Plant-soil interactions and stand decline in alfalfa: mechanisms and mitigation strategies

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ABSTRACT

In agricultural systems, effects of plants on soil microbial communities have been demonstrated to feedback over time and impact plant growth and productivity through plant-soil feedback (PSF). When negative, PSF results in productivity decline, limiting alfalfa (*Medicago sativa*) production. Unlike negative PSF, positive PSF promotes plant growth and improves productivity. Despite this, we do not fully understand the mechanisms of PSF and are thus limited in our strategies to mitigate productivity decline. Using the plant-soil feedback framework, we collected vegetation and soil samples from alfalfa stands grown to mixture (alfalfa-grass) and monoculture at stand ages 1 to 6 years old, near Saskatoon, SK. These soils were used in a completely randomized experimental design to inoculate 4 alfalfa varieties, viz. *2010*, *Foothold*, *3010*, and *Spyder*, and 5 other forage species, viz. *Onobrychis viciifolia*, *Trifolium pratense*, *Vicia americana*, *Elymus lanceolatus*, and *Agropyron cristatum*, of which traits depicting root economic spectrum and symbiosis were sampled. Additionally, next-generation amplicon sequencing was used to identify amplicon sequence variants (ASVs) of soil bacteria, oomycetes, and arbuscular mycorrhizal and other fungi in the inoculum associated with PSF. Field conditions including plant diversity, soil phosphorus, soil texture, weed abundance, and fiber content of focal crop mediated how plants condition soil microbial communities. These conditioning effects altered the relative composition of soil mutualists, plant-growth promoting microbes, saprotrophs and pathogens, all of which affected PSF. These PSFs, however, differed depending on the variety and crop species identity due to differences in how these plant types interacted with the soil microbiome. This allowed me to identify more than 30 soil microbial taxa that promoted positive or negative PSF, although the important taxa were rarely consistent among varieties or species. Root trait expressions for high resource conservative strategies and symbioses with mutualists lead to more positive PSF while the opposite traits (more resource acquisitive strategies and reduced symbioses) lead to more negative PSF. These root traits, however, varied among the species and to a smaller extent among the varieties, indicating that some crop species and cultivars can resist soil biotic stress under certain field conditions, and thus alleviate stand decline. This plant-soil feedback approach will be useful in trait-based selection during pasture rejuvenation and cultivar development for resistance to soil biotic stress. The resources provided in this study will therefore enhance sustainable management of productivity decline in agroecosystems.

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DEDICATION

To my lovely wife, Abiola, my beautiful daughter Sharon, my parents Reuben (late) and Dupe, my siblings, and my academic mentors Drs. Jonathan Bennett and Omotoso Borisade, all of whom believed in me and supported my career aspirations.

- To God almighty, in whom I can do all things.

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LIST OF ABBREVIATIONS

ADF: Acid detergent fiber

AMF: Arbuscular mycorrhizal fungi

ASV: Amplicon sequence variant

Lasso: Least absolute shrinkage and selection operator

LDA: Linear discriminant analysis

LEfSe: LDA effect size

MSE: Mean square error

NDF: Neutral detergent fiber

PERMANOVA: Permutation multivariate analysis of variance

PSF: Plant-soil feedback

RTD: Root tissue density

SEM: Standard error of mean

SRL: Specific root length

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1. INTRODUCTION

Plant-soil feedback (PSF) is the process by which plants create microbial legacies which in turn affects the growth, reproduction, and fitness of subsequent plant species over time (Bennett & Klironomos, 2019; Klironomos, 2002). This plant-soil interaction is predicted to regulate species diversity in plant communities through soil microbial effects on plant growth that either promote or prevent co-existence among species (Crawford et al., 2019; Jiang et al., 2020). Studies have proposed pathogens, beneficial microbes, saprotrophs, plant secondary chemicals, and nutrients as the dominant driving factors in PSF which may either be positive, negative or neutral (Bennett & Klironomos, 2019; Smith-Ramesh & Reynolds, 2017). There is an increasing evidence that abiotic factors including soil physical and chemical properties influence PSF through their effects on soil microbial community structure (Crawford et al., 2019; Bergmann et al., 2016). Among the biotic drivers of PSF, positive PSF is driven majorly by beneficial soil microbes, while diverse soil-borne pathogens contribute to negative PSF (Bennett & Klironomos, 2019; Crawford et al., 2019). This negative effect is generally noticed as productivity decline or soil sickness which limits crop productivity (Annicchiarico et al., 2015). However, there is a growing evidence that plants can induce the enrichment of protective beneficial soil microorganisms which can suppress the proliferation of soil-borne pathogens (Gómez Expósito et al., 2017; Schlatter et al., 2017). Thus, understanding of PSF in perennial agroecosystems may be an important strategy to improve stand productivity.

Alfalfa, *Medicago sativa* L., is the main perennial forage legume in the temperate region (Annicchiarico et al., 2015). It is widely grown for feeding livestock and is preferred for offering a greater environmental and agronomic advantage in terms of soil fertility and the rate of nitrogen fixation per cropping year, compared with annual legumes (Bues et al., 2013). Generally, perennial forage legumes are particularly suitable when seeded with grasses. This forage legume-grass mixture alleviates the risk of life-threatening bloat associated with grazing on high protein forage and promotes soil biodiversity (Annicchiarico et al., 2015). However, traits of greater values to farmers and seed industries including yield, field persistence, compatibility with companion grasses, and adaptation to biotic and abiotic stress in perennial forage pasture significantly decline over time (Annicchiarico et al., 2015). The perennial nature of alfalfa and the propensity to be

seeded with grasses can therefore influence soil microbial communities, thus creating variation in PSF (Atul-Nayyar et al., 2008; Mariotte et al., 2018; Sprunger et al., 2019).

The effects of plants on soil microbiome assembly are dependent on plant identity and environmental conditions (An et al., 2011; Hannula, et al., 2020; Wippel et al., 2021). Differences in plant traits such as root morphology, litter quality, and exudate quality and quantity among cultivars, species or functional groups create variation in microbial legacies (Gorim & Vandenberg, 2017; Williams et al., 2021). Environmental factors such as soil type can influence plant-microbes interactions, and thus shape the structure of soil microbiome in a specific way (Schlemper et al., 2017; Wagner et al., 2016). Additionally, soils determine the availability of nutrients to plants which in turn affect plant growth, root structure, root exudate, and microbiome assembly (Bulgarelli et al., 2012). Response of soil microbial communities to the conditioning effects of plants can be influenced by the age of the stands, suggesting that PSF can change over time (Hawkes et al., 2013; Orr et al., 2015; Wagner et al., 2016). Consequently, changes in cropping systems alter the diversity of soil microbiome and differentially drive PSF for cultivars and species over time (Orr et al., 2015; Wattenburger et al., 2019). There is evidence that continuous monoculture systems promote accumulation of detrimental soil organisms and contribute to negative PSF for subsequent crops (Edwards et al., 2019; Mao et al., 2021). It is not clear, however, how selection of forage species or varieties can mitigate the impact of negative soil microbial legacies in alfalfa systems. Therefore, the adoption of the plant-microbiome approach (Pérez-Jaramillo et al., 2016) for the identification of suitable forage species and varieties with positive belowground-microbial traits holds the potential to unravel the mechanisms of productivity decline and integrate adaptive ecological approaches for sustainable crop production.

Objectives

The main objectives of the study are:

1. To identify which of the alfalfa varieties and forage species will remain productive when exposed to soil microbes associated with alfalfa production
2. To explore how certain soil properties and plant traits contribute to soil microbe-mediated feedback in alfalfa production.
3. To determine how the relationship between soil microbial community structure and alfalfa productivity change with alfalfa production systems and stand age.

2. LITERATURE REVIEW

2.1. Origin of alfalfa cultivars in Canada

Alfalfa (*Medicago sativa L*. subsp. *sativa*) is the most important forage crop species in North America and the main perennial legume in most temperate regions of the world (Annicchiarico et al., 2015). It is recognized as an energy-efficient, effective source of biological (N_2) nitrogen fixation, a good source of protein yield/ha, and one of the most widely adapted agronomic crops. It is generally agreed that alfalfa originated in Vavilov's "Near Eastern Center" – Iran, Transcaucasia, Asia Minor, and the highlands of Turkmenistan (Bolton, 1962; Wilsie, 1962). These areas are known with cold winters and hot dry summers. Alfalfa was first introduced into Eastern Canada in 1871 and later spread throughout Quebec, Ontario, and the Atlantic provinces. Due to more severe winter, it was scarcely grown in Western Canada until the selection and introduction of extremely winter hardy types by Dr. L.E. Kirk of the University of Saskatchewan in 1926 (Barnes et al., 1988). Alfalfa is currently grown as both pure alfalfa and alfalfa-grass mixtures in all the Canadian provinces on a total land area increasing from two and a half million ha in 1981 (Michaud et al., 1988) to over four million ha in 2016 (Statistics Canada, 2021).

2.2. Production systems of alfalfa

Alfalfa is an autotetraploid cross-pollinated perennial species that exists naturally as a heterogeneous population of plants (Vandemark et al., 2006). Developmental stages in alfalfa can be broadly classified into vegetative, bud, flower (boom), and seed pod. Alfalfa is a deep-rooted species and can grow up to 150 cm deep in the soil depending on variety and age (Li et al., 2019). It is popularly grown to pure stand or in mixture with other forage legumes and grasses. Pure stand cropping system ensures high protein content and biological nitrogen fixation without the need for fertilization, even for the next crop (Samaddar et al., 2021). While this system is suitable for seed production and feed production for mixed rations, it is discouraged for direct grazing due to bloat resulting from a high intake of leafy legumes, and it is prone to weed invasion (Annicchiarico et al., 2015). Mixed cropping systems, on the other hand, provide higher yield due to complementarity among species in growing season and resource acquisition (Lekberg et al., 2021; Li et al., 2019; Nyfeler et al., 2009). Mixed stands have further advantages including the belowground transfer of biologically fixed N_2 from legumes to grasses, reduced risk of bloat and lower weed invasion (Annicchiarico et al., 2015). Despite the advantages of mixed stands, alfalfa

can be negatively impacted by association with grasses, which are better competitors in environments that typically support them (Kilcher & Heinrichs, 1996). For example, Li et al. (2019) reported a decrease in biological nitrogen fixation from alfalfa-grass mixture to alfalfa monoculture due to reduced efficiency, and increased nitrogen and phosphorus acquisition in monoculture relative to the mixture, which led to a greater aboveground and root biomass of alfalfa. In contrast, alfalfa persisted long-term in dual culture with Russian winter rye and this was attributed to the higher rooting depth (Atul-Nayyar et al., 2008), which suggests that the performance of alfalfa in mixture is context-dependent. The performance of crop species or cultivars, and the amount biological nitrogen fixed in the soil markedly affect legume-grass competition dynamics over time (Lekberg et al., 2021; Li et al., 2019). Conditions that may affect the yield and persistence in alfalfa pastures include stand age (Arcand et al., 2016; Sprunger et al., 2019), complimentary plant type (Bennett & Cahill, 2016), and soil fertility management (Geisseler & Scow, 2014; Lauber et al., 2013). These influence plant productivity through interactions with soil communities, implying that field conditions drive changes in soil microbial community structure and influence plant growth in a specific way (Orr et al., 2015; Wattenburger et al., 2019). Nonetheless, studies elucidating how these factors interact to mitigate productivity decline of forage stands are not presently available.

2.3. Microbial functional groups in plant-soil feedbacks

Plant-soil feedback (PSF) is the process by which plant exerts conditioning effects on soil microbial communities to create a legacy which then affects the growth of future generations of plants (Bennett & Klironomos, 2019; Hannula et al., 2020). PSF is driven by changes in the abiotic environment (Bergmann et al., 2016; van der Putten et al., 2016), and essentially, by different soil microbial functional groups including mutualists, saprotrophs, pathogens and plant-growth promoting microbes (Bennett & Klironomos, 2019; Smith-Ramesh & Reynolds, 2017). Detrimental microbes include both the major plant pathogens and minor parasitic and non-parasitic deleterious rhizosphere fungi and bacteria (Barea et al., 2008). Beneficial microbes, on the other hand, include decomposers of organic matter, plant growth-promoting microbes, and mutualist symbionts; including N_2-fixing bacteria and arbuscular mycorrhizal fungi (AMF) (Barea et al., 2005). Both soil bacteria and fungi act directly and indirectly to modify the mechanisms of PSF (Wehner et al., 2010). For instance, some fungal associations reduced the competitive ability of young seedlings but increased that of the adult plants (Bennett & Cahill, 2016). Similarly, bacterial

groups conditioned by different plant groups differentially affect PSF (Hannula et al., 2020). These microbial effects indicate that interactions of plants with bacterial and fungal groups affect plant-plant interactions and thus contribute to PSF mechanisms.

Host specific ability to form an association with plant-growth promoting microbes and mutualists such as AMF and rhizobia is associated with positive PSF (Bennett & Klironomos, 2019; Crawford et al., 2019), even in the presence of a pathogen (Hannula et al., 2020). Plant-growth promoting microbes enhance plant growth by improving nutrient uptake, stimulating root development (Bashan & Holguin, 1998), increasing plant tolerance to abiotic stress and soil contaminants (Lucy et al., 2004), and enhancing host immunity against pests and pathogens through induction of systemic resistance and other mechanisms (Smith & Goodman, 1999; Spoel & Dong, 2012; Van Loon, 2007). These beneficial effects on plant growth would be expected to promote positive PSF. AMF provide numerous benefits to their host plants. These include increased water, nitrogen and phosphorus uptake, enhanced resistance to root pathogens, and improve drought (Jacott et al., 2017; Jia et al., 2021), and salinity tolerance (Evelin et al., 2019). These beneficial effects of AMF on hosts have been demonstrated to promote positive PSF (Hannula et al., 2020; Mao et al., 2021). Rhizobia, on the other hand, improve plant productivity through biological fixation of atmospheric nitrogen which is made available to the host through symbiotic association with legume roots (Alías-Villegas et al., 2015; Barea et al., 2005). Activities of rhizobia species in enhancing growth and productivity in their hosts was demonstrated to promote positive PSF (Edwards et al., 2019). However, the ability of mutualists to promote positive PSF may depend on a number of factors including competitiveness, origin, and host specificity. For instance, rhizobia species, in the genus *Bradyrhizobium* are a poor root colonizers under competition with other mutualists (Bellabarba et al., 2021; Chalasani et al., 2021), and thus may have less capacity to generate positive PSF in some crop species. AMF on the other hand can harbor endocellular and endosymbiotic bacteria, some of which may be deleterious soil organisms (Artursson et al., 2006; Gough et al., 2021), demand greater amount of host photosynthates with minimum benefits (Burleigh et al., 2002; Jacott et al., 2017) and consequently, express negative growth effect on plants. However, the magnitude of this negative growth response may depend on the ancestral lineage and isolate of the microbes (Jacott et al., 2017; Vasar et al., 2021). Further, some AMF offer more protection against pathogens than others (Maherali & Klironomos, 2007). Consequently, some purported mutualists can generate negative PSF (Bever, 2002).

Interaction of hosts with species-specific soil-borne pathogens creates negative PSF (Bever et al., 2015; Crawford et al., 2019) which increases in strength under increased fertilization (Lekberg et al., 2021) and continuous monoculture (Mao et al., 2021; Wang et al., 2021) associated with modern agriculture. Continuous monoculture promotes accumulation of crop species- and cultivar-specific soil pathogens (Edwards et al., 2019; Mao et al., 2021; Strom et al., 2020), compared with polyculture systems (Wang et al., 2021). This accumulation of soil-borne pathogens contributes to productivity decline in perennial systems, such as alfalfa production (Annicchiarico et al., 2015; Shi et al., 2021) due to long-term interactions with soil pathogens which increases pathogen-host compatibility (Diez et al., 2010). For example, alfalfa was characterized to be susceptible to numerous soil-borne pathogenic species including *Phytophthora* sp., *Colletotrichum* sp., *Clavibacter* sp., *Fusarium* sp., *Aphanomyces* sp., *Verticillium* sp. (Annicchiarico et al., 2015; Munkvold et al., 2001). Many of these species have been found to be enriched in alfalfa soils (Annicchiarico et al., 2015; Samaddar et al., 2021). A recent study provided novel evidence of oomycetes as the major pathogens driving negative individual and pairwise PSFs (Domínguez-Begines et al., 2021). It is not clear, however, how the conditioning effects of plant stands can alter the composition of these microbial groups and whether taxa within these groups will differentially influence PSF among and within plant species.

Host preferences in plant-microbe interactions and microbial effects on plant growth vary within and among plant species (Hannula et al., 2020; Wippel et al., 2021; Xu et al., 2020). Both soil-borne pathogens and mutualists have differing specificity and their interactions vary from highly specific to generalists associations with plants (Agrawal & Heil, 2012; Horn et al., 2017), thus creating variation in PSF within and among plant species. For example, a recent study showed that variation in PSF depends on the susceptibility of the hosts to oomycetes pathogens (Domínguez-Begines et al., 2021). Similarly, Nagaraj et al. (2021) showed that mycorrhizal-dependent accumulation of biomass in pigeon pea is genotype specific, indicating that host preferences in microbial interactions can drive variation in PSF. PSF can also differ between native and non-native species (Hawkes et al., 2013). Non-native species escape from natural enemies and lack of host specific pathogens limits negative PSF (Hannula et al., 2020; Parker & Gilbert, 2007). Conversely, native species are more likely to encounter specialist pathogens and are thus prone to negative PSF (Smith-Ramesh & Reynolds, 2017). Changes in microbial community structure induced by invasive plants can contribute to a positive PSF loop by improving the performance of

the invasive species leading to the exclusion of the native plant species that is experiencing relatively poor performance under new microbial environment (Batten et al., 2008). The contribution of these soil biota to PSF, however, can be very specific to certain taxa. For instance, Montañez et al. (2012) isolated 22 putative growth-promoting bacteria and characterized them for the presence of growth-promoting traits and found that *Rhanella* sp. provided the most frequent biological N_2-fixation and highest phosphorus solubilization capacity. Differences in how plant species and varieties interact with soil microbiomes may therefore drive variation in intra- and inter-specific PSF.

Within the rhizosphere, autogenic factors such as microbe-microbe interactions play important roles in structuring the overall microbiome assembly, thus influencing the strength and direction of PSF (Berg et al., 2017; Niu et al., 2017). Co-inoculation of phosphate-solubilizing bacteria and AMF improved nutrient uptake in alfalfa by significantly enhancing nodulation, and N_2-fixation rate (Barea et al., 2002; Toro et al., 1998). Similarly, co-inoculation of 2 different strains of N_2-fixing bacteria with AMF effectively improved nutrient uptake, and plant growth in the rhizosphere of alfalfa, although an indigenous microflora competitively reduced the functions of the AMF (Biró et al., 2000). In another study, AMF promotion of phosphorus uptake in plants was suppressed by the competition with coexisting soil microbes, notably: Acidobacteria (Svenningsen et al., 2018). This indicates that competitions among soil microbes affect plant benefit, and can therefore alter the outcome of PSF.

