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Indirect Impact of Soil Microbial Communities on Plant-Aphid Interactions

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INDIRECT IMPACT OF SOIL MICROBIAL COMMUNITIES ON PLANT-APHID INTERACTIONS

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ABSTRACT

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The rhizosphere is a unique ecosystem consisting of microbial communities that have complex signaling pathways, which can influence the biological functions of plants. The community dynamics of these micro-environments are influenced by root, fungal, and bacterial exudates that can preferentially select for functional classes of microbes. Arbuscular mycorrhizal fungi (AM) fungi and plant growth promoting rhizobacteria (PGPR) are members of this community that have been extensively studied due to their ability to form symbiotic relationships with plants and play an important role in triggering induced systemic resistance (ISR), resulting in defensive "priming.' Consequently, 'primed' plants can activate stronger and faster defense responses to future attacks by pathogens and insects. The biological system in this study involved four genotypes of barrel medic plants (*Medicago truncatula*), pea aphids (*Acyrthosiphon pisum*), and microbial communities present in three field-collected soils and one commercial topsoil. In the first experiment, wild-type (WT) A17 *M. truncatula* plants grown with an inoculant harvested from a *M. sativa* field demonstrated lower aphid colony weight than the commercial Pioneer topsoil ($p = .0205$), indicating that the resident plant community of this soil was effective in eliciting defensive priming. In the second experiment, WT A17 *M. truncatula* and mutants *Mtdmi1*, *Mtdmi3*, and *Mtram1* were used to investigate the role of microbial symbionts in plant-aphid interactions. Although there were no observed differences in

aphid colony weight between genotypes, treatments with active microbial communities did have significantly lower aphid colony weight than autoclaved treatments ($p < .0001$). This combined with diversity indices in the rhizosphere and root endosphere indicated that functional microbes were more important than richness of microbes and were a greater driver of aphid resistance in *M. truncatula*. Together, these experiments demonstrated a significant effect of the soil microbial community on plant-aphid interactions.

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CHAPTER I

LITERATURE REVIEW

Aims

This research detailed the role of soil microbial diversity in plant insect resistance with a focus on functionally symbiotic rhizosphere microbes. Two separate experimental designs were implemented to complete the objectives of this study. The first system, designed to investigate the impact of rhizosphere microbial diversity on above-ground insect herbivory and plant growth, involved barrel medic (*Medicago truncatula*) plants grown with different soil inoculants and infested by pea aphids (*Acrythosiphon pisum*). The second system was designed to investigate the role of functionally symbiotic microbes within a single soil inoculant on insect resistance using four genotypes of *M. truncatula* with varying capacities to form a symbiosis with arbuscular mycorrhizal (AM) fungi and/or plant growth-promoting rhizobacteria (PGPR). The topsoil collected and used in this study included two agricultural soils where corn (*Zia mays*) and alfalfa (*Medicago sativa*) were grown organically, one grassland soil with similar soil texture characteristics as the agricultural soils, and one topsoil from a commercial sand company (Pioneer Sand Company: Landscape Supply Materials, Windsor, Colorado). All four topsoils were used as inoculants in the first system, while the commercial topsoil was the only one used in the second system. The *M. truncatula* genotypes include Jemalong A17 wild type (WT) and three mutants: required for arbuscular mycorrhization 1 (*ram1*), does not make infections 1 (*dmi1*), and *dmi3*. These two experimental systems were designed to explore plant responses to the community of bacteria and fungi present within an agricultural and commercial topsoil. The

use of the soil microbiome provides a real-world application and advocates for soil management practices that culture beneficial microbes to promote plant health.

These two experimental systems were used to evaluate the following objectives and hypotheses:

O1 Evaluate plant growth and plant interactions with aphids as they are impacted by microbes from field-collected soil inoculants.

H1 Soils with high bacterial diversity and/or high fungal diversity promote plant defense responses against aphids resulting in lower aphid fitness.

H2 *M. truncatula* plants will benefit from the soil microbial communities that are associated with the most closely related plants (*M. sativa*).

Each soil inoculant was unique in its rhizosphere microbial community as a result of the different plant communities and geologic area from which they were collected. The differences in dominant fungal or bacterial operational taxonomic units (OTUs) could indirectly impact plant induced systemic resistance (ISR), which was quantified as aphid colony weight per plant. The soil microbial community that *M. truncatula* inherited from the *M. sativa* inoculum might have more compatible plant growth promoting rhizobacteria (PGPR), which would confer a greater phenotypic response associated with PGPR including greater plant growth and a more robust ISR (1, 2). Each soil inoculant had an autoclaved control to assess if these impacts were the result of the inherent microbial communities. The internal transcribed spacer deoxyribonucleic acid (DNA) region between ribosomal subunits was amplified and quantified using automated ribosomal intergenic spacer analysis (ARISA) to assess diversity and richness for each treatments' rhizospheric soil.

O2 Assess the impact of loss of symbiosis-related genes on plant growth and aphid herbivory grown in plants grown in active or autoclaved soil.

H3 *M. truncatula* mutants unable to form symbiosis with AM fungi and/or rhizobia will exbibit less plant growth compared to wild type plants when grown in active soil.

As plants lose the ability to form functional symbioses with rhizobia and/or AM fungi, less plant growth will be observed due to their diminished nutrient uptake through the symbiosis pathway. *M. truncatula* wild type A17 can form symbioses with rhizobia and AM fungi, resulting in greater plant growth parameters. This effect is predicted to be especially prevalent in the comparison of wild type and *Mtdmi3*, which is unable to form symbioses with rhizobia and AM fungi.

H4 Aphid colony weight between mutant *M. truncatula* will be ranked from lowest to highest according to ability to form a symbiosis with wild type A17 as the lowest, then *Mtram1*, *Mtdmi1*, and *Mtdmi3* as the highest.

H5 Endophytic bacterial and/or fungal diversity is a greater driver of plant resistance against aphids than rhizospheric bacterial and/or fungal diversity.

I predicted *M. truncatula* wild type would have the greatest resistance to aphid herbivory as a result of ISR triggered by bacterial and fungal symbionts, and systemic acquired resistance (SAR) triggered by pathogenic microbes, although these pathways were not directly tested in this study. As the ability to form a symbiosis was degraded, so too would plant defensive capabilities against aphids. *Mtdmi3*, being unable to form AM and rhizobial symbioses, would only have the inherent plant defensive capacity. An insect herbivory study was conducted on each genotype and combined with genetic fingerprinting of bacteria and fungi using ARISA of surface-sterilized root tissue and of rhizosphere soil to determine which group of symbiotic microbes had the greatest positive or negative impact on aphids and plant growth.

Rhizosphere Plant Interactions

The rhizosphere is a ubiquitous term used to describe the ecosystem of microbial communities that are stimulated or inhibited by root secretions and/or root senescence and

sloughing, i.e., rhizodeposition (3, 4). Up to 40% of plant photosynthetic production is exuded and deposited as primary (carbohydrates and organic acids), or secondary (phenolics and flavonoids) metabolites (3, 5, 6). The composition of exudates from both microbes and plants can vary dramatically between different species and genetic variants of the same species, which have direct impacts on the abundance and composition of rhizosphere microbes (7-10). For example, exudates from alfalfa (*Medicago sativa*) roots have been shown to influence the abundance of common soil bacteria (7), genetic variants of cherry tomatoes (*Solanum lycopersicum*) produce unique exudate profiles that altered communities of fungi and bacteria in the rhizosphere (8), and symbiotic rhizobia and fungi have been shown to alter root exudates in multiple plant species through changes in their symbiotic carbon allocation (9). The interaction of plant and microbes has also been demonstrated in corn (*Zea mays*) inoculated with the PGPR *Azospirillum brasilense* that formed a synergistic loop in which root exudates changed to benefit the bacteria, thereby increasing bacterial abundance and the root growth promoting effect (10). The concentration of these metabolites exists as a gradient stemming from the roots (11), the extent of which defines the zone of the rhizosphere within the soil. For the purpose of this literature review, the rhizosphere is further subcategorized in terms of microbial proximity to plant roots and includes the endosphere, rhizoplane, and ectorhizosphere. The endosphere involves the cell layers of the plant itself and consists of bacterial and fungal endophytes (12). The term endophytes can have a variety of contexts, and the definition adopted by Overbeek and Saikkonen is a microbe living within the plant without causing harm, which consist of symbionts, commensals, or even pathogens and saprotrophs if in an asymptomatic state (12). The rhizoplane refers to the plant root – soil interface where bacterial and/or fungal cells or biofilms and loosely adhered soil surround plant roots, and where concentrations of root exudates

are highest (11, 13-15). The outermost zone is the ectorhizosphere and includes microbial populations at the rhizoplane interface to the bulk soil not directly adhered by the root, but within close enough proximity of the root that the community assemblage is influenced by root exudates (16-19).

The community dynamics of bacteria and fungi in these micro-environments are important factors in plant growth, resistance to environmental stress, and plant resistance to pathogens and herbivores (3, 14, 20-23). Arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPR) are members of the rhizosphere community that have significant ecological and economic value due to their functional role in plant physiological health that translate to identifiable benefits through agricultural practices (11, 23-27). These bacteria and fungi pervade the rhizosphere where they communicate and interact with plant cells via symbiotic structures, such as AM fungal arbuscules or rhizobacterial nodules (endosphere), and the root cell wall interface (rhizoplane and ectorhizosphere) (3, 5, 11, 12, 28, 29). Together, AM fungi and PGPR are some of the most prevalent organisms on the planet. For instance, AM fungi in the class Glyomeromycetes form a symbiosis with at least two-thirds of all known plant species (30) and genera of PGPR within the phyla Actinobacteria (31, 32), Proteobacteria (33), and Acidobacteria (34) are not only affiliated with a variety of plants, but have been shown to be some of the most abundant bacteria in the rhizosphere (35-39). Some PGPR species, such as *Azospirillum brasilense*, are used as biofertilizers to improve plant nutrient uptake (10), whereas others, such as *Pseudomonas putida* may be used as a biocontrol agent that boosts, or primes, a plants defense immune system (40). Elsewhere, AM fungi species have been shown to transmit defensive signaling among connecting plants (41), and have been used as biofertilizers in conjunction with PGPR to improve sugar and protein content of fava bean and wheat crops (42).

Molecular Activators of Systemic Acquired Resistance

Systemic acquired resistance (SAR) is a broad-spectrum response that is activated when plant tissue is damaged by a pathogen, creating a sustained resistance towards future attacks (43-45). The term SAR was first used by Frank Ross in studies involving tobacco mosaic virus (TMV), which highlighted increased resistance in plant tissues immediately surrounding a TMV lesion (46). In his experiments, Ross discovered that inoculation of tobacco (*Nicotiana tabacum*) L. var. Samsun NN leaves with dilute concentrations of TMV resulted in decreased numbers of lesions and distance between lesions when inoculated a second time, and both of those parameters decreased further when the days between the initial and challenge inoculation was increased (46). A breakthrough in the understanding of SAR signaling came when R.F. White demonstrated that Samsun NN tobacco leaves infected with TMV exhibited a reduction in lesions when treated with aspirin (acetylsalicylic acid) and salicylic acid (SA) (47), eventually leading to the identification of SA as an activator of SAR genes upon accumulation in damaged plant tissue (47, 48).

A review of SAR genes by Ward et al. outlined a number of pathogenesis-related (PR) genes and proteins that were associated with the onset of SAR (49). These genes have since been associated with pathogen-, microbe-, herbivore- or damage- associated molecular patterns (PAMPs, MAMPs, HAMPs, or DAMPs, respectively), of which analogs can be found across plants and animals (50-56). The PAMPs, MAMPs, and DAMPs can be in the form of proteins, lipids, and/or carbohydrates and are detected by pattern recognition receptors (PRRs) on the cell surface (rhizoplane and/or endosphere) (57-60). For instance, defensive gene expression in *Medicago truncatula* was induced when exposed to glucan-chitosaccharides isolated from the cell wall of the fungal pathogen *Aphanomyces euteiches* (57). In addition, monoterpene DAMPs

isolated from volatile organic compounds (VOCs) emitted from *Arabidopsis thaliana* infected with the bacterial pathogen *Pseudomonas syringae* were used to induce SAR in other plants (61). The first line of plant defense against pathogens in the rhizosphere occurs when the plant detects PAMPs or MAMPs called PAMP-triggered immunity (PTI), which is essentially stimulation of PRRs (62). However, some pathogens are able to bypass or suppress PTI by inhibiting or altering PRRs through effectors, pathogenic proteins that are secreted into plant cells in order to suppress or disrupt plant defensive hormone signaling (63-66). A secondary plant immune response called effector-triggered immunity (ETI) may then be triggered and can be much more specific to the pathogen effector, creating an evolutionary arms race between plants and their pathogens (45, 62, 65, 67). Both PTI and ETI share signaling pathways (68-70) and can be characterized by an accumulation of SA in tissues in the case of biotrophic pathogens (45, 71) and jasmonic acid (JA) and ethylene (ET) in the case of necrotrophic pathogens and insects (72, 73), with ETI generally inducing a stronger and more persistent response (69, 74). Activation of SAR begins with the monomerization of non-expressor of pathogenesis-related (NPR) proteins, which are transcription factors for SAR genes (75, 76). These NPR proteins increase in concentration with the accumulation of SA in local tissues and regulate SA in distal tissues (67, 75-77).

The regulation SA-dependent resistance may be important for the overall health of the plant since an individual plant may be attacked by an insect herbivore as well as a pathogen, and the defenses employed by the SA pathway are not effective against many guilds of insect herbivores (78-81). Pathogens and insect herbivores may even exist in a symbiotic relationship (82-84) that accelerates the dispersal of both (85). Some pathogens target disruption of SA-regulated defenses through induction of the ET pathway (85), which suppresses SA signaling (86) and/or disrupts the

JA defensive signaling pathway (83) important for activation of defense against insect herbivores (82, 83, 85). A major plant hormone disruptor is coronatine (COR), which is produced by many *P. syringae* pathovars (pv) as a JA conjugate mimic, effectively antagonizing the SA pathway and reducing plant resistance to the pathogen (58, 87-89). Manipulating these disruptors can be used to elucidate the mechanisms of pathogen induced plant susceptibility to insect herbivores. For instance, *A. thaliana* susceptibility to *Trichoplusia ni* herbivory may be elicited in plants through MAMPs that trigger SA and antagonize JA, and by effectors produced by coronatine-deficient *P. syringae* (pv) *tomato* that induce ET signaling and interfere with antiherbivore defenses (85). Pathogenic microbes in the rhizosphere are not the only ones that can elicit a defensive response; beneficial microbes are also capable of eliciting defensive priming (90) through MAMPs that accelerate the response to pathogens and insect herbivores through JA and ET pathways known as induced systemic resistance (ISR) (45, 91).

Molecular Activators of Induced Systemic Resistance

Defense mechanisms against insect herbivores and pathogens can be metabolically and/or ecologically costly, with the plant potentially sacrificing pathogen defense for insect herbivory defense, or photosynthates for alkaloids or protease inhibitors (92-95). This cost may be offset if the plant is in a primed state (increased alertness) without fully expressing genes associated with defensive traits, but can react quickly to stress, pathogens, and/or insect herbivory compared to unprimed plants (72, 90, 96, 97). In primed plants, the metabolic energy necessary for defense is centered around gene expression rather than the synthesis or activation of phytohormones, phytoanticipins, and phytoalexins that can increase fitness costs (72, 98, 99). The mechanisms of SAR are an example of priming after infection of a pathogen, or induction with PAMPs, which is driven by the SA pathway (44, 100). In some cases, activation of SA-dependent pathways

leading to SAR may be elicited by PGPR and non-pathogenic microbes through PAMP-triggered PTI (101, 102). For instance, Niu et al. found that *Bacillus cereus* strain AR156, a PGPR, elicited SAR against the pathogen *Pseudomonas syrnigae* pv. tomato in *Arabidopsis* through SA-dependent expression of NPR proteins (101). Similarly, the PGPR *Brevibacterium iodinum* KUDC1716 was demonstrated to induce expression of pathogenesis related (PR) proteins in pepper plants (*Capsicum annuum* L) leading to resistance against the pathogen *Stemphylium lyopersicis* (102). Conversely, induced systemic resistance (ISR) is often elicited by non-pathogenic microbes such as PGPR and AM fungi, and although some species of PGPR elicit an SA-mediated response (101-103), ISR is often referenced in the context of SA-independent pathways i.e. the JA/ET pathways (45, 104, 105). The variety of diseases that ISR and SAR are effective against does not highly overlap, but together they can provide a broad spectrum of resistance (106). Although the terms SAR and ISR are officially synonymous (45), for pragmatic reasons we refer to SAR when the induced resistance is triggered by a pathogen or demonstrated to be SA dependent and to ISR when the induced resistance is triggered by a beneficial microbe or demonstrated to be SA independent.

