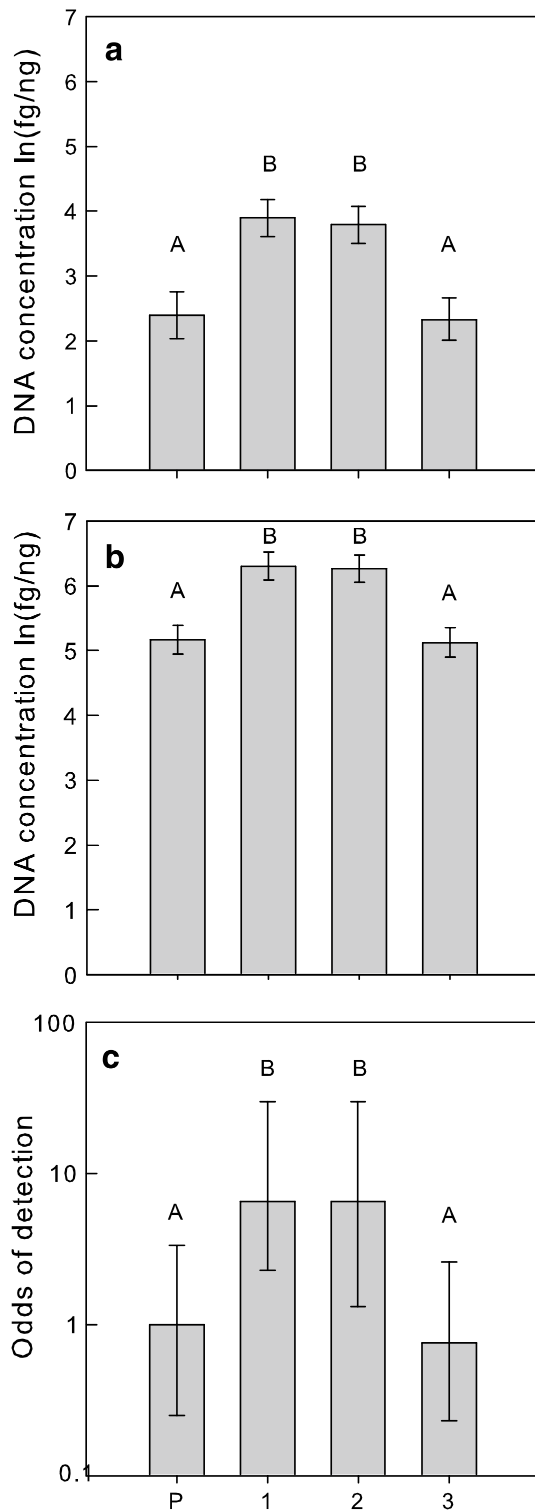


Fig. 1 Total biomass (**a**, **d**), root biomass (**b**, **e**) and root length (**c**, **f**) of M.26 (**a–c**) and CG.210 (**d–f**) plants harvested at 3, 6 and 9 weeks ($n = 5$, M.26 in pasteurized soil $n = 4$). Points are least square means (**a**, **b**, **d** and **e** back-transformed); *vertical bar* is 95 % confidence interval. Differences between plants grown in field and pasteurized soil are significantly different (*$P < 0.05$)

subsequent analysis. Three pathogens targeted in our assay were detected at concentrations that allowed quantification. *P. sylvaticum*, *Cylindrocarpon* spp., and *P. irregulare* DNA was detected in 90, 93, and 59 % of root samples, respectively. *Rhizoctonia solani* AG-5 and *Pythium ultimum*, were not recovered in any samples at detectable levels. *Phytophthora* DNA concentration was above the reliable detection limit in only six root samples, which was considered too few for further analysis.

Analysis of pathogen abundance was conducted in a two-step process to determine whether pathogens were less likely to be detected in certain branching orders and, if detected, if they were more or less abundant in one branching order versus another. *Cylindrocarpon* was equally likely to be detected across roots of different orders and trajectories ($P = 0.44$; Online Resource B4), however, lower concentrations of the pathogen were found in pioneer and third order roots than first and second order ($P = 0.002$ and $P = 0.003$, respectively; Fig. 2a; Online Resource D2). There were no differences in the likelihood of detecting *P. sylvaticum* across the three branching orders, but this pathogen was less likely to be detected in pioneer roots than first order fine-feeder roots ($P = 0.01$; Online Resource D2). In terms of abundance, *P. sylvaticum* followed the same pattern as *Cylindrocarpon*, pioneer and third order roots had lower concentrations of DNA (Fig. 1b). *P. irregulare* was less likely to be detected in pioneer and third order roots compared to first and second orders ($P = 0.009$ and $P < 0.001$, respectively; Fig. 1c; Online Resource D2). At the final harvest, no third order root samples contained detectable quantities of *P. irregulare* DNA (Fig. 3), so they were not included in the factorial analysis of pathogen abundance. For the samples in which *P. irregulare* was detected, there were no differences in the abundance



◀ **Fig. 2** Quantitative PCR estimates of replant pathogen colonization of pioneer (P), first, second and third order roots. *Pythium sylvaticum* (**a**) and *Cylindrocarpon* spp. (**b**) abundance in samples with positive detection expressed as the natural log of target DNA concentration in root DNA extract (ln(fg/ng)). *Cylindrocarpon* samples with positive detection included in analysis $n = 26$(P), 29(1st), 29(2nd) and 25(3rd). *P. sylvaticum* samples with positive detection included in analysis $n = 21$(P), 29(1st), 30(2nd), 25(3rd). *P. irregulare* (**c**) colonization is expressed as odds of detection ($p/1 - p$), where $p =$ the probability of detection ($n = 30$, third order $n = 28$). Columns are least squares means averaged across rootstocks and harvest date. *Vertical bar* is one standard error (**a**, **b**) and 95 % confidence interval (**c**). Columns with different letters are significantly different ($P < 0.05$)

among pioneer, first and second order roots ($P = 0.95$; Online Resource B5). However, the pattern indicated by the logistic analysis of *P. irregulare* and the least squares estimates of *Cylindrocarpon* and *P. sylvaticum* abundance all support decreased populations of replant pathogens in third order roots (Fig. 2).