2.4. Plant domestication and genetic instability of symbiosis traits

Efficient root colonization and host specificity in symbiosis are heritable plant traits that can evolve and impact plant performance (Fan et al., 2017; Smith & Goodman, 1999). The domestication of agricultural plant species has been implicated in the loss of symbiotic traits (Pérez-Jaramillo et al., 2016; Porter & Sachs, 2020). For example, wild relatives of pea and broad bean interact better with symbionts than their domesticated counterparts (Mutch & Young, 2004). Further, symbiosis with rhizobia increased yield potentials of older cultivars of soybean than newer cultivars (Kiers et al. 2007). This suggests that domestication and artificial selection of traits during crop improvement can contribute to inadvertent loss of symbiosis traits in some crops. Agricultural practices such as fertilization, weed control, and lower plant density can increase resource availability to plants in a way that benefits from symbiosis are reduced (Liu et al., 2020; Pérez-

Jaramillo et al., 2016). Long-term application of fertilizer to plants reduces plant reliance on soil microbes for nutrient cycling, resulting in subsequent reduction in plant investment to symbiosis (Klinger et al., 2016; Weese et al., 2015). Consequently, this effect drives the evolution of less cooperative mutualists in agricultural systems (Regus et al., 2017; Shantz et al., 2016; Weese et al., 2015). This idea can substantially impact the performance of domesticated species like alfalfa in which genetic sources of diversity used for breeding are cultivated populations rather than wild (Annicchiarico et al., 2015). Selection for resistance to root pathogens can lead to trade-offs in symbiosis with mutualists that share common signaling process (Cao et al., 2017; Rey & Jacquet, 2018). This is possible if the mode of disease resistance is by inhibiting the formation of pathogen's appressoria required to penetrate and infect host cells, leading to the inhibition of the equivalent structure (haustorium) required by mutualists to penetrate host cells (Wang et al., 2012) (Cao et al., 2017; Rey & Jacquet, 2018). In contrast, plant breeding and domestication can also inadvertently co-select for plant traits that support root colonization with beneficial soil microorganisms (Cordovez et al., 2019). This is well exemplified in the study of Mendes et al. (2018) that found that breeding for *Fusarium oxysporum* resistance in common bean co-selected for traits that promote higher abundance of specific beneficial bacteria taxa in the rhizosphere of the resistant cultivar. Despite the role of soil microbes in PSF, implication of previous selection on the responsiveness of alfalfa cultivars and forage species to soil microbes is not yet understood.

2.5. Inter-dependent impact of soil fertility in plant-soil feedbacks

Soil fertility drives PSF by reducing plant dependence upon mutualists (Lekberg et al., 2021), and enhancing plant immunity against belowground pests and pathogens (Augspurger & Kelly, 1984). Plants derive up to 80% of their phosphorus requirements and 25% of their nitrogen requirements from mycorrhizal associations (Marschner & Dell, 1994) in exchange for 4 – 20% of their total carbon budget (Rygiewicz & Andersen, 1994; Tinker et al., 1994). Plants experiencing low soil phosphorus invest more carbon in the development of AM hyphal networks (Covacevich et al., 2006; Ryan et al., 2000). However, the ability of AMF to provide nutritional benefits to host plants can shift from mutualism to parasitism, depending on prevalent environmental factors (Johnson et al., 1997). For instance, a plant will derive mutualistic benefit for carbon invested in mycorrhizal associations that increases phosphorus uptake when phosphorus is limiting, but same will become parasitic when phosphorus is supplied through fertilization. This is because the carbon that could

have been otherwise allocated to increase plant fitness is allocated to a non-beneficial symbiosis (Jacott et al., 2017; Johnson et al., 1997), resulting in negative PSF.

Adequate nutrition enhances disease resistance by inducing changes in host defense compounds (Dordas, 2008; Huber & Haneklaus, 2007). Plants produce preformed defense compounds including inhibitory phenols, flavonoids, and phytoalexins that accumulate around infection sites and offer resistance when the nutrients required for the synthesis of those compounds are adequate (Huber & Haneklaus, 2007). For example, increased calcium enhanced host resistance to macerating diseases, caused by soil-borne pathogens, by increasing the structural integrity of host cell wall and membrane to inhibit extracellular enzymes produced by the pathogens (Huber, 1994). However, efficacy of host nutrition in disease tolerance or resistance of plants to pathogens depends on a number of factors including type of association formed by the dominant pathogen (Dordas, 2008; Huber & Haneklaus, 2007). High nitrogen supply increased disease severity of obligate pathogens, but also increased host immunity against facultative pathogens (Dordas, 2008). Collectively, these results suggest that increased soil fertility and root colonization by mutualists can influence PSF in different ways. Therefore, an increased understanding of the role of soil fertility on soil microbial community will allow for manipulation of PSF mechanisms in the direction that can enhance the persistence and productivity of alfalfa pastures.

2.6. Plant diversity and soil microbial community assembly in the rhizosphere

Plant species act as selective filters and actively recruit their own microbiome from a larger community, thus shaping soil microbial composition (Gornish et al., 2020; Hannula et al., 2020). Differences in resistance to pathogens and symbiotic associations with mutualists within and among plant species (Gorim & Vandenberg, 2017; Nagaraj et al., 2021; Plett et al., 2021; Xu et al., 2020) contribute to the abundance of specific microbial group in the soil (Hannula et al., 2020; Xu et al., 2020). For example, if plant community composition changes toward taxa with more susceptible traits to root pathogens, this can promote pathogens and suppress mutualists' abundance, because susceptible plants allocate less carbon to mutualists (Cappelli et al., 2020; Grman, 2012; Xu et al., 2020). In other words, if the plant community includes more taxa with more resistant traits, beneficial microbial groups will be promoted and pathogens will be suppressed, partly because some plants resistant to soil-borne pathogens allocate greater resource to the belowground community to strengthen their association with beneficial taxa (Mendes, et al.,

2018; Xu et al., 2020). However, plant diversity effects on soil microbial community composition may depend on the ability of the dominant plants to sanction non-beneficial mutualism. Previous studies have shown that host control in sanctioning non-beneficial mutualists varies among plant species (Grman, 2012) but not genotypes (Wendlandt et al., 2019). These results imply that environmental or management processes, such as seeding different plant community compositions will influence soil microbial communities, and this change will affect PSF (Hannula et al., 2017; Putten et al., 2016).

Plant diversity shapes belowground microbial diversity and thus regulates plant growth promotion and associated ecosystem functions (Bennett et al., 2020; Beugnon et al., 2021). Increased plant diversity has been shown to minimize the proliferation of soil-borne pathogens (Vukicevich et al., 2016), increase the abundance of AMF (Bennett et al., 2020) and beneficial bacteria communities (Latz et al., 2012). Conversely, monoculture systems increase the abundance of pathogenic microbes, causing productivity decline (Mao et al., 2021; Shi et al., 2021). Because these pathogens are more specific to the monoculture crops, increasing stand diversity can restore productivity by 'dilution effects' through plant community resistance against the pathogens (Collins et al., 2020; Latz et al., 2012). Since soil microbial community structure can be shaped by plant identity, differences in the conditioning effects on soil microbes among plant family, species and genotypes (Hannula et al., 2020; Ulbrich et al., 2021; Wippel et al., 2021) can drive plant diversity effect on plant growth, and subsequently PSF. For instance, in systems conditioned by legumes, the abundance of fungal parasites, saprotrophs, and potential plant pathogens increased (Hannula et al., 2020) and the introduction of legumes to other systems reduced the abundance of disease suppressive bacteria (Latz et al., 2012, 2015). Further, in systems conditioned by forbs and grasses, the abundance AMF and disease-suppressive bacteria increased, relative to legumes (Hannula et al., 2020; Latz et al., 2015). This was attributed to the reduction of defense mechanisms e.g. saponins in grasses compared to legumes (Osbourn, 2003), thus necessitating reliance on soil microbes for defense against pathogens. Nevertheless, some legumes including alfalfa, promote specific microbial groups like nutrient mineralizers and decomposers (Menendez & Carro, 2019; Samaddar et al., 2021) which may enhance nutrient availability and promote positive PSF for the following plants.

Regardless of functional groups, plant diversity effects on PSF are affected by host-specific differences in pathogen resistance and symbiotic associations with mutualists (Crawford et al., 2019; Gornish et al., 2020). Hypothetically, heterospecific plants will experience negative PSF if susceptible to pathogens enriched in monoculture of other species. Similarly, plant species growing in soils previously conditioned by a diverse plant community will experience negative PSF if it lacks the ability to benefit from mutualists accumulating in those soils (Bever, 2002). Under both scenarios, the ability of the conditioning plant communities to alter the composition of microbial groups that affect plant growth has been neutralized by the specific interactions between the subsequent plants and the conditioned soil microbial communities (Bartelt-Ryser et al., 2005; Hannula et al., 2020; Wang et al., 2021). Combined, these findings indicate that the extent to which plant diversity influences PSF will depend on the relative contribution of both conditioning and subsequent plant species. Further study is required to understand how plant diversity, through microbial legacies feedback on growth of subsequent crop species and cultivars.

2.7. Root traits responses to soil biota and implication for plant-soil feedback

Soil microbial communities stimulate changes in the expression of belowground plant traits and these changes can enhance or limit nutrient acquisition and utilization (Guo et al., 2020; Mao et al., 2021), thus influencing PSF (Mao et al., 2021). Root traits including specific root length (SRL), root tissue density (RTD), average root diameter and root-shoot biomass ratio are linked with the whole-plant economic spectrum (Bergmann et al., 2020; Kramer-Walter et al., 2016) which affects resource allocation to growth and defense, and thus influences PSF (Bennett & Klironomos, 2019; Revillini et al., 2016). The ability of plants to regulate acquisitive-conservative tradeoffs under different soil conditions can drive positive PSF if more conservative strategies are expressed in the presence of beneficial soil microbes that can supply limiting resources, and negative PSF when more acquisitive strategies are expressed in the presence of these beneficial microbes. For example, Mao et al. (2021) showed that root traits responded to soil microbial community composition by expressing conservative syndromes (i.e. high root diameter and low SRL) which correlated positively with aboveground biomass, indicating positive PSF. However, the net effect of belowground trait expression on PSF may depend on the efficiency of the trait that changes to supply limiting resources to the plant (Revillini et al., 2016).

The effect of root traits on PSF is complex, as it depends on the relative composition of soil microbial functional groups and plant identity. Root traits can shift from resource conservative to acquisitive strategies when certain microbial groups are rare or when the microbial groups cannot form a functional association with plants (Mao et al., 2021). Expression of root traits, however, vary among plant functional groups (Williams et al., 2021), species and varieties (Gorim & Vandenberg, 2017). Trait expression also varies with the presence of neighbor, neighbor identity (Bennett & Cahill, 2016; Hendriks et al., 2015), and environmental conditions (Bergmann et al., 2020). Interaction of these factors with trait expression has been shown to influence PSF (Bergmann et al., 2016; Crawford et al., 2019; Hendriks et al., 2015). Plant species can experience negative PSF when less biomass is allocated to the root system (Hendriks et al., 2015). Conversely, high root diameter can, indirectly, enhance pathogen resistance (Laliberté et al., 2015) by producing tougher roots with more structural components, or by supporting colonization of symbionts such as AMF that offer resistance against pathogens (Jia et al., 2021; Maherali & Klironomos, 2007). This can result in positive PSF. Because of the inverse relationship between SRL and root diameter, expression of high SRL in grass species (Bergmann et al., 2016; Bever, 1994) can cause negative PSF by increasing susceptibility to pathogens through exploration of greater soil volume (Bever, 1994) or having less defended tissue (Laliberté et al., 2015). High SRL can also reduce the ability of plants to form association with AMF and thus drive negative PSF (Bergmann et al., 2016). Variation in root trait expression is therefore an important, yet not well studied, mechanism of PSF.

3. DETERMINING THE ROLE OF FIELD CONDITIONS AND BELOWGROUND PLANT TRAITS MECHANISMS IN PLANT-SOIL FEEDBACK

3.1. Preface

Productivity decline in perennial stands results when monoculture systems promote species-specific pathogens, or when some companion crops in mixed systems favor deleterious soil microbes. Seeding of new crops into existing stands is commonly practiced in perennial systems based on the assumptions that new growth will restore productivity. It is not known, however, how abiotic components can influence the conditioning effects of plants on soil biota and how these soil biota affect the growth of newly seeded crops. In order to unravel the mechanisms of productivity decline and work towards potential mitigation strategies, there is a need to understand how varieties and forage species respond to soil microbial legacy effects. In this study, we tested the growth responses of four varieties of alfalfa and five forage species to soil biota in alfalfa monoculture and mixed stands, and their mechanisms of resisting negative soil biota effects.

Publication statement

This chapter except the 'mechanisms of plant-soil feedback' is a modified part of a manuscript titled "*Soil microbial legacies of alfalfa production vary with field conditions and among varieties and species*". It is currently under revision at Agriculture Ecosystems & Environment Journal on July 2021 and is currently under review. Contributors of the manuscript are Awodele S.O (lead author) & Bennett J.A. (Corresponding Author). The other part 'mechanisms of plant-soil feedback' is being modified and will be published in near future.

3.2. Abstract

Plants have strong effects on soil microbial communities and thus plant growth in those soils. These microbe-mediated plant-soil feedbacks (PSFs) can positively or negatively impact the growth of subsequent crops. However, the role of multiple factors such as root trait expression, species identity, crop diversity, and soil characteristics in PSF is not clear. This study is focused on alfalfa (*Medicago sativa*), the globally most common forage legume. Plants and soils from 24 alfalfa stands evenly split between mixtures and monocultures of varying ages were sampled within 300 km of Saskatoon, SK. Using the soils from these sites, an experiment was conducted to quantify how the conditioning factors altered PSF effects on four alfalfa varieties and five native

and tame grass and legume forage species, and compared their belowground traits. Alfalfa monocultures generated more negative PSF than mixtures overall, indicating dilution of antagonistic soil biota in mixture. Differences among varieties and species were idiosyncratic: one alfalfa variety had positive PSF in mixture soils, whereas one legume species (*Trifolium pratense*) had positive PSF in monoculture and another (*Onobrychis viciifolia*) had positive PSF in mixture. PSFs were also mediated by the plant community and soil characteristics of the conditioning stand. PSFs among varieties were mediated by soil texture, soil phosphorus and alfalfa fiber content. Similarly, PSFs among species were mediated by alfalfa fiber content, alfalfa nitrogen content, stand age, plant species richness and weed abundance. These relationships, however, were highly dependent on the variety or species selected and whether the field was seeded to monoculture or mixture. Interestingly, PSFs of native species were negatively impacted by weed abundance, indicating that weeds promote soil microbial antagonists of native plants and thus limit their utility for pasture rejuvenation. Among the measured root traits, only root tissue density differed among the varieties. Forage species expressing conservative resource-use strategies (high root diameter, high root-shoot ratio, low specific-root length, etc.) and symbiosis promoting traits (nodulation and arbuscular mycorrhizal fungi) had more positive PSF relative to those expressing more acquisitive resource-use strategies (opposite traits); however, root traits varied significantly among the species. As PSF is context dependent, belowground trait-based variety and species selection combined with diversity-related management practices are critical to sustainably reduce the impact of negative soil microbial legacies in agroecosystems**.**

3.3. Introduction

Plant-soil feedback (PSF) is the process by which plants influence soil microbiota and other soil components to the extent that it changes the growth of subsequent generations (Bennett & Klironomos, 2019; Crawford et al., 2019). Depending on whether beneficial or antagonistic soil biota accumulate, PSF can be either positive or negative and thus promote or impede plant population growth (Crawford et al., 2019). Consequently, PSFs can alter plant communities and even ecosystem functioning, depending on the strength and direction of the feedbacks, as well as the dominance of the species experiencing these feedbacks (Bennett & Cahill, 2016). Understanding PSFs is therefore critical to both basic and applied ecology, yet this is challenging as PSFs can be highly context dependent (Crawford et al., 2019).

Alfalfa, *Medicago sativa*, is the world's most common forage crop, covering cropping area of about 30 million ha in Europe, and North and South America (Cash & Yuegao, 2009). As a perennial species, alfalfa is subject to numerous soil-borne diseases (Annicchiarico et al., 2015). These antagonists cause steep decline in productivity, indicating that negative PSF is a serious concern for alfalfa production. Alfalfa is also reliant on multiple beneficial soil microbes (Biró et al., 2000), including rhizobacteria and arbuscular mycorrhizal fungi, which may counteract negative PSFs (Bennett & Klironomos, 2019). These below-ground plant-microbe interactions shape the soil microbial community and influence the direction and magnitude of PSF (Crawford et al., 2019).

Agricultural management practices can have strong effects on both disease accumulation and the abundance of beneficial microbes, and are critical to managing PSF in alfalfa and other crops (Mariotte et al., 2018). Increasing plant diversity can increase beneficial and decrease antagonistic soil microbes within mixed stands (Bennett et al., 2020) and rotation systems can further dilute any negative PSFs (Mariotte et al., 2018). Perennial forages are commonly grown as grass-legume mixtures to increase productivity (Serajchi et al., 2017). Although the effects of mixture diversity on PSF are expected to be positive, there is evidence for disruption of mycorrhizal functions in some mixtures due to the ability of some mixture grasses to attract fungivorous nematodes that feed on mycorrhizal fungi (Atul-Nayyar et al., 2008). While rotation systems are less common in perennial forages, seeding new species into declining stands is a popular means of pasture rejuvenation (i.e. restoration of stand productivity) (Khatiwada et al., 2020). Consequently, preceding crops create legacies in biological and chemical soil properties (Hallama et al., 2019) which ameliorate or promote negative PSF for subsequent species (Bennett & Klironomos, 2019; Crawford et al., 2019). Moreover, plant identity can have strong effects on soil microbial community structure, and thus PSF, with significant variation among plant families, species, and even genotypes (Pregitzer et al., 2013; Wagner et al., 2016). Temporal changes in plant-soil interactions can also impact the direction of PSF (Hannula et al., 2019; Hawkes et al., 2013), and as these interactions become strengthened over time, large variation in PSF is expected to develop for establishing species during stand rejuvenation.

Differences among plant family, species or genotypes to modulate belowground communities are thought to drive many of the effects of plant diversity on PSFs (Bartelt-Ryser et al., 2005). For

example, accumulation of antagonistic and beneficial microbes can differ between tame forage species, such as alfalfa, and host native species. Species and genotype selection should therefore have strong effects on PSF within perennial pastures. Even the abundance and composition of weedy plant species can alter PSF for desirable species by altering soil biota (Gornish et al., 2020), although effects depend on how the seeded genotypes or species respond to those changes. Moreover, native and non-native plant species are expected to exhibit differences in PSF dynamics (Perkins & Nowak, 2013) because non-native species will experience less negative feedbacks due to escape from their specific pathogens (Klironomos, 2002). Martín-Robles et al. (2020) showed that domesticated plants promoted the abundance of antagonists relative to mutualists. However, while non-native species may escape their specific pathogens, some domesticated crops can exhibit enhanced interactions with beneficial soil microbes if this trait is inadvertently co-selected (Mendes et al., 2018).