The identification of SA-independent defensive pathways described in ISR was discovered using transgenic tobacco plants that were transformed with the nahG gene from the PGPR *Pseudomonas putida*, which encodes salicylate hydroxylase (107, 108). These transgenic nahG tobacco plants could not synthesize SA and were originally used to demonstrate that SA was necessary to induce SAR (107). Other experiments involving the PGPR *Pseudomonas flourescens* strain WCS417r and transgenic nahG *A. thaliana* demonstrated a SA-independent systemic resistance pathway exists (109). It was found that the JA/ET pathway was involved in the ISR elicited by WCS417r *P. flourescens* using *A. thaliana* mutants compromised in JA or ET

(105). Since then, a number of studies have unveiled the mechanisms in which PGPR and AM fungi activate ISR and has been extensively reviewed elsewhere (45, 72, 77, 104, 110). Both AM fungi and PGPR produce extracellular MAMPs, such as lipopolysaccharides (64, 111) and flagellin (58, 60, 91), that are recognized by PPR and may trigger PTI similar to PAMPs, and use similar strategies as pathogens to overcome the plant immune response and form a symbiosis (72, 112). It was found that three species of *Pseudomonas* comprised of a pathogen, an opportunist, and a commensal employ a flagella regulator that inhibit flagellin synthesis to evade PTI in the close relative of tobacco *Nicotiana benthamiana*, and *A. thaliana* (112). Cross-talk between the plant and symbiont, often involving microbial lipochitooligosaccharides (LCOs) and plant strigolactones and flavonoids is necessary to further establish the symbiosis (45, 111, 113-115). These signaling pathways originated in the evolution of AM fungi-plant symbiosis within the phylum Glomeromycota (116) in which LCOs produced specifically by mycorrhizal fungi (myc-LCOs also known as Myc factors or Sym factors) induce the formation of a pre-penetration apparatus by the plant (111, 117). In the legume-rhizobacterial symbiosis involving *M. truncatula* and *Rhizobium meliloti*, these LCOs are known as nodulation (Nod) factors that are species-specific and are perceived by plants and induce the formation of infection threads that guide the bacteria to nodule primordium cells (111, 118-120). The homology in activated gene pathways in which AM fungi and PGPR enhance plant defenses gives further evidence for the need of a community approach when considering soil and crop health. Advanced knowledge of these pathways will someday identify the systemic signaling molecule involved in ISR and may be exploited for sustainable agriculture which has remained elusive (45, 72, 121).

Induced Systemic Resistance Mediated by Arbuscular Mycorrhizal Fungi and Plant Growth-Promoting Rhizobacteria Against Aphids

There are numerous examples of ISR mediated by AM fungi and PGPR against aphids, but the identification of specific PGPR and AM fungi were not conducted and these instances are beyond the scope of this review. Rather, this section briefly describes the defenses elicited by plants against aphids and the dichotomy that exists within AM fungi and PGPR mediated ISR. While plants colonized with AM fungi have enhanced resistance to generalist and root-feeding insects (122), the effect on specialist insect herbivores is variable and may even benefit the insect (123, 124). The defenses associated with the SA pathway involve increased concentrations of defensive metabolites in leaf tissue, which is ingested by generalist and root feeding insects that generally exhibit chewing feeding behavior (20, 125). Aphids, however, use a specialized mouthpart called a stylet to follow a sugar gradient to phloem cells, and do not pierce any other plant cells that would elicit a SA dependent defensive response (123, 126). This points towards induction of JA by the plant in response to aphid herbivory as the main defensive signaling hormone for aphid resistance (127-129). In fact, aphid saliva may also contain effectors that modulate the plant immune system through JA/SA antagonism similarly to the pathogens associated with the aphid microbiome described above (128). By avoiding defensive metabolites in plant tissue, aphids can directly benefit from the increased nutritional status that is gained by AM fungi colonization (123, 125). For instance, in the tripartite plant-microbe-insect interactions involving broad bean (*Vicia faba* L), a mixture of several AM fungal inocula, and pea aphids (*Acyrthrosiphon pisum*) lead to increased attractiveness to pea aphids by AM fungi through host location via an increase in VOCs, and in turn suppressed AM fungi symbiosis through aphid herbivory (130). In contrast, *Epichloë* fungal symbionts reduced plant VOC

emissions and inhibited population growth of the generalist aphid *Rhopolasiphum padi* (131). The positive, or negative, interactions between rhizosphere microbes and aphids advances the argument that microbial diversity is crucial for inducing a robust systemic resistance (122, 132). For example, *M. truncatula* and rice (*Oryza sativa*) demonstrated aphid susceptibility when inoculated with AM fungi, and aphid resistance in canola (*Brassica napus*) and wheat (*Triticum aestivum*) (122, 133-135). In another study, Tétard-Jones et al. found that a rhizobacteria supplementation (*Pseudomonas aeruginosa* 7NSK2) on barley (*Hordeum vulgare*) had a negative or positive impact on English grain aphid (*Sitobion avenae*) population size depending on the plant genotype (132). The susceptibility of aphids with symbionts may be dependent on abiotic factors as well as demonstrated by Wang et al. (2020) who found variations in grain aphid (*Sitobion avenae*) that correlated with AM fungal colonization in winter wheat (*T. aestivium* L.) according to the ratios of phosphorous and nitrogen (136). Conversely, Wilkinson et al. found no difference in *S. avenae* abundance in barley (*Hordeum vulgare*) inoculated with AM fungi and grown with or without access to supplemental nitrogen (137). To further understand how beneficial microbes can elicit robust defensive signaling, tripartite plant-microbe-insect interactions must be taken in the context in which they exist in natural and agricultural systems.

Rhizosphere Microbial Diversity Drives Plant Growth and Induced Systemic Resistance

Bacteria and fungi, whether symbiotic or not, release exudates that, in combination with plant exudates and abiotic soil properties, shape the microbial community through a resource supply (exudates) that can disproportionately favor one species (3, 5, 138-140). For instance, *A. thaliana* inoculated with *Pseudomonas putida* KT2440 demonstrated ISR against *P. syringae* pv. *tomato* DC3000 via an extracellular haem-peroxidase (PP2561) that also produced unique root

exudate patterns compared to uninoculated plants and functioned in competitive colonization by the beneficial rhizobacteria (141). In another example, probiotic rhizobacteria in the root microbiome of *A. thaliana* were not only resistant to scopoletin, an antimicrobial root exudate associated with iron mobilization, they demonstrated ISR through elicitation of the MYB72 transcription factor that regulates scopoletin (91). Root exudate patterns may even change in the presence of pathogenic soils to recruit symbionts for protection. In a study by Berendsen et al. (2018), a consortium of three biofilm-forming rhizobacteria capable of ISR in *A. thaliana* against powdery mildew (*Hyaloperonospora arabidopsidis*) was recruited after infection by the mildew (142). Interestingly, the ISR against *H. arabidopsidis* was not significant with individual inoculation of the three rhizobacteria isolates indicating a synergistic community relationship that benefited plant growth and disease resistance (142). Understanding these plant-microbe interactions in the community spectrum is important since the degree of ISR is dependent on the specificity and diversity of the microbes present in the soil surrounding the plant (143-146).

Although many three-way plant-microbe-pathogen/herbivore studies focus on the mechanisms of individual microbial species, numerous efforts are now being made to understand these mechanisms in consortiums of inoculants. In another study involving a mixed inoculant of bacterial isolates previously found to increase plant growth (including several *Pseudomonos* species) demonstrated that sorghum (*Sorghum bicolor*) inoculated with the consortium of these isolates performed better against charcoal root rot (*Macrophomina phaseolina*) than any single isolate (147). This was also seen in *Nicotiana attenuate* in which a consortium of five bacterial isolates were more successful against pathogenesis by the fungi *Fusarium* sp. U3 and *Alternaria* sp. U10 than individual inoculants or consortiums missing one to two of the five species (148). A subsequent study showed that the consortium of five bacterial isolates conferred ISR via

complementary traits including biofilm formation, siderophore production, and production of antifungal compounds (149). The translation of consortium inoculations to field-based studies is crucial for the exploitation of these microbes for agricultural use. For instance, a field study by Raklami et al. demonstrated that a consortium of PGPR, rhizobia, and AM fungi inoculants improved growth in faba bean (*V. faba* L.) and wheat (*Triticum durum* L.) compared to separate inoculations of these symbiotic groups (42). Similarly, Nidhi et al. found that a mixed inoculation of a PGPR (*Exiguobacterium oxidotolerans*) and AM fungi (*Glomus fasciculatum*) combined with vermicompost fertilizer improved plant growth in wild mint (*Mentha arvesis* L) more than any singular use or combination of these amendments, with similar results between greenhouse and field studies (150). Another field study involving paricà (*Schizolobium parahyba* var. *amazonicum*) demonstrated similar results in which mixed inoculations of PGPR and AM fungi combined with fertilization improved plant growth (151). Many studies involving consortiums of inoculants are also being tested against insect herbivory.

Recent information has shown that a microbial community can further influence, or be influenced by, insects feeding on spatially relevant plants (41, 130, 152-155). The degree of this influence on plant-insect interactions has also been linked to bacterial diversity (134). For instance, a field investigation by Naeem et al. found that bread wheat (*Triticum aestivum* L.) inoculated with a consortium of *Bacillus* sp. strain 6 and *Pseudomonas* sp. strain 6K resulted in the lowest aphid population than inoculation either species alone (134). Similarly, a comparison of corn (*Zea mays* var Jacobsen 4704) inoculated with single species PGPR and blends of PGPR (blend 8 and blend 9) altered corn VOCs and resulted in significantly lower egg deposition by the European corn borer (*Ostrinia nubilalis*) (156). Although the blends of PGPR did not have significantly lower egg deposition compared to the single species inoculant in a choice

comparison of all three inoculants and the control, the two blends did demonstrate lower egg deposition when compared in choice comparisons between control and one of the inoculants alone (156). A study involving the inoculation of mustard (*Brassica juncea*) with single species and consortiums of AM fungi and PGPR demonstrated ISR via increases in oxidative stress enzymes in AM fungi-inoculated plants and AM fungi/PGPR- inoculated plants, but with variable results in PGPR-inoculated plants alone (157). In another study involving *A. thaliana* and a variety of managed (corn and potato) and unmanaged (*Arabidopsis* and pine) soil inoculum found that the soils with lowest beet armyworm larval weight were from managed soils, namely potato soils with among the highest bacterial diversity and richness, potentially indicating a correlation between ISR and bacterial richness in lieu of functional groups that were found in unmanaged *Arabidopsis* soil (153). A single plant species that dominates a soil, such as a monoculture crop, has the potential to alter the microbial community for plant biological function over time, resulting in increasing ISR capability and providing a potential explanation for greater resistance in managed potato soils compared to conspecific soils (153, 158, 159). The rhizosphere microbial community is dynamic and cultivating a diverse and biased community must take into consideration the land use history of the soil, including pesticide use and crop rotation techniques.

Toward Development of a Functional Rhizosphere Microbiome

A functional rhizosphere can be cultured through crop rotation, selection practices, and fertilization (158, 160, 161) that may increase plant yield through plant growth promotion and defensive priming against pathogens and insect herbivores (16, 25, 162). Modern agriculture is centralized on obtaining maximum plant yield, but has been shown to significantly decrease rhizosphere microbial diversity (163-167). Numerous studies have pointed toward detrimental

effects of modern agriculture such as tillage (36, 165, 168), over-cropping (164), monoculture (169), and herbicide application (166, 167) on AM fungi and PGPR. However, rotation with pulse crops, such as alfalfa (163, 170), and management practices that focus on the evolutionary availability of plant nutrients, such as grazing after harvest and no-till practices (171), can restore non-productive soils towards greater resiliency to extreme weather, disease, and insect herbivory. For example, a rotation sequence can influence beneficial microbes, such as promoting the abundance of Glomeromycota by preceding with sunflower (*Helianthus annus*) or maize (*Zea mays*) (172). The effect of conventional tillage on rhizosphere diversity has been illustrated where a negative impact on AM fungi diversity and colonization was observed, potentially due to soil perturbation that destroys existing hyphal networks that can colonize maize and wheat seedlings (165). Another effect of conservation tillage is retention of crop roots that increase carbon substrates for the survival of functional microorganisms (173). Similarly, the addition of biochar in tomato not only increased taxonomic rhizosphere bacteria diversity, it promoted a functional assemblage of biocontrol and PGPR (161). Organically produced molecular patterns are not the only chemicals able to induce ISR in plants, there are a number of analogs that can elicit the same response (174-177). There is strong potential for the commercialization of SAR and ISR for sustainable agriculture via plant and/or soil inoculation and through harvesting of elicitors from symbionts (121, 144). DAMPs have been used to trigger plant immune responses and have been proposed as plant vaccines (178). Among these are benzothiadiazole that was demonstrated to elicit SAR in tomatoes (175), 2,4-Didcholorophyenoxy acetic acid induced SAR in potatoes (*Solanum tuberosum*) (177), and even simple treatments such as calcium salt and SA have been demonstrated to induce SAR (176). To move forward in sustainable agriculture, we must view soil as a functional ecosystem which we can manipulate towards better food security.

In these experiments, I attempt to display how varying soil inoculum consisting of *in situ* collected rhizosphere microbiomes confer ISR against aphids by using the model plant *M. truncatula* and the phloem-feeding insect *A. pisum*. The diploid model legume *M. truncatula* has been used as an analog for alfalfa (*M. sativa*) because the tetraploid nature of alfalfa makes it difficult to use in genetic studies (179). *M. truncatula* has previously shown moderate resistance to *A. pisum* and has been a model for tripartite plant-insect-microbe interactions in previous studies (133, 180, 181). In addition to demonstrating the effect of varying microbiomes on aphid-plant-beneficial microbe interactions, I used mutants of *M. truncatula* to identify functional segments of the microbial species assemblage in one soil inoculum, and their potential impact on *A. pisum* herbivory.

Significance

Modern agriculture is centralized on obtaining maximum plant yield, but has been shown to significantly decrease rhizosphere microbial diversity, potentially resulting in the loss of functional rhizosphere microbes (163-167). A functional rhizosphere microbial community can be cultured and through crop rotation, crop selection, and fertilization techniques (158, 160, 161), which if applied correctly, may increase plant yield through plant growth promotion and microbially induced defensive priming against pathogens and insect herbivores (16, 25, 162). The rhizosphere microbial community is dynamic and cultivating a functional, diverse, and biased community must take into consideration the land use history of the soil. There is strong potential for the commercialization of microbially induced SAR and ISR for sustainable agriculture through crop and soil manipulation, ultimately leading toward global food security (121, 144). The model in this experiment is designed to showcase how fungal and bacterial diversity impact plant defensive signaling and alter herbivory success by insects. To further

understand and validate the effect of beneficial microbes on defensive signaling, tripartite plant-microbe-insect interactions must be taken in the context in which they exist in natural and agricultural systems.

CHAPTER II

INDIRECT IMPACT OF RHIZOSPHERE MICROBIAL COMMUNITIES FOUND IN NATURAL SOILS ON APHID-PLANT INTERACTIONS

Abstract

The rhizosphere is a unique ecosystem consisting of microbial communities that can influence the biological functions of plants. The community dynamics of these micro-environments are influenced by root, fungal, and bacterial exudates that can preferentially select for functional classes of microbes. Arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPR) are functional symbionts of the rhizosphere microbial community that have been extensively studied due to their ability to form symbiotic relationships with plants. Symbiosis with PGPR and/or AM fungi can promote plant growth and trigger induced systemic resistance (ISR), resulting in defensive 'priming' of host plants that demonstrate stronger and faster responses to future attacks by pathogens and insects. The biological system in this tripartite plant-insect-microbe herbivory study involves barrel medic plants (*Medicago truncatula*), pea aphids (*Acyrthosiphon pisum*), and microbial communities present in field-collected soils from Greeley, Colorado. The objective of this experiment was to evaluate plant growth and plant interactions with aphids as they are impacted by microbes from four field-collected soil inoculants with varying plant communities including corn (*Zea mays*), alfalfa (*Medicago sativa*), a natural prairie, and a commercial topsoil (Pioneer) from an unknown field. Plant growth among active soil inoculants demonstrated significantly greater root fresh weight in corn inoculated soils compared to Pioneer inoculated soils. Aphid colony weight was

negatively impacted by *M. truncatula* grown in alfalfa inoculated soil compared to Pioneer inoculated soil (p =.0205), indicating that the resident plant community of this soil was effective in eliciting defensive priming. This research demonstrates how resident plant communities may impact plant growth and resistance to aphid herbivory.

Introduction

The zone of soil that is impacted by plant root secretions, known as the rhizosphere, harbors a microbial community of bacteria and fungi that perform essential functions related to nutrient availability and long-term resiliency of agricultural crops (11, 14, 25, 26, 54). The ecosystem services these microbes provide ranges from plant growth as a result of increased nutrient bioavailability (182-185), suppression of plant pathogens within the soil (161, 186), and defensive priming resulting in increased defensive metabolites within plant tissues (45, 90, 187). Members of the rhizosphere microbial community such as plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal (AM) fungi have been shown to impact their plant hosts' growth and phytohormones (27, 42, 69, 188), resulting in increased plant biomass and innate immune responses analogous to the responses observed with the human gut microbiome (50, 52, 189). Plant defensive priming is a type of immune response characterized by systemically enhanced defensive capabilities that confer resistance to pathogens or herbivores (45). Systemic acquired resistance (SAR) is a type of plant defensive priming dependent on activation of the salicylic acid (SA) defensive pathway associated with pathogen infection and defined by enhanced resistance to pathogens upon challenge inoculation (45, 46, 62). Conversely, induced systemic resistance (ISR) is a type of defensive priming characterized by the jasmonic acid and ethylene (JA/ET) defensive pathway and is associated with beneficial rhizosphere microbes such as PGPR and AM fungi (21, 45, 46, 77, 190). While the terms SAR and ISR may be used

synonymously for mutualistic microbes, for the purpose of this paper SAR will be defined as pathogen dependent, even in the case of SA-dependent acquired resistance found in some beneficial bacteria and fungi (44, 45, 191, 192). In SAR, the SA pathway is elicited by pathogen triggered immunity (PTI) in which microbe-, damage-, or pathogen- associated molecular patterns (M/D/PAMPs) in the form of proteins, lipids, and/or carbohydrates are detected by pattern recognition receptors (PRRs) on the plant cell surface (58, 60, 193). A second layer of defense, called effector triggered immunity (ETI), is a more specific defense that is activated by intracellular effector molecules, usually produced by the pathogen to circumvent pathogen triggered immunity (PTI) (45, 62, 65, 67). In rhizosphere mediated ISR, MAMPs are similarly detected by PPRs on the root cell surface, but are recognized as symbiotic factors that activate SA-independent defenses (45, 105, 106, 190). SA and JA/ET plant defensive signaling pathways are antagonistic to one another and differ in the type of damage associated response (46, 78, 79, 106). For instance, the SA pathway is more effective against biotrophic pathogens while the JA/ET pathway is more effective against insect herbivores and necrotrophic pathogens (78-81). The regulation of the SA and JA/ET pathways are important for the overall health of the plant since an individual plant may be attacked by an insect herbivore as well as a pathogen, and the defenses employed by the SA pathway are not effective against many guilds of herbivores (78-81). Pathogens that are dispersed within the saliva of insect herbivores may even complement each other by accelerating the dispersal of both (82-85). Many of these pathogens target disruption of the SA regulated defense through induction of the ET pathway (85), which suppresses SA signaling (86) and/or disrupts the JA defensive signaling pathway (83) important for the defense against insect herbivores, such as aphids (82, 83, 85). The pea aphid (*Acyrthrosiphon pisum*) used in the present study is a model organism for insect-microbe-plant

interactions and has similarly been shown to harbor facultative bacteria that aid the aphid in host plant specialization, suppression of plant volatiles that attract aphid parasitic wasps, and disruption of plant defense against aphids (194-196).

