FINAL PROJECT REPORT
WTFRC Project Number: AP-16-106

Project Title: Managing rhizosphere/soil microbiology via apple rootstock biochemistry

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Total Project Funding: $150,000

Other funding sources: None

WTFRC Collaborative expenses: None

Budget 1
Organization Name: **Contract Administrator:**
Telephone: **Email address:**

Item	2016	2017	2018
Salaries[1]	$30,000	$31,000	$32,000
Benefits	$10,000	$10,200	$10,400
Wages			
Benefits			
Equipment			
Supplies	$7,500	$8,300	$9,100
Travel	$500	$500	$500
Miscellaneous			
Plot Fees			
Total	$48,000	$50,000	$52,000

Footnotes: [1]Salary support is requested from 0.5 FTE of a postdoctoral research associate.

OBJECTIVES

This report summarizes results in the final year of a three-year project assessing the impact of rootstock cultivar on the soil microbiome, specifically examining root exudates and rhizosphere (root-zone) soil microbiome changes. The project objectives per the project proposal are below:

1. Characterize the effect of apple rootstock genotype on composition of the rhizosphere and orchard soil microbiome.
2. Define differences in the natural chemical compound profile produced by rootstock cultivars that differ in inhibiting deleterious (pathogenic) or attracting beneficial rhizosphere microorganisms.
3. Test the composite and independent (single compound instead of natural suite of compounds) impacts of natural chemical compounds on specific microbes or the soil microbiome to verify functional role in inhibition of deleterious microbes or attraction of beneficial microbes.
4. Determine effects of apple rootstock genotype on rhizosphere soil pH, contrasting rootstocks harboring different rhizosphere microbiomes of functional importance.

The significant **year one** findings reported in 2016, included a) defining specific root exudate metabolites differing among rootstocks G.41, G.935, M.9Nic29, and M.26, b) establishing that root exudate quantity correlates to rootstock vigor / tree size, c) delineating preliminary data regarding rhizosphere pH, and d) demonstrating that new apple seedling growth in replant soil is altered according to the genotype of the previously planted rootstock.

Significant findings for **year two** (2017) included a) determining that rootstock genotype-specific fungal rhizosphere communities differing from the no-tree soil control developed within 6 weeks after planting, b) phenolic compounds exuded from roots can inhibit pathogen growth, c) root exudates can lower soil pH, presumably due to contributions from organic acids and hydrogen ions.

SIGNIFICANT FINDINGS YEAR 3

Year three expanded upon Objectives 1, 2, and 3, to incorporate the assessment of the impact of the scion on the rhizosphere microbiome. Objective 4 was completed year two.

The significant findings for year three were:

1. In controlled environment/greenhouse studies, apple scion cultivar did not have a detectable cultivar-based effect on the root-zone microbial community during the first two seasons of growth after bud-grafting. Scions assessed were ‘Honeycrisp’, ‘Granny Smith’, and ‘G.41’, grafted onto rootstock cultivar ‘G.41’.
2. Evidence indicates that rootstocks can maintain fungal endophytes inside root tissue without symptoms of disease, even under sterile tissue culture conditions. Rootstock core fungal endophytes included genera *Ilyonectrica*, *Serendipita*, *Lasiosphaeria*, *Leptodontidum*, and *Paraglomus*. Potential beneficial biological functions are detailed in results and discussion. Rootstocks tested were ‘G.935’ and ‘M.26’.
3. In a greenhouse experiment, metabolites released by tree roots within the first growing season were shown to differ with the scion cultivar bud-grafted on the apple rootstock G.41 (even though no impact of these compounds was detected for the associated root-zone microbial community). Cultivar-based differences were more profound in metabolites with potential to inhibit pathogen growth than in metabolites that would promote overall microbial growth in

the root-zone. Scions assessed were ‘Honeycrisp’, ‘Granny Smith’, and ‘G.41’, grafted onto rootstock cultivar ‘G.41’.

4. When microbes were specifically excluded from the experiment (that is, micropropagated trees grown in sterile conditions with no natural root-zone microbial populations), more compounds that could be utilized as substrate by the rhizosphere microbiome than substrates with potential to inhibit pathogens differed between rootstock cultivars M.26 and G.935.
5. Assessment of metabolites produced by rootstocks in axenic conditions indicated that phloridzin and sorbitol are among the more abundant metabolites produced by apple roots; testing the impacts of these metabolites on the soil microbial community indicates that sorbitol has a significant effect on both the bacterial and fungal community structure, while phloridzin influenced only the fungal community structure and to a lesser extent than did sorbitol.
6. Extension roots and fibrous roots of apple rootstocks possess different phenolic compound profiles which also differ according to rootstock cultivar. Cultivars tested were ‘M.26’ and ‘G.41’.