Variation in root traits within and among plant species (Gorim & Vandenberg, 2017; Plett et al., 2021) could influence interactions with microbes and thus, PSF (Mao et al., 2021). Soil microbial communities can mobilize nutrients, extend root nutrient uptake zone, and protect plants against pathogens (Barea et al., 2005; Mao et al., 2021). Consequently, expression of conservative resource-use strategies such as low specific root length (SRL), and high root tissue density (RTD), root-shoot ratio, and root diameter, in the presence of soil biota, may conserve resources and improve efficient allocation of photosynthates to plant growth (Guo et al., 2020; Kramer-Walter et al., 2016). For example, a conservative trait (i.e. high RTD) correlated positively with total plant biomass in maize growing in living soils, relative to sterile, a condition indicative of positive PSF (Mao et al., 2021). High root diameter, on the other hand, promotes collaboration with beneficial microbes and reduces pathogen attack (Sweeney et al., 2021), suggesting that higher root diameter will drive positive PSF by enhancing the colonization of AMF and rhizobia (Crawford et al., 2019). More acquisitive root traits are associated with high levels of root exudations, while high conservative root traits are associated with lower root exudation (Bergmann et al., 2020; Williams et al., 2021), implying that plant variety or species expressing acquisitive root traits will incur greater morphological and structural costs under biotic stress (Guo et al., 2020; Kramer-Walter et al., 2016) and thus experience negative PSF due to reduction in belowground resource allocation. Thus, investigating root trait response patterns of crop varieties and species to soil biota will enhance a more mechanistic understanding of plant-soil feedbacks in perennial agroecosystems.

In addition to the composition of plant community, soil characteristics and forage quality of standing plants can affect soil biota and modify PSF. Soil texture can impact soil microbial community structure and induce changes in the diversity and richness of soil microbial groups (Ma et al., 2016; Obayomi et al., 2021), and then modify PSF. Increases in soil nutrient supply can favor soil pathogens over mutualists such as arbuscular mycorrhizal fungi (AMF) which are less essential to plants under fertile conditions (Revillini et al., 2016), but can also enhance plant immunity against pathogens through efficient uptake (Smith-Ramesh & Reynolds, 2017). Forage quality traits such as nitrogen and fiber content in alfalfa affect crop growth (Ke et al., 2015; Yan et al., 2018). Increase in nutrient supply improves litter quality, decomposition rates, and soil nutrient availability, resulting in positive PSF (Ke et al., 2015). Therefore, differences in forage quality traits in alfalfa could impact, or be impacted, by PSFs.

We collected soils from 24 alfalfa fields that had been seeded to either alfalfa monocultures or grass-alfalfa mixtures and ranged between one and six years old. Using these soils as inoculum we had two objectives:

(1) to quantify the effects of plant diversity, stand age and plant identity on intra- and inter-specific PSF in alfalfa production.

For this we conducted a greenhouse experiment to test for variety and species differences in PSF effects on four alfalfa varieties and five additional native and tame legume and grass species. These data were then combined with field collected estimates of plant diversity, weed abundance, alfalfa quality traits, soil characteristics, stand productivity, and root traits:

(2) to determine the role of biotic and abiotic factors as well as the mechanisms of intra- and inter-specific PSF and the consequences for pasture rejuvenation in perennial agroecosystems.

3.4. Materials and Methods

3.4.1 Site selection

Twenty-four (24) established alfalfa stands evenly divided between monocultures and mixtures (plant diversity hereafter) were selected. The stands were chosen to represent a range of ages, evenly split among 1, 2, 3, or 4-year-old stands. Three-year-old monoculture stands were unavailable so six-year-old stands were selected instead (see Appendix A for site description). All

sites were within 300 km of Saskatoon, Saskatchewan and were primarily under commercial production, with one experimental mixture site. Management history, soil nutrient and texture, abundance of crop species, and other field factors varied among sites (Table 3.1).

Table 3.1. Descriptive summary of actual values of field variables in alfalfa monoculture and mixture stands. Sample taken near Saskatoon, SK., Western Canada in August, 2019.

Variable	Mean ± S.D		Minimum		Maximum	
	Monoculture	Mixture	Monoculture	Mixture	Monoculture	Mixture
Acid detergent fiber (%)	30.08 ± 4.95	26.75 ± 3.58	22.40	18.32	43.33	33.58
Neutral detergent fiber (%)	41.25 ± 5.57	38.94 ± 7.47	31.15	28.44	54.36	58.29
Nitrogen content (%)	17.29 ± 1.98	17.36 ± 2.55	11.97	11.79	20.93	22.29
Soil organic carbon (%)	3.12 ± 1.28	3.02 ± 0.97	1.13	1.68	8.52	5.94
Soil phosphorus (ppm)	2.31 ± 0.59	2.05 ± 0.35	1.50	1.44	3.71	2.99
Soil nitrogen (ppm)	10.55 ± 4.10	9.12 ± 2.35	4.30	5.67	26.61	14.41
Soil clay content (%)	38.33 ± 11.64	33.31 ± 11.26	14.00	14.00	58.00	58.00
Soil sand content (%)	23.33 ± 12.39	31.24 ± 13.90	2.00	10.00	66.00	66.00
Soil silt content (%)	38.33 ± 6.98	35.45 ± 6.47	20.00	20.00	52.00	44.00
Weed abundance	0.10 ± 0.11	0.13 ± 0.15	0.00	0.00	0.44	0.46
Grass abundance	0.01 ± 0.01	0.38 ± 0.17	0.00	0.09	0.06	0.73
Legume/Alfalfa abundance	0.89 ± 0.11	0.48 ± 0.15	0.55	0.25	1.00	0.80

3.4.2. Vegetation and soil sampling and analysis

Field sampling was completed in summer 2019 during which we randomly selected three sampling locations at least 50 m apart in each stand, except for the experimental site where three separate replicates were selected. The sites were at least two kilometers apart. At each sampling location, we placed a $1m^2$ quadrat in which we estimated percent cover to plant species level. This data was used to calculate plant species richness and relative abundance of three functional groups: legumes (primarily alfalfa), grasses and weeds. Following cover estimation, we clipped all vegetation to a stubble heights of 2 cm. Samples were dried at 60°C for 72h and weighed to estimate stand productivity.

To assess alfalfa forage quality traits, we ground the alfalfa biomass from each quadrat. Using these samples, we determined the percent acid and neutral detergent fiber (ADF and NDF) using an ANKOM 2000 Fiber Analyzer™ (ANKOM Technology, New York, USA) and the percent nitrogen using a Leco CN628 analyzer (LECO, Michigan, USA). In each quadrat, we also collected 12 soil cores spread evenly across the plot. These soil cores from each plot were homogenized as one sample prior to use in the experiment, totaling 72 samples (3 samples × 24

sites). Each soil core was 2 cm wide and 15 cm deep, except at two sites where excessive rockiness prohibited sampling beyond this depth. In such locations, additional cores were collected to ensure sufficient soil to inoculate the growth chamber experiment (maximum 15). We transported the soil samples to the lab on ice, and stored them at -20°C for molecular analysis of soil microbes. Other soil subsamples were preserved in the refrigerator (4°C) prior to use for inoculation of growth chamber experiments. Due to labelling errors, only 66 out of 72 samples were used for the experiment. All the 24 sites were represented in the experiment, except that the 3 samples per site design was not feasible for 4 sites (Appendix A). For these soil samples, we measured soil texture by the hydrometer method (Bouyoucos, 1962), total nitrogen and total phosphorus by Kjeldahl digestion (Bremner & Mulvaney, 1982), followed by analysis on AA2 Autoanalyzer (SEAL Analytical, Inc. Wisconsin, USA) and percent soil organic carbon by combustion (Yeomans & Bremner, 1991) using a Leco TruMac™ elemental analyzer (LECO, Michigan, USA).

3.4.3. Feedback phase

For the plant-soil feedback experiment, we selected four varieties of alfalfa and six additional forage species. The four varieties included Foothold, Spyder, 2010, and 3010 selected by the seed producer (BrettYoung Seeds Ltd.) for different traits including growth, disease resistance and root type (Table 3.2). The additional six species included two tame legumes (*Trifolium pretense* and *Onobrychis viciifolia*) and two native legumes (*Vicia Americana and Dalea purpurea*), and one tame grass (*Agropyron cristatum*) and one native grass (*Elymus lanceolatus*). For one of the native legumes (*Dalea purpurea*), few seeds germinated in the experiment (<1%), so this species was not considered further.

To quantify how plant diversity and stand age in alfalfa production affected plant-soil feedbacks of conspecific and heterospecific plants, a growth chamber experiment was conducted. The experiment was conducted between August and December, 2019, and split between two Conviron™ growth chambers (model: GR48 and PGV36) located at the University of Saskatchewan. The chambers were set with an average temperature of 24°C, and humidity of 13%. Light availability was 472µM PAR on average for 16 h per day. We prepared a 2:1 topsoil-sand mixture and heat-sterilized them in two 45 minute cycles in the autoclave at 121°C for use as background soil in 594 (66 field soil samples × 9 plant types) pots (Deepots D40L, volume: 656mL; Stuewe and Sons Inc., Tangent, Oregon, USA). Each pot was inoculated with 30mL of

field soil limiting the amount of soil inocula to < 5% of total volume per pot to isolate the role of soil microbes from any soil fertility differences among the sampled soils (Bever, 1994). Five seeds of each of the 9 plant types were then added to 66 pots containing the different soil inoculums. As a control, five seeds of each plant type were also added to three pots containing only sterile background soil. The pots were arranged in a completely randomized design. After germination, plants were thinned to 1 individual per pot to reduce competition. Plants were watered to field capacity at 48-hour intervals until harvest at approximately four months. At this time, we harvested the shoots and roots from each pot, washed the roots thoroughly with water, and then dried them at 60°C for 72h, and weighed them.

Table 3.2. Experimental plant materials in the study of microbe-mediated plant-soil feedback under greenhouse conditions.

Alfalfa (*Medicago sativa*)					Other forage species			
Variety	**DRI***	**Fall dormancy**	**Root type**	**Key feature**	**Species**	**Common name**	**History**	**Functional Group**
3010	30	2.5	Sunken Crown	High forage yield	*Trifolium pratense*	Red clover	Tame	Legume
2010	29	2.4	Creeping	Extensive rhizomes	*Onobrychis viciifolia*	Sainfoin	Tame	Legume
Foothold	30	2	Spreader	High leaf to stem ratio	*Vicia americana*	American vetch	Native	Legume
Spyder	27	1	Creeping	High forage yield	*Dalea purpurea*	Purple prairie clover	Native	Legume
					Agropyron cristatum	Crested wheatgrass	Tame	Grass
					Elymus lanceolatus	Northern wheatgrass	Native	Grass

*DRI = Disease Resistance Index (out of 30)

For each plant grown in live soils, soil feedback effects were calculated as the log ratio of biomass of plants grown in live soils (inoculated pots) to the average biomass in sterile soils (Brinkman et al., 2010). Negative and positive feedbacks reflect greater net effects of antagonists and beneficial microbes, respectively. By standardizing feedback effects in this way, we can compare the direction and magnitude of PSF effects among alfalfa varieties (intraspecific PSF) and between the other plant species (interspecific PSF) regardless of any differences in the absolute size of the varieties or species.

3.4.4. Mycorrhizal colonization and root nodulation

For arbuscular mycorrhizal fungi (AMF) colonization, roots were rinsed to remove soils, and fine root (< 0.5 mm diameter) were randomly subsampled and stored in glass vials containing 70% ethanol and kept at room temperature until use. Five 4 cm – long fine root fragments were selected at random and cleared with 10% KOH at 96°C for 2 hours for the legumes, and 1.5 hours for the grasses. Clearing times varied to optimize the procedure for the different root thickness. The roots

were rinsed with water and transferred to 2% HCl at 96 C ° for 20 minutes for the legumes and 15 minutes for the grasses to acidify and ensure that stain would bind better to the roots. After rinsing the root samples in clear water, roots were transferred to a staining solution (1:1:1 proportion of lactic acid, glycerol, and water) with 5% ink solution at 96°C for 20 minutes for the legumes and 15 minutes for the grasses. The root samples were then rinsed in water with few drops of lactic acid as de-staining solution. Samples were stored in de-staining solution overnight (Vierheilig et al., 1998). Microscope slides were prepared and mycorrhizal colonization rate was measured using the gridline intersect methods at ×40 magnification (Giovannetti & Mosse, 1980). Quantification of total AMF colonization rate was expressed as the percentage of root intercepts colonized by AMF structures i.e. arbuscules, vesicles or hyphae from 50 intercepts per sample (McGonigle et al., 1990). For root nodulation, dried root samples were washed and rehydrated in clear water at 4°C for 48 hours and scanned with EPSON Perfection V800/V850 Pro Scanner (EPSON America, Inc., CA, USA) and analyzed using WinRHIZO software (Regent Instruments Inc., Quebec, Canada). Quantification of percent nodulation (%) was expressed as the ratio of number root nodules to root length, to standardize measurements for all species and varieties.

3.4.5. Root morphological trait measurements

Root-shoot biomass ratios were calculated as total root dry mass divided by shoot dry mass. Root morphological traits were measured from random subsample of roots (averaged at < 0.5mm diameter and 0.14g) harvested from each pot and scanned using EPSON Perfection V800/850 Pro Scanner (Epson America Inc., CA, USA) and analyzed using WinRHIZO software (Regent Instruments Inc., Quebec, Canada) which measures and returns root parameters including total root length, total root volume, and average root diameter. We avoided tap roots and focused on lateral root systems given their greater potential for resource acquisition potentials (McCormack et al., 2015). After the analysis, root subsamples were oven dried at 60°C for 72 hours and weighed to obtain dry root mass. Specific root length (SRL) was calculated as total root length divided by root dry mass, root tissue density (RTD) as root dry weight divided by total root volume (Kramer-Walter et al., 2016).

3.4.6. Statistical analysis

All the data were analyzed using R software version 1.3.9 (R Core Development Team, 2020). Differences in PSF among forage species and alfalfa varieties were evaluated using separate

models. For the forage species models, PSF data was averaged for each sample across alfalfa varieties. The models included PSF as response variable, plant species (or varieties) × plant diversity × stand age as factorial fixed effects, with stand age included as a quadratic continuous variable to account for non-linearity in the relationship. For the random variables, soil sample identity nested within site was included. Initially, growth chamber identity was included as well; however, this random variable explained zero variance and the two growth chambers had identical intercepts for the plants, so this was excluded from subsequent models. These models, and all subsequent mixed models were run using the lme4 package (Bates et al., 2014). Degrees of freedom were estimated using Satterthwaite's approximation in the lmerTest package (Kuznetsova et al., 2017).

To identify which biotic and abiotic predictors influenced variation in plant soil feedback, model selection was used. To reduce multicollinearity and the dimensionality of the covariates prior to model selection, principal component analysis (PCA) was conducted. Separate PCAs was run on the data for the forage quality, soil texture, soil nutrients, and abundance of plant functional groups (Appendix A) using the psych package (Revelle, 2018). For each data set, the first two components as they best summarized the data were selected. For plant functional groups, PC1 represented transition from legume to grass dominance while PC2 represented increasing weed abundance. For soil texture, PC1 represented increasing clay and decreasing sand content, and PC2 represented decreasing silt content. For forage quality, PC1 represented increasing acid and neutral detergent fiber (fiber content hereafter), and PC2 represented decreasing nitrogen content. For soil nutrients, PC1 represented increasing percent carbon and total nitrogen while PC2 represented increasing total phosphorus (Fig. 3.1; Appendix B). These principal component (PC) scores were extracted and used as predictor variables in subsequent analysis.

For model selection, separate models were run for mixtures and monocultures due to inherent differences between these groups in the relative abundance of plant functional groups. For each model, a global model structured similarly to the previous PSF model but lacking plant diversity and its interactions was first generated. It included PSF as response variable, and each of the 6 principal components, stand age, species richness, and stand productivity as continuous predictors. Each of these variables was fitted as an interaction with the plant species (or variety) variable. The dredge function in the MuMIn package (Barton, 2020) was used to run all possible combinations of the fixed effects.

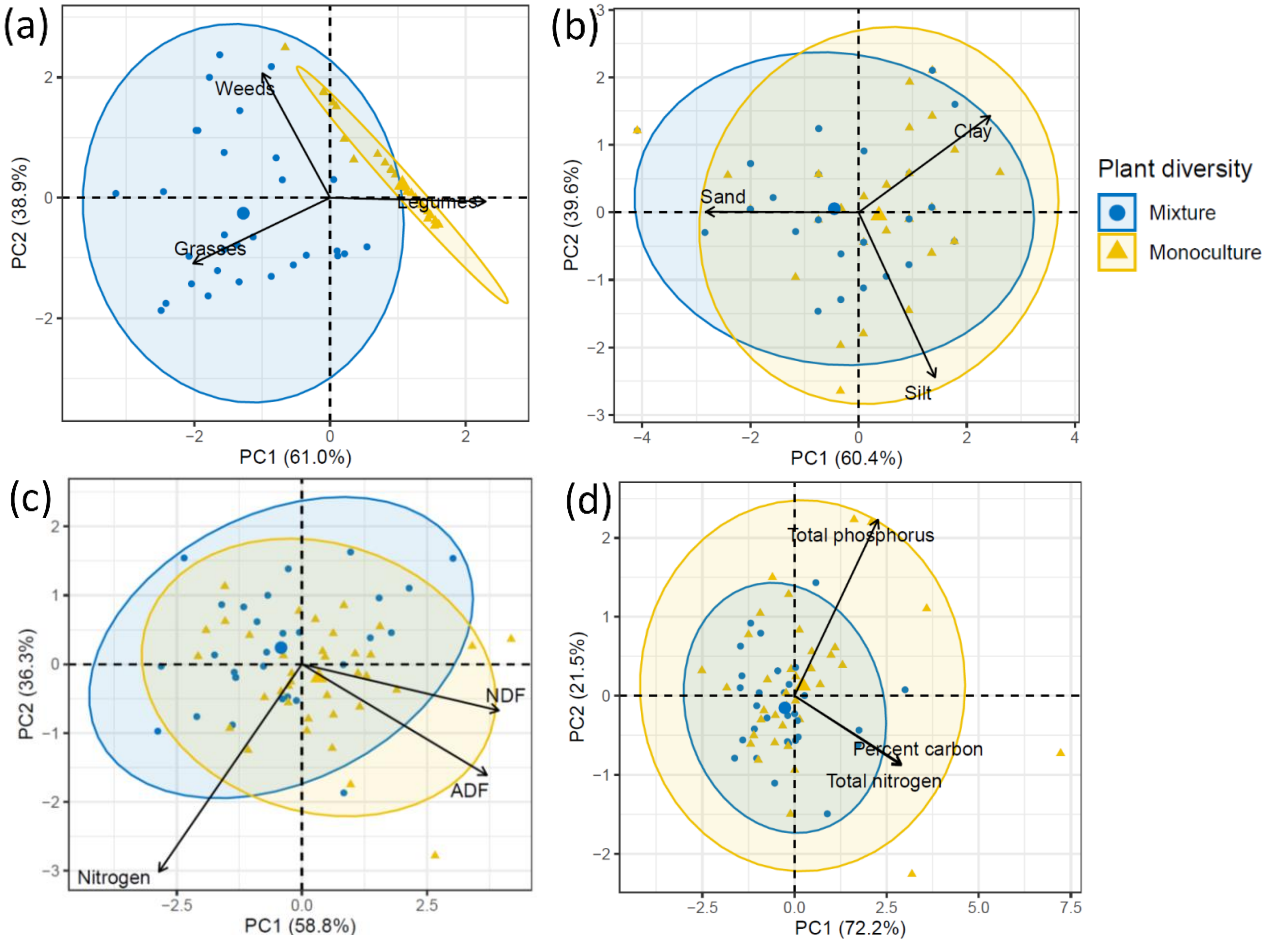

Figure 3.1. Principal component analysis of the abundance of species functional group **(a)**, soil texture class **(b)**, percent forage quality (nitrogen and fiber) in alfalfa **(c)** and soil nutrient (phosphorus, nitrogen and soil organic carbon) **(d)** observed in alfalfa monoculture and associated mixture stands. ADF = acid detergent fiber, NDF = neutral detergent fiber.