Aphids are important crop pests commonly studied for the significant economic impact and specialized feeding behavior (197, 198). Aphids use a specialized mouthpart called a stylet to follow a sugar gradient to phloem cells, avoiding disruption of plant cells that would elicit a SA-dependent defensive response (123, 126). This points towards induction of JA by the plant in response to aphid herbivory as one of the defensive signaling hormones for aphid resistance (127-129). Aphid saliva has endogenous effectors that, like facultative bacteria, modulate the plant immune system through JA/SA antagonism (199-201). By avoiding defensive metabolites in plant tissue, aphids can directly benefit from the increased nutritional status that is gained by rhizosphere microbes (123, 125, 130). For this reason, rhizosphere microbes may have negative or positive indirect effect on aphid population size depending on rhizosphere community composition, and plant genotype (122, 132). For instance, Tétard-Jones et al. found that supplementation with *P. aeruginosa* 7NSK2, a PGPR isolated from barley (*Hordeum vulgare*), had a negative or positive impact on aphid population size depending on plant genotype (132). Other studies have shown aphid susceptibility in barrel clover (*Medicago truncatula*) and rice (*Oryza sativa*) inoculated with AM fungi, and aphid resistance in other plants inoculated PGPR including canola (*Brassica napus*) and wheat (*Triticum aestivum*) (122, 133-135). AM fungi can be particularly variable in regard to aphid resistance depending on the phosphorous and nitrogen ratios in the rhizosphere (136, 137). For example, Wang et al. found a correlation between AM fungi colonization in winter wheat (*Triticum aestivum* L.) and the grain aphid (*Sitobion avenae*) population abundance that varied depending on the ratios of phosphorous and

nitrogen (136). In another study, Wilkinson et al. found no difference in *S. avenae* abundance in barley (*Hordeum vulgare*) inoculated with AM fungi and grown with or without access to supplemental nitrogen (137). The variation in aphid responses conferred between functional microbes and crop species illustrates the need to study microbe-plant-insect interactions using natural and agriculture systems where these factors can be manipulated for the greatest plant benefit.

Many soil microbiology studies are centered on the benefits of a specific functional group, or inoculum. Although these studies have significantly advanced our knowledge of plant defensive signaling pathways and the impacts that specific symbiotic rhizosphere microbes may have on plant biological function, they do not describe ecological interactions that can exist within complex consortiums of rhizosphere microbes as they might exist *in situ.* For instance, many root-associated beneficial microbes have been implicated in JA-regulated ISR (21, 45, 202), while others may induce the SA pathway during initial contact with plant roots prior to recognition and formation of the symbiosis (203). Two previous studies involving plant-microbe-insect interactions used dilutions of a single soil to demonstrate the effect of species richness as it relates to plant-insect interactions (204, 205). By using oilseed rape (*Brassica napus*), the cabbage root fly (*Delia radicum*), and a liquid inoculum harvested from a cultivated field, Lachaise et al. 2017 demonstrated a significantly lower larval emergence rate in plants grown in soils with high and low levels of dilution compared to a medium level of dilution (205). In this study, it was suggested that the high level of diversity in the medium dilution may have included rare species that induced a more robust plant defense (205). In another example, Hol et al. demonstrated that a reduction in rare microbes resulted in increased aphid (*Brevicoryne brassicae*) body size after feeding on beets (*Beta vulgaris*) (204). In this study, the authors also

demonstrated significantly higher levels of glucosinolates that are plant defensive compounds against insects, indicating that this negative effect against aphids may have been indirectly induced by rare microbes (204). The diversity and richness of microbes within the rhizosphere may be associated with the effectiveness of ISR and warrants further research. A better understanding of rhizosphere diversity and richness and their indirect impact on insect pests, would allow manipulating these soil metrics through inoculation and crop selection for agricultural benefit (143-146).

This experiment was designed to evaluate aphid abundance and plant growth responses using varying rhizosphere microbial inoculants. The soils were collected in the fall of 2016 from a natural prairie ecosystem (N, Natural), a corn field (C, Corn), an alfalfa field (A, Alfalfa), and a control commercial topsoil (P, Pioneer) from Pioneer Sand Co. (Windsor, Colorado). The natural soil was chosen based on the observation of having a diverse plant community of grasses and shrubs, which should in turn have greater rhizosphere microbial diversity due to the specificity observed between plants and microbes. Alfalfa (*Medicago sativa*) is a leguminous plant that is closely related to the model plant used in this experiment, *M. truncatula* (barrel medic clover). As legumes, both *M. sativa* and *M. truncatula* are known to form symbioses with PGPR and AM fungi. The diversity and richness of bacteria and fungi in a soil can be determined through a variety of methods that have been previously reviewed (206). Automated ribosomal intergenic spacer analysis (ARISA) was chosen with the objective of surveying the diversity and richness of the experimental rhizosphere microbiome without species- or genera-specific classification (206, 207). This technique has been used to describe shifts in microbial communities in agricultural systems such as the transition of highland forests to agriculture, and to assess treatments effects on crops (208, 209).

In the present study, four different soil inoculants with varying resident plant communities were used to demonstrate the indirect effect of the rhizosphere microbiome collected from agronomic, natural, and commercial topsoil on pea aphid (*Acrythrosiphon pisum*) - barrel medic clover interactions. It was hypothesized that a) soil inoculants with high fungal and/or bacterial diversity and richness promote a stronger plant defense response against aphids resulting in reduced aphid colony weight per plant, and b) *M. truncatula* plants will benefit from the soil microbial communities that are associated with the most closely related plants (*M. sativa*).

Methods

Site Selection and Soil Inoculum

Three of the four soils used were predicted to include different microbial communities based on the plant species that were growing in these soils. Two of the soils were collected from fields with alfalfa (A; *M. sativa*) and corn (C; *Zea mays*) crops from Monroe farm, the oldest organic farm in NE Colorado (Figure 1). The alfalfa and corn fields are rotated at least biennially according to the farm owner and may represent a microbial community from this rotation and not necessarily the current crop. However, for the purposes of this experiment the dominant crop that existed at the time of harvest was assumed to be the greatest determinant of the bacterial and fungal community. A third soil was collected in close geographic range of Monroe Farm from a natural (N) prairie field (Figure 1). All three soils were classified as sandy loam using the web soil survey website provided by the National Resources Conservation Service (https://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx). The top organic horizon (O horizon) was taken for each soil by collecting the top 10 cm for the alfalfa and natural soils and the top 5 cm in the corn field. The corn field sits atop a hill that has experienced erosion from

decades of farming and the organic (O) horizon was not as deep as in the other sample locations. The soil was collected using a 2-3/4" soil auger every half meter for ten meters and combining the contents into autoclave bags. A subsample of each, as well as a fourth topsoil deemed Pioneer (P) from Pioneer Sand Co. (Windsor, Colorado) were dried for two days in a greenhouse, crushed, and filtered using a 2 mm sieve. The Pioneer topsoil is described as a screened sandy loam representing the top 5" of a field (https://www.pioneersand.com/products/garden_bed_solutions/topsoil_fill_dirt/a_topsoil) and is likely an aggregate of several soils. The resulting filtered soil was used as an inoculum under the pretense that a resemblance of the original soil microbial community was present. It is important to note that by manipulating the soil to normalize the conditions for all treatments, this likely altered the bacterial and fungal community to some degree, although this was not quantified. For instance, many mycorrhizal species are not able to regenerate from hyphal fragments (165) and any plant colonization by AM fungi would depend on germination of viable AM fungal spores in the soil inoculum. The soil substrate consisted of seven parts sterile sand, one part soil inoculum, and one part sand in which seedlings were grown which contains the root exudates of the developing plants. Two "soil conditions" were used: a control group of each soil inoculum was sterilized by autoclaving two times (60 min, 121°C, at 15 psi) and prepared in the same ratio, while the other group were not autoclaved. This created eight soil treatment groups, four autoclaved and four active with most of the microbial community in the soil upon collection. The treatments were as follows: alfalfa (A), corn (C), natural (N), pioneer (P), autoclaved alfalfa (AA), autoclaved corn (AC), autoclaved natural (AN), and autoclaved pioneer (AP). All mixtures were then saturated with half-strength modified Hoagland's nutrient solution (100 μM P). The low ratio of soil inoculum combined with filtering of larger substrates and saturation with Hoagland's nutrient solution

were used to eliminate indirect effects of inherent soil texture and nutrient composition on the microbial communities cultured throughout the experiment. Each treatment initially consisted of ten biological replicates that included one plant per pot. Eight soil treatments (four active and four autoclaved) received aphids and eight soil treatments did not (160 plants total).

Figure 1. Site locations for Natural (N), alfalfa (A), and corn (C)

Plant Growth Conditions

Wild type A17 *Medicago truncatula* Jemalong seeds were scarified, surface-sterilized, and germinated for one week as previously described (210). Seedlings were planted in sterile sand that was saturated with half strength modified Hoagland's nutrient solution (100 µM P) (133). Plants were placed in a growth chamber with an 8/16 dark (22 °C)/light (25 °C) regimen for 10 days with 40% humidity. Seventeen-day old plants were divided based on size and

distributed evenly among treatments. Subsets of these plants were transferred into individual 6.35cm x 6.35cm x 8.89cm pots with a soil volume of 330 cm^3 of soil substrate consisting of 7 parts of autoclaved sand, 1 part sand in which seedlings were grown in, and 1 part of a soil inoculum (P-Pioneer, A-Alfalfa, C-Corn, or N-Natural). All ten replicates for any given treatment were grown on planting trays designed for the specified pot size. Plants were grown for an additional 40 days in a growth chamber as described above, and were fertilized twice a week with 10 mL of ½ strength modified Hoagland's solution (100 µM P) per pot to encourage AM fungus colonization, if present (211, 212). When plants were 57 days old, they were covered with domes, transferred to a greenhouse with supplemental light using an 8 h dark/16 h light cycle, and were fertilized twice a week. Each plant was covered with fine mesh bag and plants were transferred into insect proof cages and grown in a greenhouse with a light cycle of 8/16 dark/light for an additional two weeks, or at 71 days old. Plants were harvested at 81 days old. Root fresh weight (RFW) was taken at the time of harvest and shoot dry weight (SDW) was taken using an analytical balance after dehydration overnight in an oven at 60°C.

Pea Aphid Herbivory

Aphids used in this experiment were parthenogenetic pea aphids (*Acyrthosiphon pisum*) that were provided by Dr. Kenneth Korth (University of Arkansas, Fayetteville, AR, USA) and were reared on fava bean (*Vicia faba* L.) plants as previously described (133). Three apterous pea aphid female adults from a seven-day old synchronized colony were allowed to feed for ten consecutive days on plants that included an herbivory treatment. Plants were allowed to acclimate to greenhouse conditions from growth chamber conditions for two weeks before aphids were added at day 71. All the surviving aphids from each plant (referred to as a colony) that included an herbivory treatment were collected via fine paintbrushes and transferred to petri

dishes on the day of harvest. Aphids were weighed the same day as they were harvested by immobilizing them at -20 °C, transferring the entire colony to a foil bowl using a fine paint brush, and weighing using a microbalance (SE-2F Sartorious balance, Denver, Colorado, USA).

Microbial Deoxyribonucleic Acid Isolation and Community Analysis

The rhizosphere fraction of the soil was collected for each plant replicate by removing the plant from the bulk soil of the pot and disturbing the roots with a sterilized spatula. All soil collected from each replicate were pooled by treatment and thoroughly mixed (n =1 per treatment). Autoclaved Milli-Q water was used to rinse the soil. Soil and water were disturbed and decanted into a vacuum filter flask with a Whatman #1 filter paper and sterilized ceramic filters until the all fine soil was extracted, which was determined by a clear decant. The filter paper and contents were ground with a mortar and pestle before DNA extraction using the DNeasy PowerSoil® kit (QIAGEN®). Automated ribosomal intergenic spacer analysis (ARISA) was conducted on internal transcribed spacer (ITS) regions of bacteria and fungi as previously described by Ranjard et al. (213). PCR amplification using 16S ribosomal sequences of bacteria and the 18S/28S sequences in other soil organisms, such as fungi, was conducted using the fluorescently labeled HEX primers S-D-Bact-1522-b-S-20 (small ribosomal subunit)/L-D-Bact-132-a-A-18 (large ribosomal subunit) for bacteria and fluorescently labeled FAM primers 2234C/3126T for fungi (207, 213). The sequence for S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18 are 5’-TGCGGCTGGATCCCCTCCTT-3’/ 5’-CCGGGTTTCCCCATTCGG-3’, respectively, and the sequence for 2234C/3126T are 5’-GTTTCCGTAGGTGAACCTGC-3’/5’-ATATGCTTAAGTTCAGCGGGT-3’, respectively (207). Two PCR technical replicates per sample were used for endophytic DNA using fungal and bacterial primers, and rhizosphere DNA using fungal and bacterial primers (8 samples total). Each rhizosphere PCR sample consisted of

0.6 uL of each primer (10 µM each), 2.4 uL GoTaq buffer (Promega), 0.7 uL dNTPs (10mM each), 1 uL $MgCl_2$, 0.06 uL Taq polymerase (5U/ uL), 3.96 uL DNA-free water, 0.06 uL of bovine serum albumin (BSA, 100X), and 3 uL of DNA template as described previously for 'shotgun' PCR protocols (213). Thermal profiles used in PCR were as previously described for ribosomal intergenic spacer analysis (207). PCR amplification was verified via gel electrophoresis before drying, shipping and fragment analysis on an Applied Biosystems 3500 Genetic Analyzer by the DNA Lab at Arizona State University. The spectrograph profiles were analyzed for peak size in base pairs and peak area in base pair*reflective fluorescence units (RFUs) using Thermo-Fisher Connect™ microsattelite analysis (MSA) online application (https://apps.thermofisher.com/editor-web/#/app/app-microsatellites-web). PCR products generated using bacterial and fungal primers from the active soil treatments (16 profiles) were analyzed using Peak Window Sizes 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, and 31 (in datapoints) within the MSA app. The automatic_binner.r script produced by Ramette (214) was used to determine optimal Peak Window Size, which was chosen based on the size in which the correlation factor was over 60 (window size of 25 for bacteria and 17 for fungi). All treatments were run with the respective window size and replicates were merged using Microsoft Excel. The peak size was rounded to the first integer using Excel round function and Highlight Duplicates was used to color fill cells in which peak sizes were found in both replicates. The peaks were then filtered by fill color and the peak areas were averaged to create one ARISA profile from the two technical replicates for each treatment.

Statistical Analysis

All statistical analyses were performed using R studio (215). ARISA profiles were analyzed for Shannon-Weiner diversity (I), species richness, Jaccard Index of similarity,

nonmetric multidimentional scaling (NMDS), and analysis of similarities (ANOSIM) using treatment, aphid herbivory, and autoclave soil treatment as factors using the vegan package in R studio (216). The NMDS method is ideal for condensing and visualizing large datasets like ARISA profiles and the vegan package implements a Bray-Curtis similarity for the rank-based correlations which includes presence/absence and abundance as opposed to methods like Jaccard that only compare presence/absence (216, 217). However, NMDS is not a statistical test for differences between populations, although the Goodness of Fit (R-squared) statistic produced by the envfit() function in the R package was used to visually describe the fit of the grouping ellipsis (216). Analysis of similarity (ANOSIM) was used to statistically test differences in ARISA profiles using the anosim() function with Bray-Curtis distances and 9999 permutations in the vegan package (216). Post-hoc analysis of ASNOSIM by soil was not possible due to the number of replicates. ARISA profiles of autoclaved and active soil treatments were used to verify a significant change in bacterial and fungal communities as a result of autoclaving. The Shapiro-Wilk test was conducted on plant and aphid data to confirm normality using the rstatix package (218). Raw data that were not normally distributed were visualized for skewedness, and transformed according to the ladder of powers until normality was achieved using Shapiro-Wilk tests (219). This resulted in a log transformation for aphid colony weight and a square root transformation for shoot dry weight. Two-factor analysis of variance (ANOVA) was conducted on aphid colony weight using soil inoculum and autoclave soil treatment as factors. Correlations between plant and aphid parameters were conducted using the car package in R (220). Three-factor ANOVA was conducted on shoot and root data with herbivory (with and without aphids), soil inoculum (Pioneer soil, alfalfa soil, corn soil, natural soil), and autoclaved soil treatment (autoclaved and active) as factors. When an interaction term was statistically significant ($P <$

0.05), the Tukey honest significant difference (HSD) test was used for pairwise comparisons using the pairs() function (220). All ANOVA tests were conducted using car package in R studio (220). Individual plots were produced using the package ggplot2 in R studio

Results

Impact of Soil Microbes on Plant Growth

The number of surviving plant replicates in each soil type is represented in Table 1. Shoot dry weight and root fresh weight measurements were taken to assess the impact of soil microbes and aphid herbivory on plant growth. The interaction between soil inoculum, aphid herbivory, and autoclave treatment did not have a statistically significant effect on shoot dry weight, but the interaction between autoclave treatment and soil inoculum and the main effects of autoclave treatment and soil inoculum were statistically significant (Figure 2). Shoot dry weight of plants grown in the Corn soil inoculum, both active and autoclaved, as well as in the autoclaved Alfalfa and Natural soil inocula were significantly higher than those of plants grown in the active Pioneer soil inoculum (Figure 2). Shoot dry weight of plants grown in the autoclaved Pioneer soil inoculum were also significantly higher than those of plants grown in the active soil Pioneer and Natural soil inocula (Figure 2). Similar results were observed for root fresh weight with the interaction between soil inoculum, aphid herbivory, and autoclave treatment being statistically non-significant, while the interaction between autoclave treatment and soil inoculum as well as the main effects of soil inoculum and autoclave treatment were significant (Figure 3). Root fresh weight of plants grown using Pioneer and Natural soil inocula were significantly different when autoclaved versus active soils were compared, this was not observed using the Alfalfa and Corn soil inocula (Figure 3). Root fresh weight of plants grown

in the different active inoculum was the same, however, root fresh weight of plants grown in the Alfalfa autoclaved inoculum was less compared to those of plants grown in Pioneer and Natural autoclaved inoculum (Figure 3).