The discussion will consider these results in the context of previous studies on the apple soil and microbiome, address how results impact future studies on the soil/rhizosphere/endosphere microbiome, rootstock breeding, and future directions.

METHODS

Finding 1: Apple scions ‘Granny Smith’ and ‘Honeycrisp’ were grafted on rootstock cultivar ‘G.41’, with ‘G.41’ grafted onto ‘G.41’ serving as a control. Root-zone (rhizosphere) soil was collected and analyzed for differences in the fungal microbial communities utilizing molecular methods. Briefly, soil DNA was extracted and the internal transcribed region of ribosomal DNA (a region commonly used to identify fungi) was specifically amplified from the soil fungal community via polymerase chain reaction. The resulting amplicons were digested with restriction enzymes and DNA fragments were subjected to terminal fragment length polymorphism (T-RFLP) analysis to establish a fungal community profile.

Finding 2: DNA extraction and molecular analysis to assess endophyte presence in axenically micro-propagated trees was performed using previously published methods [1]. Extracted DNA was subsequently submitted to a metagenomics sequencing service (Molecular Research, Shallowater, TX).

Finding 3: Nursery grown 3/8” diameter G.41 rootstock liners were planted in sterilized sand in pots and maintained under greenhouse conditions. Scions (Honeycrisp, Granny Smith, and G41) were bud-grafted onto the rootstock liners shortly after planting. Several months after grafting, root exudates were collected via a root dip method and processed similar to methods in outlined in Leisso et al. [2]. Samples were analyzed for biochemical compounds that could function either as growth substrate for microbes or could potentially inhibit microbes in the root zone.

Finding 4: M.26 and G.935 rootstock stem tissue was multiplied axenically via sterile micropropagation and treated to induce rooting (**Figure 1**). Three months after root initiation, rooted plantlets were subjected to a sterile root dip process to collect exudates. Exudates were filtered, flash frozen, concentrated, and analyzed via liquid chromatography – mass spectrometry. Full methods are detailed in a published article [1].

Figure 1. A sterilely propagated rootstock and collection of root exudate metabolites via a 'root dip' system.

Finding 5: Soil from two locations was treated periodically with phloridzin and sorbitol solutions. DNA was extracted from the soil and ribosomal DNA fragments from fungal and bacterial microorganisms were specifically amplified via polymerase chain reaction, digested with restriction enzymes, and fragments analyzed to establish a community profile (T-RFLP analysis).

Finding 6: rootstock liners ('M.26' and 'G.41') were planted in pasteurized sand and maintained in a growth chamber. Six weeks after planting trees were removed from the pots, and roots were divided into separate samples classed as either "extension" or "fibrous" roots, taking 3 samples of each root type per tree resulting in a total of 36 samples. "Extension" roots for metabolite assessment were attached to the main stem and generally larger than 1 mm in diameter; "fibrous" roots were attached to extension roots and had a greater number of branches per length and overall were smaller than 0.5 mm in diameter with a fibrous morphology. Roots were flash frozen in liquid nitrogen and metabolites expected to have inhibitory activity toward plant pathogenic organisms were analyzed. The experiment was repeated with a longer tree growth period prior to root tissue collection.

RESULTS & DISCUSSION

Significant finding 1. The apple scion did not have profound detectable effect on the fungal rhizosphere microbiome in terms of overall diversity based upon species count (**Figure 2**) or in composition (**Figure 3**) over the first two seasons of growth after bud-grafting. For brevity, data on bacteria are not shown but overall results have a similar interpretation. Scions assessed were 'Honeycrisp', 'Granny Smith', and 'G.41', grafted onto rootstock cultivar 'G.41'.