The pattern of pathogen abundance by root order shifted with harvest date (Fig. 3). By the final harvest both *P. sylvaticum* and *P. irregulare* species had increased sharply in abundance in pioneer roots ($P = 0.0004$ and $P = 0.001$, respectively; Online Resource D3). In contrast, *P. sylvaticum* DNA was recovered at lower concentrations in third order roots at the final harvest (2.86 fg/ng) compared to the first (65 fg/ng) ($P < 0.0001$; Online Resource D3) and, as described above, *P. irregulare* was not detected in third order root samples at the final harvest.

Rootstock resistance to replant pathogen colonization

Rootstock resistance to ARD pathogen colonization was evaluated by comparing pathogen abundance between the rootstocks by branching order (Fig. 3). Within first order roots, CG.210 had lower abundance of both *P. irregulare* and *P. sylvaticum* ($P = 0.04$ and $P = 0.05$, respectively; Online Resource B6) and a lower abundance of *Cylindrocarpon* at the final harvest compared to M.26 ($P = 0.03$; Online Resource D4). In pioneer roots, CG.210 also had a lower abundance of *Cylindrocarpon* ($P = 0.03$; Online Resource B6), though for the latter, this was mainly attributable to increased levels of target DNA in roots of M.26 at the final harvest. There was no difference between the rootstocks in levels of *P. irregulare* DNA recovered from pioneer roots ($P = 0.90$; Online Resource B2). The difference between rootstocks in the abundance of *P. sylvaticum* in Pioneer roots was not significant ($P = 0.08$; Online Resource B2), though few samples of CG.210 Pioneer roots contained quantifiable levels of the pathogen (Fig. 3, inset), limiting the power of this comparison. In second order roots there were no overall differences between the rootstocks in populations of *P. sylvaticum*, *P. irregulare*, or *Cylindrocarpon* ($P > 0.05$; Online Resource B6), though populations in both rootstocks were highly variable over time and *P. sylvaticum* was found at higher concentrations in M.26 at the final harvest ($P = 0.05$; Online Resource D4). In third order roots higher concentrations of *P. sylvaticum* DNA were recovered in CG.210 roots compared to

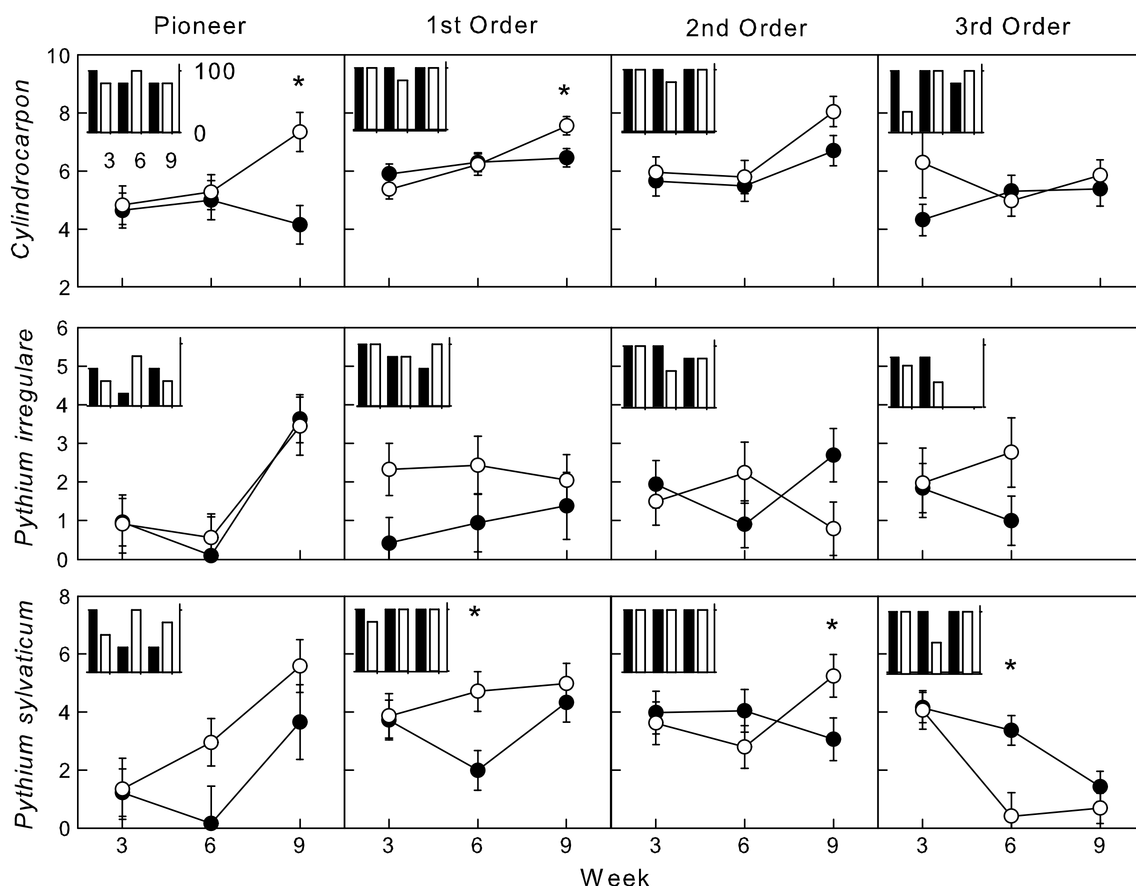


Fig. 3 Quantitative PCR estimates of replant pathogen abundance in roots of M.26 (*solid circles*) and CG.210 (*open circles*) at 3, 6 and 9 weeks. Points are least square means of pathogen DNA concentration in roots with positive detection from five replicate plants (see *inset bars* for frequency of detecting sample 0–100 %). *Vertical bars* are one standard error of the mean. Means are significantly different (*$P < 0.05$) between M.26 and CG.210

M.26 ($P = 0.02$; Online Resource B6), mainly attributable to differences at the second harvest (Fig. 3). There was no difference between the rootstocks in the levels of *Cylindrocarpon* or *P. irregulare* DNA in third order roots (Online Resource B6). Differences between the rootstocks in level of *P. sylvaticum* DNA in third order roots were transient and CG.210 had higher levels at the second harvest compared to M.26 ($P = 0.007$; Online Resource D4). Across all root orders, pathogen concentration was not significantly correlated with observed growth suppression in terms of biomass or root length (Online Resource C1).