Table 1. Number of replicates from experiment 1 at the time of greenhouse transfer (57 days old)

Treatment	With Aphids Autoclaved	Without Aphids Autoclaved	With Aphids Active	Without Aphids Active
A	7	7	6	6
C	8	9	7	6
N	10	7	10	9
P	9	10	9	9

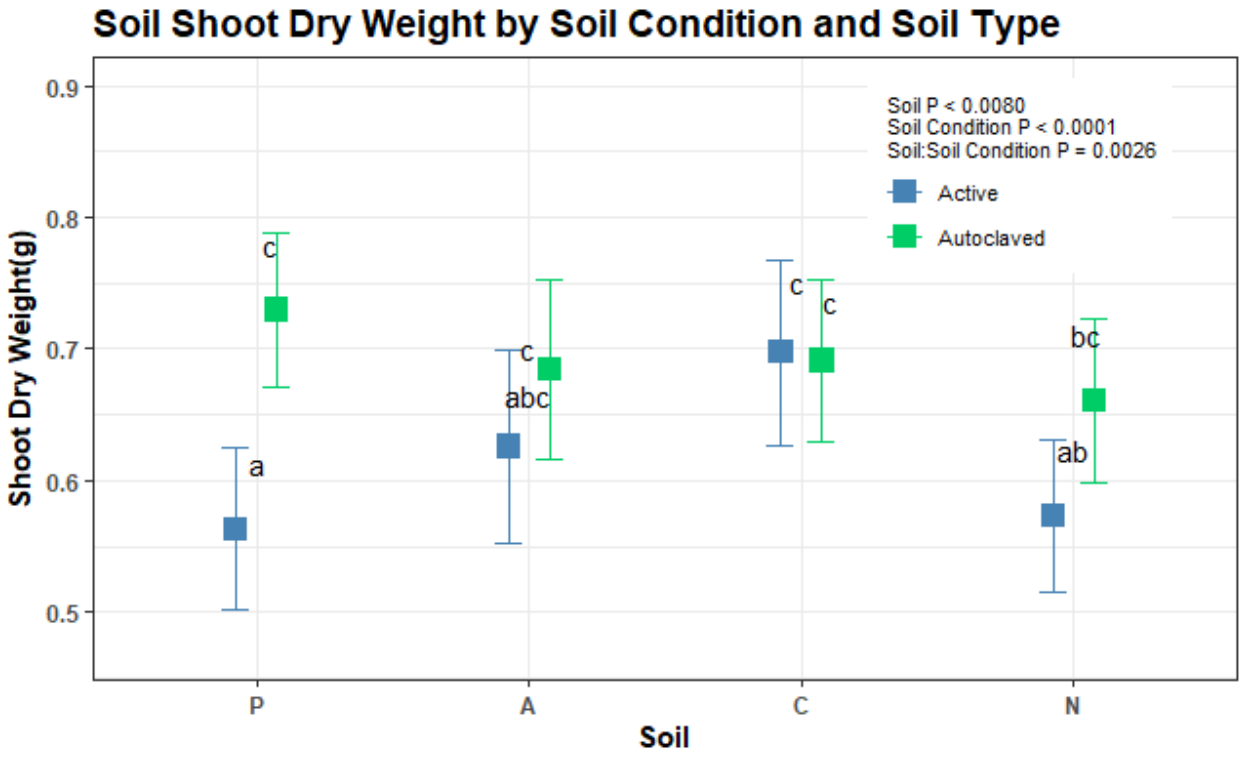

Figure 2. Mean shoot dry weight of *Medicago truncatula* plants grown in four types of soil inocula and two types of soil conditions (autoclaved vs active). The interaction between soil inoculum and soil condition and the main effects were statistically significant ($P < 0.05$) according to a two-factor ANOVA. Different letters represent statistically significant differences among treatments based on Tukey HSD test ($P < 0.05$). Values represent the mean of ten biological replicates ± standard deviation. P = Pioneer, A = Alfalfa, C = Corn, N = Natural.

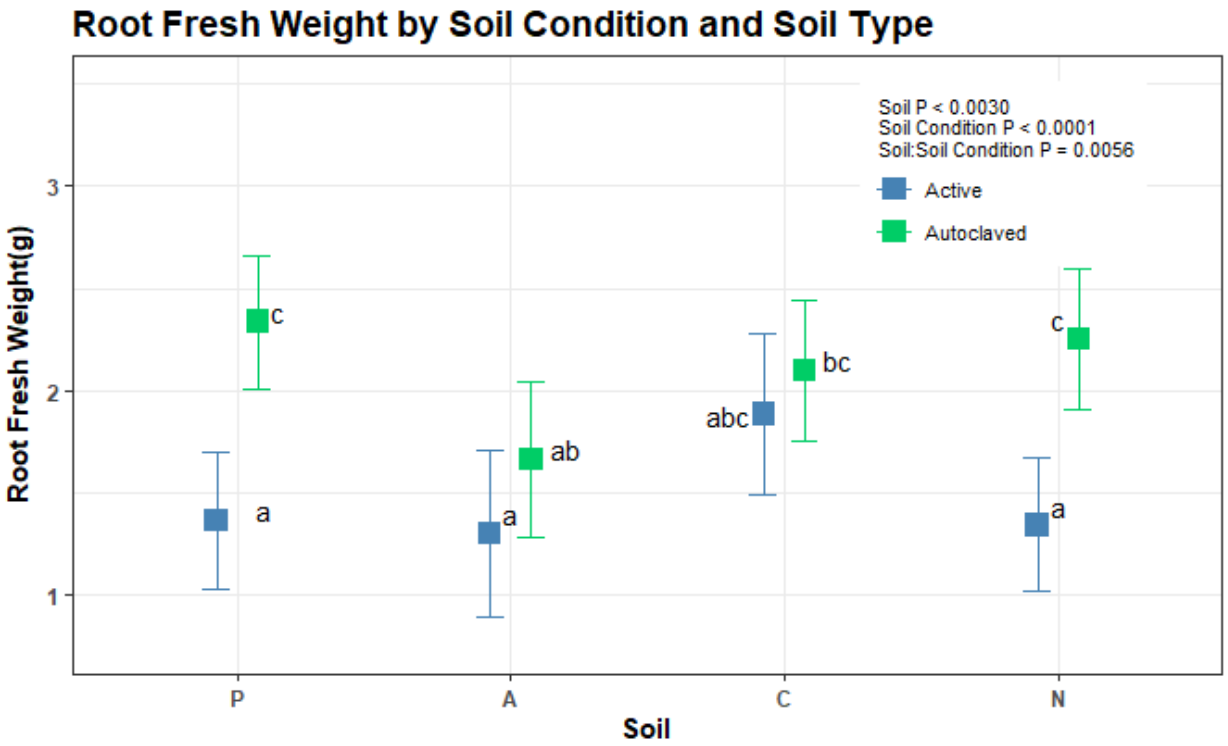

Figure 3. Mean root fresh weight of *Medicago truncatula* plants grown in four types of soil inocula and two types of soil conditions (autoclaved vs active). The interaction between soil inoculum and soil condition and the main effects were statistically significant ($P < 0.05$) according to a two-factor ANOVA. Different letters represent statistically significant differences among treatments based on Tukey HSD test ($P < 0.05$). Values represent the mean of ten biological replicates ± standard deviation. P = Pioneer, A = Alfalfa, C = Corn, N = Natural.

Indirect Impact of Soil Inoculum on Aphid Fitness

The results of two-factor ANOVA for aphid colony weight showed a significant impact of the interaction between soil inoculum and soil condition (Figure 4). The main effect of soil inoculum was also significant, but the main effect of soil condition was not (Figure 4). There was significantly lower aphid colony weight in plants with active alfalfa soil than active pioneer soil and autoclaved natural soil (Figure 4). Correlations between normalized aphid colony weight and normalized plant parameters showed no significant relationship, indicating that significant differences were a result of indirect aphid- microbe effects.

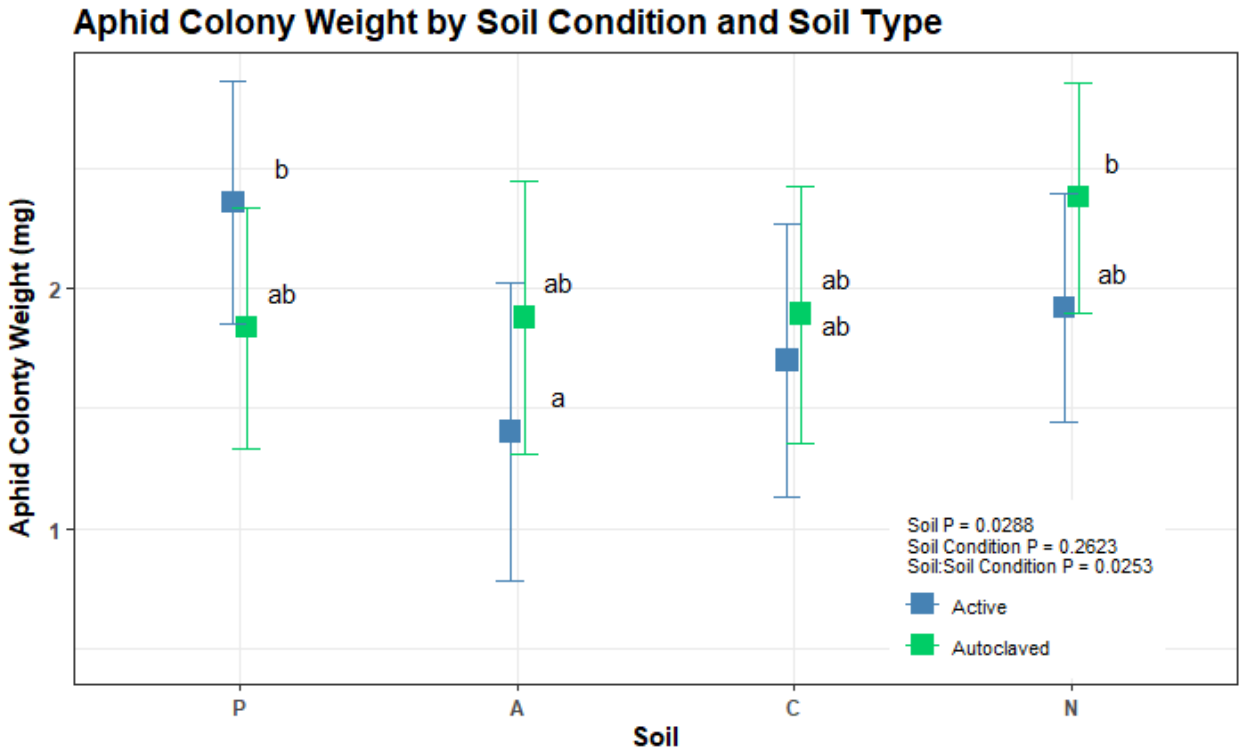

Figure 4. Indirect effect of soil inoculum and soil condition (autoclaved vs active) on aphid colony weight after 10 days of feeding on *Medicago truncatula* plants. The interaction between soil inoculum and soil condition, and the main effect of soil inoculum were statistically significant ($P < 0.05$) according to a two-factor ANOVA. Different letters represent statistically significant differences among treatments based on the Tukey HSD test ($P < 0.05$). Values represent the mean of ten biological replicates ± standard deviation. P = Pioneer, A = Alfalfa, C = Corn, N = Natural.

Non-metric multidimensional scaling (NMDS) of fungal OTUs showed significant differences between active and autoclaved treatments in both ANOSIM (Table 2, $p = .0307$) and NMDS visualization (Figure 5B, goodness of fit $p = .04$), but no significance between aphid herbivory. The same effect was observed in bacterial OTUs, with autoclaved treatments significantly different than active treatments in both ANOSIM (Table 2, $p = .0004$) and NMDS visualization (Figure 5D, goodness of fit $p = .001$) and no significance between aphids and no aphids. The stress for bacterial and fungal OTU NMDS ordinations were 0.135 and 0.117, respectively. Soil type was only compared with ANOSIM between active profiles with and

without aphids and was significant for bacterial communities (Table 2, p = .0094), but not fungal communities (Table 2, p = .1389).

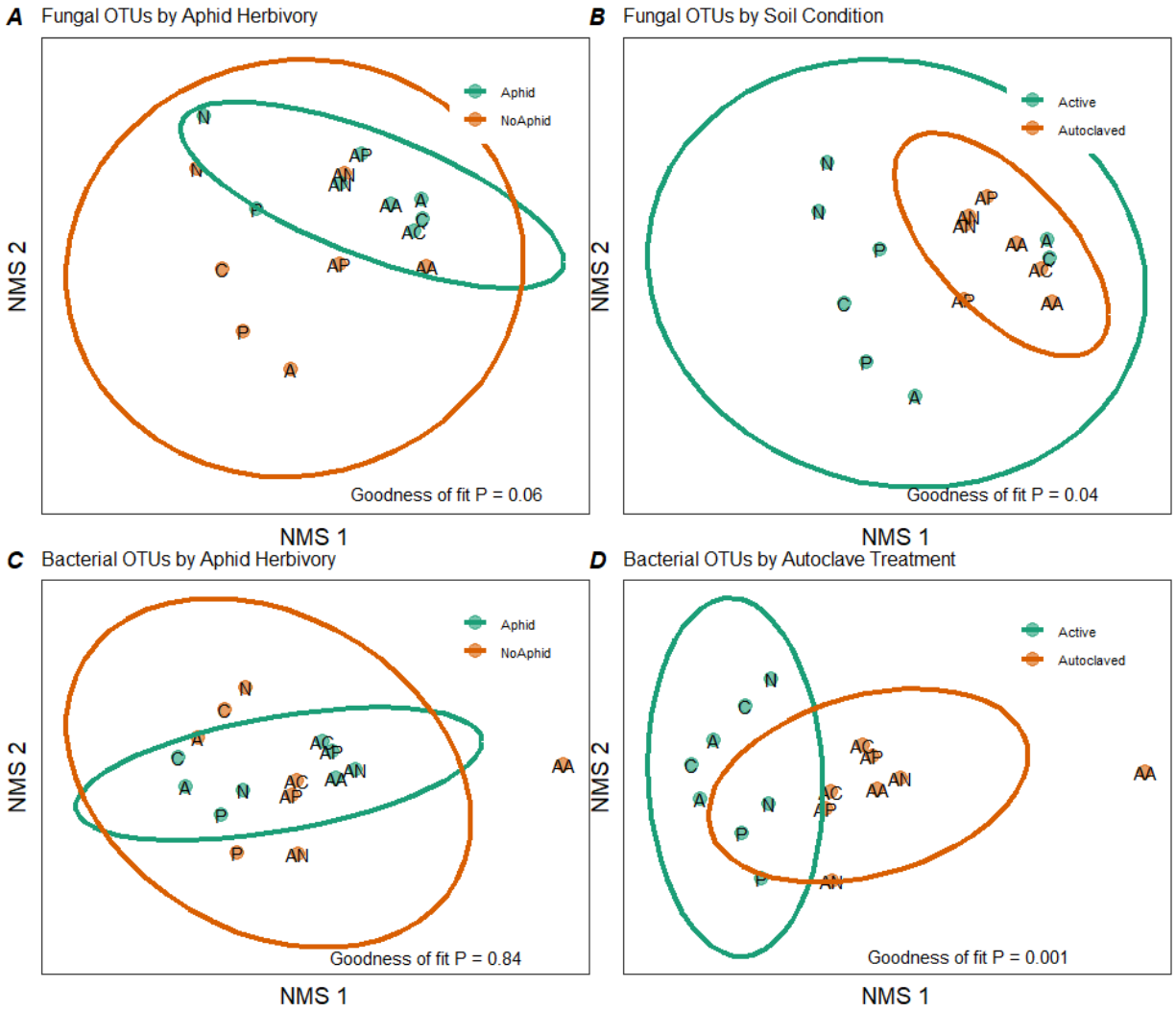

Figure 5. Non-metric multidimensional scaling (NMDS) of operational taxonomic units for fungi grouped by aphid herbivory (A), fungi grouped by soil condition (autoclaved vs active) (B), bacteria grouped by aphid herbivory (C), and bacteria grouped by soil condition (autoclaved vs active) (D). Goodness of fit *P* values represent the dissimilarity of the treatments plotted by aphid herbivory (A and C) and active/autoclaved soil (B and D). Treatment labels are as follows: P = Pioneer, A = Alfalfa, C = Corn, N = Natural, AP = Autoclaved Pioneer, AA = Autoclaved Alfalfa, AC= Autoclaved Corn, AN = Autoclaved Natural.