Then the relative AICc (Akaike information criteria corrected for small sample size) weight of each predictor variable and the average parameter estimate were calculated using model averaging of all models with ΔAICc < 2 (Burnham & Anderson, 2002) which averages the weight of the models in which a predictor variable appeared. To explore significant interactions denoting differences among varieties or species, a reduced mixed effects model was run using only the variables with an importance value of 0.5 or greater and post hoc tests using Tukey's HSD method in the emmeans package (Russell, 2018).

To identify below-ground plant traits contributing to variation in plant-soil feedbacks among alfalfa varieties and forage species, separate mixed-effects model analysis was conducted on each trait. Structure of the model included plant trait as response variable, varieties (or species) as fixed effect and samples identity nested within sites as random effects. At the species level, traits for *Medicago sativa* were derived by calculating the average score of the varieties for each trait. To test the effects of stand age, plant diversity, and their interaction on root trait expression in living soils, another mixed effects model analysis was conducted. Each model included a root trait as response variable, plant species (or varieties) × plant diversity × stand age as factorial fixed effects, and sample nested within site as random effects. All the analyses were conducted using lme4 package (Bates et al., 2014). Mean differences were tested using anova function from lmerTest package (Kuznetsova et al., 2017) followed by TukeyHSD with the *emmeans* package (Russell, 2018). To meet normality assumption, response variables were log or square-root transformed where required.

3.5. Results

3.5.1. Intraspecific plant-soil feedbacks

Both variety and plant diversity had significant effects on PSF of alfalfa ($P<0.001$ and $P = 0.025$ respectively), with a marginal interaction between these two terms ($P = 0.054$; Fig. 3.2a). Negative PSF was stronger in monoculture than mixture soils (-0.185 vs -0.046). Stand age and associated interactions were not significant in the initial model (Table 3.3); although this changed once more covariates were included. Based on model selection, PSF varied among alfalfa varieties in monoculture soils (Fig. 3.2a; Table 3.4; Appendix C). Variety Foothold had more negative PSF than the other varieties (Table 3.5).

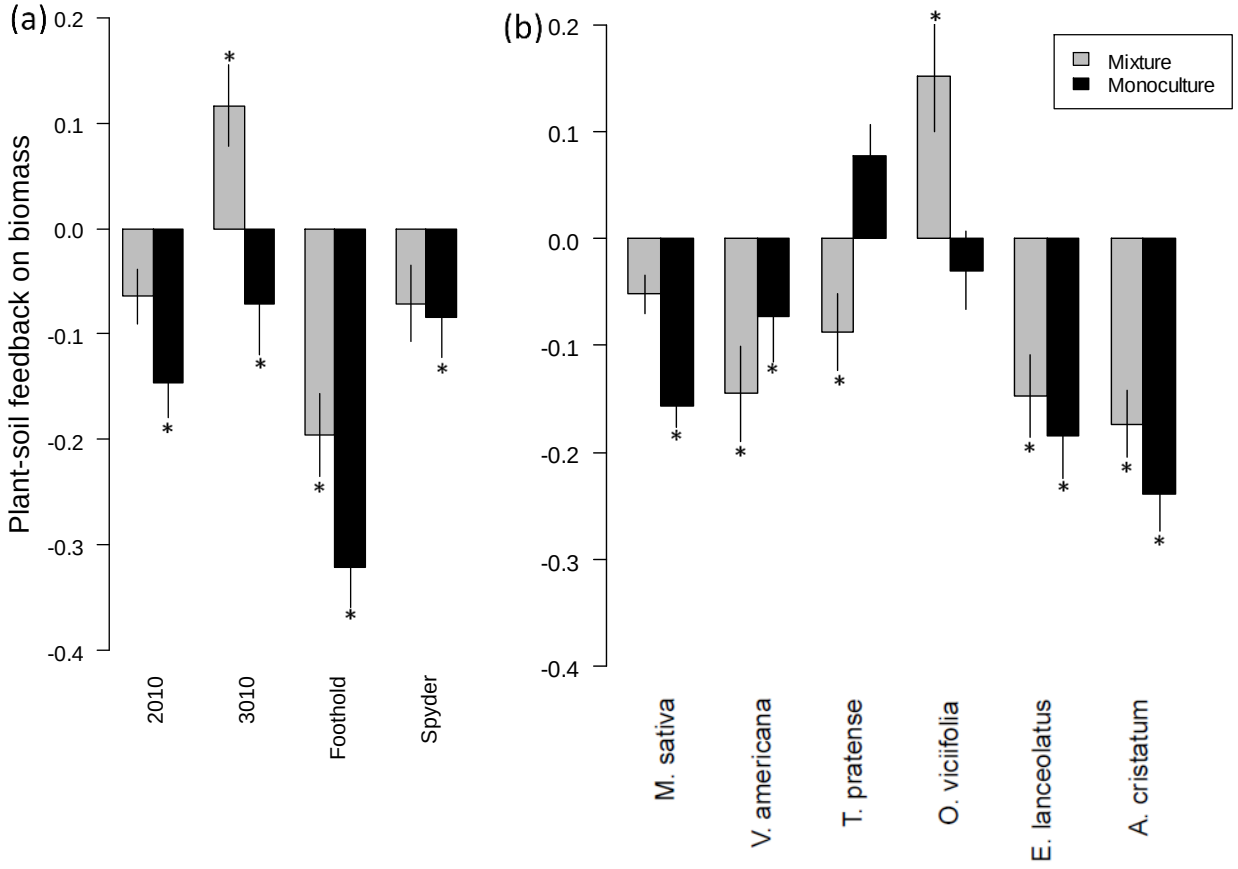

Figure 3.2. Intra- and inter-specific plant-soil feedback in alfalfa-associated agroecosystem. Mean feedback of different alfalfa varieties and plant species in soils previously conditioned by alfalfa in monoculture and mixed stands. Panels show **(a)** plant-soil feedback among four alfalfa varieties and **(b)** among six different forage species. Bars indicate the mean feedback and error bars the standard error. Asterisks represent feedback scores with significant differences from zero.

Table 3.3. Mixed model results testing the effects of alfalfa variety, stand age and plant diversity on mean biomass plant-soil feedback of alfalfa varieties in alfalfa monoculture and mixture soils. n = 242.

Effect	Sum Sq	Mean Sq	Df (num, den)	F	Pr (>F)*
Variety	0.9114	0.3038	3, 164.04	7.0753	**0.0001**
Stand age	0.1074	0.0537	2, 19.518	1.2508	0.3084
Plant diversity	0.2478	0.2478	1, 20.382	5.7718	**0.0259**
Variety × stand age	0.4616	0.0769	6, 165.03	1.7917	0.1037
Variety × plant diversity	0.3337	0.1112	3, 166.04	2.5901	0.0547
Stand age × plant diversity	0.1838	0.0918	2, 19.518	2.1396	0.1445
Variety × stand age × plant diversity	0.4172	0.0695	6, 165.03	1.6191	0.1448

**Treatment with boldface type indicates P-values < 0.05*

Table 3.4. Model-averaged values for biotic and abiotic predictors of intraspecific plant-soil feedbacks of alfalfa in alfalfa monoculture and mixture soils[a]

Effect in Monoculture	Estimate	Std. Error	z-value	p-value	Relative Importance
(Intercept)	-0.3509	0.052	6.697	<0.0001	--
Variety	--	--	--	--	**1.00**
Fiber content PC	0.0308	0.0461	0.663	0.5075	**1.00**
Soil clay:sand PC	-0.0092	0.0103	0.884	0.3766	0.06
Soil silt PC	0.0165	0.0149	1.096	0.273	0.06
Soil phosphorus	-0.047	0.0273	1.474	0.1405	**0.92**
Stand age (poly 1)	-0.3871	0.2301	1.666	0.0956	--
Stand age (poly 2)	0.4056	0.221	1.818	0.0691	**0.54**
Plant species richness	0.0218	0.0125	1.72	0.0854	0.44
Variety × soil phosphorus	--	--	--	--	**0.58**
Variety × fiber content PC	--	--	--	--	**1.00**
Effect in Mixture					
(Intercept)	-0.1794	0.0391	4.511	<0.0001	--
Variety	--	--	--	--	**1.00**
Fiber content PC	0.0452	0.0284	1.565	0.1175	0.25
Nitrogen content PC	0.0149	0.0155	0.948	0.343	0.18
Soil clay:sand PC	-0.027	0.014	1.905	0.0568	**0.65**
Soil silt PC	0.0375	0.0196	1.893	0.0583	**0.65**
Soil carbon:nitrogen PC	-0.0257	0.0244	1.037	0.2998	0.14
Plant species richness	-0.0068	0.0064	1.035	0.3007	0.06
Pasture productivity	<-0.0001	<0.0001	0.911	0.3625	0.11

[a]Summary of conditional average results of change in site-level means of plant-soil feedback for alfalfa varieties after model averaging: Model was fitted separately for monoculture and mixture. The level of predictors included in the intercept terms in addition to the ones listed above are weed abundance PC, alfalfa nitrogen PC, soil carbon:nitrogen PC and pasture productivity in monoculture model. Stand age, weed abundance PC, legume:grass abundance PC and soil phosphorus PC in mixture model. Only the predictors in models with a delta AIC < 2 are provided in the table. The relative importance of the predictor is the total sum of the weights of the model in which predictor appears. 1 indicates that the predictor appeared in all models. Predictors with an importance of 0.50 or greater (bolded) are selected for a mixed model analysis. PC = principal components. Poly 1 = polynomial factor. Poly 2 = polynomial factor raised to power 2.

Table 3.5. Within group mean comparison of intra-specific plant-soil feedback of alfalfa varieties in monoculture and mixture soils from the analysis of reduced models with important predictors.

Contrast in Monoculture	**Estimate**	**SE**	**df**	**t-ratio**	**p-value***
Foothold - 2010	-0.1697	0.0521	119	-3.257	**0.0079**
Foothold - 3010	-0.2468	0.0516	118	-4.78	**<0.0001**
Foothold - Spyder	-0.2377	0.5521	118	-4.564	**0.0001**
2010 - 3010	-0.0770	0.0524	118	-1.469	0.4594
2010 - Spyder	-0.0679	0.0529	120	-1.283	0.5752
3010 - Spyder	0.0090	0.0525	119	0.173	0.9981
Contrast in Mixture					
Foothold - 2010	-0.1241	0.049	81	-2.53	0.0628
Foothold - 3010	-0.2971	0.049	81.2	-6.069	**<0.0001**
Foothold - Spyder	-0.1359	0.0496	81.9	-2.74	**0.0372**
2010 - 3010	-0.1731	0.0491	80.9	-3.528	**0.0038**
2010 - Spyder	-0.0118	0.0496	82.1	-0.238	0.9952
3010 - Spyder	0.1613	0.0496	81.8	3.25	**0.0089**

**Treatments with boldface type indicates P-values < 0.05*

P-value adjustment: tukey method of comparing family estimates

Other differences among the varieties were dependent on the fiber content of alfalfa in the field and, to a lesser extent, phosphorus content in the conditioned soil (Table 3.4; Appendix C). For fiber content, PSF for 3010 exhibited a positive relationship, whereas the remaining varieties showed no significant relationships (Fig. 3.3a, Table 3.6). PSF also trended negatively with soil phosphorus for all varieties except 3010 (only significant for Spyder; Table 3.7). Consequently, 3010 had less negative PSF than the other varieties, but only when inoculated with soil microbes from fields with high fiber alfalfa or high phosphorus soils (Fig. 3.3b, Table 3.7). For all varieties, stand age was a marginal predictor of PSF in monoculture soils (Table 3.4; Appendix C), with a negative relationship that plateaued by year 4 (Fig. 3.3c).

PSF also varied among varieties in mixture soils (Fig. 3.2a; Table 3.4; Appendix C). Most varieties exhibited negative PSF; however, negative PSF was greatest for Foothold and 3010 exhibited positive PSF (Table 3.5; Fig. 3.2a). PSF was also dependent on soil texture in the conditioning soil but there was no evidence this differed among varieties (Table 3.4; Appendix C).

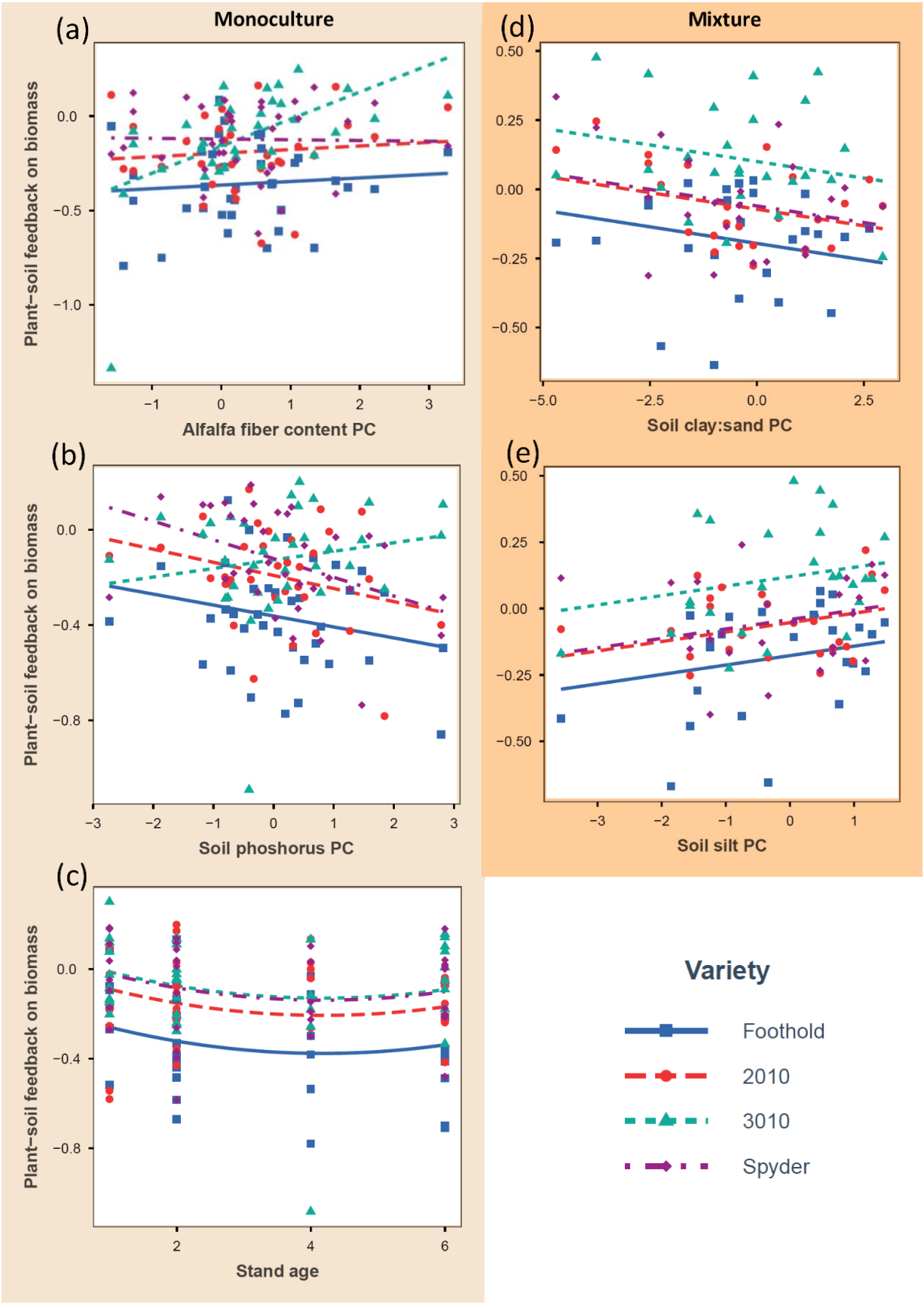
(a)
Monoculture
Plant−soil feedback on biomass
Alfalfa fiber content PC
(b)
Soil phoshorus PC
(c)
Stand age
(d)
Mixture
Soil clay:sand PC
(e)
Soil silt PC
Variety
Foothold
2010
3010
Spyder

Figure 3.3. Relationship between plant-soil feedback of alfalfa varieties and **(a)** fiber content (ADF and NDF) (P = 0.507), **(b)** soil phosphorus (P = 0.140), **(c)** stand age (P = 0.069), **(d)** soil clay and sand (P = 0.056), **(e)** soil silt content (P = 0.058) in alfalfa monoculture (panel a-c) and mixture (panel d,e) soils. Model averaging was used to analyze predictors with an importance value of 0.50 or greater influencing intraspecific PSF of alfalfa varieties (Foothold, 2010, 3010 and Spyder), calculated as the ln ratio of plant biomass in soils previously conditioned by alfalfa-associated soil inoculum vs sterile soils.

Table 3.6. Post hoc analysis of the relationship between fiber content and intraspecific plant-soil feedback of alfalfa in monoculture soils.

Variety	Slope	SE	df	Lower CL	Upper CL	t-ratio	p-value*
Foothold	0.0234	0.0486	137	-0.0726	0.1195	0.483	0.6298
2010	0.0224	0.049	135	-0.0745	0.1194	0.458	0.6477
3010	0.1850	0.0487	136	0.0888	0.2814	3.800	**0.0002**
Spyder	-0.0066	0.0487	138	-0.103	0.0897	-0.137	0.8914

**Boldface type indicates P-values < 0.05; Confidence level at 0.95*

Table 3.7. Post hoc analysis of the relationship between soil phosphorus and intraspecific plant-soil feedback of alfalfa in monoculture soils.

Variety	Slope	SE	Df	Lower CL	Upper CL	t-ratio	p-value*
Foothold	-0.0461	0.0334	152	-0.112	0.0199	-1.379	0.1698
2010	-0.0554	0.0371	154	-0.1286	0.0179	-1.493	0.1375
3010	0.0359	0.0338	152	-0.0308	0.1026	1.063	0.2893
Spyder	-0.0789	0.0339	152	-0.1458	-0.0119	-2.326	**0.0213**

**Boldface type indicates P-values < 0.05; Confidence level at 0.95*

PSF became more negative in soils with greater clay relative to sand content (Fig. 3.3d) and less negative with greater silt content (Fig. 3.3e).

3.5.2. Interspecific plant-soil feedbacks

PSF effects varied greatly among forage species (P < 0.001), with differences dependent on whether the soils were sampled from monocultures or mixtures (interaction P < 0.001; Fig. 3.2b). Neither the main effect of plant diversity nor stand age and associated interactions were significant in the initial model (Table 3.8).

Table 3.8. Mixed-effects model results testing effects of forage species, stand age, and field diversity on mean biomass plant-soil feedback of forage species in alfalfa monoculture and mixture soils. n = 378.