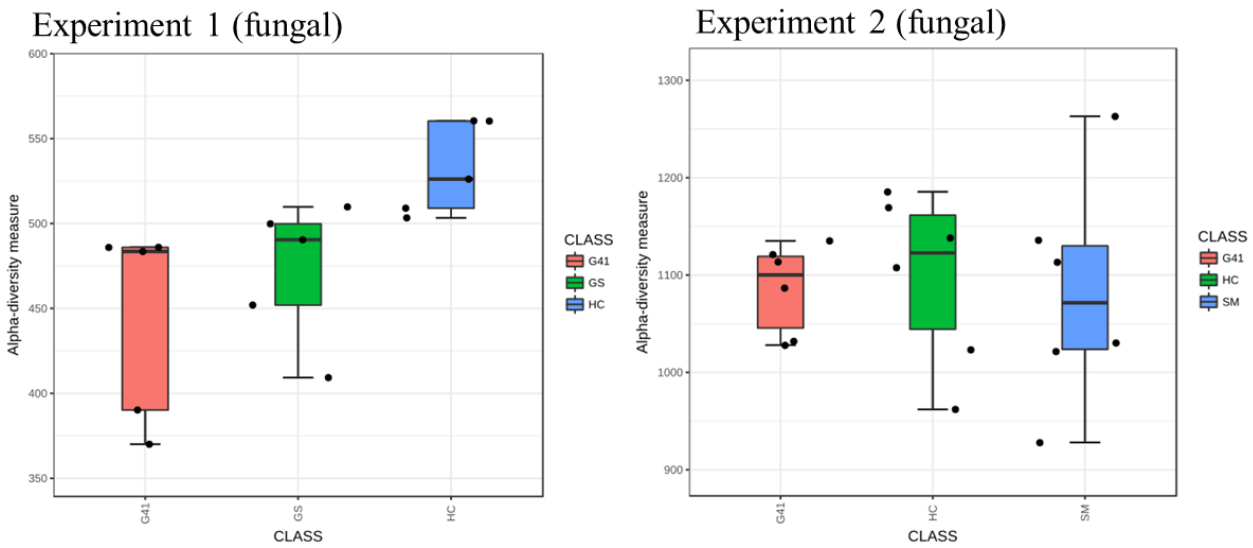


Figure 2. Fungal microbiome diversity in terms of species count.

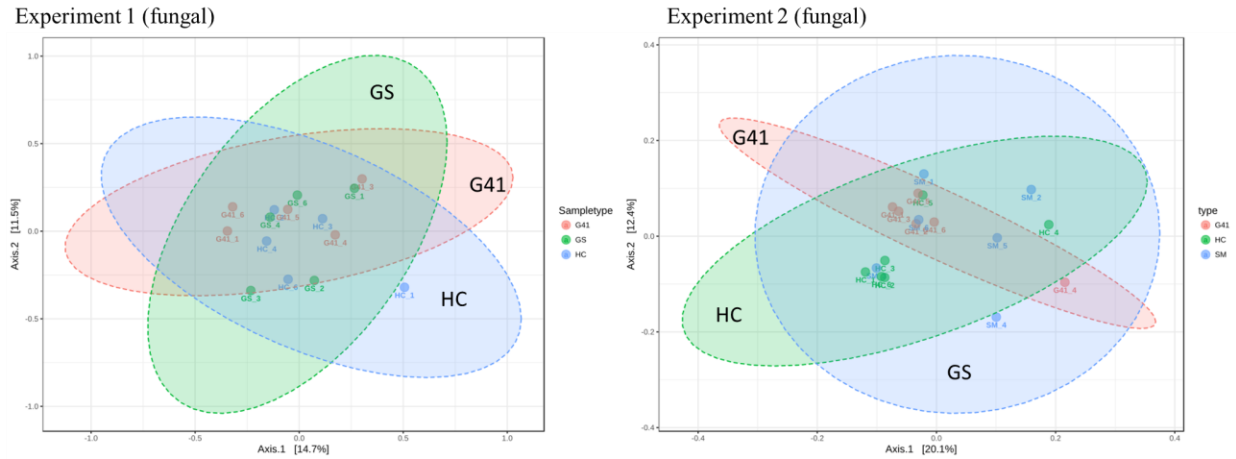
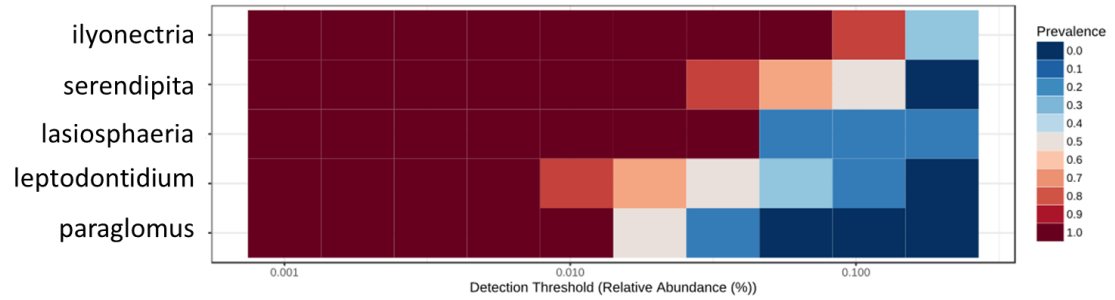