Table 2. ANOSIM results for fungal and bacterial community profiles in the rhizosphere. Soil inoculum ANOSIM were conducted with data from active soil microbes only. * represents statistically significant P values

Factor	R^2	P-value	R^2	P-value
ANOSIM:	Fungal		Bacterial	
Soil Condition (Active / Autoclaved)	0.2383	0.0307*	0.5173	0.0002*
Aphid Herbivory (With Aphids / Without Aphids)	0.1086	0.1163	0.03376	0.2869
Soil Inoculum (Alfalfa / Corn / Natural / Pioneer)	0.3958	0.1389	0.875	0.0094*

Bacterial diversity and richness indices are represented in Table 3. Shannon-Weiner Diversity index (H) was relatively uniform. All diversity and richness values were higher with the addition of aphids in active soil inoculum (Table 3). Jaccard index of similarity (J) for bacteria ranged between 0.83 (Alfalfa inoculated soil without aphids and Alfalfa inoculated soil with aphids) and 0.97 (Pioneer inoculated soil without aphids and Natural inoculated soil without aphids).

Table 3. Shannon-Weiner Index (H) and Species Richness (S) for bacterial operational taxonomic units (OTUs) after ARISA analysis. N = Natural, P = Pioneer, C= Corn, A = Alfalfa. Only one species was detected using DNA samples collected from alfalfa soil inoculum (autoclaved) of plants that had aphids, therefore, H was not reported.

Inoculant	Active No Aphid		Active Aphid		Autoclaved No Aphid		Autoclaved Aphid	
	H	S	H	S	H	S	H	S
A	3.5	86	3.8	70	NA	1	2.7	24
C	3.2	83	3.4	80	3.1	43	2.6	21
N	3.5	88	3.8	73	2.8	33	2.9	40
P	3.4	75	3.6	73	3.3	45	2.8	28

Overall, Pioneer and Natural soil inoculum with aphids were least similar to all other soil inoculum (Table 5).

For the active fungal soil inocula, corn soil had the lowest reported diversity, followed by Alfalfa, Pioneer, and Natural with the highest (Table 4). Richness between the four active soil inoculants without aphids was similar between Alfalfa, Corn, and Pioneer, but Natural soil reported nearly twice the number of species (Table 4). Corn soil inocula, both with and without aphids, was similar in richness to Pioneer and Alfalfa but with lower diversity indicating more evenness within the Corn soil inocula. The larger richness of fungal OTUs in autoclaved Alfalfa soil inocula without aphids compared to active Alfalfa soil inoclua without aphids indicates contamination (Table 4). The Jaccard similarity values for fungal OTU profiles ranged between 0.45 (Corn inoculated soil with aphids and alfalfa inoculated soil without aphids) to 0.89 (Corn inoculated soil with aphids and Natural inoculated soil with aphids), and were more variable among soil inocula than those of bacterial OTU profiles, likely due to larger numbers of bacterial OTUs detected (Table 5). Corn soil inocula without aphids was the least similar to all other profiles while Natural soil inocula with aphids was the most similar (Table 5).

Table 4. Shannon-Weiner Index (H) and Species Richness (S) for fungal operational taxonomic units (OTUs) after ARISA analysis. N = Natural, P = Pioneer, C= Corn, A = Alfalfa. No fungal peaks in consensus within the two PCR replicates were detected using DNA samples collected from Corn soil inoculum (autoclaved) of plants without aphids, therefore, indices were not reported.

Inoculant	Active No Aphid		Active Aphid		Autoclaved No Aphid		Autoclaved Aphid	
	H	S	H	S	H	S	H	S
A	1.9	35	2.1	38	1.9	43	1.6	20
C	1.6	34	1.5	37	NA	NA	1.6	28
N	2.5	62	1.9	49	2.0	35	1.8	26
P	2.3	34	2.2	36	2.2	32	1.7	15

Table 5. Jaccard index of similarity for fungal and bacterial operational taxonomic units. Above values (grey) cells are bacterial OTU similarities, below cells are fungal OTU similarities. Only plant genotypes grown in active soil were compared. N = Natural, P = Pioneer, C= Corn, A = Alfalfa, Aph= with Aphids

	A	**AphA**	**C**	**AphC**	**N**	**AphN**	**P**	**AphP**
A		0.83	0.90	0.88	0.91	0.88	0.96	0.91
AphA	0.77		0.92	0.91	0.92	0.84	0.91	0.89
C	0.68	0.80		0.87	0.86	0.93	0.95	0.91
AphC	0.82	0.45	0.80		0.95	0.92	0.94	0.90
N	0.78	0.85	0.63	0.87		0.85	0.97	0.95
AphN	0.83	0.88	0.74	0.89	0.58		0.86	0.85
P	0.72	0.79	0.65	0.79	0.81	0.83		0.75
AphP	0.70	0.81	0.62	0.83	0.63	0.68	0.67	
Ave Fungal Similarity	0.76	0.76	0.70	0.77	0.74	0.78	0.75	0.71
Ave Bacterial Similarity	0.90	0.89	0.91	0.91	0.92	0.88	0.91	0.88

Discussion

The present study explored the impact of the rhizosphere microbiome on plant growth and insect herbivory. It was hypothesized that a) soil inoculants with high microbial diversity and richness promote a stronger plant defense response against aphids resulting in reduced aphid colony weight per plant, and b) *M. truncatula* plants will benefit from the soil microbial communities that are associated with the most closely related plants (*M. sativa*). Several studies have linked rhizosphere bacterial and fungal diversity to reduced soil functioning including plant biomass and nutrient retention, plant nutrient, disease suppression (42, 153, 221, 222). Under the

hypothesis above, a greater number of microbial species provide complementary plant benefits and that increased numbers of observed OTUs would translate to a stronger defense response against aphids triggered by ISR. This complementation has been demonstrated in studies involving consortiums of PGPR and AM fungi which observed greater plant growth and disease resistance (42, 148, 149). In some cases these complementary consortia are recruited by the plant during and/or after an attack (149, 223, 224). Although the effect of plant growth and defense against herbivory have been demonstrated by a number of bacterial and fungal inoculants, very few studies have examined the impact of the microbial community as it exists in-situ, or used field collected soils as inoculants to evaluate the effect on specialist herbivores such as *A. pisum* (26, 41, 130, 152-155, 161, 225).

The hypothesis that *M. truncatula* would benefit from soil inoculum from a *M. sativa* field was not supported in terms of plat growth. Among active soils, only plants grown in Corn soil inoculum demonstrated significantly greater root fresh weight than plants grown in Pioneer soil inoculum, demonstrating some effect of the resident plant community (Figure 2). Most active soil inoculated treatments showed a trend towards lower plant biomass in both shoots (Figure 2) and roots (Figure 3). This was an interesting result since most autoclaved treatments trended towards lower diversity and richness while previous studies have reported greater plant growth parameters in treatments with active inoculated soils, or with greater microbial diversity (153, 221, 226). This may be explained by the way the soil inoculant was introduced, such as a direct soil (v/v) transfer, soil suspensions, or microbial suspensions that are commonly used to evaluate field soils in a laboratory setting. In Badri et al., plant growth was greatest in plants with active inoculated soils compared to a control with no inoculant, despite the source soil and plant assemblage previously inhabiting the soil (153). The method of inoculation in Badri et al.

(2013) involved a non-filtered soil suspension with separate soil inoculant controls for each soil tested filtered through a 0.45um sieve, which allows for any microbial or plant exudates and some bacterial and fungal spores (153). Many plant growth parameters were only significantly greater in active soils compared to the non-inoculated control and not the 0.45um filtered control, indicating some inherent soil effect or microbiota in the 0.45um inoculants that positively impacted plant growth (153). In a study comparing soil inoculation methods of a field soil, van de Voorde et al. found that plants grown in a microbial suspension that was filtered through several sieves including a 20um sieve demonstrated the greatest plant biomass compared to a direct soil inoculum sieved through a 1mm screen, and a soil suspension in which the soil was pelleted and the supernatant passed through 1mm mesh (226). In that study, the authors also demonstrated the presence of nematodes almost exclusively in directly sieved soil compared to the two suspensions (226). While the presence of nematodes was not determined in the present study, the method of inoculation may have introduced organisms that negatively affected plant growth. For the purposes of this study, the intended inoculants were introduced in a manner that more closely represents the soil microbial community from which they were collected while attempting to control for the effect of inherent soil properties, which were not determined. For instance, in a comparative field inoculation study by Howard et al., the authors demonstrated that soil microbes were most similar to the initial soil in the 5% (v/v) inoculant compared to 1% and 0.5% (v/v) mixture and a soil wash (suspension) (227). In the present study, the 2mm sieve, and 10% (v/v) mixture of field soil should have good representation of the field soil, although the similarity to the initial soil was not determined.

The hypothesis that the soil inoculum with the highest microbial diversity would exhibit a stronger plant defensive response, measured as reduced aphid colony weight was not supported.

The diversity and richness indices for all field soil inoculants were very similar, and no conclusion could be drawn regarding diversity and aphid resistance. In the case of the presence of rhizosphere fungi, the plants grown with the Natural soil inoculum had the greatest fungal diversity and richness (Table 3) but did not show reduced aphid colony weight (Figure 4). Bacterial diversity was very similar in both species' diversity and richness and if anything, the opposite was true. Active Alfalfa inoculated soil had one of the lowest bacterial richness and significantly lower aphid colony weight compared to active Pioneer inoculated soil and autoclaved Natural inoculated soil. This points towards rhizosphere species specificity rather than diversity alone as descriptors of ISR against aphids, giving support for the importance of resident plant community and plant species-specific microbiomes over bacterial and fungal diversity as a determinant of aphid suppressive soil.

The hypothesis that the resident plant community alters the soil microbial diversity impacting defense response of succeeding plants was supported. Under this assumption, Alfalfa soil would have a microbial community that exhibits the greatest ISR response for the model plant *M. truncatula* given their evolutionary relatedness to one another, including the genetic framework to form symbiotic structures (nodules) with some PGPR. This has been demonstrated with other species including *Arabidopsis* with the generalist cabbage looper (*Trichoplusia ni*) (153). In this exploration of plant-microbe-insect interactions, Badri et al. demonstrated significantly less *T. ni* weight gain in some soils inoculated with field soil suspensions, including an unmanaged *Arabidopsis* field soil (153). Interestingly, the least *T. ni* weight gain was in managed potato soils, indicating that the importance of resident plant community on the rhizosphere microbiomes ability to resist insect herbivory, while important and predictable, may not be specific to related plant species (153). This effect was seen in another plant-microbe-

insect study in which Howard et al. demonstrated significantly less leaf area eaten on cucumber plants by *T. ni* in soils inoculated with older succession fallow field inoculants (5% v/v) compared to earlier succession years and soil taken from active maize field plots, despite the inclusion of maize itself in the study design (228). Although other plant species were evaluated with the same soil inoculants, there was no other significant effects on *T. ni* herbivory (228). Another generalist herbivore, the fall armyworm (Spodoptera frugiperda) was evaluated in this study, but no significant negative effects were demonstrated in any of the plant species tested, indicating that soil suppressiveness of insect herbivores may be specific to the source soil as well as the target herbivore (228).

In a study by Kos et al., ragwort (Jacobaea vulgaris) was grown in ten different soils conditioned with other plants representing functional groups, including one soil conditioned with the model plant of the study, J. vulgaris, to determine the effect on generalist (Brachycaudus cardui) and specialist (Aphis jacobaeae) aphid herbivory (225). Although the entire microbiome was not characterized, the authors found significant differences in fungal communities in both the soil condition treatment and the functional group type (forbe, grass, or legume) (225). The aphid performance for both species in the study by Kos et al. was lowest for plants grown in soil inoculants conditioned by Leucanthemum vulgare, not by the plants conditioned with the same species as the host plant, although performance was lowest for A. jacobaeae when grouped by forbes indicating a potential evolutionary relationship similar to the model in the present study with Alfalfa inoculated soil (225).

Aphid colony weight on *M. truncatula* was similarly lowest in Alfalfa inoculated soil, suggesting that the relatedness of *M. sativa* impacted the ability to resist aphids (Figure 4). This is further supported by significantly different bacterial rhizosphere communities grouped by soil

in ANOSIM. Although no post hoc test was conducted for pairwise comparisons of soil, Corn inoculated soil had the highest dissimilarity rank, followed by Natural, Alfalfa, and Pioneer. Nodule forming rhizobacteria are likely the bacterial elicitor of ISR in the present study given both *M. truncatula* and *M. sativa* able to form a symbiosis, although the presence of nodule forming PGPR was not quantified. PGPR as a culprit of ISR in Alfalfa inoculated soil may be supported through Jaccard similarities. which only compare presences of an OTU, and NMDS ordinations, which take into account abundance as well as OTU presence. Corn and Alfalfa inoculated soils with aphids are much closer in proximity in fungal NMDS plots compared to Pioneer and Alfalfa inoculated soils (Figure 5A) but reported the lowest Jaccard similarity for Fungal OTUs (Table 4). This points towards larger differences in fungal abundances and not composition between Alfalfa and Corn inoculated soils compared to Pioneer and Alfalfa inoculated soils. Given the non-significance in ISR against aphids between Alfalfa inoculated soil and Pioneer inoculated soil, it seems likely that bacterial composition was a greater factor than fungal composition in suppressing aphid herbivory. Although Corn and Alfalfa did not have significantly different aphid colony weights, The possibility that Pioneer inoculated soils harbored pathogens may have also impacted the significance between soil inoculants and is indicated by significantly lower shoot dry weight and root fresh weight in active soils compared to autoclaved soils in this treatment, although Natural inoculated soils demonstrated the same significance for root fresh weight without the effect on aphid herbivory (Figure 2 and Figure 3). This may impact the results if the Pioneer soil inoculant harbored pathogens with compounding effects on aphid success, such as defensive signaling disruption.

The rhizosphere community has a direct impact on plant growth and defense, but the diversity and richness of this community alone does not determine the degree to which positive

interactions occur (11, 29, 143). It is well established that the microbial community of the rhizosphere is correlated with the resident plant community, mostly through modulation of root exudates by the plant and availability of bioavailable nutrients inherent in the soil (7, 8, 10). Soil microbial management designed to promote ecosystem services have garnered enormous support given increased pest immunity and topsoil erosion in commercial agricultural practices (22, 26, 27, 36, 62, 121, 122, 163, 168). Taken together, this research demonstrates how inherent plant assemblages may alter the soil microbial community and induce ISR depending on the succeeding crop. Further research is needed to define how crops may be rotated to minimize losses to aphid herbivory between various crop species.

CHAPTER III

INDIRECT IMPACT OF SOIL MICROBIAL DIVERSITY ON APHID-PLANT INTERACTIONS IN SYMBIOSES MUTANTS AND WILD TYPE *MEDICAGO TRUNCATULA*

Abstract

The rhizosphere is a unique ecosystem consisting of microbial communities that have complex signaling pathways, which can influence the biological functions of plants. The community dynamics of these micro-environments are influenced by root, fungal, and bacterial exudates that can preferentially select for functional classes of microbes. Arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPR) are functional symbionts of the rhizosphere microbial community that have been extensively studied due to their ability to form symbiotic relationships with plants. Microbial symbiosis with PGPR and/or AM fungi can promote plant growth and trigger induced systemic resistance (ISR), resulting in defensive 'priming' of host plants. Consequently, 'primed' plants can activate stronger defensive responses and respond faster to future attacks by pathogens and insects. The biological system in this tripartite plant-insect-soil microbe study involves four genotypes of barrel medic plants (*Medicago truncatula*), pea aphids (*Acyrthosiphon pisum*), and microbial communities present in a commercial topsoil. The four genotypes used include wild-type A17, does not make infections 1 and 3(*Mtdmi1*, *Mtdmi3*), and reduced arbuscular mycorrhization (*Mtram1*). There were no significant differences in fungal or bacterial microbial populations according to genotype, however most genotypes demonstrated a grouping effect in non-metric multidimensional scaling. Although there were no observed differences in aphid colony weight

between genotypes, active treatments did have significantly lower aphid colony weight than autoclaved treatments. Plant growth in *Mtram1* inoculated with active soil demonstrated significantly lower root fresh weight and shoot dry weight than plants grown in autoclaved soil, demonstrating a negative relationship with this genotype and the soil inoculum used that was not present in any other genotype. This research demonstrated that rhizosphere composition and not richness alone was an important factor in determining plant growth and resistance to insects.

Introduction

Plants and the microbiome surrounding their roots interact with each other via complex signaling pathways. Plants exude up to 40% of their photosynthesized carbohydrates into the soil through their roots (3, 6). Together with sloughed and dead plant cells, these carbohydrates create a zone of plant root exudate influence called the rhizosphere. This microbial community consists of plant pathogens, mutualists, and symbionts that are constantly communicating and interacting with the plant. Symbiotic microbes, such as plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal (AM) fungi, exchange limiting nutrients for plant carbohydrates through root structures called nodules (rhizobacteria) and arbuscules (AM fungi) (30, 32-34, 229). The oldest documented case of a microbial symbiotic relationship with plants is with the AM fungi within Glomeromycota, of which members form a relationship with up to 80% of all land plants (30). These fungi not only provide limiting nutrients, such as phosphate, they provide drought resistance and communicate signals from other plants through a dense hyphal network (29, 41). More recently on the evolutionary timeline, legumes have formed a novel symbiotic relationship with nitrogen fixing rhizobacteria in which ammonia is exchanged for carbohydrates within symbiotic structures called nodules (230-232). Collectively, AM fungi

and PGPR not only promote plant growth, they bolster plant defensive capabilities through microbial induced systemic resistance (ISR) (49, 105, 233).