Root development as a constraint on pathogen habitat

Distinct stages of tissue development were associated with each root order (Table 1). Root diameter increased with root order ($P < 0.0001$; Online Resource B1). Meanwhile, an increase in stele diameter ($P < 0.0001$; Online Resource B7) combined with the senescence and loss of the cortex in second and third order roots (Fig. 4) resulted in a higher stele-to-root ratio ($P < 0.0001$; Online Resource B7). Sections from first order roots had an intact cortex while second order roots varied considerably in the state of the cortex, ranging from intact to absent. Concurrent with this transition, periderm initiation and secondary development of the vascular cylinder was evident in 75 % of second order sections. The cortex in third order roots was either senescent or absent entirely and remnants of a cortex were only present in one fourth order root sections.

By definition, pioneer roots were of greater diameter than first order fine-feeder roots. There was a small, though statistically significant difference in the stele-to-root ratio between these groups ($P < 0.0001$, Table 1 and Online Resource D5). The percentage of pioneer root samples exhibiting periderm initiation was slightly greater than first order fine-feeder roots, consistent with the assertion that these roots are destined to become higher branching orders.

When sampling symptomatic and asymptomatic roots for comparison, few third order symptomatic roots could be obtained from either rootstock. Across branching orders, almost without exception, a cortex was present (either fully intact or in the process of senescence) in roots that

Table 1 Anatomical measurements of root cross-sections taken from pioneer, first, second, third, and fourth order roots

	Pioneer	1	2	3	4
Diameter (μm)	545 (141)c	270 (67)a	431 (163)b	592 (231)c	1055 (301) d
Cortex (μm)	165 (51)e	79 (23)c	90 (52)d	44 (62)b	1 (5)a
Stele (μm)	157 (41)b	72 (22)a	198 (102)c	440 (219)d	990 (301)e
Stele:root	0.3 (0.08)b	0.26 (0.05)a	0.46 (0.17)c	0.74 (0.19)d	0.93 (0.03) e
Cortex condition[a]	80/7/1	87/3/0	51/42/11	5/32/40	0/1/31
Periderm[b] (%)	10	2	74	95	100
n	88	90	104	75	32

Mean (SD) of roots sampled from three replicate plants of M.26 and CG.210 rootstocks growing in pasteurized and field soil at 3, 6 and 9 weeks. Columns with different letters are statistically different (*$P < 0.05$)

[a] Number of samples with cortex present/senescent/missing

[b] Percent of samples with periderm initiation

displayed visual symptoms; in contrast, there was a higher frequency of sections without a cortex when sampled from asymptomatic roots (Fig. 4). This localization of symptoms corresponded with fungal and oomycete colonization of the cortex that was observed in multiple root cross-sections.

Anatomical differences between rootstocks

Anatomical differences between the root branching system of CG.210 and M.26 were evaluated on plants growing in pasteurized soil for the harvests at 6 and 9 weeks to reflect roots that developed in the assay soil. Overall, CG.210 roots had a finer branching structure with a smaller diameter ($P = 0.0001$) and a thinner cortex ($P = 0.03$) than M.26 (Online Resource B8).

Chemical defenses as a mechanism of tissue resistance

The composition of root phenolic compounds was investigated to determine patterns of chemical defenses on the root branching system in relation to patterns of pathogen abundance. Roots of different order and trajectory differed considerably in their phenolic profile, accounting for the greatest portion of variation in the multivariate analysis of chromatogram peaks ($R^2 = 0.28$; $P < 0.001$; full-factorial ADONIS, $R^2 = 0.66$; Online Resource B9). The factor analysis resulted in five synthetic factors that captured 86 % of the variance in peak area among samples (Table 2). Phloridzin, the largest peak in the chromatogram, loaded heavily on F2. Of the compounds loading on F1, four shared similar spectra as phloridzin, with peak absorbance at 285 nm, which may indicate precursors or breakdown products, while the spectra of one resembled a hydroxycinnamic acid and had a peak absorbance at 315 nm. Factor 3 also included compounds with spectra similar to phloridzin, while single compounds with peak absorbance at 325 nm (putatively a caffeic acid) and 255 nm loaded on F4 and F5, respectively.

Branching order (and trajectory) influenced the concentration of each factor ($P < 0.001$) except for F5 ($P = 0.19$; Online Resource B10) (Fig. 5b–f) and two notable patterns emerged. Factor 2 was found at highest levels in higher order roots and phloridzin itself reached concentrations over 10 % of root dry weight (Fig. 5a), peaking in third order roots. In all orders, phloridzin was present in concentrations that far exceeded other peaks, and represents the majority of phenolics found in these apple roots. Second, in contrast to F2, the other factors were found at higher concentrations in first order, second order or pioneer roots, indicating a shift in composition of phenolic compounds, in addition to the shift in total concentration, among branching orders.

The levels of phenolic compounds shifted in roots in field soil compared to the pasteurized control, and the direction and magnitude of the shifts were highly dependent on root order and trajectory (Fig. 5b). There was a significant increase for each factor except F4 in first order roots in field soil ($P < 0.05$; Online Resource D6) (Fig. 5b–f). In contrast, in second and third order roots all factors were either the same or lower in the field soil as compared to the pasteurized control.

First order fine-feeder and pioneer roots also differed in their phenolic profile. Pioneer roots had lower concentrations of F2 (phloridzin) ($P = 0.0007$) and F3 ($P < 0.0001$) than smaller diameter fine-feeder roots, but a higher concentration of F4 ($P < 0.0001$; Online Resource D7). Additionally, there was a significant increase for pioneer roots in the level of F4 when growing in field soil compared to the pasteurized control ($P = 0.0006$), but no increase in the level of this compound in first order roots ($P = 0.95$; Online Resource D6). While there was not a significant effect of growth trajectory on F5, this compound was found