Figure 3. Fungal community composition detected in the rhizosphere of G.41 rootstock was not influenced by scion cultivar. Data represent principal component analysis of operational taxonomic unit (OTU) data derived through amplicon sequence analysis of fungal ribosomal DNA. GS = Granny Smith; HC = Honey Crisp; G.41 = G.41 as scion grafted on G.41.

Significant finding 2. Rootstock core fungal endophytes included members of the genera *Ilyonectrica*, *Serendipita*, *Lasiosphaeria*, *Leptodontidum*, and *Paraglomus*. Biological functions are detailed **Figure 4.** Rootstocks tested were G.935 and M.26.



The most common fungal genera present as endophytes in both G.935 and M.26 are indicated above. Counts of each genera and relative presence in all samples are shown: relative number of sequence reads above the detection threshold are indicated on the x-axis combined with color to indicate the prevalence of the genera in the samples assessed. Genera present in a greater number of samples are indicated in red; genera present in fewer samples are indicated in blue.

	Ilyonectria sp.	*Seredipita sp.*	*Lasiosphaeria sp.*	*Leptodontidium sp.*	*Paraglomus sp.*
Biological function	Genera include non-symptomatic root endophtyes [3] Can be plant pathogens Can be associated with diverse hosts [4]	Genera has been demonstrated to enhance biomass yield and drought tolerance [5]	Endophtyic fungus; biological function unknown [6]	Endophytic fungal genera; biological impacts of some genera include increasing root length; genera tends to be host specific [7]	Related genera can increase nitrogen and phosphorus uptake [8] More common in organically managed systems [9]

Figure 4. The core community of fungal endophytes detected by molecular methods in apple roots and respective biological function according to published literature.

Significant finding 3. Differential effects of the scion on apple root exudate compounds derived from G.41 rootstock were detectable in the first season of growth according to scion for bud-grafted apple trees. In general, total exudates were more profound in metabolites that would inhibit pathogen growth than those that would promote overall growth in the microbiome (**Figure 5**). Scions assessed were 'Honeycrisp', 'Granny Smith', and 'G.41', grafted onto rootstock cultivar 'G.41'.