Effect	Sum Sq	Mean Sq	df (num, den)	F	Pr (>F)*
Forage species	1.3110	0.2622	5, 289	7.0798	**<0.0001**
Stand age	0.0406	0.0203	2, 18	0.5477	0.5873
Plant diversity	0.0249	0.0249	1, 19	0.6711	0.4224
Forage species × stand age	0.3162	0.0316	10, 286	0.8538	0.5772
Forage species × plant diversity	0.8023	0.1605	5, 289	4.3326	**0.0008**
Stand age × plant diversity	0.0479	0.0240	2, 18	0.6471	0.5349
Forage species × stand age × plant diversity	0.2865	0.0287	10, 287	0.7738	0.6541

**Treatment with boldface type indicates P-values < 0.05*

PSF differences among species; however, were also dependent on multiple biotic and abiotic contexts, although these contexts differed between monoculture and mixture soils (Table 3.9; Appendix D).

In monoculture, PSF of *M. sativa* differed significantly from other tame legume species (*T. pratense* and O. *viciifolia*) but not the other forage species. PSF also differed idiosyncratically between the native and tame legumes: the native legume (*V. americana*) differed from *T. pratense* but not *O. viciifolia*. PSF did not differ between the native and tame grass species either (Fig.3.2b, Table 3.10). Interestingly, native and tame forage species did differ in how PSF responded to weed abundance in the conditioning community (Table 3.9; Appendix D): PSF was negatively related to weed abundance for the two native species *E. lanceolatus* ($P < 0.001$) and *V. americana* ($P = 0.040$), but not the tame species (Fig. 3.4a, Table 3.11). Independent of species, PSF was also positively related to alfalfa fiber content (Fig. 3.4b) as well as stand age and plant species richness, although these latter relationships were not significant (Table 3.9; Appendix D).

In mixture, PSF differed among species, although these differences were not consistent within functional groups or by species origin. *Onobrychis viciifolia* was the only species that benefitted from mixture soils and differed from all other species. Otherwise, PSF ranged from neutral to negative and did not differ among species (Fig. 3.2b, Table 3.10). Beyond species differences, PSF was negatively related to plant species richness ($P = 0.013$; Fig. 3.5a) and positively related to alfalfa nitrogen content during conditioning ($P = 0.051$; Fig. 3.5b).

Table 3.9. Model-averaged values for biotic and abiotic predictors of interspecific plant-soil feedback of forage species in alfalfa monoculture and mixture soils[a]

Effect in Monoculture	Estimate	Std. Error	z-value	p-value	Relative Importance
(Intercept)	-0.2000	0.0420	4.7400	<0.0001	--
Forage species	--	--	--	--	**1.00**
Fiber content PC	0.0622	0.0206	3.0000	0.0027	**1.00**
Weed abundance PC	-0.0023	0.0381	0.0620	0.9509	**1.00**
Stand age (poly 1)	0.3730	0.2389	1.5520	0.1206	--
Stand age (poly 2)	0.3732	0.2274	1.6310	0.1028	**0.60**
Plant species richness	0.0150	0.0089	1.6830	0.0924	**0.58**
Soil phosphorus PC	-0.0201	0.0143	1.3970	0.1624	0.41
Soil carbon:nitrogen PC	0.0098	0.0122	0.7980	0.4248	0.07
Forage species × weed abundance	--	--	--	--	**1.00**
Effect in Mixture					
(Intercept)	0.0260	0.0576	0.4490	0.6531	--
Forage species	--	--	--	--	**1.00**
Legume:grass abundance PC	0.0375	0.0237	1.5680	0.1168	**0.55**
Alfalfa nitrogen PC	0.0286	0.0146	1.9470	0.0515	**0.75**
Plant species richness	-0.0178	0.0071	2.4770	0.0132	**1.00**
Fiber content PC	-0.0496	0.0260	1.8930	0.0583	**0.76**
Weed abundance PC	0.0183	0.0175	1.0420	0.2975	0.11

[a]Summary of conditional average results of change in site-level means of plant-soil feedback for forage species after model averaging: To capture the specific predictors in monoculture and mixed stands respectively, model was fitted separately for monoculture and mixture. The level of predictors included in the intercept terms in addition to the ones listed above are soil clay:sand PC, soil silt PC, alfalfa nitrogen PC and pasture productivity in monoculture model. Stand age, soil clay:sand PC, soil silt PC, soil phosphorus PC, soil carbon:nitrogen PC and pasture productivity in mixture model. Only the predictors in models with a delta AIC < 2 are provided in the table. The relative importance of the predictor is the total sum of the weights of the model in which predictor appears. 1 indicates that the predictor appeared in all models. Predictors with an importance of 0.50 or greater are bolded and selected for a mixed model analysis. PC = principal components. Poly 1 = polynomial factor. Poly 2 = polynomial factor raised to power 2.

Table 3.10. Within group mean comparison of interspecific plant-soil feedback of forage species in alfalfa monoculture and mixture soils.

Contrast in Monoculture	**Estimate**	**SE**	**df**	**t-ratio**	**p-value***
M. sativa - A. cristatum	0.0824	0.0429	185	1.920	0.3931
M. sativa - E. lanceolatus	0.0311	0.0436	186	0.714	0.9801
M. sativa - O. viciifolia	-0.1290	0.0436	186	-2.961	**0.0399**
M. sativa - T. pratense	-0.2420	0.0436	186	-5.549	**<0.0001**
M. sativa - V. americana	-0.0848	0.0429	185	-1.977	0.3595
A. cristatum - E. lanceolatus	-0.0513	0.0436	186	-1.176	0.8477
A. cristatum - O. viciifolia	-0.2114	0.0436	186	-4.850	**<0.0001**
A. cristatum - T. pratense	-0.3244	0.0436	186	-7.437	**<0.0001**
A. cristatum - V. americana	-0.1671	0.0429	185	-3.897	**0.0019**
E. lanceolatus - O. viciifolia	-0.1601	0.0443	188	-3.618	**0.0051**
E. lanceolatus - T. pratense	-0.2731	0.0443	187	-6.164	**<0.0001**
E. lanceolatus - V. americana	-0.1159	0.0436	186	-2.659	0.0886
O. viciifolia - T. pratense	-0.1130	0.0442	186	-2.555	0.1140
O. viciifolia - V. americana	0.0443	0.0436	186	1.016	0.9123
T. pratense - V. americana	0.1572	0.0436	186	3.605	**0.0053**
Contrast in Mixture					
M. sativa - A. cristatum	0.1333	0.0502	146	2.654	0.0912
M. sativa - E. lanceolatus	0.0910	0.0502	146	1.812	0.4614
M. sativa - O. viciifolia	-0.2054	0.0518	149	-3.964	**0.0016**
M. sativa - T. pratense	0.0407	0.0498	146	0.817	0.9640
M. sativa - V. americana	0.1188	0.0498	146	2.388	0.1673
A. cristatum - E. lanceolatus	-0.0423	0.0491	145	-0.861	0.9550
A. cristatum - O. viciifolia	-0.3387	0.0507	147	-6.676	**<0.0001**
A. cristatum - T. pratense	0.0927	0.0487	144	-1.905	0.4035
A. cristatum - V. americana	-0.0145	0.0487	144	-0.289	0.9997
E. lanceolatus - O. viciifolia	-0.2967	0.0507	147	-5.843	**<0.0001**
E. lanceolatus - T. pratense	-0.0504	0.0487	144	-1.035	0.9053
E. lanceolatus - V. americana	0.0278	0.0487	144	0.057	0.9927
O. viciifolia - T. pratense	0.2461	0.0502	147	6.453	**<0.0001**
O. viciifolia - V. americana	0.3242	0.0502	147	6.453	**<0.0001**
T. pratense - V. americana	0.0782	0.0482	144	1.622	0.5852

**Treatments with boldface type indicates P-values < 0.05*

P value adjustment: tukey method of comparing family estimates

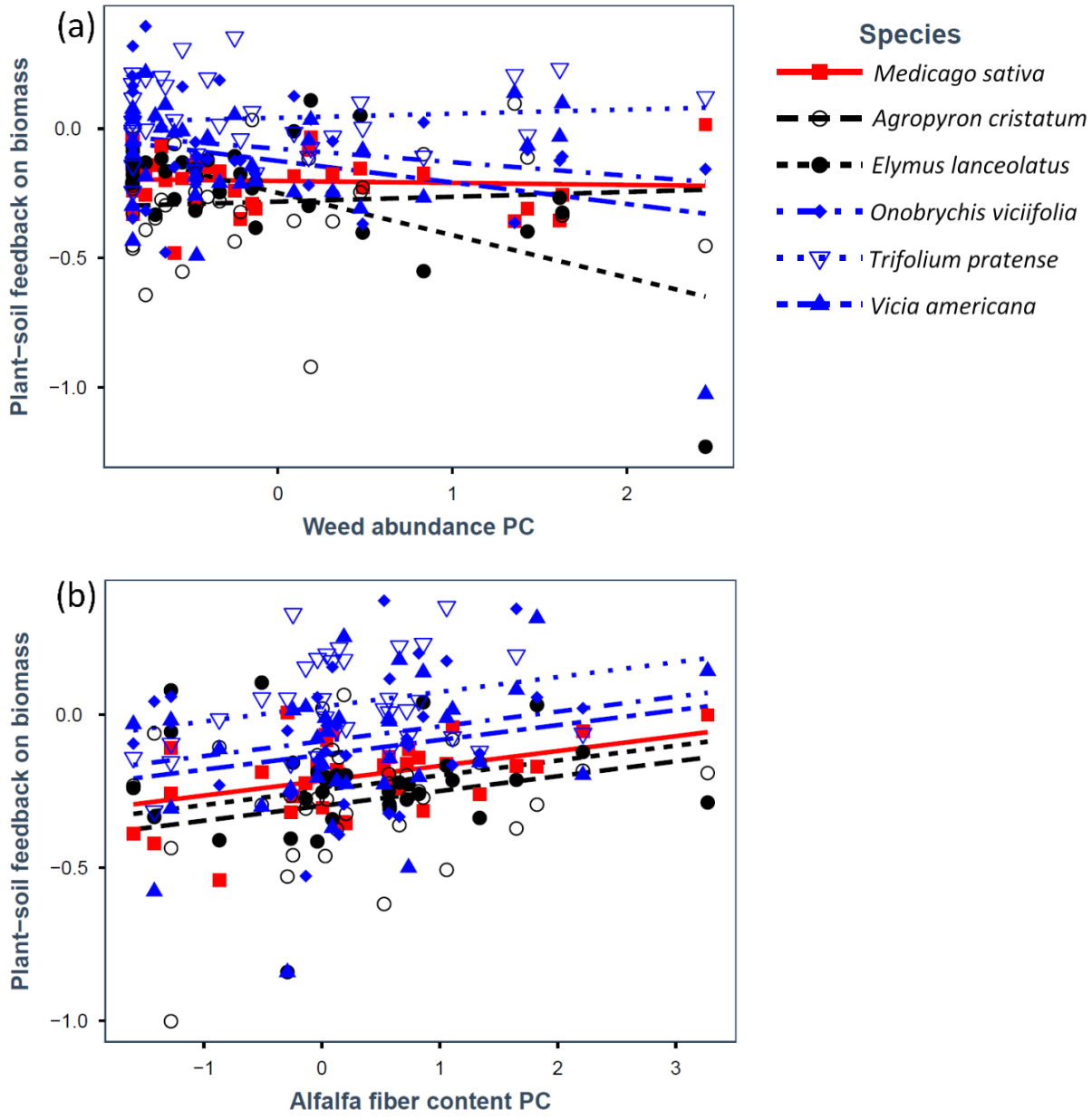

Figure 3.4. Relationship between plant-soil feedback of forage species and weed abundance (main effect in model selection: $P = 0.950$; Interaction in reduced model: $P = 0.002$) **(a)** and alfalfa fiber content (ADF/NDF) in alfalfa ($P = 0.002$) **(b)** in alfalfa monoculture soils. Model averaging was used to analyze predictors with an importance value of 0.50 or greater influencing interspecific PSF of *Medicago sativa*, *Agropyron cristatum*, *Elymus lanceolatus*, *Onobrychis viciifolia*, *Trifolium pretense and Vicia americana*, calculated as the ln ratio of plant biomass in soils previously conditioned by alfalfa associated soil inoculum vs sterile soils.

Table 3.11. Post hoc analysis of the relationship between weed abundance and interspecific plant-soil feedback of forage species in alfalfa monoculture soils.

Species identity	Slope	SE	df	Lower CL	Upper CL	t-ratio	p-value*
M. sativa	-0.0069	0.0400	203	-0.0858	0.0718	-0.175	0.8616
A. cristatum	0.0192	0.0400	203	-0.0595	0.0980	0.482	0.6303
E. lanceolatus	-0.1627	0.0420	215	-0.2455	-0.0800	-3.877	**0.0001**
O. viciifolia	-0.0516	0.0404	204	-0.1314	0.0280	-1.279	0.2025
T. pratense	0.0162	0.0425	209	-0.0675	0.0999	0.382	0.7028
V. americana	-0.0826	0.0400	203	-0.1614	-0.0038	-2.067	**0.0400**

**Boldface type indicates P-values < 0.05; Confidence level at 0.95*

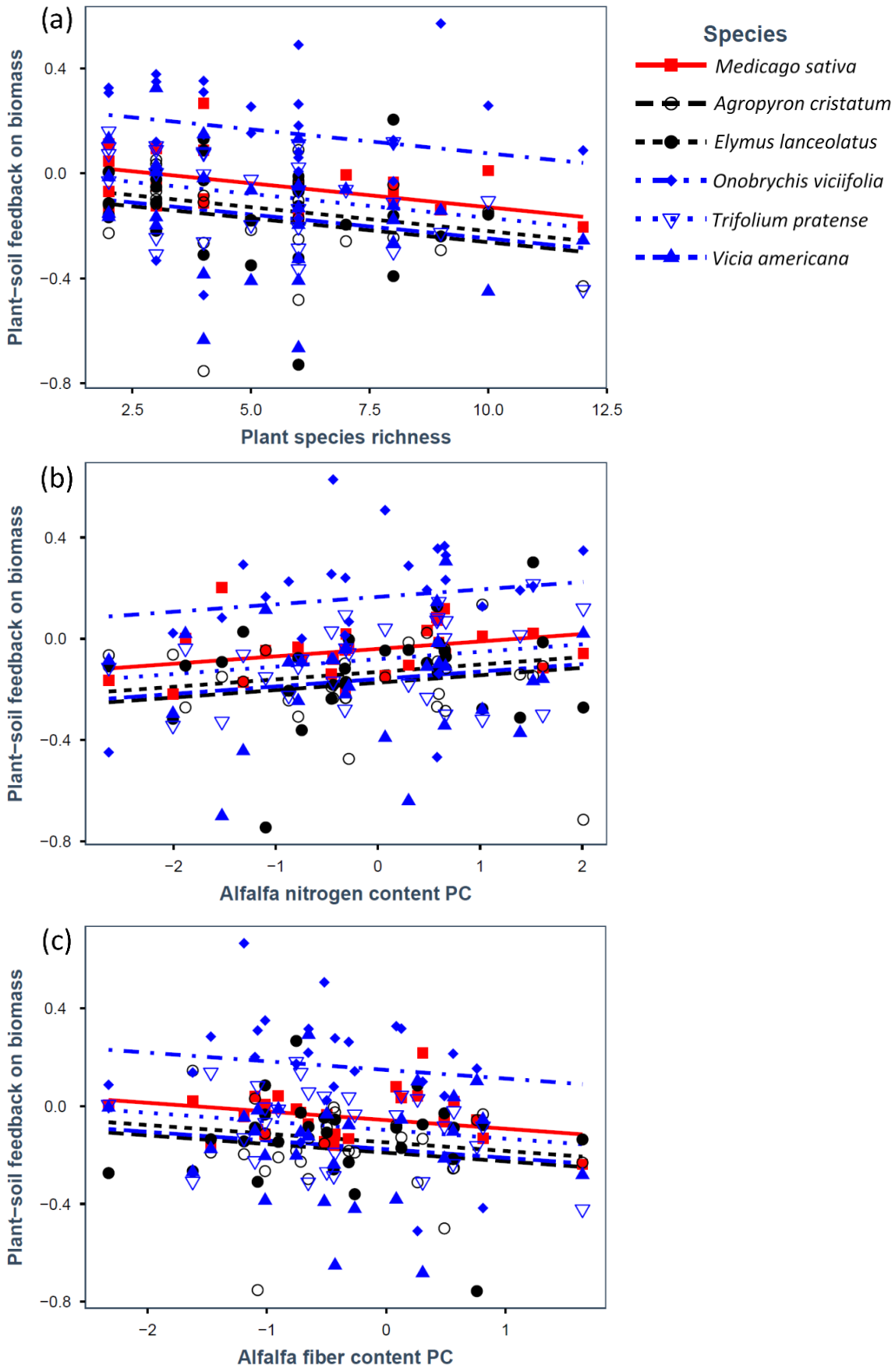
(a)
(b)
(c)
Plant−soil feedback on biomass
Plant species richness
Alfalfa nitrogen content PC
Alfalfa fiber content PC
Species
Medicago sativa
Agropyron cristatum
Elymus lanceolatus
Onobrychis viciifolia
Trifolium pratense
Vicia americana

Figure 3.5. Relationship between plant-soil feedback of forage species and **(a)** plant species richness (P = 0.013), **(b)** field nitrogen content in alfalfa (P = 0.051), and **(c)** field fiber content (ADF/NDF) in alfalfa (P = 0.058) in alfalfa-associated mixture soils. Model averaging was used to analyze predictors with an importance value of 0.50 or greater influencing interspecific PSF of *Medicago sativa, Agropyron cristatum, Elymus lanceolatus, Onobrychis viciifolia, Trifolium pretense and Vicia americana,* calculated as the ln ratio of plant biomass in soils previously conditioned by alfalfa associated soil inoculum vs sterile soils.

Although more marginal, PSF was negatively related to alfalfa fiber content (P = 0.058; Fig. 3.5c). PSF also increased with legume abundance, although not significantly (P = 0.116).

3.4.3 Mechanisms of plant-soil feedback

Root tissue density (RTD) differed significantly among the varieties (P = 0.014; Appendix E). RTD was significantly higher in Foothold, Spyder and 2010, while 3010 had the lowest RTD but not significantly different from 2010 (Fig. 3.6a). There was no significant difference in other root traits among the varieties (P > 0.05; Appendix E).

Conditioning effects of stand age and plant diversity did not have strong effects on the expression of belowground traits among the varieties (P > 0.05), however, the root-shoot ratio of varieties differed between mixture and monoculture (P = 0.031) with 2.62% increase in monoculture relative to mixture (Fig. 3.7a; Appendix F).

All root traits differed significantly among the species, usually by plant functional group (Fig. 3.6b-g; Appendix G). Root nodulation by rhizobia was higher in *T. pretense* and lower in *V. americana,* while *M. sativa* and *O. viciifolia* were intermediate (Fig. 3.6b). Root colonization by AMF was higher in the legumes than the grasses, although *T. pratense* and *O. viciifolia* were intermediate (Fig. 3.6c). Average root diameter was higher in the legumes than the grasses, although *T. pratense* was intermediate (Fig. 3.6d). SRL was significantly higher in the grasses compared to the legumes, although *T. pratense* had a considerable higher SRL among the legumes (Fig. 3.6e). Root-shoot ratio was higher in *M. sativa* and *V. americana* than the grasses and other legumes, although *O. viciifolia* was intermediate (Fig. 3.6f). RTD for the species was significantly higher in *M. sativa* and lower in *O. viciifolia,* but no clear differences were observed between the grasses and other legumes (Fig. 3.6g).