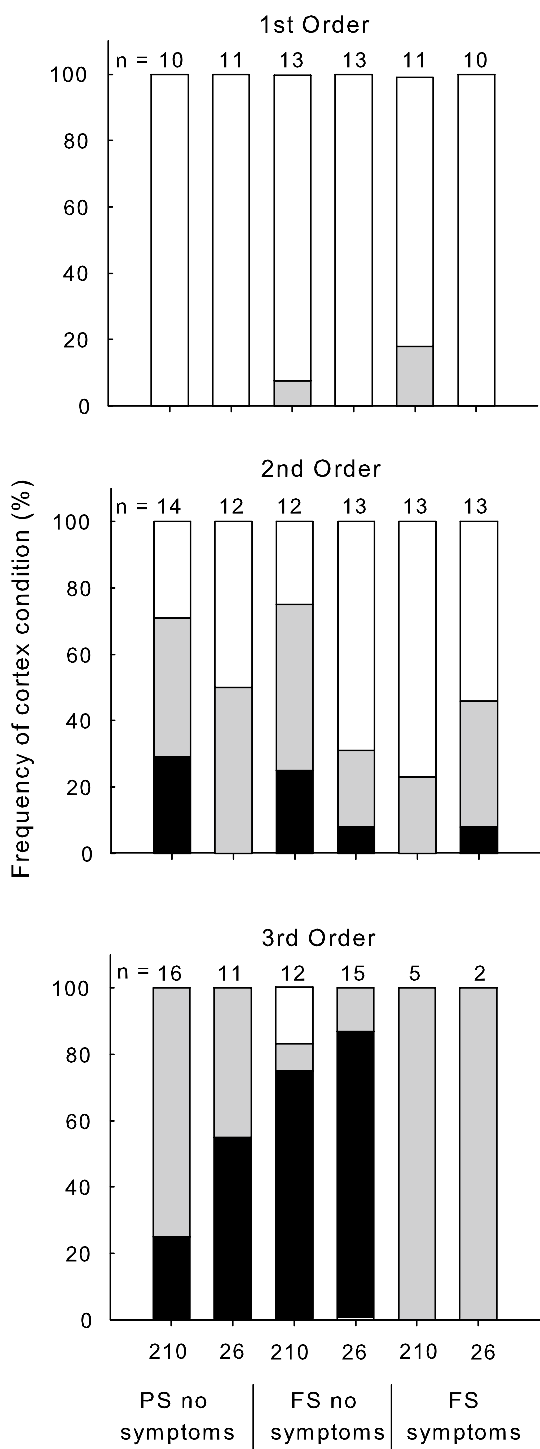


Fig. 4 Frequency of first, second and third order root cross-sections with intact (*white bars*), senescent (*grey bars*), or absent (*black bars*) cortex. Samples were taken from three replicate plants during harvests at week 6 and 9 and grouped by soil treatment (PS and FS), appearance of visual symptoms and rootstock ((M.)26 and (CG.)210). *Values above each column* are the number of root segments sectioned and imaged

Table 2 Loadings of *z*-transformed chromatogram peaks (rows) on principle component factors (columns)

Retention time (min)	Peak absorbance λ (nm)	F1	F2	F3	F4	F5
13.3	285	0.80				
13.85	285	0.88				
14.5	285	0.92				
14.8	285		0.51	0.63		
15.9	315	0.85				
16.9	285			0.93		
17.1	285	0.47	0.65			
18.6 (phloridzin)	285		0.90			
19.2	285		0.88			
27.6	255					0.92
31.9	325				0.92	
% of total variance explained		31	22	14	11	10
Eigenvalue		4.20	2.63	1.12	0.83	0.77

To ease interpretation, only peaks that load above |0.40| on a factor are shown

Factors extracted from principle component analysis with varimax rotation

in higher concentrations in pioneer roots growing in field soil than first order fine-feeder roots in the same soil (Student's $t_{0.05,107.23} = 2.61$, $P = 0.01$).

Contrasting phenolic profile between rootstocks

The two rootstocks differed in their profile of phenolic compounds both in terms of their constitutive concentration in pasteurized soil, and their shifts in production in response to growing in field soil. The two rootstocks differed in the concentrations of each group of compounds represented by the five factors in first order roots ($P < 0.01$; Online Resource B12; Fig. 6). The same pattern was observed for pioneer roots, except that there was no difference in F3 for the two rootstocks ($P = 0.81$; Online Resource B12). Concentrations of phenolic compounds in first order fine-feeder roots responded differently to growth in field soil compared to the pasteurized control for both F2 and F3 (soil × rootstock: $P = 0.02$ and $P = 0.002$, respectively; Online Resource B12). First order roots of M.26 in field soil had an increase in concentration of F2 compared to the same roots in pasteurized soil, while CG.210 roots had low concentrations of this factor in both soils. In contrast, the direction of change in the two rootstocks was different for F3 with a much greater induction observed in CG.210 (Fig. 6).