The resistance conferred by symbiotic microbes is a type of defensive priming in which the plant reacts faster to subsequent pathogen infection or insect herbivory than it would without a symbiosis (72, 96, 234). Another type of defensive priming, deemed systemic acquired resistance (SAR), is conferred after pathogenic attack which requires infection and recovery before resistance to future pathogen attack (43, 46). This type of defensive priming is usually in response to detection of pathogen-, microbe-, or damage- associated molecular patterns (P/M/DAMPs) in which cell wall components of genera-specific compounds are detected by pattern recognition receptors (PRRs) on the plant cell wall, resulting in PAMP-triggered immunity (PTI) and/or effector triggered immunity (ETI) (34, 51, 52, 54-56, 58, 62, 235, 236). SAR is associated with a salicylic acid (SA) accumulation in distal tissues and is defined as being pathogen induced (47, 48). Many PGPR and AM fungi similarly produce MAMPs that are detected by PPRs, but these are recognized by the plant as symbiotic factors that activate SA-independent defense (40, 174, 177, 237). Instead, the ISR in plants by symbiotic microbes is typically characterized by induction of the jasmonic acid/ ethylene (JA/ET) defensive pathway (45, 109, 202, 238, 239). The JA/ET defensive pathway is associated with resistance to necrotrophic whereas the SA pathway is more effective against and biotrophic pathogens (46, 71, 72, 240). Both JA and SA act in the defense against insects depending on the plant, insect herbivore, and mechanism of feeding (241-244) The crosstalk between SA and JA/ET plant defensive pathways is important because the plant is often under attack by pathogens and herbivores simultaneously, and the two pathways are often antagonistic (92, 128, 245, 246). Some insects, such as aphids, may contain effectors in their saliva that modulate the plant

immune system through JA/SA antagonism (128). Aphids also use specialized mouthparts called a stylet to follow a sugar gradient to phloem cells while avoiding puncturing other cells as much as possible (123, 126). Plants defend themselves against aphids through induction of JA and/or SA mechanisms that increase plant concentrations of callose, glucosinolates, protease inhibitors, and methyl salicylate (129, 242-244). This delicate balance of SA and JA is often a target for disruption by aphid saliva and symbiotic aphid bacteria injected into plants through the stylet (199-201). Through this feeding behavior and JA/SA antagonism, aphids can directly benefit from the increased nutritional status conferred by beneficial rhizosphere microbes (123, 125, 130).

The impact of the rhizosphere microbiome on induced plant defenses against aphids is important for agriculture as aphids are a major crop pest and the impacts of their economic damage may be offset by managing agricultural systems for the promotion of PGPR and AM fungi (14, 25, 27, 122, 185, 247, 248). Models and experiments that explore three-way insect-plant-microbe interactions are important for understanding and implementing microbial inoculants and agricultural practices that exploit the enhanced defenses conferred by rhizosphere microbes. This experiment was designed to investigate the role of symbiotic microbes found in a commercial topsoil on *Medicago truncatula* plant defense against pea aphids, *Acyrthrosiphon pisum*. Four genotypes of *M. truncatula* with varying capacities to form a symbiosis with AM fungi and/or PGPR were used to explore how each of these symbionts, if present in the soil inoculum, impact plant defense against aphids. The four genotypes used are wild type Jemalong A17, *Mtram1* (reduced arbuscular mycorrhization) (249), *Mtdmi1* (does not make infections), and *Mtdmi3* (250, 251). The *dmi* mutants are not able to form nodules with certain rhizobacteria (250, 251). MTDMI1 is a membrane spanning protein that is necessary for the induction of

calcium spiking in plant root hairs resulting in lateral root formation that occur during both nodule and arbuscule formation (250, 252). MTDMI3 functions downstream of MTDMI1 and is a calcium/calmodulin dependent protein kinase involved in decoding the calcium spiking induced by nodulation and mycorrhization factors (250-252). The *Mtdmi3* mutants are not able to form a symbiosis with either rhizobia or AM fungi while the *Mtdmi1* mutants are also not able to form a symbiosis with rhizobia, but have demonstrated reduced AM fungal root colonization (253-255). The mutation in the *Mtram1* genotype results in abnormal function of plant specific GRAS-domain (GIBBERELLIC-ACID INSENSITIVE, REPRESSOR of GAI, and SCARECROW) transcription factor that results in no AM symbiosis due to a defect in hyphopodium formation, but retains the ability to form nodules (256, 257). By using these *M. truncatula* mutants that interfere with AM symbiosis (*Mtram1*) and rhizobial symbiosis (*Mtdmi1*), or both (*Mtdmi3*), in addition to the wild type (A17), we can explore how each of these guilds of rhizosphere microbes modulate plant defense against aphids.

The objective of this study was to assess the impact of loss of AM fungal and rhizobial symbioses on plant growth (with and without soil microbes), soil microbial populations, and plant-aphid interactions. With the full ability to benefit from AM fungi and rhizobial functional symbioses through increased nutrition, wildtype A17 *M. truncatula* grown in active soil are hypothesized to have the greatest root fresh weight and shoot dry weight. Rhizosphere diversity has been previously attributed to increased plant growth, although the specificity of microbe and host plant and not just species richness often determines the impact of the rhizosphere microbiome (153, 221, 226, 258).

Similarly, plant defense against aphid herbivory was hypothesized to be greatest in wildtype A17 due to the potential ISR conferred by both AM fungi and rhizobia whose presence

were not confirmed specifically in this study, but illustrated through endophytic diversity and richness, plant ability to form a symbiosis, and aphid colony weight. This hypothesis was formed under the assumption that as the ability to form a symbiosis is degraded, so too will plant defensive capabilities against aphids. Lastly, it was hypothesized that the ability to form a functional symbiosis would impact the rhizosphere and endosphere microbial community. An insect herbivory study was conducted on each genotype and combined with automated ribosomal intergenic spacer analysis (ARISA) of surface sterilized root tissue to determine which symbiosis mutation had the greatest positive or negative impact on plant induced systemic resistance against aphids.

Methods

Soil Substrate

The soil inoculum used in this experiment was a topsoil from Pioneer Sand Co. (Windsor, Colorado), which was dried, crushed, and filtered using 2mm mesh and mason sand (Pioneer Sand Co., Windsor, Colorado. This topsoil was readily available and has been used as an autoclaved substrate in previous studies involving AM fungi-plant interactions (133). The Pioneer topsoil is described as a screened sandy loam representing the top 5” of a field (https://www.pioneersand.com/ products/garden_bed_solutions/topsoil_fill_dirt/a_topsoil) and is likely an aggregate of several soils. The sand was rinsed with tap water and decanted until the water was clear (8-10 times), bagged, and autoclaved twice (60 min, 121°C, at 15 psi). The soil substrate was prepared by mixing seven parts of sterile sand, one part of topsoil, and one part of sand in which seedlings were grown containing root exudates. Each genotype was also grown in the same topsoil that was sterilized by autoclaving two times (60 min, 121°C, at 15 psi) and prepared in the same ratio. This creates eight soil/ genotype treatment groups in total, four

"autoclaved" and four "active" with the microbial community in the topsoil that was used as inoculum. All soil substrates were saturated with half strength modified Hoagland's nutrient solution (100 µM P) and placed in 6.35cm x 6.35cm x 8.89cm pots for an approximate soil volume of 330 cm^3. Each soil/genotype treatment consisted of ten biological replicates that included one plant per pot. Eight soil/genotype treatments received aphids and eight soil/genotype treatments did not receive aphids (160 plants total).

Plant Growth Conditions

Seeds of *Medicago truncatula* Jemalong A17 (wild type), and the mutant lines *Mtdmi1*, *Mtdmi3*, and *Mtram1* were scarified and surface-sterilized, and germinated for 7 days as previously described (210). The *Mtdmi1* and *Mtdmi3* seeds were kindly provided by Dr. Maria Harrison (Boyce Thompson Institute for Plant Research, Ithaca, NY, USA) and *Mtram1* seeds were provided by Dr. Giles Oldroyd (University of Cambridge, Cambridge, UK). Seedlings were planted in sterile sand that was saturated with half strength modified Hoagland's nutrient solution (100 µM P) which encourages AM fungi colonization, if present (211, 212). Plants were placed in a growth chamber with an 8/16 dark (22 °C)/light (25 °C) regimen for 10 days with 80% humidity. Seventeen-day old plants were divided based on size and distributed evenly among treatments. Subsets of these plants were pots with the volume of soil listed above consisting of 7 parts of autoclaved sand, 1 part sand in which seedlings were grown in, and 1 part of active or autoclaved topsoil (Pioneer Sand, Windsor, Colorado). All plant replicates for a single treatment were grown on a single tray designed for the size of the pot. Plants were then grown for an additional 40 days in a growth chamber as described above and were fertilized with 10 mL of ½ strength modified Hoagland's solution twice a week. Each plant was covered with fine mesh bags and plants were transferred into insect proof cages inside of a greenhouse with a

light cycle of 8/16 dark/light for an additional two weeks, or until plants were 71 days old (Table 5). Plants were harvested at 81 days old. An analytical balance was used to measure root fresh weight (RFW) and shoot dry weight (SDW) at the time of harvest. SDW was determined after dehydration overnight in an oven at 60°C.

Pea Aphid Herbivory

Aphids used in this experiment were parthenogenetic female pea aphids (*Acyrthosiphon pisum*) that were provided by Dr. Kenneth Korth (University of Arkansas, Fayetteville, Arkansas, USA) and were reared on fava bean (*Vicia faba* L.) plants as previously described (133). Three apterous pea aphid female adults from a synchronized colony (seven days old) were allowed to feed for ten consecutive days on plants that included an herbivory treatment. Plants were allowed to acclimate to greenhouse conditions from growth chamber conditions for two weeks before aphids were added. All the surviving aphids from each plant (referred to as a colony) that included an herbivory treatment were collected via fine paintbrushes and transferred to petri dishes on the day of harvest before being frozen at -20 °C. Aphids were weighed the day after plants were harvested by immobilizing them at -20 °C, transferring the entire colony to a foil bowl using a fine paint brush, and weighing using a microbalance (SE-2F Sartorious balance, Denver, CO, USA).

Microbial Deoxyribonucleic Acid Isolation and Community Analysis

The rhizosphere fraction of the soil was collected for each plant replicate by removing the plant from the bulk soil of the pot and disturbing the roots with a sterilized spatula. All rhizosphere soil collected from each replicate were pooled by treatment and thoroughly mixed. Autoclaved Milli-Q water was used to rinse the soil. Soil and water were disturbed and decanted into a vacuum filter flask with a Whatman #1 filter paper and sterilized ceramic filters until the

all fine soil is extracted, which was determined by a clear decant. The filter paper and contents were ground with a mortar and pestle before DNA extraction using the DNeasy PowerSoil® kit (QIAGEN®). Endophytic bacterial and fungal DNA were extracted from plant roots that were surface sterilized. Root surfaces were sterilized by rinsing with tap water, immersing in formaldehyde for 7 minutes, immersing in sodium hydroxide for 10 minutes, and rinsing three times with autocalved Milli-Q water by vortexing for 2 minute each time (259). Automated ribosomal intergenic spacer analysis (ARISA) was conducted on internal transcribed spacer (ITS) regions of bacteria and fungi as previously described by Ranjard et al. (2003) (213). PCR amplification using 16S ribosomal sequences of bacteria and the 18S/28S sequences in other soil organisms, such as fungi, was conducted using the fluorescently labeled HEX primers S-D-Bact-1522-b-S-20 (small ribosomal subunit)/L-D-Bact-132-a-A-18 (large ribosomal subunit) for bacteria and fluorescently labeled FAM primers 2234C/3126T for fungi (207, 213). The sequence for S-D-Bact-1522-b-S-20/L-D-Bact-132-a-A-18 are 5'-TGCGGCTGGATCCCCTCCTT-3'/ 5'-CCGGGTTTCCCCATTCGG-3', respectively, and the sequence for 2234C/3126T are 5'-GTTTCCGTAGGTGAACCTGC-3'/5'-ATATGCTTAAGTTCAGCGGGT-3', respectively (207). Two PCR technical replicates per sample were used for endophytic DNA using fungal and bacterial primers, and rhizosphere DNA using fungal and bacterial primers (8 samples total). Each rhizosphere PCR sample consisted of 0.6 uL of each primer (10 µM each), 2.4 uL GoTaq buffer (Promega), 0.7 uL dNTPs (10mM each), 1 uL $MgCl_2$, 0.06 uL Taq polymerase (5U/uL), 3.96 uL DNA-free water, 0.06 uL of bovine serum albumin (BSA), and 3 uL of DNA template as described previously for 'shotgun' PCR protocols (213). Each endophyte PCR sample consisted of the same mixture, but with 1.96 uL DNA-free water, and 5 uL of DNA template. Thermal profiles used in PCR were as

previously described for ribosomal intergenic spacer analysis (207). PCR amplification was verified via gel electrophoresis before drying, shipping, and running fragment analysis on an Applied Biosystems 3500 Genetic Analyzer by the DNA Lab at Arizona State University. The spectrograph profiles were analyzed for peak size in base pairs and peak area in base pair*reflective fluorescence units (RFUs) using Thermo-Fisher Connect™ microsattelite analysis (MSA) online application (https://apps.thermofisher.com/editor-web/#/app/app-microsatellites-web). Both technical replicates of the spectrographs of PCR products generated using bacterial and fungal primers from the active soil treatments (16 profiles) were analyzed using Peak Window Sizes 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, and 31 (in datapoints) within the MSA app. Only active ARISA profiles were used for window comparison due to the low number of detection in autoclaved treatment ARISA profiles. The automatic_binner.r script produced by Ramette (214) was used to determine optimal Peak Window Size, which was chosen based on the size in which the correlation factor was over 60 (window size of 23 for bacteria and 21 for fungi). All treatments were run with their respective window size and replicates were merged using Microsoft Excel. The peak size was rounded to the first integer using Excel round function and Highlight Duplicates was used to color fill cells in which peak sizes were found in both replicates. The peaks were filtered by fill color and the peak areas were averaged to create one ARISA profile for each treatment. All peaks that were not detected in both replicates were discarded.

Statistical Analysis

All statistical analysis were performed using R studio (215). ARISA profiles were analyzed for Shannon-Weiner diversity (H), species richness (S), Jaccard (J) Index of similarity, and non-metric multidimentional scaling (NMDS) using genotype, aphid herbivory, and

autoclave treatment as factors using the vegan package in R studio (216). Shapiro-Wilk test was conducted on plant and aphid data to confirm normality using the car package (220). Raw data that were not normally distributed were visualized for skewedness, and transformed according to the ladder of powers (219) until normality was achieved using Shapiro-Wilk tests. This resulted in a cube root transformation for shoot dry weight, a square root transformation for root fresh weight, and a log transformation for aphid colony weight. Three-factor analysis of variance (ANOVA) was conducted on shoot and root data, having aphid herbivory, plant genotype, and soil condition (autoclaved or active) in R studio (220, 260). When a three factor ANOVA was non-significant, a two factor ANOVA was conducted. The Tukey HSD test was used for pairwise comparisons using the pairs ( ) function (220). Individual Plots produced by NMDS and ANOVA were visualized using the packages ggplot2 and cowplot in R studio (260, 261). To demonstrate the effect of the soil condition (active vs autoclaved) on plant growth, pairwise t tests were conducted on root fresh weight and shoot dry weight using the package car (220).

Results

Impact of Loss of AM and Rhizobial Symbioses on Aphid Herbivory and Plant Growth

Shoot dry weight and root fresh weight measurements were taken to compare the effect of microbes present in active soil and absent in autoclaved soil on plant growth of *M. truncatula* wild type (A17) and symbioses mutants (*Mtdmi1*, *Mtdmi3*, and *Mtram1*) on remaining replicates (Table 5). For shoot dry weight, the interaction between genotype, aphid herbivory, and soil condition was statistically significant, as well as the interaction between genotype and soil condition, and the main effect of soil condition (Figure 6). Among active soil, *Mtram1* plants without aphids demonstrated significantly lower shoot dry weight than WT A17 without aphids,

Mtdmi1 without aphids, and WT A17 with aphids (Figure 6). Active *Mtram1* without aphids was also significantly lower than all other autoclaved genotypes with and without aphids except for autoclaved *Mtdmi1* with aphids (Figure 6). Both active *Mtram1* were significantly lower than both autoclaved *Mtram1*, regardless of aphid herbivory (Figure 6). For root fresh weight, the three-factor interaction between soil condition, aphid herbivory, and genotype was not statistically significant. However, two factor ANOVA demonstrated significant interaction between soil condition and genotype as well as the main effects of soil condition and genotype (Figure 7). The interaction between genotype and soil condition was also significant for root fresh weight, as was the main effects of genotype and soil condition (Figure 7). *Mtram1* plants grown in active soil inoculum weighed less than all other treatments except for active WT A17 (Figure 7). Mean aphid colony weight represents all live aphids that were present on a plant. The interaction between genotype and soil condition (active vs autoclaved) was not significant, nor was the main effect of genotype, but the main effect soil condition was significant (Figure 8).