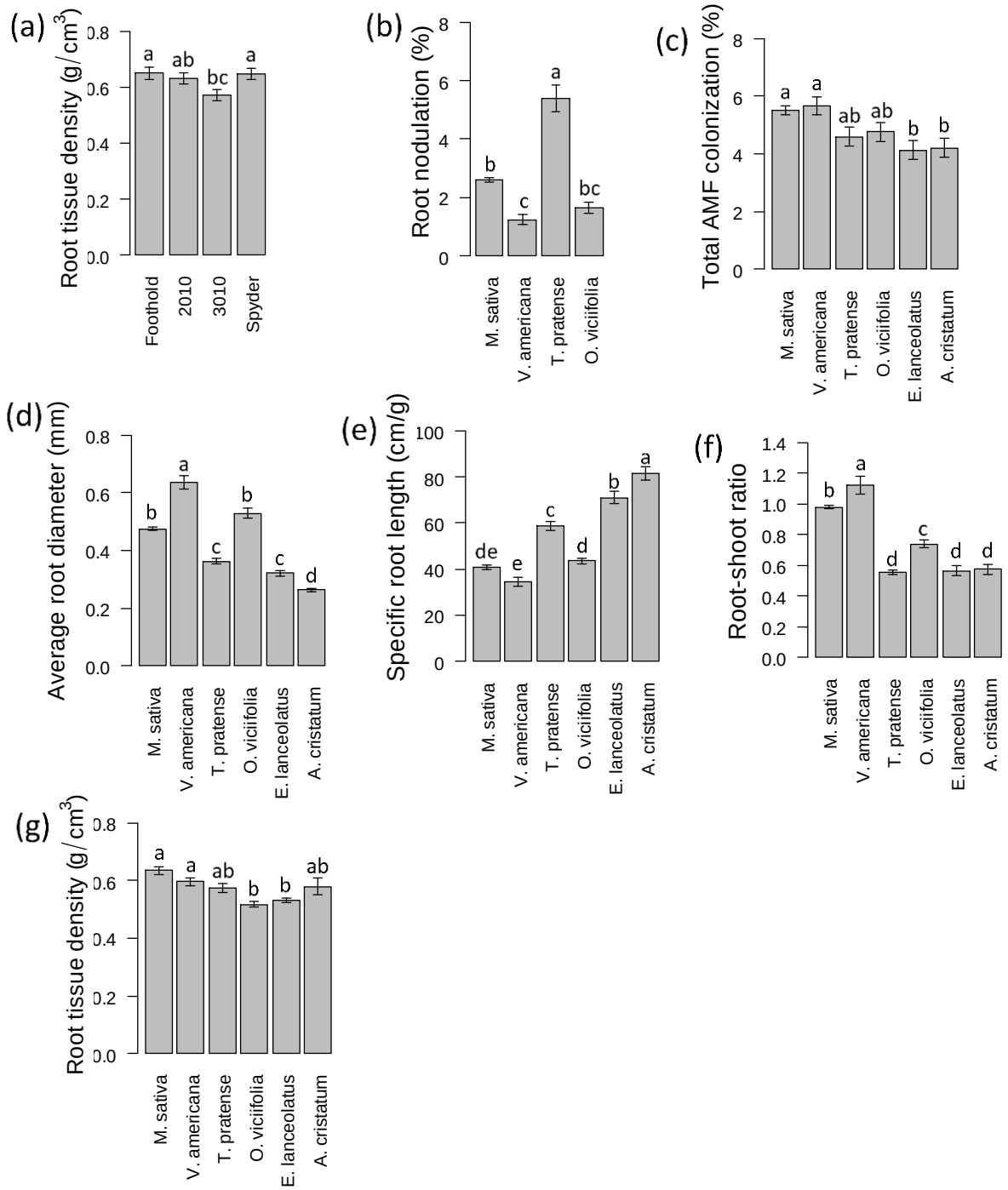

Figure 3.6. Significant root traits responses of alfalfa varieties **(a)** and forage species **(b-g)** to inoculation with live soil (microbial communities) collected from alfalfa monoculture and mixture stands. n = 231 (variety) and n = 360 (species). Effects of variety and species identity were determined by mixed-effects models ($P < 0.05$). Different letters indicate significant mean differences among plants according to Tukey's HSD post hoc test. Error bars represent standard error of mean.

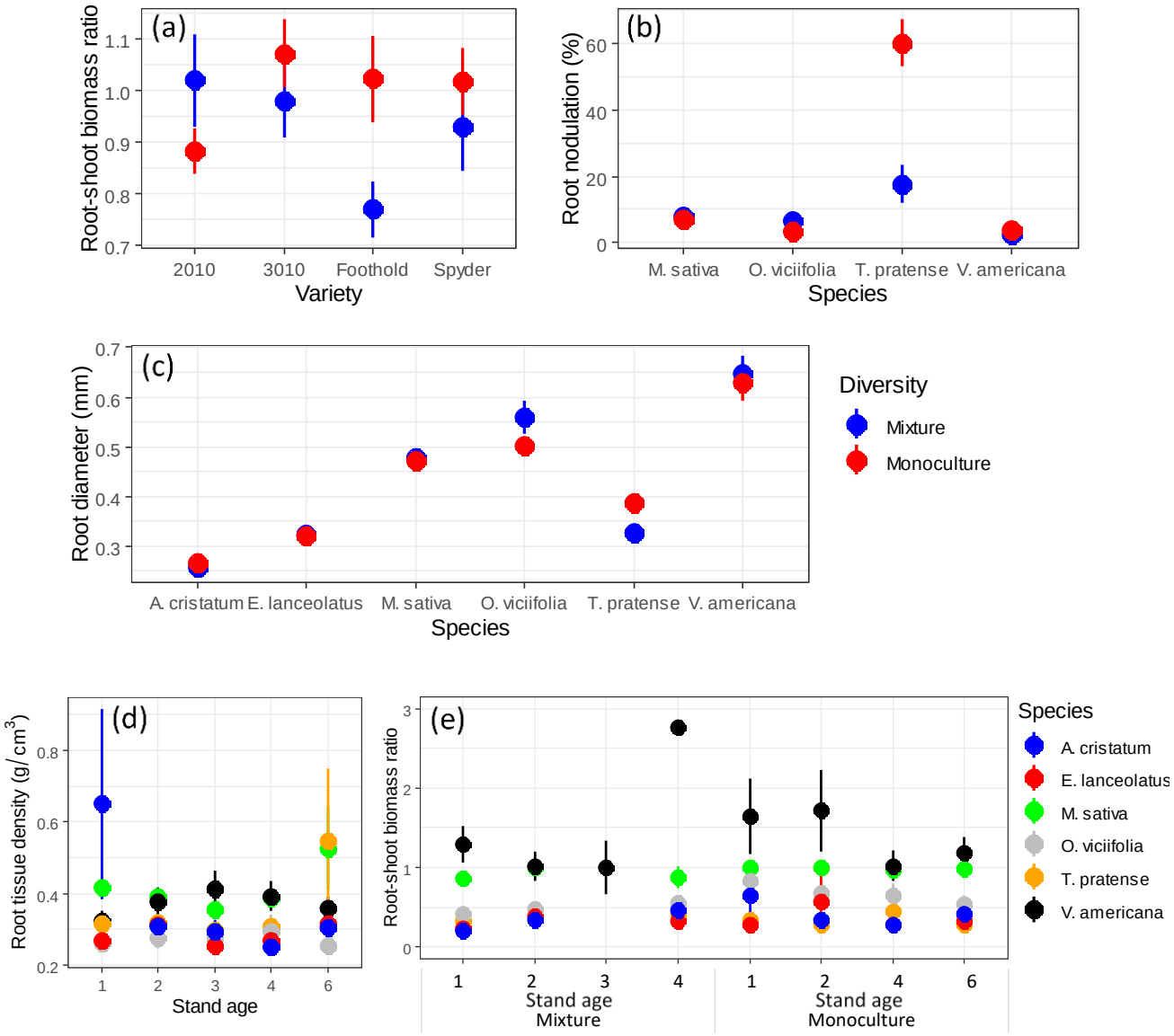

Figure 3.7. Significant changes in root trait responses of alfalfa varieties (a) and forage species (b-e) to inoculation with live soil (microbial communities) collected from alfalfa monoculture and mixture stands. n = 231 (variety) and n = 360 (species). **(a)** Field diversity effect on root-shoot biomass ratio of the varieties (P = 0.031). Species-dependent effect of field diversity on **(b)** root nodulation (P < 0.001), **(c)** average root diameter (P = 0.044), and **(d)** species-dependent effect of stand age on root tissue density (P < 0.001). **(e)** Species-dependent effects of stand age and field diversity on root-shoot biomass allocation (P = 0.012). Error bars represent standard error of mean.

Expression of many root traits was affected by plant diversity, and stand age depending on species identity (Fig. 3.7b-e; Appendix H). *T. pratense* had greater root nodulation in monoculture than mixture, while *O. viciifolia* had slightly higher but non-significant nodulation in mixture compared to monoculture (Fig. 3.7b). *T. pratense* also had higher root diameter in monoculture than mixture, while root diameter of *O. viciifolia* was higher in mixture than monoculture (Fig. 3.7c). However, root nodulation and diameter of other species did not differ between mixture and monoculture soils (Fig. 3.7b&c). *T. pratense* and *M. sativa* had significantly higher root tissue density in older stands while the opposite was true for *A. cristatum* (Fig. 3.7d). The differences observed in root-shoot ratio was dependent on stand age and diversity. Expression of this trait by *V. americana* increased in year 4 in mixture, while it was lower in year 3 in monoculture. However, other species did not show a distinct pattern in this trait (Fig. 3.7e).

3.6. Discussion

Both variety and species identity strongly affected the strength and direction of PSF, consistent with variation in pathogen resistance and mutualist benefits (Crawford et al., 2019; Gornish et al., 2020). Intraspecific feedback was more negative in monoculture than mixture soils suggesting that heterospecific neighbors dilute strong PSF effects (Bennett & Klironomos, 2019; Mao et al., 2021). There was no evidence, however, that this dilution effect was consistent for other species grown in alfalfa-conditioned soil, likely because they were unaffected by alfalfa specific microbes. Strength of PSF was reduced by stand age especially for conspecifics in monoculture, but otherwise stand age had little effects. This indicates that plant species effects on the soil microbial community can change over time, causing changes in yield. These effects, however, are diluted in mixture and unimportant for heterospecific crops (Bartelt-Ryser et al., 2005; Hannula et al., 2020).

PSF was consistently negative for the alfalfa varieties in monoculture soils but less so in mixture soils, consistent with dilution of negative biotic effects on conspecific crops in more diverse mixtures (Edwards et al., 2019; Mao et al., 2021). Nevertheless, the varieties differed in PSF within monoculture and mixture soils, due to their abilities to resist pathogens, benefit from mutualists (Eck et al., 2019; Gornish et al., 2020), or adjust root trait expression (Mao et al., 2020). Root trait response patterns to soil microbes were, in most cases, similar among the varieties. While this is surprising, it suggests that these traits have not been selected for, thus reflecting their ancestral state in the varieties (Annicchiarico et al., 2015; Wang et al., 2011). However, RTD varied among the

varieties and the overall expression was high, a trait indicative of conservative syndrome (Bergmann et al., 2020; Kramer-Walter et al., 2016). Low RTD is an acquisitive strategy associated with a fast relative growth rate and a rapid resource acquisition that enables root system to expand rapidly with a low investment on dry matter (Bergmann et al., 2020; Kramer-Walter et al., 2016). Interestingly, RTD was slightly lower in the variety (3010) with most positive PSF, implying that lower RTD contribute to positive PSF for alfalfa cultivars in forage stands. Moreover, allocation of resources to root biomass in alfalfa varieties differed between monoculture and mixture, suggesting that there is genetic, yet unexplored, potential in alfalfa varieties to change root trait expression under different microbial effects. Whether these traits were specifically selected for or whether such selection was incidental is unknown; however, it does suggest that directed plant breeding for lower root tissue density and higher root biomass can increase positive PSF and improve rejuvenation of alfalfa stands.

Unsurprisingly, given their strong effect on microbial communities, soil characteristics during the conditioning phase affected PSFs. Phosphorus rich soils in monoculture generated negative PSF for most varieties, except one. Increased phosphorus availability often reduces mutualists benefits (Revillini et al., 2016) and thus impedes alfalfa growth in monoculture. Interestingly, this study found that some alfalfa varieties can still benefit from soil microbial communities under increased nutrient supply. Such varieties should be most successful in high input systems. Additionally, soil texture during conditioning also influenced intra-specific PSF in mixture soils. Soils with greater silt and sand content (i.e. coarser-textured) positively influenced PSF effects on the growth of alfalfa, compared to soils with higher clay content (finer-textured) where PSF effects were negative. Coarser-textured soils promote higher microbial diversity relative to finer-textured soils (Ma et al., 2016; Obayomi et al., 2021), and are better drained. Both rhizobia and mycorrhizal fungi can be more beneficial under these conditions (Revillini et al., 2016), and may thus be promoted by the plant. This indicates that beneficial effects of soil microbial communities associated with mixtures are further enhanced in coarser-textured soils.

Plant-soil feedback varied among the legume species and the variation was explained by root trait expression. Some species performed better in monoculture (*T. pratense*) and others in mixture (*O. viciifolia*). The mechanism for this variation may be mediated by a number of factors including differences in the accumulation of host specific pathogens and mutualists, ability to withstand generalist pathogens and accumulate mutualists (Crawford et al., 2019; Gornish et al., 2020), or

regulate root conservative-acquisitive tradeoffs (Kramer-Walter et al., 2016; Mao et al., 2020). Interestingly, root traits varied significantly among the species, consistent with previous observation (Gorim & Vandenberg, 2017). Root nodulation rate and average root diameter were higher for *T. pratense* in monoculture and for *O. viciifolia* in mixture where their PSFs were most positive. This result is in line with observations that changes in root traits induced by soil biota drives variation in PSF among species (Hendriks et al., 2013; Hendriks et al., 2015).

Thicker roots support colonization with mutualists and can enhance pathogen resistance (Sweeney et al., 2021). Therefore, this result indicates that *T. pratense* is more tolerant of alfalfa specific pathogens, allowing it to benefit from their mutualists, whereas *O. viciifolia* will benefit less from alfalfa-specific microbes than generalist microbes, which are more common in heterogeneous plant communities (Benítez et al., 2013). RTD also varied among the species and was lowest in the species (*O. viciifolia*) with the most positive PSF. This suggests that the rapid resource acquisition at a lower cost of root biomass associated with low RTD (Kramer-Walter et al., 2016; Mao et al., 2020) contributes to positive PSF for species under biotic stress conditions, consistent with the varieties. Legumes in current study typically expressed conservative syndromes (low SRL, high root diameter, high root-shoot ratio) and experienced less negative PSF relative to the grasses. Consequently, conservative strategies are important mechanism of positive PSF in agroecosystems (Hendriks et al., 2015; Mao et al., 2021).

Plant-soil feedback was consistently negative for the grass species regardless of the diversity of the seeded stand. The two grasses (*E. lanceolatus* and *A. cristatum*) allocated less resources to root biomass compared to the legumes. Reduction in root-shoot ratio is an acquisitive strategy associated with reduced access to available soil biotic and abiotic resources (Kulmatiski et al., 2017) and has been linked with negative PSF (Hendriks et al., 2015). This is in line with the grasses in this study having more negative PSF relative to the legumes. Although there was significant variation among the species, grass species had the thinnest root diameter and consequently lowest AMF colonization, indicating that lower AMF colonization exacerbates negative PSF. This is consistent with previous observations that grasses usually have low AMF responsiveness (Bergmann et al., 2016; Reinhart et al., 2012), and that AMF play an important role in PSF (Gough et al., 2021; Mao et al., 2021). These grasses typically expressed more acquisitive syndromes (high SRL, low root diameter, low root-shoot ratio) relative to the legumes that expressed high conservative syndromes (opposite traits). This suggests that acquisitive strategies are important

mechanism of negative PSF for the grasses in agroecosystems (Hendriks et al., 2015; Mao et al., 2021). The practical implication of this result is that some grass species are not promising for rejuvenation of alfalfa stands, especially when previous stand had greater density of alfalfa.

The ability of plant species to survive under biotic and abiotic stress conditions has been long attributed to optimal and efficient allocation of resources especially to those related to the root economic spectrum (Guo et al., 2020; Kramer-Walter et al., 2016). Microbial induced changes in trait expression, however, are not always related to changes in PSF (Kramer-Walter et al., 2016). Greater allocation of biomass to root and higher average root diameter in some species in the current study were not clearly related to positive PSF, contrary to previous observations (Hendriks et al., 2013; Hendriks et al., 2015; Mao et al., 2020). This inconsistency may be attributed to species-specific differences in growth responses to specific soil microbes or optimal allocation of resources to structure that can best supply limiting resources (Gorim & Vandenberg, 2017; Hendriks et al., 2015). This result suggests that the efficiency of microbial induced root trait expression to drive positive PSF depends on the identity of the plant species.

Native plant species are expected to experience stronger negative PSF than non-native species due to the presence of specific natural enemies (Gornish et al., 2020; Klironomos, 2002). Inconsistent differences between native and tame species in the current study could be attributed to inherent differences in how these species interact with soil microbes, or the fact that wheatgrass species are ubiquitous throughout the study region which may lead to a higher probability of encountering their pathogens (Bennett & Cahill, 2016). While such differences are not ubiquitous, they depend on plant identity rather than origin (Perkins & Nowak, 2013). Interestingly, increased weed abundance generated more negative PSF for the native species only, *V. americana* and *E. lanceolatus,* suggesting that weeds serve as reservoir of pathogens which 'spillback' upon native plant species (Flory & Clay, 2013). Consequently, the efficacy of native species for pasture rejuvenation depends on the prevalence of weeds in the field.

Independent of the responding varieties or species, the traits of the conditioning alfalfa plants affected PSFs, potentially due to effects on decomposition and nutrient cycling or as proxies for defense allocation (Revillini et al., 2016; Yan et al., 2018). Positive effects of alfalfa fiber content on PSF in monoculture suggest that plant traits related to low decomposability (i.e. higher fiber content) can mediate shifts in soil microbial communities (Yan et al., 2018) that promote plant

growth. This was not consistent among alfalfa varieties, however. In contrast, increased fiber content, though marginal, negatively impacted PSF among the forage species in mixture soils, suggesting that decomposability traits can impair the positive soil microbial effects associated with higher plant diversity (Bennett et al., 2020). Interestingly, alfalfa nitrogen content positively influenced the growth of forage species in mixture soils, suggesting that both nitrogen content and mutualists increase with plant diversity (Furey & Tilman, 2021). This result is consistent with previous observations that litter with higher decomposability (i.e. higher nitrogen than carbon content) promote the abundance of beneficial soil microorganisms (Ke et al., 2015; Yan et al., 2018). Our results, therefore, indicate that in addition to pathogens and mutualists, focal crop species influence how soil microbes support the growth of subsequent crops through decomposability-related traits.