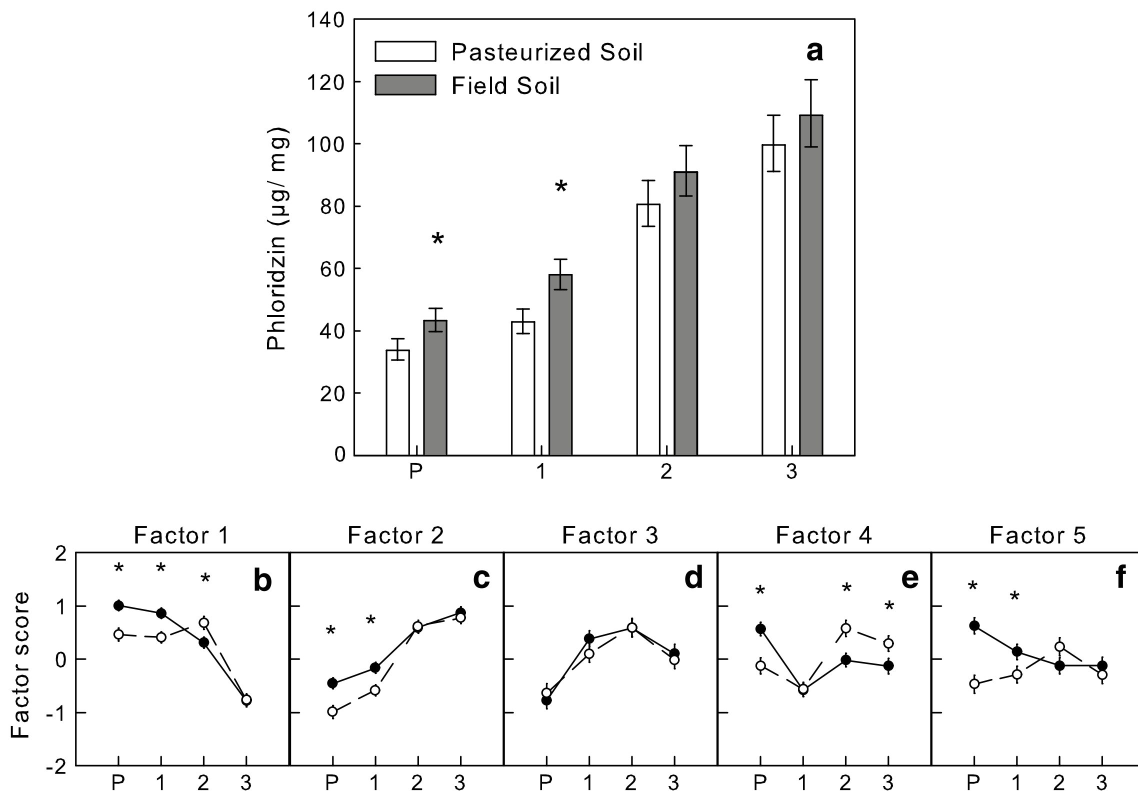


Fig. 5 **a** Phloridzin concentration (µg/mg) in pioneer (P) first, second, third and fourth order roots growing in field soil and pasteurized soil. **b–f** LS mean factor scores by root order in field soil (*black circles*) and pasteurized soil (*open circles*). Columns are back-transformed least square means averaging across rootstocks (M.26 and CG.210) and harvest dates (3, 6, and 9 weeks). *Vertical bar* is 95 % confidence interval of the mean (**a**) and one standard error of the mean (**b–f**) ($n = 30$, where missing values $n \geq 22$). Differences between pasteurized and field soil are significantly different (*$P < 0.05$)

Phenolic factors were tested to determine if they were positively or negatively correlated with pathogen colonization (Table 3). Factor 1 (putative phloridzin derivatives or precursors and a hydroxycinnamic acid) was negatively correlated with *Pythium* DNA of both species ($P = 0.01$, Table 3 and Online Resource C2). This factor was found at higher levels in M.26, but induced in field soil in both rootstocks (Fig. 6). F4 (caffeic acid) was also negatively correlated with *Cylindrocarpon* DNA ($P = 0.005$); however, this effect was not significant if rootstock and root order were included in the model (Online Resource C3). This compound was found at greater concentrations in roots of CG.210 ($P < 0.0001$; Online Resource B12), and particularly high concentrations in CG.210 pioneer roots in field soil (Fig. 6). In contrast, F2 was positively correlated with both *Cylindrocarpon* and *P. sylvaticum* ($P = 0.005$ and $P = 0.05$, respectively; Online Resource C2). This compound was found at higher levels in M.26 than CG.210 ($P < 0.0001$; Online Resource B12), and increased dramatically in the field soil compared to the pasteurized control in M.26 (Fig. 6). The significance of the correlation between F2 and both pathogens was lost when rootstock is included in the model (Online Resource C3) as F2 was found in greater concentrations in M.26 roots, which also had higher concentrations of *P. sylvaticum* and *Cylindrocarpon* DNA. Finally, F5, which was found at highest concentration in pioneer roots in field soil, was also positively correlated with both *Pythium* species ($P = 0.04$ and $P = 0.0003$; Online Resource C2). However, the correlation between F5 and *P. sylvaticum* was not significant when rootstock was added to the model (Online Resource C3). For M.26 there was a positive relationship between with *Cylindrocarpon* and levels of Factor 5, while there was a weak negative relationship in CG.210 roots (rootstock × pathogen, $P = 0.007$; Online Resource C3).

Discussion

Our results provide support for the hypothesis that root development results in tissue-level resistance to replant pathogen colonization. Each of the three pathogen taxa detected in our study were less abundant in third order compared to first and second order roots (Fig. 1). Similar patterns observed in herbaceous species (English and Mitchell 1988) have been attributed to restriction of initial

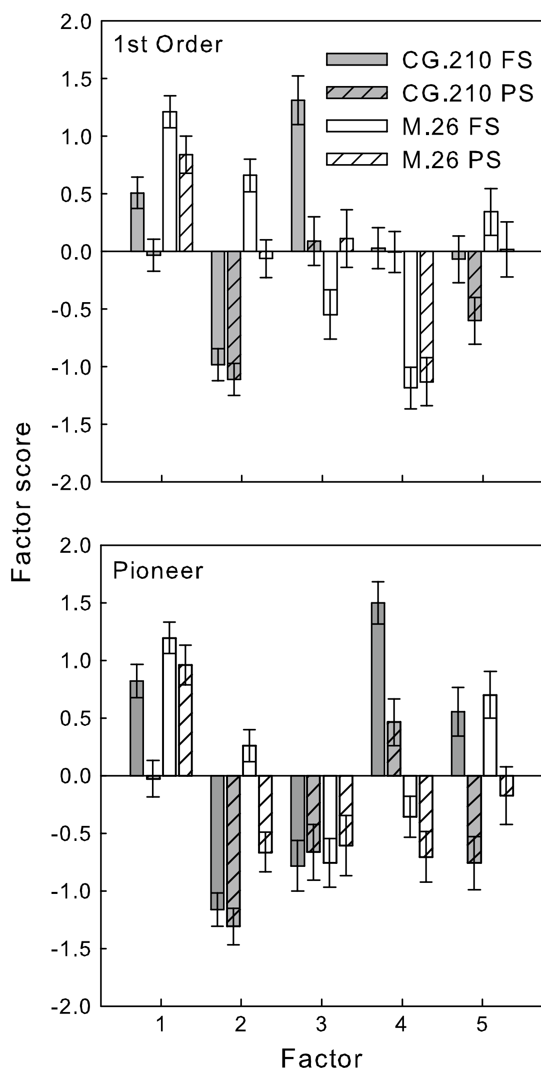


Fig. 6 Phenolic factor scores in pioneer and first order roots by rootstock (M.26 and CG.210) and soil treatment (pasteurized and field) averaged across harvest dates (3, 6, and 9 weeks) ($n = 15$, where missing values $n \geq 10$). *Zero line* represents centroid of PCA extracted factors and *columns* indicate the magnitude and direction of least square mean separation from the overall mean. *Vertical bar* is one standard error of the mean

infection(s) to root tips (English and Mitchell 1989). However, in the absence of mechanisms that restrict spread, this would result in increasing pathogen populations in higher order roots over time. This was not observed for any of the organisms in this study. Rather, populations of *Pythium* species declined sharply in third order roots by the final harvest, and *Cylindrocarpon* populations remained stable (Fig. 5). This is consistent with a model of replant disease where pathogens do not colonize entire root systems, but are concentrated on distal branches (Fig. 7). Expression of disease symptoms in this context may result from attrition of root tips, disruption of uptake and feedbacks on carbon limitation (Jönsson 2006).