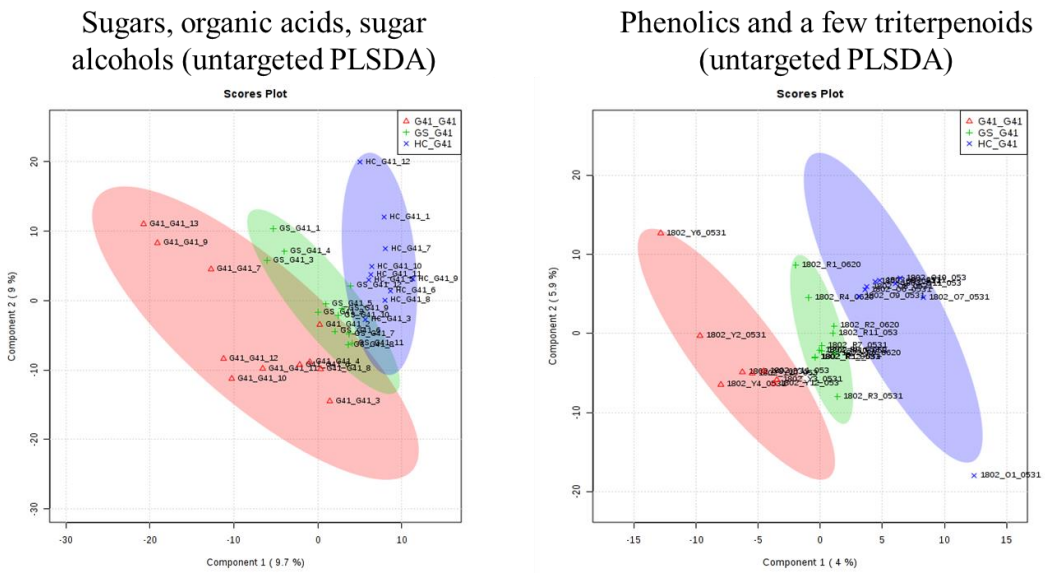


Figure 5. Apple scions had an effect on rootstock G.41 root exudate composition, however these differences did not bring about corresponding differences in composition of the rhizosphere microbiome. GS = Granny Smith; HC = Honeycrisp

Significant finding 4. In a separate experiment, where microbes were specifically excluded from the experimental system, more compounds that could feed the rhizosphere microbiome than inhibit pathogens differed between rootstock cultivars. Rootstock cultivars assessed were 'M.26' and 'G.935' (data not shown; see publication [1]).

Significant finding 5. Assessment of metabolites produced by rootstocks in axenic conditions indicated phloridzin and sorbitol are among the more abundant metabolites produced by roots; testing the impacts of these metabolites on the soil microbial community indicated that sorbitol has a significant effect on both the bacterial (**Figure 6**) and fungal community structure, while phloridzin influenced only the fungal community structure and to a lesser extent than did sorbitol.

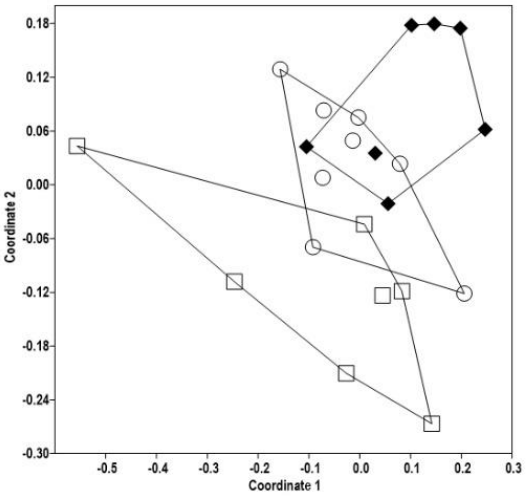


Figure 6. Effect of apple rootstock metabolites phloridzin and sorbitol on relative composition of the soil bacterial community. Phloridzin and sorbitol were added (0.5 ml) to independent orchard soil samples and bacterial community composition was determined by T-RFLP analysis. The bacterial community in sorbitol treated soil (♦) differed significantly in structure from both the control (P = 0.0021) and phloridzin treated (P = 0.0162) soil. □ = control; ○ = phloridzin; ♦ = sorbitol

Significant finding 6. Extension roots and fibrous roots have different phenolic compound profiles which also differ according to rootstock cultivar (**Figure 7**). Cultivars tested were M.26 and G.41.