3.7. Conclusion

The past plant community can have strong effects on subsequent crops via changes in soil microbial communities. Plants responding to these microbial communities can adjust the expression of their root traits as a coping mechanism to acquire limiting resources for growth and protection against pathogens. Strong variation observed in intra- and inter-specific PSF suggests that some varieties of alfalfa and forage species are more tolerant of negative soil microbial legacies than others, and that careful selection of crops can enhance productivity when re-seeding existing stands. PSF, however, is context dependent as many abiotic and biotic components can influence how plants condition soil microbial communities. Root trait expression such as high root diameter, symbiosis with nitrogen-fixing bacteria, root biomass, and lower root tissue density are useful in predicting how crop species and varieties tolerate biotic stress. Future research should focus on the genetic basis of these traits, and evaluate their expression in response to soil biota under field conditions. These are needed in directed microbiome-assisted plant breeding efforts to increase positive PSF and productivity of perennial crops. Selection of variety and species selection are therefore critical to successful pasture rejuvenation.

4. IDENTIFICATION OF FUNCTIONAL SOIL MICROBIAL COMMUNITIES ASSOCIATED WITH INTRA- AND INTER-SPECIFIC PLANT-SOIL FEEDBACKS

4.1. Preface

In Chapter 3 we saw that field conditions such as seeded plant diversity, stand age and soil characteristics affect how standing crops condition soil microbial communities. When exposed to these soil biota, some varieties and forage species grew better, while others grew worse depending on root trait expression. This implies that these plants are responding differently to specific soil microbial groups, and that simply seeding new crops into existing pasture may not always mitigate productivity decline. It is thus imperative to understand how plant-specific interactions with soil microbial groups can serve as a guide to mitigate productivity decline. In this study, specific interactions of alfalfa varieties and forage species with different soil microbial groups were assessed and systems that promote plant-specific beneficials and pathogens were identified.

Publication statement

This chapter is being modified as a manuscript to be submitted for publication. Contributors of the manuscript are Awodele S.O. (Lead Author), Tremblay J., Bainard L., Biligetu B. & Bennett J.A. (Corresponding Author).

4.2. Abstract

Soil microbial communities play important roles in plant health due to their potential capabilities to promote or inhibit plant growth and drive plant-soil feedback (PSF). However, our knowledge of how diversity-related cropping systems change the composition of soil microbial communities and how these changes drive PSF among crop varieties and species in perennial agroecosystems is still in its infancy. We sampled soils from alfalfa monoculture and mixture stands (plant diversity) near Saskatoon, SK., at stand ages 1 to 6 years old and used targeted amplicon sequencing to quantify soil microbial community composition, focusing on bacteria, general fungi, arbuscular mycorrhizal fungi, and oomycetes. Using these soils as inoculum, we grew four varieties of alfalfa (*Medicago sativa*) and five additional native and tame forage grasses and legumes. We then tested how soil microbial community composition affected plant growth as an estimate of PSF, calculated as the log ratio of plant growth in live soil versus sterile. Community composition of soil oomycetes, arbuscular mycorrhizal and other fungi differed between

monocultures and mixtures and this effect varied as a function of stand age; however, community composition of soil bacteria was not affected. The diversity of arbuscular mycorrhizal fungi (AMF) was greater in mixture than monoculture, while the diversity of oomycetes in mixture decreased as stands aged, suggesting that greater plant diversity favors the proliferation of diverse AMF and reduces potential pathogens over time, thus reducing the strength of negative PSF. The changes induced by plant communities affected the abundance of many beneficial and pathogenic soil microbes with little effects on saprotrophs, indicating that plants initiate PSF process by altering the availability of soil microbes that can impact the growth of subsequent crops. More than 30 soil microbial taxa, including both beneficial and pathogenic microbes, were associated with positive and negative PSFs, although these associations were variety and species dependent. Soil microbes associated with positive PSFs were mostly enriched in mixture, while those associated with negative PSFs were mostly enriched in monoculture. This indicates that soil microbes conditioned in heterogeneous stands enhance crop growth relative to those in alfalfa monoculture stands. Negative associations with soil microbes were more common for the native than non-native species, suggesting that natural enemies of native species are abundant in alfalfa stands and thus limit their suitability for rejuvenation of alfalfa stands. Collectively, these results indicate that some varieties and species are more promising than others for rejuvenation of alfalfa stands than others due to the possibilities of encountering their mutualistic or pathogenic soil microbes present in either mixture or monoculture stands.

4.3. Introduction

Plant-soil interactions involve complex relationships between plant roots and soil chemical, physical and biological components, with strong effects on plant and soil health (Beugnon et al., 2021; Dias et al., 2015; Lekberg et al., 2021). Growing plants alter the structure and composition of soil microbial communities, including pathogens, saprotrophs, and mutualists (Nguyen et al., 2016; Putten et al., 2016). The net effects of these altered microbial communities on plant growth, called plant-soil feedbacks (PSFs), which varies from positive to negative and affects the growth of natural plant populations and crop yields (Edwards et al., 2019; Mao et al., 2021). Identifying soil microbial taxa affecting PSF is critical for both sustainable crop production and ecosystem functioning, yet the factors determining their assembly and relative abundance in agroecosystems are poorly understood.

Many bacterial (Edwards et al., 2019b; Mendes et al., 2011) and fungal taxa (Harman et al., 2004; Hossain et al., 2007; Säle et al., 2021) have been identified to promote plant growth, and thus positive PSF (Crawford et al., 2019; Mao et al., 2021). On the other hand, soils also harbor plant pathogenic groups including oomycetes and some fungi (Colavolpe, 2020; Domínguez-Begines et al., 2021) and bacteria (Nguyen et al., 2021), which drive negative PSF (Crawford et al., 2019; Domínguez-Begines et al., 2021). Community structure and composition of these soil microbes can be influenced by crop management, and thus crop management can affect PSF (Jangid et al., 2008; Mariotte et al., 2018; Orr et al., 2015). Plant species and varieties can differ in their growth response to soil microbes, however, resulting in variation in PSF among those plant groups (Hannula et al., 2020; Ulbrich et al., 2021).

Seeded plant diversity with different plant functional groups, such as grass-legume mixtures increases soil microbial diversity (Bartelt-Ryser et al., 2005; Beugnon et al., 2021), and thus enriches soils with microbial communities that influence PSF (Bartelt-Ryser et al., 2005). The inclusion of legumes can increase the abundance of beneficial soil microbes such as arbuscular mycorrhizal fungi (AMF; Samaddar et al., 2021) and rhizobacteria (Chalasani et al., 2021), thus improve their availability for following crops (Samaddar et al., 2021). However, legumes may also decrease the abundance of disease suppressive bacteria in mixture (Latz et al., 2012, 2015), thus increase the frequency of fungal parasites, saprotrophs, and potential plant pathogens (Hannula et al., 2020). Inclusion of grasses, however, can restore the abundance of disease-suppressive bacteria in such soils (Latz et al., 2015). Even within plant functional groups, plant species and varieties can act as a selective filter and recruit specific groups from large soil microbial communities (Berendsen et al., 2012; Samaddar et al., 2021; Ulbrich et al., 2021). Consequently, there is a growing evidence that host preferences in selecting microbial taxa differ among crop varieties (An et al., 2011; Ulbrich et al., 2021), species and functional groups (Hannula et al., 2020; Wippel et al., 2021).

Host preferences in plant-microbial interactions can also be influenced by plant origin (Kama et al., 2020; Kendig et al., 2020). Native plants have greater adaptation to the resident soil microbes as they share a greater amount of evolutionary history (Wippel et al., 2021). Consequently, while native and non-native species may share generalist pathogens, non-native species may suffer less disease severity due to escape from specialist pathogens (Kendig et al., 2020). These host-specific differences in microbial interactions are associated with variation in resistance to certain pathogens

and mode of actions (An et al., 2011; Mendes et al., 2018), root traits, and exudate composition which create unique niches that select specific rhizosphere microbiome (Chaparro et al., 2014; Williams et al., 2021). Despite the observed effects of microbiome legacies on subsequent generations of plant varieties and species (Hannula et al., 2020), how specific soil-borne pathogens, mutualists, and saprotrophs feedback on these plant types remains largely unknown.

Conditioning effects of plant functional groups on soil microbial communities, however, change over time (Orr et al., 2015; Wagner et al., 2016). Soil bacterial communities can shift over time due to changes in the relative contribution of certain taxa (Hannula et al., 2019). Soil fungal communities, on the other hand, are relatively more stable, although they may vary with conditioning plant species or functional groups (Hannula et al., 2019). Plant developmental stage and age can change root exudate composition (Chaparro et al., 2014; Hamlen et al., 1972) which in turn alters soil microbial community (Williams et al., 2021; Zhalnina et al., 2018). Additionally, susceptibility to pathogens can also allow for selection of beneficial mutualists (Friman et al., 2020) and can progressively shift the microbial communities as the plant develops (Chaparro et al., 2014; Williams et al., 2021). These induced changes in soil microbial communities through time can create overlapping niches within soil microbial communities (Moroenyane et al., 2021) and thus resulting in temporal variation in PSF (Hannula et al., 2019; Hawkes et al., 2013).

Alfalfa, *Medicago sativa* is an important perennial forage crop in Canada, occupying cropping area of approximately 1.2 million hectares in mixture and monoculture systems (Statistics Canada, 2016). Over time, alfalfa production declines in productivity due to deleterious rhizosphere microorganisms and abiotic factors such as soil nutrient depletion (Annicchiarico et al., 2015). Alfalfa has been identified to leave a significant microbial footprint on soils (Bidellaoui et al., 2019; Menendez & Carro, 2019; Samaddar et al., 2021). For example, microbial taxa known for producing phytohormones and improving plant growth (Menendez & Carro, 2019; Samaddar et al., 2021), and some pathogenic taxa that causes vascular wilt (Samaddar et al., 2021) were carried over from alfalfa to subsequent crops in different systems. However, we do not understand how host preferences in microbial interactions may influence growth responses of subsequent crops to changes in soil microbial communities. This knowledge will facilitate the selection of promising varieties and species for the successful rejuvenation of alfalfa stands.

Soils were collected from 24 alfalfa fields grown to monoculture and alfalfa-grass mixture at one to six years old (chapter 3), and sequenced using 16S (bacteria) and 18S ribosomal DNA (rDNA; AMF), and internal transcribed spacer (ITS) amplicons (general fungi and oomycetes). This study had three main objectives:

(1) to assess how the structure and composition of soil microbial communities in alfalfa production change with plant diversity and stand age.

We then combined these data with the results from growth chamber experiment conducted using these soils.

(2) to determine whether the changes in soil microbial community composition is associated with growth responses of different varieties or species.
(3) to identify soil microbial functional groups driving PSF among alfalfa varieties, and forage species belonging to different functional groups and origin.

4.4. Materials and Methods

4.4.1. Site selection

A total of 24 existing alfalfa pastures under monoculture and mixed cropping systems within 300 km of Saskatoon were identified. For a more thorough site description see Section 3.4.1. They were separated by stand age generally at 1, 2, 3, and up to 6 years. The sampling fields belong to 10 producers and one experimental site. The mixture sites were split at 1, 2, 3, and 4 years old from fields cultivated for cattle producers. We were unable to identify any monoculture stands used by cattle producers, therefore, we sampled alfalfa fields cultivated for seed production of 1, 2, 4, or 6 years old, due to the loss of three-year-old field to a flooding event in the region.

4.4.2. Vegetation and soil sampling

Field sampling was completed in summer 2019 during which we selected three sampling locations separated by at least 50 m at each site, except for the experimental site where we selected three separate replicates. The sites were at least two kilometers apart. At each sampling location, a 1 m^2 plot was placed within which 12 soil cores at 2 cm wide and 15 cm deep spread evenly across the plot were collected. In locations where soil depth less than 15 cm was encountered, we collected additional soil cores to ensure sufficient volume of soil inocula for the growth chamber experiment.

We mixed the soil samples from each plot, transported them to the lab on ice, and stored at -20°C for ~16 months before they were retrieved for molecular analysis of soil microbes. Other soil subsamples were preserved in the refrigerator (4°C) prior to use for inoculation of growth chamber experiments. Due to some mislabeling and field sampling errors, the total number of locations sampled was reduced from 72 (3 samples × 24 sites) to 66.

4.4.3. Plant-soil feedback

We used four varieties of alfalfa and six additional forage species to estimate feedback variation among varieties and species. The four varieties included Foothold, Spyder, 2010, and 3010 selected by the seed producer (BrettYoung Seeds Ltd.). They represent cultivars that vary in growth, disease resistance and root morphology in my study (see Chapter 3). The additional six species included two tame legumes (*Trifolium pretense* and *Onobrychis viciifolia*), two native legumes (*Vicia Americana and Dalea purpurea*), one tame grass (*Agropyron cristatum*) and one native grass (*Elymus lanceolatus*). For one of the native legumes (*Dalea purpurea*), few seeds germinated in the experiment (<1%), so this species was not considered further. Seeds for each variety and species were seeded into each pot containing each of the 66 soil samples as inoculum, and into pots containing sterilized soils in phytotron growth chambers. The pots were arranged in a completely randomized design, after 4 months plants were harvested and dried at 60°C for 72h for aboveground biomass. For each plant grown in live soils, PSF was calculated as the natural log of the ratio of biomass of plants grown in live soils (inoculated pots) to the average biomass in sterile soils (Brinkman et al., 2010).

4.4.4. Soil microbial DNA extraction and high-throughput amplicon sequencing

For each of the soil samples collected from the field, soil microbial DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Quantification of extracted DNA was done using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, U.S.A.). Soil bacteria, oomycetes, and fungi, with an additional focus on AMF, were sequenced to understand how field conditions change their relative abundance and how these changes influence PSF. Specifically, for fungi, the primers ITS1F/58A2R targeting the fungal intergenic transcribed spacer (ITS) region were used (Gardes & Bruns, 1993; White et al., 1990). For bacteria, the primers 515F/806R targeting the V4 region of the 16S rDNA genes were used (Bergmann et al., 2011). AMF SSU ribosomal RNA gene, and oomycetes ITS1 region were

amplified using the primers WANDA/AML2 in SSU (Vasar et al., 2021) and ITS6/ITS7ae (Taheri et al., 2017), respectively. The libraries were sequenced using Illumina MiSeq PE250 and PE300 platforms (at Genome Quebec, Montreal, Canada).

4.4.5. Bioinformatics analysis

Sequencing data was analyzed using AmpliconTagger (Tremblay & Yergeau, 2019). Briefly, raw reads were scanned for sequencing adapters and PhiX spike-in sequences. Primer sequences were removed using pTrimmer v1.3.4 (Zhang et al., 2019). The remaining sequences were processed to generate Amplicon Sequence Variants (ASVs) in DADA2 v1.12.1 (Callahan et al., 2016). Chimeras were removed with DADA2 followed by UCHIME reference (Rognes et al., 2016). Bacterial ASVs were assigned a taxonomic lineage with the RDP classifier (Wang et al., 2007) using training sets containing the complete SILVA release 138 database (Quast et al., 2012) supplemented with a customized set of mitochondria and plastid sequences. The fungi ITS, AMF, and oomycetes training sets were constructed from the UNITE database (Abarenkov et al., 2010). Taxonomic lineages were combined with the cluster abundance matrix obtained above to generate raw ASV tables. Five-hundred 1,000 reads rarefactions were then performed on these ASV tables and the average number of reads of each ASV for each sample was computed to obtain consensus rarefied ASV tables. Taxonomic summaries were computed with RTK v0.93.2 (Saary et al., 2017) and microbiomeutils v0.9.3 (Yergeau et al., 2020), respectively using the consensus rarefied ASV tables.

4.4.6. Statistical analysis

Data analysis was performed in R environment version 1.3.9 (R Core Development team, 2020). To compare differences in alpha diversity of soil microbial communities between mixture and monoculture (plant diversity) and among stand ages, Shannon's diversity index was estimated at the ASV level separately for each amplicon using the *vegan* package (Oksanen et al., 2013). Mixed-effects models were conducted with the diversity scores as response variables, age × plant diversity as fixed effects, and sample nested within site as random variables. Mean differences were tested using the *anova* function in lmerTest package (Kuznetsova et al., 2017) followed by TukeyHSD in the *emmeans* package (Russell, 2018). Permutation multivariate analysis of variance (PERMANOVA) was performed to assess the effects of plant diversity and stand age on soil microbial community composition using *Adonis* function in *vegan* package based on a Bray-Curtis

dissimilarity matrix, and the strata within permutations were conducted (Oksanen et al., 2013). To visualize changes in the structure of these soil microbial communities based on relative abundance, I used sample scores from principal coordinate analysis (PCoA) axes computed from the Bray-Curtis distance (Lozupone et al., 2007).

To determine which taxa at the order and genera taxonomic levels were significantly different between mixture or monoculture soils, I used the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011) with an LDA threshold of 4 and α value of 0.01 to reduce the chance of false positive (Type I error) associated with multiple tests using the *microeco* package (Liu et al., 2021). LEfSe provides the list of ASV features (microbial taxa in this case) that are different between mixture and monoculture sites with statistical significance. Using the Kruskal-Wallace test, differences ($P < 0.01$) between taxa in mixture and monoculture sites are determined simultaneously in a sum test, followed by pairwise Wilcoxon tests to determine taxa that are similar within mixture, and monoculture sites. Taxa with $P < 0.01$ or with equal variation among all comparisons are then retained. The LDA then ranks the taxa using their relevance according to the effect size (Segata et al., 2011).

To identify soil microbial genera with the potential to affect PSF for alfalfa varieties and forage species, least absolute shrinkage and selection operator (lasso) multiple regression was performed using the *caret* package (Kuhn et al., 2018). This method prevents model overfitting, controls for multicollinearity among predictors, and selects only the most important predictor variables based on k-fold cross-validation which identifies the lambda value that produces the lowest test mean squared error (MSE) to improve model prediction accuracy (Ranstam & Cook, 2018). Each model included PSF of a variety or species as the response variable, and the relative abundance of genera within different microbial groups (bacterial, oomycetes, AMF or other fungi) as predictor variables. All taxa with low total abundance (<0.001%) and found in less than 40% of the 66 samples were excluded from this analysis (Hannula et al., 2019). While such taxa may not have any effect on the dependent variable (PSF), their inclusion may lead to biased estimation of the coefficients for other independent variables and can thus reduce the prediction accuracy of the model (Philippi, 1993). To test the statistical significance of taxa in predicting PSF, the important taxa identified in lasso regression models for each variety or species were included as fixed-effects in mixed-effects models with PSF as the response variable. Sample nested within site were

included as random variables. To visualize the important predictors of PSF, parameter estimates were extracted from the lasso regression models for all varieties and species. The resulting data was loaded into *ClustVis* online web tool to generate a heatmap (Metsalu & Vilo, 2015).

4.5. Results

4.5.1. Effects of stand age and plant diversity on the diversity and composition of soil microbial communities

Plant diversity and stand age affected the community composition of soil oomycetes, AMF and other fungi, as well as the diversity of AMF and oomycetes (Fig. 4.1, Appendix I). AMF diversity was significantly higher in mixture soils compared to monoculture, however, it was not affected by stand age and associated interaction (Fig. 4.1a). Stand age and its interaction with plant diversity significantly affected oomycete diversity; in mixed stands, oomycete diversity declined as the stand aged (Fig. 4.1b). Stand age, plant diversity and their interaction did not affect the diversity of soil bacteria and other fungal communities (Appendix I). PERMANOVA showed that stand age, plant diversity and their interaction significantly influenced the community composition of all the microbial groups except soil bacteria with plant diversity consistently accounting for most of the variation, followed by stand age and their interactions across the microbial groups (Fig. 4.2; Appendix I).