In addition to root classes that result from development of the branching system, first order roots were defined as either large diameter, fast growing pioneer roots, or smaller diameter, fine-feeder roots (Zadworny and Eissenstat 2011). Overall, pathogen colonization was lower in pioneer roots than in first order fine-feeder roots (Fig. 1). This pattern was dependent on harvest date, and colonization by pathogens was equal by the final harvest. As we did not estimate root age in this study, it is possible differences in age distribution, and not colonization potential, led to the observed increase in pathogen abundance in pioneer roots. Nonetheless, the different patterns of pathogen abundance in these two root classes provide further support for the hypothesis that first order fine-feeder roots and pioneer roots differ in the rate and extent of colonization by common soil-borne pathogens.

Shifts in anatomy and chemical defenses were observed among root orders and growth trajectories, concurrent with shifts in pathogen abundance. Similar to other authors, classification by root order revealed distinct stages of root development characterized by increasing diameter, increasing stele-to-root ratio, and senescence of the cortex. These changes are indicative of a shift in function from resource uptake to resource transport (Guo et al. 2008; Pregitzer et al. 2002). First order roots had an intact cortex, meanwhile, the senescence and loss of cortical tissues occurred in second and third order roots as the secondary vascular system developed. If the organisms targeted in our assay were not infecting the vascular system of higher order roots, the loss of cortex would explain the decrease in pathogen DNA concentration observed in third order roots. Circumstantial evidence for this includes the observation that visual symptoms were restricted to roots retaining or in the process of shedding their cortex (Fig. 2). While our anatomical sectioning was not designed to evaluate the infection of specific tissues, we observed no indication of pathogen colonization inside the endodermis or periderm. Disease symptoms result from a disruption of tissue function, suggesting that these structural changes may also reflect a shift in the way colonization of host tissue may manifest disease symptoms (Fig. 7). Heavy colonization of first and second order roots may directly interfere with uptake function as the cortex provides surface area for the uptake of solutes and is also site of mycorrhizal colonization (Esau 1965). Approximately 50 % of the second order roots had an intact cortex and the initiation of the periderm was evident in 74 % of the samples. The periderm will isolate the cortex and seal its fate as senescent tissue making it likely the contribution of second order roots to resource uptake will become significantly decreased. As a result, pathogen colonization of the remaining cortical tissues in second and third order roots may not constitute a disruption of future root function, and thus the recovery of pathogens

Table 3 Pairwise correlations between pathogen DNA (ln(fg/ng)) and phenolic factors in first order and pioneer roots of M.26 and CG.210 rootstocks harvested at 3, 6, and 9 weeks

	n	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
P. irregulare	41	−0.38***	**0.18**	**−0.27**	**0.04**	0.54***X
Cylindrocarpon	55	**−0.04**	0.37	**0.10**	−0.38	0.17X**
P. sylvaticum	50	**−0.25****	0.28	**0.05**	**−0.25**	0.30

X following value indicates a significant interaction between rootstock and pathogen concentration

Values in bold are significant at $P = 0.05$

* Effect of target DNA concentration on phenolic factor is significant when root order and rootstock effects are included in mixed model ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

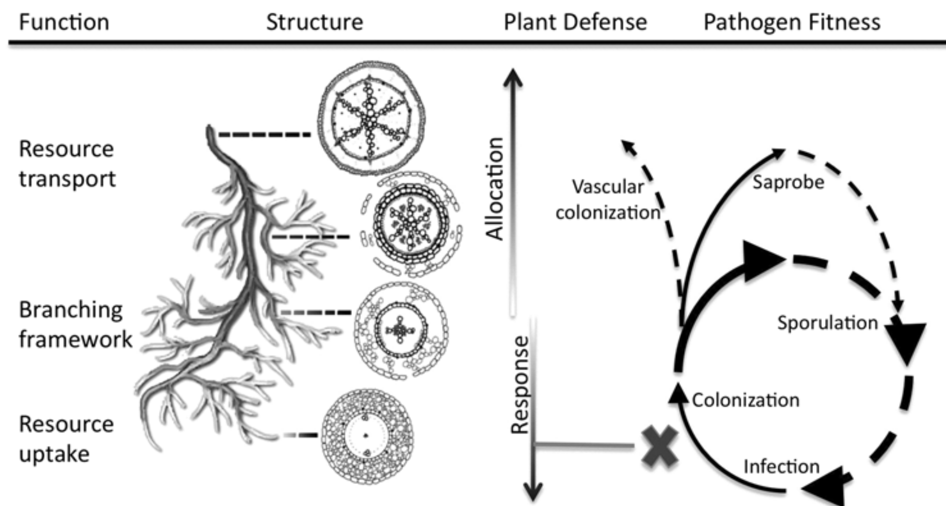


Fig. 7 Conceptual model of replant pathogen interaction with roots of heterogeneous structure and function. Roots of distinct stages of development are found on the branching system. Roots in primary development and transitioning to secondary development have the highest measure of pathogen abundance (*solid lines*), which may disrupt resource uptake (first order roots) and the further branching and establishment of the root system (pioneer roots). Detection of pathogens on roots in secondary development may result from vascular colonization or saprobic growth on senescent tissue (*dashed lines* represent unknowns). Plant chemical defenses are constitutively allocated to higher order roots and may protect the vascular tissues. Plant induction of defense is highlighted in distal branches. Pathogen resistance and chemical defenses in distal branches may contribute to rootstock performance in replant soil. Root cross-sections not to scale. Drawing by Alex Paya; cross-sections adapted from Hishi (2007)

from these roots may not be directly related to disease symptoms. This conclusion is based on root anatomy, not on the branching order itself. Therefore, the relevant root orders to a pathogenic interaction of interest may include first and second order roots in species or settings where the periderm initiates in third order roots. For example, Eissenstat and Achor (1999) found that fibrous first and second order roots of citrus did not undergo secondary development, while seedling second order roots did develop secondary xylem and periderm. Cursory investigation of roots growing in experimental soil can inform decisions of relevant branching orders, which can be then be employed to sample roots relevant to the interaction of interest.