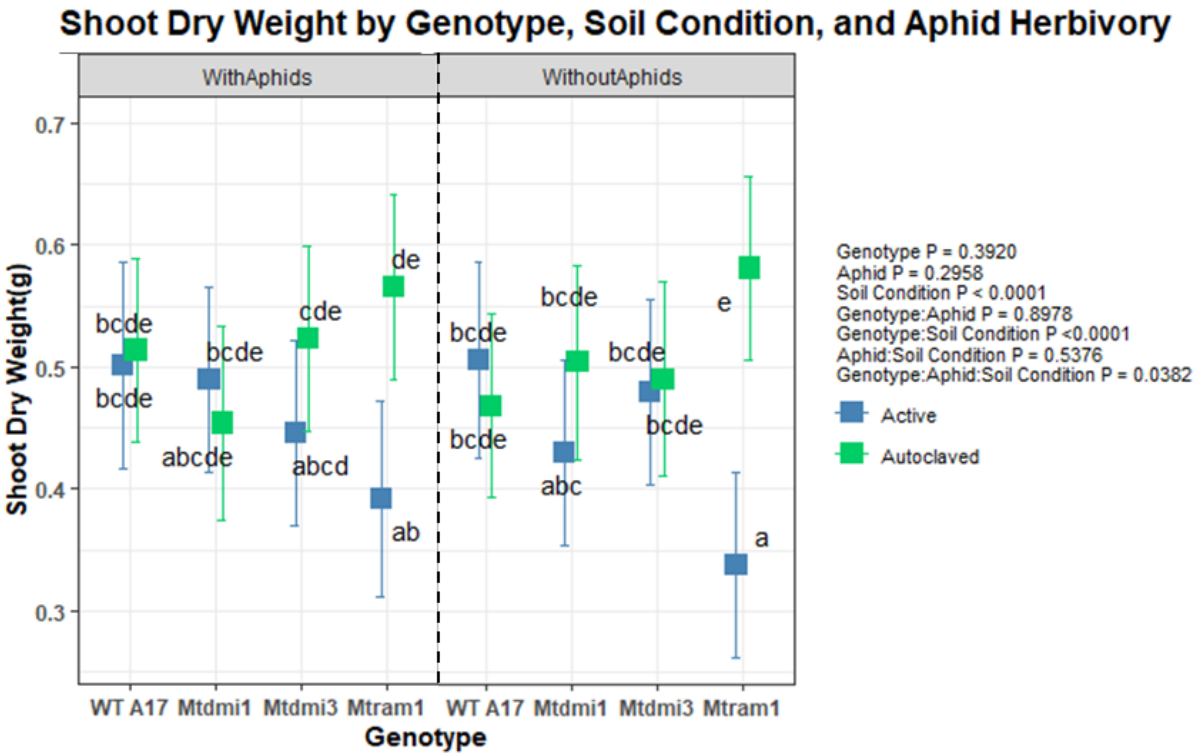

Figure 6. Effect of pea aphid (*Acrythrosiphon pisum*) herbivory, *Medicago truncatula* genotye, and soil condition (active vs autoclaved) on shoot dry weight of wild type (A17) and symbioses mutants, *Mtdmi1*, *Mtdmi3*, and *Mtram1*. The *p* values shown represent the results of a three-factor ANOVA. The interaction between genotype, aphid herbivory, and soil condition had a statistically significant ($p < .05$) impact on shoot dry weight. Treatments that share the same letter are not statistically different from each other based on Tukey HSD test ($p < .05$).

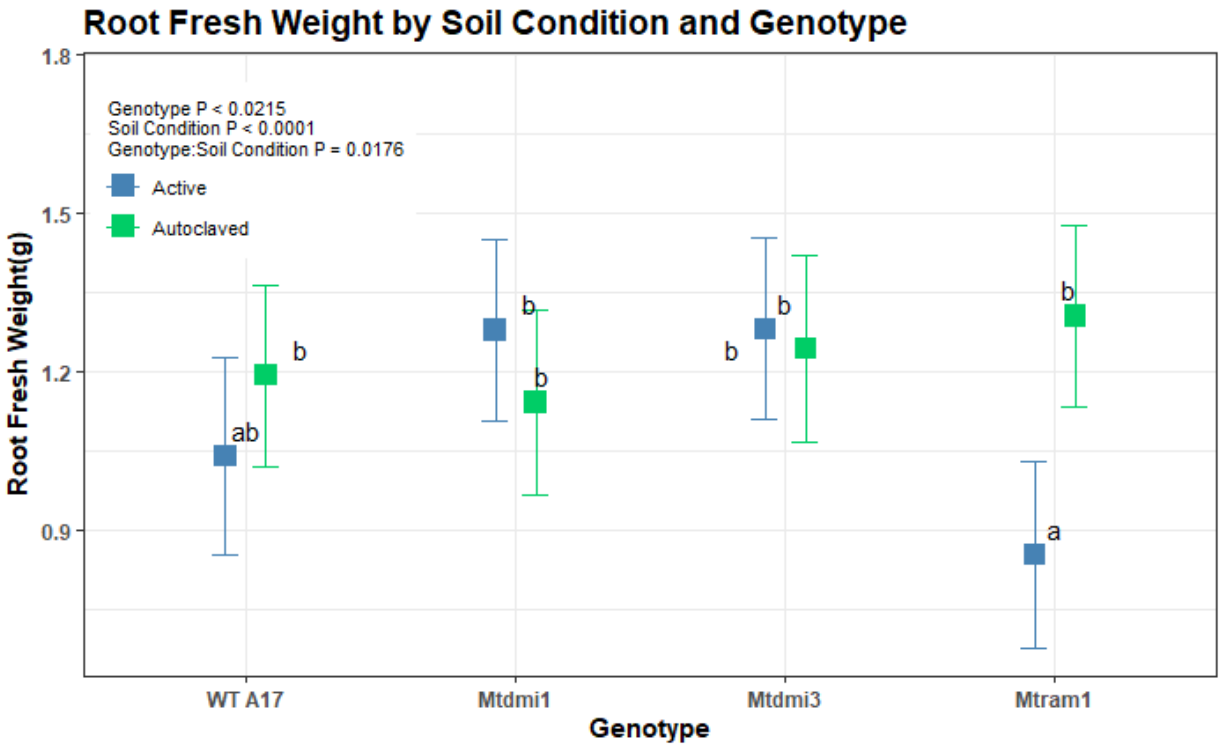

Figure 7. Effect of *Medicago truncatula* genotype and soil condition (active vs autoclaved) on mean root fresh weight of wild type (A17) and symbioses mutants, *Mtdmi1*, *Mtdmi3*, and *Mtram1*. The *p* values shown represent the results of a two-factor ANOVA. The interaction of plant genotype and soil condition had a significant ($p < .05$) impact on mean shoot fresh weight. Different letters represent statistically significant differences among treatment groups based on Tukey HSD test ($p < .05$).

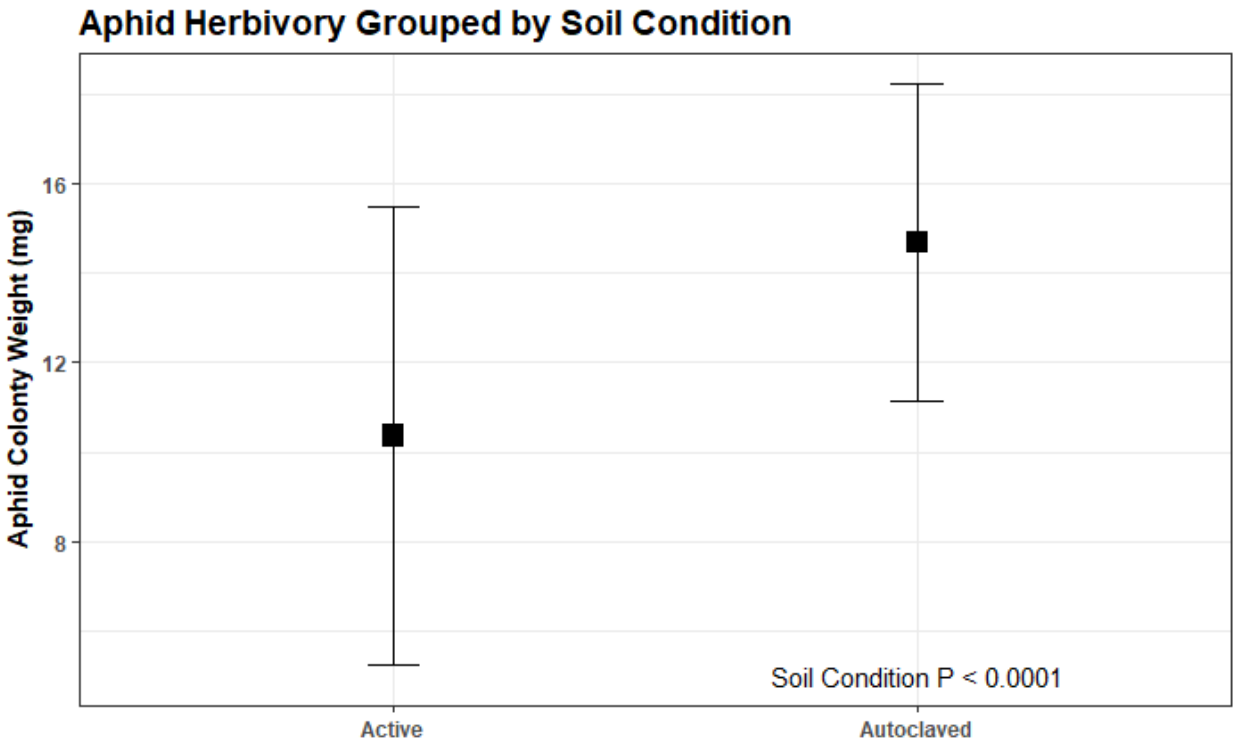

Figure 8. Mean pea aphid (*Acrythrosiphon pisum*) colony weight after feeding for 10 days on *Medicado truncatula* plants grown in active soil versus autoclaved soil.

Endospheric and Rhizospheric Microbial Communities

Endophytic and rhizospheric OTUs for bacteria and fungi were measured via ARISA for all treatments to further describe the effect that plant genotype, aphid herbivory, and soil condition had on the microbial population. As expected, soil condition (active vs autoclaved) had a significant effect on endosphere bacterial and fungal communities and on rhizosphere bacterial communities (Table 6). Neither aphid herbivory nor genotype had a significant effect on endosphere or rhizosphere bacterial and fungal communities (Table 6). The highest number of endophytic bacterial OTUs was observed in *Mtdmi1* plants grown in active soil without aphids, which also had the highest diversity among all endophytic OTU profiles (Table 7). The

lowest endophytic bacterial OTU diversity and richness was seen in wild type A17 plants grown in active soil with aphids, wild type plants grown in autoclaved soil with no aphids, and wild type plants grown in autoclaved soil with aphids. (Table 7).

Table 6. ANOSIM results for fungal and bacterial community profiles. Soil type ANOSIM were conducted with active data only. * represents significant values

Factor	**R^2**	**P-value**	**R^2**	**P-value**
	Fungal Rhizosphere		Bacterial Rhizosphere	
Soil Condition (Active / Autoclaved)	0.7589	0.0001*	0.731	0.0003*
Aphid Herbivory (With Aphids / Without Aphids)	-0.1161	0.9591	-0.1007	0.9831
Genotype	0.0773	0.2328	0.0560	0.2588
	Fungal Endosphere		Bacterial Endosphere	
Soil Condition (Active / Autoclaved)	0.0010	0.3777	0.2996	0.0037*
Aphid Herbivory (With Aphids / Without Aphids)	-0.0690	0.7860	0.0204	0.3674
Genotype	-0.0913	0.7425	-0.0215	0.5443

Among rhizospheric bacterial OTUs, there were more OTUs detected using autoclaved soil treatments for all plant genotypes with aphids than any other treatment, suggesting potential contamination (Table 7). Diversity indices of rhizosphere bacteria did not change drastically between genotypes, but lower species richness was observed in mutants grown in active soil with and without aphids compared to wild type (Table 7).

Table 7. Shannon-Weiner Index (H) and Species Richness (S) for endophytic and rhizospheric bacterial operational taxonomic units (OTUs) after ARISA analysis. No OTUs were detected using DNA from wild type plants grown in autoclaved soil with aphids.

Endospheric Bacterial OTUs								
Genotype	**Active w/o Aphids**		**Active with Aphids**		**Autoclaved w/o Aphid**		**Autoclaved with Aphids**	
	H	**S**	**H**	**S**	**H**	**S**	**H**	**S**
WT A17	3.7	60	0	1	0.1	2	NA	NA
Mtdmi1	3.8	91	0.6	2	0.9	5	0	1
Mtdmi3	2.9	43	2.9	33	2.1	16	2.9	69
Mtram1	2.5	39	2.5	40	2.5	78	2.3	53
Rhizospheric Bacterial OTUs								
Genotype	**Active w/o Aphid**		**Active with Aphid**		**Autoclaved w/o Aphid**		**Autoclaved with Aphids**	
	H	**S**	**H**	**S**	**H**	**S**	**H**	**S**
WT A17	3.6	106	3.6	117	3.6	123	3.6	132
Mtdmi1	3.4	80	3.4	107	0.7	2	3.3	147
Mtdmi3	3.3	81	3.3	71	3.0	96	3.6	145
Mtram1	3.6	80	3.3	73	3.1	80	3.5	123

The highest diversity and richness within fungal profiles was observed in *Mtram1* plants grown in active soils without aphids, and the lowest was detected in *Mtdmi1* plants grown in autoclaved soils with aphids (Table 8). The greatest rhizospheric fungal species richness among active soil treatments was observed in wild type plants with aphids, but the highest overall was observed in wild type plants grown in autoclaved soil with aphids (Table 8). The highest rhizospheric diversity indices among active soils were seen in *Mtdmi1* and *Mtram1* grown in active soils with aphids, despite these mutant lines inability (*Mtram1*), or limited ability (*Mtdmi1*) to form AM symbioses (Table 8). The highest fungal rhizosphere richness overall was observed in wild type plants grown in autoclaved soil with aphids (Table 8) indicating that there was some type of contamination.

Table 8. Shannon-Weiner Index (H) and Species Richness (S) for endophytic and rhizospheric fungal operational taxonomic units (OTUs) after ARISA analysis. No OTUs were detected using DNA from wild type plants grown in autoclaved soil with aphids.

Endospheric Fungal OTUs								
Genotype	**Active w/o Aphids**		**Active with Aphids**		**Autoclaved w/o Aphid**		**Autoclaved with Aphids**	
	H	**S**	**H**	**S**	**H**	**S**	**H**	**S**
WT A17	2.3	30	0.8	45	1.3	35	2.5	40
Mtdmi1	1.0	28	1.2	39	1.9	30	0.3	6
Mtdmi3	NA	NA	0.8	15	0.6	17	3.4	41
Mtram1	3.5	56	NA	NA	0.6	23	0.8	16
Rhizopsheric Fungal OTUs								
Genotype	**Active w/o Aphid**		**Active with Aphid**		**Autoclaved w/o Aphid**		**Autoclaved with Aphids**	
	H	**S**	**H**	**S**	**H**	**S**	**H**	**S**
WT A17	2.4	23	3.1	86	2.9	64	3.0	138
Mtdmi1	2.8	33	3.2	74	2.3	55	2.5	81
Mtdmi3	2.7	53	2.8	46	3.1	60	2.3	44
Mtram1	2.7	62	3.2	33	3.0	73	2.6	34

Similarity and Non-metric Multidimensional Scaling of Rhizospheric and Endospheric Microbes

The Jaccard index of similarity was used to compare OTU detections among bacteria and fungi within and among the rhizosphere and endosphere (Table 9). Similarity between rhizospheric bacteria and fungi between plant genotypes was high overall, with the greatest similarity being between wild type and *Mtdmi1* for bacteria, *Mtdmi1* and *Mtdmi3* for bacteria, and *Mtram1* and *Mtdmi3* for fungi (Table 9). The lowest similarity for rhizospheric microbes was between *Mtdmi1* and *Mtdmi3* for bacteria, and wild type and *Mtdmi3* for fungi (Table 9). Endophytic similarity was highest among *Mtdmi1* and *Mtram1* for bacteria, and *Mtdmi3* and *Mtram1* for fungi (Table 9). The lowest endophytic similarity observed was between wild type and *Mtram1* for fungi, and between *Mtdmi1* and *Mtdmi3* for endophytic bacteria (Table 9).

Table 9. Jaccard index of similarity for fungal and bacterial operational taxonomic units. Above values (grey) cells are Fungal OTU similarities, below cells are Bacterial OTU similarities. Only plant genotypes grown in active soil without aphids were compared. en = endophytic samples. There were no reported OTUs for endophytic fungi in *Mtdmi3*.

	A17	***Mtdmi1***	***Mtdmi3***	***Mtram1***	**enA17**	**enDMI1**	**enDMI3**	**enRAM1**
A17		0.85	0.71	0.82	0.99	0.93	NA	0.99
Mtdmi1	0.83		0.84	0.85	0.98	0.94	NA	0.96
Mtdmi3	0.76	0.73		0.86	0.98	0.94	NA	0.99
Mtram1	0.81	0.83	0.81		0.99	0.97	NA	0.98
enA17	0.94	0.97	0.96	0.96		0.92	NA	0.73
enDMI1	0.96	0.95	0.96	0.96	0.85		NA	0.95
enDMI3	0.94	0.95	0.95	0.97	0.84	0.82		NA
enRAM1	0.94	0.95	0.95	0.97	0.90	0.91	0.86	

Non-metric multidimensional scaling (NMDS) was conducted to compare fungal and bacterial profiles between different factors such as aphid herbivory, autoclave or active soil, and plant genotype. There were no significant differences in the ordination of endophytic fungi grouped by active and autoclaved soil (Figure 9A), but there was a significant difference in the relationship of rhizospheric fungal OTUs grouped between active and autoclaved soil treatments (Figure 9B). Rhizospheric fungal groupings by mutants seemed to follow a pattern of relation starting with wild type, then *Mtdmi3*, *Mtdmi1*, and *Mtram1* (Figure 9 B). It is also noteworthy that wild type rhizospheric fungi seemed to undergo a large shift with the addition of aphids, indicated by a green arrow (Figure 9B). The stress for endophytic fungal OTU ordination was 0.1333 and the stress for rhizospheric fungal OTU ordination was 0.1446.