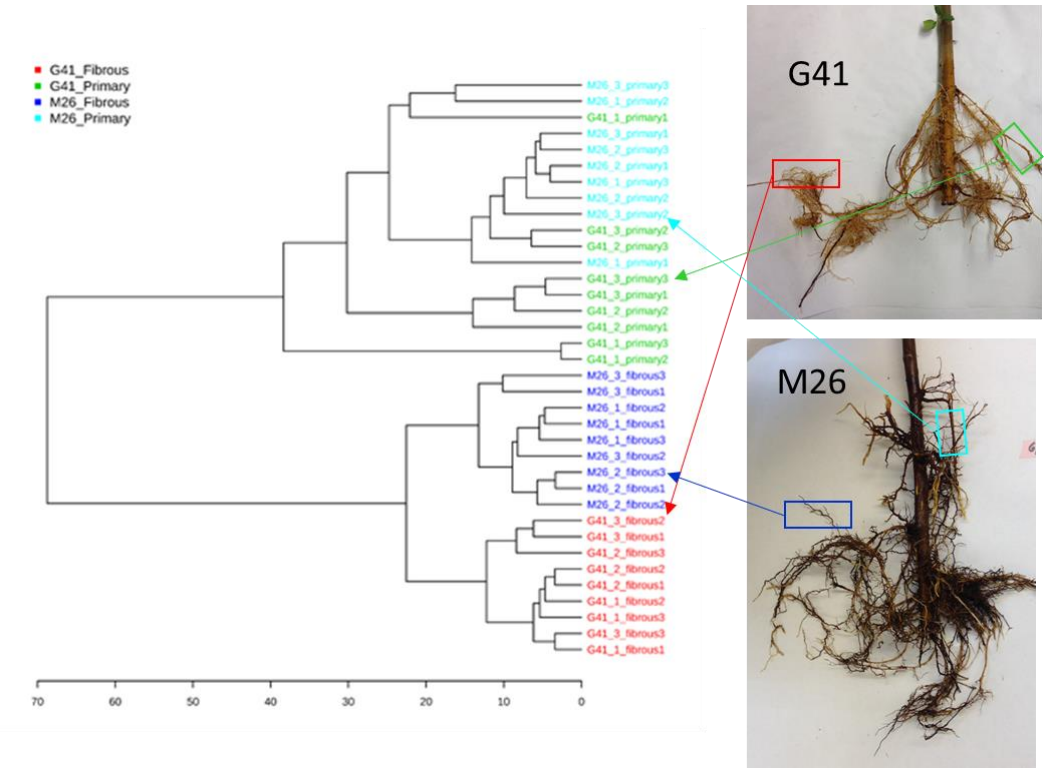


Figure 7. A dendrogram summarizes differences among phenolic root metabolite samples according to both root type (extension and fibrous) and rootstock cultivar (M.26 and G.41).

Discussion and summary

In the final year of the project, a number of experiments were completed to address project goals. *Scion* cultivar-specific impacts on the rhizosphere microbiome were not readily detected within the first two years of growth after bud-grafting. We previously established that *rootstock* cultivars do impact root-zone microbial communities [11], and that effects of rootstock cultivars on soil microbial communities carry over in the soil to affect newly planted trees [12]. The potential practical implications of the latter finding may include more intensive pre-plant treatments and post-treatment testing for soils where the previous orchard was grafted onto disease-intolerant rootstocks, e.g., many of the Malling series.

The present study indicated that the apple scion can affect root exudates qualitative and quantitatively, likely through translocation of photosynthetic products from the leaves to the roots. However, the differential metabolic composition of root exudates did not result in a detectable differentiation in composition of the rhizosphere microbiome of young trees. This remains an area where further research could lend insight into scion x rootstock cultivar compatibility, and into

tailoring scion and rootstock cultivar choices based upon knowledge of site-specific soil conditions and biology.

Endophytes (microorganism which grow within the tissues of a plant) were detected in roots of apple rootstock cultivars even when reared under axenic conditions. Cataloging and determining possible function for these microorganisms remains an area of active research. Ongoing research has demonstrated that composition of the endophytic microbiome differs in a rootstock genotype-dependent manner (Mazzola and Van Horn, unpublished). The assertion that there are additional (microorganism's) genomes, with potential impacts on tree growth, to consider when growing and obtaining trees for an orchard planting is a fascinating outcome.

Analysis of metabolic composition of extension roots and fibrous roots revealed differing levels of phenolic compounds according to both type of root and rootstock cultivar. Phenolic compounds can be involved in root disease resistance, and as it has been demonstrated that the fibrous roots are generally more involved in pathogen attack [10], this result offers a target for breeding and disease tolerance assessment.

In addition to the significant findings detailed above, results indicated that environmental factors also impact apple root exudates, both quantitatively and qualitatively. While unintentional in the present study, this result calls for continued assessment of environmental variables, especially temperature and water relations, in relation to tree and soil interactions.