In addition to the shifts in anatomy that may restrict tissue susceptibility to pathogen colonization, a number of patterns were observed between the spectrum and abundance of root phenolic compounds. First, phloridzin concentration increased with root order (Fig. 5a), with the highest concentrations observed in third order roots. The buildup of phloridzin in higher order roots may act directly to inhibit certain organisms or contribute to defenses that isolate the vascular tissues from infection. Second, the relative composition of tissue phenolics other than phloridzin, were found at their highest concentration in first order and pioneer roots. Finally, increased phenolic compound concentrations in roots grown in the field soil compared to the pasteurized control suggest induced phenolic production as defense or stress response to pathogen infestation. However, this induction was only observed in first order and pioneer roots. It is therefore possible that phloridzin

allocation may play an important role in protecting the vascular tissues whereas other compounds may be primarily involved in inhibiting initial infection and spread.

Zadworny and Eissenstat (2011) hypothesized that optimal defense allocation could account for differences in both mycorrhizal and non-mycorrhizal infection rates between first order fine-feeder and pioneer roots of several hardwood tree species. We observed distinct phenolic profiles in pioneer as compared to first order fine-feeder roots. While the functional significance of these differences is unclear, evidence of differences in the relative abundance of phenolic compounds is consistent with altered allocation of defenses to these two classes of roots. Combined with different levels of colonization by replant pathogens, the data support the hypothesis that pioneer roots receive differential investments of defenses than first order fine-feeder roots and outcomes of interactions between roots and pathogens may differ for these classes of roots (Zadworny and Eissenstat 2011).

Rootstock resistance

Support for the hypothesis of rootstock resistance to replant pathogens comes primarily from first order and pioneer roots. CG.210 roots had lower populations of all three target pathogens in these roots compared to M.26, while in higher order roots there was no clear trend in pathogen concentration between rootstocks. This suggests that resistance mechanisms limiting initial infection and colonization of distal branches may contribute to the improved performance of this rootstock in replant soil. These results add to previous studies that demonstrated lower recovery of *Pythium* species and root lesion nematodes from the roots of CG rootstocks compared to those more susceptible to replant disease (Isutsa and Merwin 2000; Mazzola et al. 2009).

There is evidence to support a relationship between pathogen abundance on the root system and plant growth outcomes, though this relationship is not always straightforward. A link has been demonstrated between pathogen biomass and virulence for *P. irregulare* and *P. sylvaticum* (Tewoldemedhin et al. 2011a). Additionally, Bent et al. (2009) found that the abundance of *P. ultimum* in root tips was inversely related to shoot biomass in a study of peach in replant soil. We did not observe a correlation between plant growth suppression and pathogen abundance in any root order. Similarly, Tewoldemedhin et al. (2011b) did not find a correlation between pathogen abundance in seedling roots and growth response following soil pasteurization in replant soil from multiple orchards. Biologically, differences in virulence of isolates may not correlate with their abundance on the root system, as observed for *Cylindrocarpon* (Tewoldemedhin et al. 2011c). Further complicating these measures, infection with multiple pathogen species with synergistic or antagonistic effects (Braun 1995; Tewoldemedhin et al. 2011b) would obscure correlations between individual organisms and plant growth suppression.

We investigated tissue phenolics as a mechanism of resistance against replant pathogens and observed differences between the two rootstocks in both the spectrum and concentration of phenolic compounds in first order and pioneer roots. A number of these phenolics, including phloridzin, hydroxycinnamic acids, and caffeic acid, have been found at higher levels in apple cultivars resistant to apple scab (*Venturia inaequalis*) (Petkovsek et al. 2009), and phloridzin has demonstrated toxicity to *Phytophthora cactorum* in vitro (Alt and Schmidle 1980; Gosch et al. 2010). The lower levels of the principle component factor associated with phloridzin in CG.210 than the more susceptible M.26, suggests that the overall concentration of this abundant phenolic is not supporting this rootstock's resistance to replant pathogens. This is consistent with a previous report of concentrations of phloridzin in root exudates peaking at the onset of ARD symptoms (Hofmann et al. 2009). Of the other principle component factors, only F4, a caffeic acid, was found in higher levels in CG.210. It also was negatively correlated with the abundance of *Cylindrocarpon*, suggesting it may deserve further attention for its role in rootstock resistance to replant pathogens.

While our observations of pathogen colonization are consistent with a level of tissue resistance of CG.210, they do not contradict hypotheses regarding vigor and tolerance-based mechanisms of plant defense. Belowground biomass and root length accumulation were twofold greater for CG.210 than M.26. The production of a more extensive root system may compensate for proportional loss. There is evidence for such vigor-based tolerance of root herbivory (Bauerle et al. 2007). However, plant vigor has not been significantly correlated with growth response in replant soil (Isutsa and Merwin 2000). Nonetheless, CG.210 allocated a greater portion of its biomass below ground, which may have contributed to its ability to maintain shoot growth in the replant soil. CG.210 roots were of smaller diameter in the lower branching orders and theoretically a finer and more highly branched root system would be cheaper to construct (Eissenstat et al. 2000), also contributing to tolerance of root loss. The interplay of resistance and tolerance in diseases of the fine-root system deserves further attention. Moreover, given the previous reports of this and other CG rootstock performance in replant soil, direct inoculations with specific replant pathogens would help to elucidate the mechanisms of plant defense against replant pathogens.

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Conflict of interest The authors declare no conflict of interest.