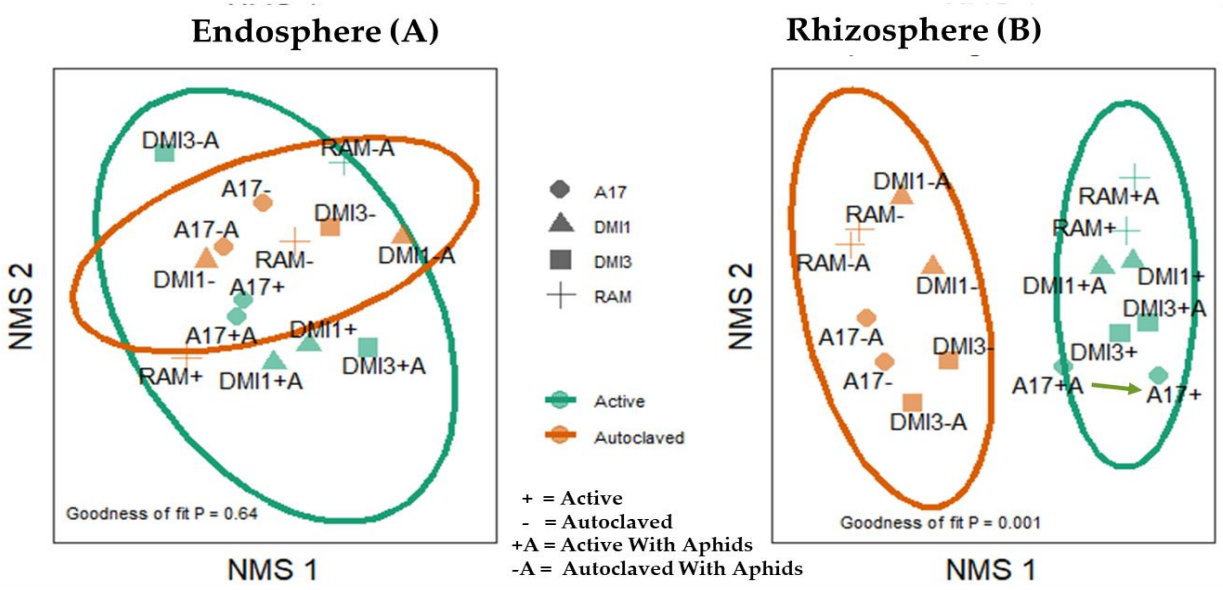

Figure 9. Non-metric multidimensional scaling (NMDS) of operational taxonomic units grouped by endophytic (A) and rhizospheric (B) fungi present in active or autoclaved soil. Treatments with an "A" at the end of the label represent treatments with aphids, while those without an "A" at the end represent treatments without aphids. Treatments with a "-" represent autoclaved soil, while "+" denotes active soil. Goodness of fit *P* values represents the

Goodness of fit linear models were not significant for endophtyic bacterial OTU profiles for plant genotype or aphid herbivory but was statistically significant between active and autoclaved soil treatments (Figure 10B), a result that was not shared with fungal endophytes. Rhizospheric bacterial OTU data were insufficient for ordination and are not shown.

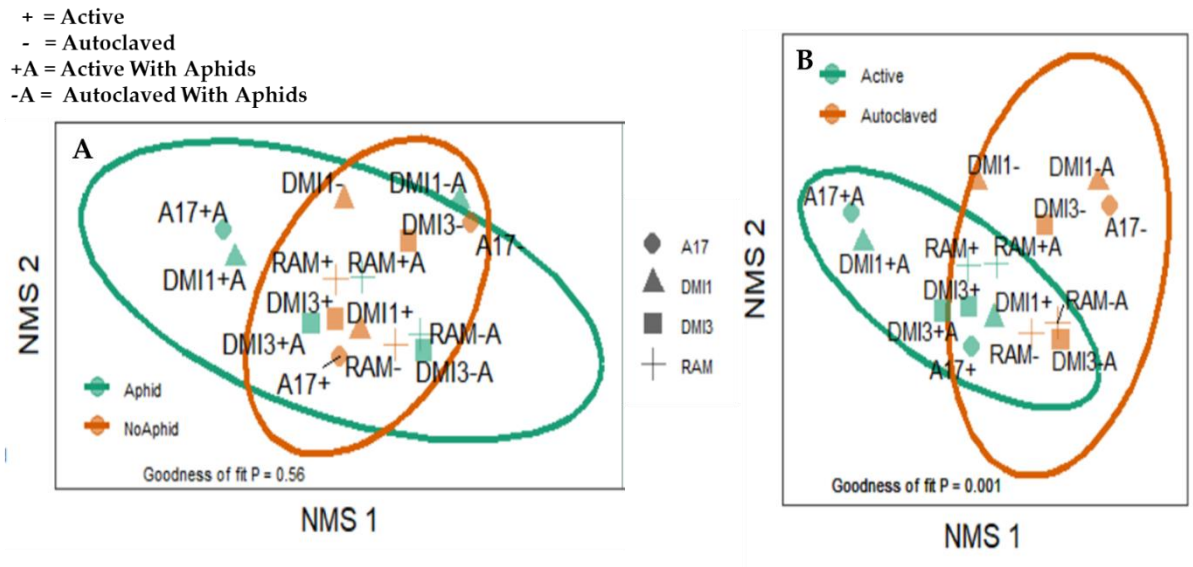

Figure 10. Non-metric multidimensional scaling (NMDS) of operational taxonomic units for endophytic bacteria grouped by aphid herbivory (A) and by active or autoclaved soil (B). Treatments with an "A" at the end of the label represent treatments with aphids, while those without an "A" at the end represent treatments without aphids. Treatments with a "-" represent autoclaved soil, while "+" denotes active soil. Goodness of fit *P* values represents the

Discussion

The present study was designed to assess the role of AM and rhizobial symbioses in determining rhizosphere bacterial and fungal communities, and how these changes translate to plant growth (with and without soil microbes) and defense against aphids. It was hypothesized that mutations in plant symbiotic genes would impact the microbial community of both the endosphere and rhizosphere. A study that used *Lotus japonicus* found significant changes in both the endosphere and rhizosphere when symbiotic genes were knocked out, including *Mtram1* (262). In this study the authors demonstrate reductions in members of Glomeromycota in *Mtram1* roots, although some Glomeromycetes were present, followed by increases of other fungi in the endosphere (262). Interestingly, this increase in other fungal endophytes, including Helotiales and Nectriaceae, was attributed to niche replacement rather than the loss of

RAM1(262). This niche replacement may have been evident in the present study through the richness between active treatments without aphids in which rhizosphere fungi were greatest in *Mtram1* (Table 8) and endosphere bacteria were greatest in active *Mtdmi1* (Table 7), although the specific composition was not identified. The present study found no significant differences in the rhizosphere or endosphere microbial communities grouped by genotype via ANOSIM, but there were interesting groupings in the NMDS ordinations (Table 6). There was a clear grouping of active fungal OTUs by genotype for all mutants in the rhizosphere (Figure 9b) whereas only *Mtdmi1* and WT A17 grouped in active endospheric fungi (Figure 9a). This points towards either a direct impact on the fungal community as a result of the symbiosis mutation, or an indirect effect in which the available symbiont altered the fungal rhizosphere, or both, although the presence of specific symbiotic microbes was not confirmed in this study. This also is supported by autoclaved fungal rhizosphere groupings in NMDS ordination in which genotypes similarly grouped together (Figure 9b). Robust detections in autoclaved treatments for both bacteria and fungi suggests contamination and there was a significant difference in fungal endosphere communities grouped by soil condition. These groupings by genotype in both active and autoclaved soils, despite significantly different composition, suggests that the symbiosis genes impact a variety of life history traits, although the specific composition of these fungal communities were not tested. For instance, in *Mtdmi3* no endophytic fungal symbionts were detected suggesting that no relationships could be formed without a functional gene (Table 7). Conversely, bacterial endospheric communities also demonstrated a grouping by genotype in active *Mtdmi3* and *Mtram1* and autoclaved *Mtdmi1* and *Mtram1*, although the mechanisms behind these groupings remains to be elucidated (Figure 10).

Plant growth parameters were hypothesized to be greatest in WT A17 plants with the ability to form AM and rhizobial symbioses. This was partially supported for shoot dry weight with active WT A17 with and without aphids being significantly greater than *Mtram1* without aphids, but not *Mtdmi1* or *Mtdmi3* with or without aphids (Figure 6). It is of note that the greatest shoot dry weight was seen in autoclaved *Mtram1* with and without aphids (Figure 6), suggesting that the effect of the altered microbial population was the cause of reduced growth, and not plant genotype alone. This reduced plant growth could be indicative of a pathogenic effect in *Mtram1* that was not present in other mutants, although the presence of pathogens was not confirmed in this study. In a study evaluating colonization by an AM fungi and by a pathogenic oomycete, Gobbato et al. (2013) demonstrated that while *Mtram1* had reduced mycorrhization, it had no effect on the pathogenic colonization (256). Few studies have explored the effect of pathogen performance on symbiotic mutants and those that have demonstrate little to no effect on susceptibility compared to wild type in *Mtram1* (256, 263), or *Mtdmi1* and *Mtdmi3* for the pathogens tested (263). This further suggests that the lack of effect seen in WT A17 and the other mutants tested was due to an either ISR against the pathogen(s) that infected *Mtram1*, or changes in the microbial community that led to the suppression of pathogens, although gene expression associated with ISR was not tested.

Aphid colony weight in *M. truncatula* mutants was expected to be significantly lower than in WT A17. Since there were no significant differences among genotypes regarding aphid colony weight, this hypothesis was rejected. However, it is worth noting that when all genotypes were grouped by active or autoclaved soil treatment, aphids weighed less in active soil treatments. In many instances, species richness in both the rhizosphere and endosphere was greater in autoclaved soil treatments and diversity was generally greater in active soil treatments

(Table 6 and Table 7). This is also supported by differences between active and autoclaved bacterial and fungal communities in the rhizosphere and bacterial communities in the endosphere (Table 6). This points toward specific soil microbes in the Pioneer topsoil that were functionally important to the indirect effect on aphids and that were reduced or eliminated via autoclave treatment. In this instance, the number of species present was less relevant to plant resistance to aphids than functionality of those microbes. This effect has been demonstrated in several studies involving conditioned soils and insect herbivory and while it can be predictable, it is often not intuitive. For instance, Badri et al. 2013 demonstrated significantly lower cabbage looper (*Trichoplusia ni*) weight gain in *Arabidopsis* grown in soil collected from an unmanaged *Arabidopsis* field, they also found the same effect with plants grown in managed potato fields. In another study, Raklami et al. 2019 found that a consortium of AM fungi and PGPR were more beneficial for plant growth than either inoculum alone, suggesting that diversity is an important driver in rhizosphere functionality (42). In an herbivory choice study, Howard et al. (2020) demonstrated greater microbial biomass in later succession soils that also decreased probability of herbivory (258). In a separate study that did not quantify the microbial community, the same authors demonstrated a negative impact on a generalist insect in the same old succession soil (228). Plants have been shown to recruit specific microbes after herbivory to aid in defense, which could explain the detrimental effect seen in the study by Howard et al. (2020) rather than the increased microbial biomass as a whole(228, 264). While diversity seemed to play a role in plant growth via autoclaved treatments, this study showcases that diversity alone is not determinant in aphid resistance.

This experiment demonstrated an overall positive impact of active rhizosphere microbes inherent in the experimental soil on plant growth promotion and aphid resistance. Further

experiments using this model and known consortiums of symbiotic and pathogenic inoculum would have clarified this tripartite interaction between the loss of symbiosis and the subsequent effect on plant defense against aphids. Regardless, the significance of reduced aphid colony weight in active soil demonstrates at least one of the ecological services provided by rhizosphere microbes, which has greater significance in the context of agricultural systems and global food security. Overall, species composition and not just high diversity and richness was determinant in aphid herbivory. Although rhizosphere and endosphere microbial communities were not significantly different among genotypes, many of them grouped together in NMDS ordination suggesting that the plant genotype altered the microbial communities. This is further supported by *Mtram1* having significantly lower shoot dry weight and root fresh weight compared to its autoclaved counterpart both with and without aphids, suggesting some pathogenesis that was suppressed in the other genotypes. This research further advances the importance of the soil rhizosphere community regarding plant growth and resistance to herbivory.

CHAPTER IV

CONCLUSION AND FUTURE DIRECTION

Conclusion

The 2015 United Nations assessment on the status of the world's soil resources states that a majority soil resources worldwide are in very poor to fair condition (265). Conventional agricultural practices such as tilling (36, 165, 168), over-cropping (164), monoculture (169), and herbicide application (166, 167) are major contributors to this global problem. For example, conventional plowing of agricultural fields is resulting in the loss of farmable topsoil up to twice as fast as it can be produced (266). Not only is this erosion economically costly for farmers, it threatens the global food supply and exacerbates climate change through loss of sequestered carbon (267, 268). Although conventional farming and fertilizing produces greater crop yield than organically or progressively managed farms, it is clear that these practices are unsustainable and eventually lead to loss of productivity (267). Regenerative agriculture seeks to amend soil erosion while promoting rhizosphere microbial ecosystem services such as reduction of insect pests at no cost to the farmer, potentially offsetting the cost of reduced crop yield (269). The experiments outlined here support this statement by demonstrating how resident plant communities, or crops, can be used to promote rhizosphere microbial ecosystem services for succeeding plants, including plant growth and defense against insects. Data that support this finding include:

- In Chapter II, aphid colony per plant was reduced on *M. truncatula* wild type plants grown in alfalfa soil inoculum compared to plants grown on the commercial Pioneer topsoil inoculum.
- In Chapter III, active treatments in experiment 2 exhibited lower aphid colony weight than autoclaved treatments with significant differences between microbial populations when grouped by soil condition. This indicates that inherent soil microbes conferred resistance while contaminants in autoclaved treatments did not.
- In Chapter III, *M. truncatula* wild type plants grown in corn soil inoculum accumulated more shoot dry weight compared to plants grown in Pioneer soil inoculum.
- In Chapter III, *M. truncatula ram1* mutants grew less in active soil inoculum compared to autoclaved soil inoculum and other genotypes, both active and autoclaved. The difference in shoot dry weight was greatest between *ram1* mutants and WT A17, and between *dmi1* and *dmi3* mutants and *ram1* mutants in root fresh weight.

In experiments in Chapters II and III, the active soil inoculum, or inherent rhizosphere community significantly reduced aphid colony weight, although whether this priming was ISR was not explored. The reduced aphid colony weight in the alfalfa soil inoculated treatments not seen in any other soil inoculum used exemplifies how crop rotation, in this case alfalfa (*M. sativa*), could be used to support the rhizosphere ecosystem services of the next crop (Figure 5). Further research is warranted with this model in a field setting to determine if this effect is conferrable to agricultural management. The corn inoculum also demonstrated this effect, but for the ecosystem service of enhanced plant growth rather than insect resistance (Figures 2B and

3B). The Pioneer soil used in Chapter III was chosen based on preliminary data that demonstrated a unique relationship with aphids in the experiment in Chapter II involving the four soil inoculum, such as increased aphid susceptibility and greater shoot dry weight and root fresh weight in autoclaved soils than in active soils. Although not particularly informative in regard to elucidating AM fungi and rhizobial tripartite interactions, it did show significantly reduced aphid colony weight in active soils over autoclaved soils, potentially supporting the notion that the specificity of rhizosphere microbes and not only diversity and richness is a factor in plant resistance to insect herbivory. Conventional tilling methods have demonstrated consistent negative impacts on rhizosphere diversity and garners support for regenerative agriculture through maintenance of inherent soil communities (165, 270, 271). Furthermore, this study supported the ability to predict the impacts of the microbial population on plant growth in relation to the previous resident plant community and presence of symbionts. To move forward with a sustainable global food supply, we must explore new ways in which we can support and modify the rhizosphere microbiome to further benefit from naturally occurring plant-microbe interactions.

Future Directions

This research added to a rapidly growing wealth of research that explores plant-insect-beneficial soil microbe interactions. The future of this field has great potential to solve the global soil erosion and food supply crisis. Outside of further understanding specific plant-soil microbe communication and interaction, emphasis needs to be put into more in situ studies on farms that explore types of crop rotations and their benefit to succeeding plants. While the study described in Chapter II indicated that crop rotation could have varying effects on plant growth and aphid resistance, further field studies would be needed to confirm the results and feasibility

in agriculture. Additionally, while the active Pioneer soil inoculum conferred plant resistance when genotypes were grouped together, the soil choice did not appear to be ideal for describing the relationships between symbiotic genes and aphid resistance. Future studies involving varying soil inoculum from agricultural fields, such as those used in Chapter II, combined with confirmation of AM fungi and PGPR presence and abundance would more greatly define the role of symbiotic communities in conferring insect resistance. While ISR and/or SAR might be inferred through aphid resistance, categorizing the exact type of resistance through activation of PR genes and SA/JA spiking would have further indicated whether the resistance to aphids seen in Chapters II and III was conferred by symbionts or pathogens. Modelling regenerative agriculture on microbial communities that exist in field settings may also provide insight into ways in which these practices can be implemented on a large scale. As this field expands, an increasing number of microbes and plant succession combinations will likely be found that promote specific ecosystem services with specific crops.

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