REFERENCES

1. Leisso, R., D. Rudell, and M. Mazzola, *Targeted Metabolic Profiling Indicates Apple Rootstock Genotype-Specific Differences in Primary and Secondary Metabolite Production and Validate Quantitative Contribution From Vegetative Growth.* Frontiers in Plant Science, 2018. **9**(1336): p. 15.
2. Leisso, R., D. Rudell, and M. Mazzola, *Metabolic composition of apple rootstock rhizodeposits differs in a genotype-specific manner and affects growth of subsequent plantings.* Soil Biology and Biochemistry, 2017. **113**: p. 201-214.
3. Chaverri, P., et al., *Delimitation of Neonectria and Cylindrocarpon (Nectriaceae, Hypocreales, Ascomycota) and related genera with Cylindrocarpon-like anamorphs.* Stud. Mcyol., 2011(68): p. 57-78.
4. Toju, H., A.S. Tanabe, and H. Sato, *Network hubs in root-associated fungal metacommunities.* Microbiome, 2018. **6**(1): p. 116.
5. Ray, P., et al., *Non-targeted Colonization by the Endomycorrhizal Fungus, Serendipita vermifera, in Three Weeds Typically Co-occurring with Switchgrass.* Frontiers in Plant Science, 2017. **8**: p. 2236.
6. Ma, X., et al., *Non-mycorrhizal endophytic fungi from orchids.* Curent Science, 2015. **109**(1): p. 36-51.
7. Fernando, A.A. and R.S. Currah, *A comparative study of the effects of the root endophytes Leptodontidium orchidicola and Phialocephala fortinii (Fungi Imperfecti) on the growth of some subalpine plants in culture.* Canadian Journal of Botany, 1996. **74**(7): p. 1071-1078.
8. George, E., et al., *Water and nutrient translocation by hyphae of Glomus mosseae.* Canadian Journal of Botany, 1992. **70**(11): p. 2130-2137.
9. Gosling, P., et al., *Distribution and diversity of Paraglomus spp. in tilled agricultural soils.* Mycorrhiza, 2014. **24**(1): p. 1-11.
10. Emmett, B., et al., *Fine-root system development and susceptibility to pathogen colonization.* Planta, 2013: p. 1-16.

11. Wang, L., and Mazzola, M. 2018. *Interaction of Brassicaceae seed meal soil amendment and apple rootstock genotype on microbiome structure and replant disease suppression*. Phytopathology doi/pdf/10.1094/PHYTO-07-18-0230-R
12. Mazzola, M. and Hewavitharana, S. S. 2018. *Advances in understanding tree fruit-rhizosphere microbiome relationships for enhanced plant health*. Chapter 1, *In*, Achieving sustainable cultivation of temperate zone tree fruits and berries. Burleigh Dodds Science Publishing. Cambridge, UK.

Executive summary: Managing rhizosphere/soil microbiology via apple rootstock biochemistry

This research program addressed three priorities outlined in the 2016 Apple Horticulture and Postharvest research needs assessment: i.) Understanding and management of soil health and productivity in conventional and organic systems (critical priority) and ii.) Soil health and productivity – Interaction of rootstocks with rhizosphere microbiology (high priority) and contributes to additional priorities including iii.) apple replant (high priority) and iv.) improved scion and rootstock genetics (medium priority).

Results contributed to addressing all of the targeted priorities. The project determined that environmental factors, as well as scion genotype and vigor impact apple root exudates, both quantitatively and qualitatively. Additionally, the project demonstrated that rootstock genotype is a critical factor in determining composition of the rhizosphere microbiome, which has implication for functional activities of these microbes and their effects on tree growth and health, including the inhibition of pathogens by certain phenolic compounds. Effects of the scion on composition of the rhizosphere microbiome are not consistently detectable, at least when examined using rootstock liners over the first two seasons of growth under controlled environment conditions. Rootstock cultivar choices also influence soil pH, an attribute reported to be a primary determinant of bacterial community composition. Rootstock vigor impacts the quantity of exudates added to the soil, with more vigorous rootstocks releasing more exudates. Root exudate metabolic profiles can differ among both scion and rootstock genotypes in several biochemical classes, including phenolic compounds, sugars, sugar alcohols, organic acids, amino acids, and triterpenoids.

Practical implications include:

1. When considering orchard planting decisions, rootstock has greater immediate impact on soil / tree interface, but the scion can impact compounds released through the roots into the soil. The full implications of the latter remain to be discerned.
2. In an orchard replant situation, the previous rootstock cultivar can impact soil biology, with apple replant disease susceptible cultivars (including many of the Malling series) leading to a more “pathogen-rich” microbiome.
3. Future research could assess the efficacy of tailoring rootstock decisions to site-specific conditions including soil type, soil biology, general climactic trends regarding temperature and precipitation; a subtext to this vision is further defining rootstock cultivar characteristics according to their optimal growth and health conditions.
4. Rootstock metabolite contrasts among genotypes can inform apple rootstock breeding programs either for disease tolerance or for supporting a beneficial microbiome.
5. The knowledge that endophytes persist in apple trees opens further questions regarding their influence on tree nutrient sequestration, growth, and transmission mechanisms, especially from a nursery production perspective.