References

Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. Biophotonics Int 11:36–42
Alt D, Schmidle A (1980) Untersuchungen uber mögliche Resistenzfaktoren des Apfels gegen *Phytophthora cactorum* (Leb. et Cohn) Schroet. Angewandte Botanik 54:139–156
Bauerle TL, Eissenstat DM, Granett J, Gardner DM, Smart DR (2007) Consequences of insect herbivory on grape fine root systems with different growth rates. Plant Cell Environ 30:786–795
Bednarek P, Osbourn A (2009) Plant-microbe interactions: chemical diversity in plant defense. Science 324:746–748
Bennett RN, Wallsgrove RM (1994) Tansley Review No. 72. Secondary metabolites in plant defence mechanisms. New Phytol 127:617–633
Bent E, Loffredo A, Yang J-I, McKenry MV, Becker JO, Borneman J (2009) Investigations into peach seedling stunting caused by a replant soil. FEMS Microbiol Ecol 68:192–200
Braun PG (1995) Effects of *Cylindrocarpon* and *Pythium* species on apple seedlings and potential role in apple replant disease. Can J Plant Pathol 17:336–341
Chaverri P, Salgado C, Hirooka Y, Rossman AY, Samuels GJ (2011) Delimitation of (*Neonectria* and *Cylindrocarpon Nectriaceae*, *Hypocreales*, *Ascomycota*) and related genera with *Cylindrocarpon*-like anamorphs. Stud Mycol 68:57–78
Eissenstat DM, Achor DS (1999) Anatomical characteristics of roots of citrus rootstocks that vary in specific root length. New Phytol 141:309–321
Eissenstat DM, Wells CE, Yanai RD, Whitbeck JL (2000) Building roots in a changing environment: implications for root longevity. New Phytol 147:33–42
English JT, Mitchell DJ (1988) Relationships between the development of root systems of tobacco and infection by *Phytophthora parasitica* var. *nicotianae*. Phytopathology 78:1478–1483
English JT, Mitchell DJ (1989) Use of morphometric analysis for characterization of tobacco root growth in relation to infection by *Phytophthora parasitica* var. *nicotianae*. Plant Soil 113:243–249
Enstone DE, Peterson CA, Ma FS (2002) Root endodermis and exodermis: structure, function, and responses to the environment. J Plant Growth Reg 21:335–351
Esau K (1965) Plant anatomy, 2nd edn. Wiley, New York
Fitter AH (1982) Morphometric analysis of root systems: application of the technique and influence of soil fertility on root-system development in two herbaceous species. Plant Cell Environ 5:313–322
Gosch C, Halbwirth H, Stich K (2010) Phloridzin: biosynthesis, distribution and physiological relevance in plants. Phytochemistry 71:838–843
Guo D, Xia M, Wei X, Chang W, Liu Y, Wang Z (2008) Anatomical traits associated with absorption and mycorrhizal colonization are linked to root branch order in twenty-three Chinese temperate tree species. New Phytol 180:673–683
Hishi T (2007) Heterogeneity of individual roots within the fine root architecture: causal links between physiological and ecosystem functions. J For Res 12:126–133
Hofmann A, Wittenmayer L, Arnold G, Schieber A, Merbach W (2009) Root exudation of phloridzin by apple seedlings (*Malus* × *domestica* Borkh.) with symptoms of apple replant disease. J Appl Bot Food Qual-Angewandte Botanik 82: 193–198
Højsgaard S, Halekoh U, Yan J (2006) The R package geepack for generalized estimating equations. J Stat Softw 15(2):1–11
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
Jaffee BA, Abawi GS, Mai WF (1982a) Fungi associated with roots of apple seedlings grown in soil from an apple replant site. Plant Dis 66:942–944
Jaffee BA, Abawi GS, Mai WF (1982b) Role of soil microflora and *Pratylenchus penetrans* in an apple replant disease. Phytopathology 72:247–251
Jönsson U (2006) A conceptual model for the development of Phytophthora disease in *Quercus robur*. New Phytol 171:55–68
Leinfelder MM, Merwin IA (2006) Rootstock selection, preplant soil treatments, and tree planting positions as factors in managing apple replant disease. HortScience 41:394–401
Mai WF, Abawi GS (1981) Controlling replant diseases of pome and stone fruits in northeastern United States by pre-plant fumigation. Plant Dis 65:859–864
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, Brown J, Zhao XW, Izzo AD, Fazio G (2009) Interaction of brassicaceous seed meal and apple rootstock on recovery of *Pythium* spp. and *Pratylenchus penetrans* from roots grown in replant soils. Plant Dis 93:51–57
McCall AC, Fordyce JA (2010) Can optimal defence theory be used to predict the distribution of plant chemical defences? J Ecol 98:985–992
McGarigal K, Cushman S, Stafford S (2000) Multivariate statistics for wildlife and ecology research. Springer, New York
McKey D (1974) Adaptive patterns in alkaloid physiology. Am Nat 108:305–320
Noyer C, Thomas OP, Becerro MA (2011) Patterns of chemical diversity in the mediterranean sponge *Spongia lamella*. PLoS One 6:e20844
Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H (2012) vegan: Community Ecology Package, R package version 2.0-3. http://CRAN.R-project.org/package=vegan
Packer A, Clay K (2003) Soil pathogens and *Prunus serotina* seedling and sapling growth near conspecific trees. Ecology 84:108–119
Petkovsek MM, Stampar F, Veberic R (2009) Seasonal changes in phenolic compounds in the leaves of scab-resistant and susceptible apple cultivars. Can J Plant Sci 89:745–753
Pregitzer KS, DeForest JL, Burton AJ, Allen MF, Ruess RW, Hendrick RL (2002) Fine root architecture of nine North American trees. Ecol Monogr 72:293–309
R Development Core Team (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Reinhart KO, Clay K (2009) Spatial variation in soil-borne disease dynamics of a temperate tree, *Prunus serotina*. Ecology 90:2984–2993

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York

Tewoldemedhin YT, Mazzola M, Botha WJ, Spies CFJ, McLeod A (2011a) Characterization of fungi (*Fusarium* and *Rhizoctonia*) and oomycetes (*Phytophthora* and *Pythium*) associated with apple orchards in South Africa. Eur J Plant Path 130:215–229

Tewoldemedhin YT, Mazzola M, Labuschagne I, McLeod A (2011b) A multi-phasic approach reveals that apple replant disease is caused by multiple biological agents, with some agents acting synergistically. Soil Biol Biochem 43:1917–1927

Tewoldemedhin YT, Mazzola M, Mostert L, McLeod A (2011c) *Cylindrocarpon* species associated with apple tree roots in South Africa and their quantification using real-time PCR. Eur J Plant Pathol 129:637–651

Watt M, Silk WK, Passioura JB (2006) Rates of root and organism growth, soil conditions, and temporal and spatial development of the rhizosphere. Ann Bot 97:839–855

Wells CE, Eissenstat DM (2002) Beyond the roots of young seedlings: the influence of age and order on fine root physiology. J Plant Growth Regul 21:324–334

Wilcox HE (1968) Morphological studies of the root of red pine, *Pinus resinosa* I. Growth characteristics and patterns of branching. Am J Bot 55:247–254

Yao SR, Merwin IA, Brown MG (2006) Root dynamics of apple rootstocks in a replanted orchard. HortScience 41:1149–1155

Zadworny M, Eissenstat DM (2011) Contrasting the morphology, anatomy and fungal colonization of new pioneer and fibrous roots. New Phytol 190:213–221