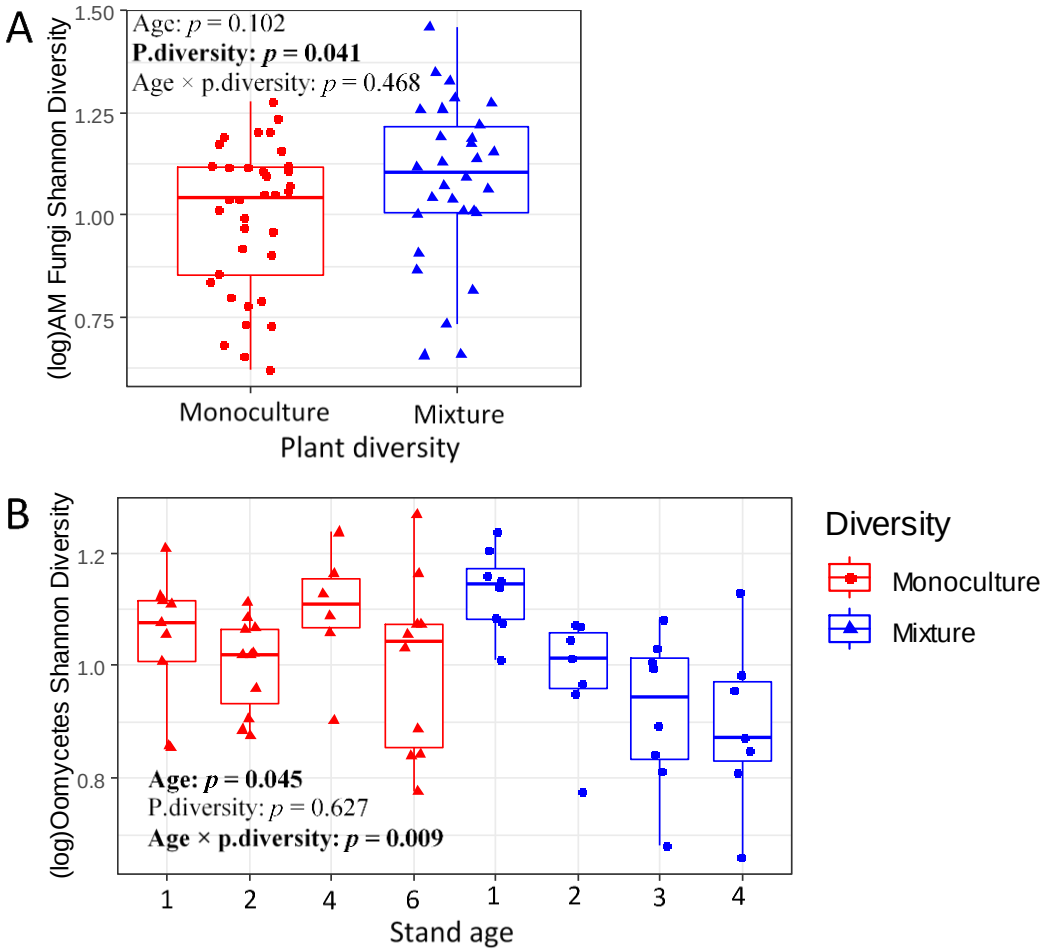

Figure 4.1. Significant changes in arbuscular mycorrhizal fungi **(a)**, and oomycetes **(b)** diversity in alfalfa monoculture and mixture soils at stand ages 1 to 6. Statistical significance of the effects of stand age (Age), plant diversity (p. diversity) and their interaction was derived from mixed effects model.

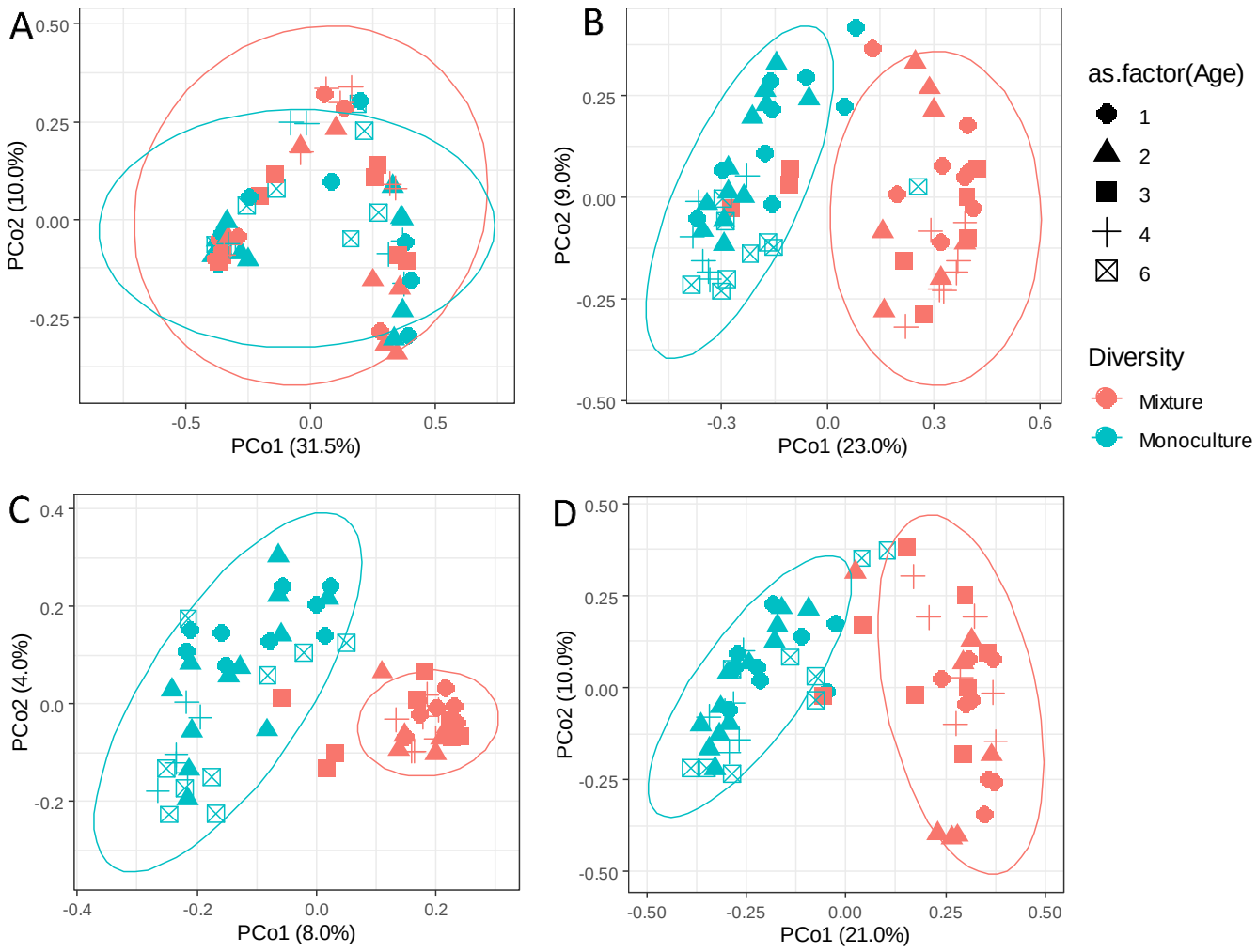

Figure 4.2. PCoA of the Bray-Curtis distance showing community composition shifts over time for soil bacteria **(a)**, arbuscular mycorrhizal **(b)** and other fungi **(c)**, and oomycetes **(d)** in alfalfa monoculture and mixture soils at stand ages 1 to 6.

At the order taxonomic level, most of the dominant microbial orders were enriched in mixture or monoculture (Table 4.1-4; Appendix J). For example, Rhizobiales, Burkolderiales, and Frankiales were significantly enriched in monoculture soils, while Bacillales, Glomerales, Hypocreales, and Pythiales were significantly enriched in mixture soils (Table 4.1-4; Appendix J). Relative abundance also varied between mixture and monoculture soils for most genera (Fig. 4.3; Table 4.1-4). For example, potential mutualists (*Rhizophagus* sp., *Glomus* sp., *Bradyrhizobium* sp.), and plant-growth-promoting microbes (PGPMs; *Haliangium* sp., *Mortierella* sp.) increased in monoculture, while other mutualists (*Dominikia* sp., *Funneliformis* sp.) and plant-growth promoting microbes (*Micromonospora* sp., *Fusicolla* sp., *Microvirga* sp. grouped among "OTHER") increased in mixture (Fig. 4.3; Table 4.1-2). Although many potential pathogens were detected, *Gibberella* sp. and *Pythium* sp. were the dominant pathogenic genera found in the fungal and oomycetes communities, respectively (Fig. 4.3c-d; Table 4.3-4).

These two genera, along with multiple other potential pathogens were differentially enriched in mixture and monoculture soils; *Gibberella* sp., *Alternaria* sp., and *Phytophthora* sp. were enriched in mixture soils, while *Pythium* sp. *Aphanomyces* sp., and *Colletotrichum* sp. (classified among "OTHER") were enriched in monoculture soils according to the differential analysis (Fig. 4.3c-d; Table 4.3-4). Of all the specific pathogens of *M. sativa*, only *Phytophthora* sp., *Aphanomyces* sp., and *Colletotrichum* sp. were differentially enriched in mixture and monoculture soils. Several putative saprotrophs including *Psathyrella* sp., and *Stenotrophobacter* sp. (Fig. 4.3a&c; Table 4.1&3) were enriched in mixture soils.

Table 4.1. Differential analysis with LDA Effect Size (LEfSe) testing bacteria taxa differentially enriched in alfalfa monoculture and mixture soils at stand ages 1 to 6 years old taken from sites near Saskatoon, SK., in August 2019.*

Phylum	Class	Order	Family	Genus	System Enriched	P - value	LDA
Actinobacteriota	Actinobacteria	0319-7L14	0319-7L14OR	Actinobacteria. 1 (Uncl)	Mixture	1.71E-08	3.0295
Acidobacteriota	Acidobacteriae	Acidobacteriales	AcidobacterialesOR	Acidobacteriales (Uncl)	Monoculture	1.68E-09	3.9005
Actinobacteriota	MB-A2-108	Actinobacteriota (Uncl)			Mixture	1.68E-12	3.5701
Acidobacteriota	AT-s3-28	AT-s3-28CL	AT-s3-28CL	Acidobacteriota 3 (Uncl)	Mixture	9.15E-03	-1.3483
Firmicutes	Bacilli	Bacillales			Mixture	1.68E-12	3.9007
Acidobacteriota	Blastocatellia	Blastocatellales	Blastocatellaceae	Stenotrophobacter	Mixture	2.45E-11	3.0210
Proteobacteria	Gammaproteobacteria	Burkholderiales			Monoculture	1.68E-12	3.6720
Proteobacteria	Gammaproteobacteria	Burkholderiales	BurkholderialesOR	Burkholderiales (Uncl)	Monoculture	1.68E-12	3.5371
Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	Hylemonella	Monoculture	2.52E-08	2.7158
Chloroflexi	Anaerolineae	Caldilineales	CaldilinealesOR	Caldilineales (Uncl)	Mixture	1.68E-12	2.5859
Chloroflexi	Chloroflexia	Chloroflexales	Roseiflexaceae	Kouleothrix	Monoculture	1.68E-12	3.6702
Chloroflexi	Gitt-GS-136	Chloroflexi 1 (Uncl)			Mixture	1.68E-12	3.7768
Chloroflexi	KD4-96	Chloroflexi 2 (Uncl)			Mixture	1.68E-12	3.0975
Verrucomicrobiota	Verrucomicrobiae	Chthoniobacterales			Monoculture	1.68E-12	4.3467
Actinobacteriota	Actinobacteria	Frankiales			Monoculture	1.68E-12	3.8305
Actinobacteriota	Actinobacteria	Frankiales	Nakamurellaceae	Nakamurella	Monoculture	3.61E-12	2.6773
Actinobacteriota	Thermoleophilia	Gaiellales			Monoculture	1.68E-12	3.7291
Actinobacteriota	Thermoleophilia	Gaiellales	Gaiellaceae	Gaiella	Monoculture	1.68E-12	2.8995
Actinobacteriota	Thermoleophilia	Gaiellales	GaiellalesOR	Gaiellales (Uncl)	Monoculture	1.68E-12	3.6611
Gemmatimonadota	Gemmatimonadetes	Gemmatimonadales			Monoculture	1.68E-12	3.8449
Gemmatimonadota	Gemmatimonadetes	Gemmatimonadales	GemmatimonadalesOR	Gemmatimonadales (Uncl)	Monoculture	2.45E-11	3.0381
Myxococcota	Polyangia	Haliangiales	Haliangiaceae	Haliangium	Monoculture	2.46E-12	3.1560
Actinobacteriota	Acidimicrobiia	IMCC26256	IMCC26256OR	Acidimicrobiia (Uncl)	Monoculture	1.68E-12	3.2893
Actinobacteriota	Actinobacteria	Micrococcales			Mixture	1.68E-12	3.5702
Actinobacteriota	Actinobacteria	Micrococcales	Micrococcaceae	Pseudarthrobacter	Mixture	1.68E-12	2.9447
Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae	Galbitalea	Monoculture	1.83E-06	2.0173
Actinobacteriota	Actinobacteria	Micromonosporales	Micromonosporaceae	Micromonospora	Mixture	2.59E-07	2.4106
Verrucomicrobiota	Verrucomicrobiae	Pedosphaerales	Pedosphaeraceae	Pedosphaeraceae (Uncl)	Monoculture	3.82E-07	2.9032
Actinobacteriota	Actinobacteria	PeM15	PeM15OR	Actinobacteria (Uncl)	Mixture	4.00E-06	3.0990
Myxococcota	Polyangia	Polyangiales	PolyangialesOR	Polyangiales (Uncl)	Monoculture	5.29E-12	2.0595
Actinobacteriota	Actinobacteria	Propionibacteriales			Mixture	1.68E-12	3.4853
Actinobacteriota	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides	Mixture	1.68E-12	2.8775
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Monoculture	2.46E-10	2.2727
Actinobacteriota	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	Monoculture	1.68E-12	2.3858
Acidobacteriota	Blastocatellia	Pyrinomonadales			Mixture	1.68E-12	3.8006
Acidobacteriota	Blastocatellia	Pyrinomonadales	Pyrinomonadaceae	Pyrinomonadaceae (Uncl)	Mixture	1.68E-12	3.8006
Proteobacteria	Alphaproteobacteria	Rhizobiales			Monoculture	1.68E-12	3.8881
Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Rhodoplanes	Monoculture	1.68E-12	3.7610
Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Bradyrhizobium	Monoculture	1.68E-12	3.5849
Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Microvirga	Mixture	3.61E-12	3.5182
Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae	Devosia	Mixture	1.67E-11	2.2375
Methylomirabilota	Methylomirabilia	Rokubacteriales	RokubacterialesOR	Rokubacteriales (Uncl)	Mixture	1.68E-12	2.7951
Actinobacteriota	Rubrobacteria	Rubrobacterales	Rubrobacteriaceae	Rubrobacter	Mixture	1.14E-11	4.0981
Actinobacteriota	Thermoleophilia	Solirubrobacterales			Mixture	1.68E-12	4.0850
Actinobacteriota	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	Solirubrobacter	Mixture	1.68E-12	3.9201
Actinobacteriota	Thermoleophilia	Solirubrobacterales	Solirubrobacteraceae	Conexibacter	Monoculture	1.68E-12	3.4005
Actinobacteriota	Thermoleophilia	Solirubrobacterales	67-14	Solirubrobacterales (Uncl)	Mixture	1.68E-12	3.8022
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Mixture	1.68E-12	2.4130
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonadaceae (Uncl)	Mixture	2.45E-11	2.8763
Acidobacteriota	Subgroup 11	Subgroup 11CL	Subgroup 11CL	Acidobacteriota 2 (Uncl)	Mixture	1.16E-08	1.8599
Acidobacteriota	Subgroup 20	Subgroup 20CL	Subgroup 20CL	Acidobacteriota 1 (Uncl)	Mixture	5.92E-03	-0.7052
Acidobacteriota	Subgroup 5	Subgroup 5CL	Subgroup 5CL	Acidobacteriota 4 (Uncl)	Monoculture	2.45E-11	2.6456
Acidobacteriota	Holophagae	Subgroup 7	Subgroup 7OR	Holophagae (Uncl)	Monoculture	1.68E-12	3.2546
Planctomycetota	Phycisphaerae	Tepidisphaerales			Monoculture	1.68E-12	3.6311
Planctomycetota	Phycisphaerae	Tepidisphaerales	TepidisphaeralesOR	Tepidisphaerales (Uncl)	Monoculture	1.68E-12	3.6311
Acidobacteriota	Thermoanaerobaculia	Thermoanaerobaculales	Thermoanaerobaculaceae	Thermoanaerobaculaceae (Uncl)	Mixture	5.29E-12	2.6918
Chloroflexi	Chloroflexia	Thermomicrobiales			Mixture	1.68E-12	3.9598
Chloroflexi	Chloroflexia	Thermomicrobiales	ThermomicrobialesOR	Thermomicrobiales (Uncl)	Mixture	1.68E-12	3.9687
Acidobacteriota	Vicinamibacteria	Vicinamibacterales			Mixture	1.68E-12	4.2172
Acidobacteriota	Vicinamibacteria	Vicinamibacterales	VicinamibacteralesOR	Vicinamibacterales (Uncl)	Mixture	1.68E-12	4.1213

*****Only the differentially abundant taxa from top 17 most abundant orders, 29 most abundant genera, and important genera affecting PSF according to multiple regression are shown. (Uncl) represents unclassified taxa.

Table 4.2. Differential analysis with LDA Effect Size (LEfSe) testing arbuscular mycorrhizal fungi taxa differentially enriched in alfalfa monoculture and mixture soils at stand ages 1 to 6 years old taken from sites near Saskatoon, SK., in August 2019.

Phylum	Class	Order	Family	Genus	System Enriched	P - value	LDA
Mucoromycota	Glomeromycetes	Diversisporales			Mixture	2.59E-07	4.1038
Mucoromycota	Glomeromycetes	Diversisporales	Diversisporaceae		Mixture	4.00E-06	3.9282
Mucoromycota	Glomeromycetes	Diversisporales	Diversisporales (Uncl)		Mixture	4.30E-05	3.7109
Mucoromycota	Glomeromycetes	Diversisporales	Diversisporaceae	Diversispora	Mixture	0.0003	3.3158
Mucoromycota	Glomeromycetes	Diversisporales	Diversisporaceae	Diversisporaceae (Uncl)	Mixture	0.0002	3.9452
Mucoromycota	Glomeromycetes	Diversisporales	Diversisporales (Uncl)	Diversisporales (Uncl)	Mixture	4.30E-05	3.7144
Mucoromycota	Glomeromycetes	Glomerales			Mixture	1.64E-12	4.8095
Mucoromycota	Glomeromycetes	Glomerales	Glomeraceae		Mixture	1.68E-12	4.6896
Mucoromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae		Mixture	2.46E-12	4.2172
Mucoromycota	Glomeromycetes	Glomerales	Glomeraceae	Dominikia	Mixture	1.31E-05	4.9634
Mucoromycota	Glomeromycetes	Glomerales	Glomeraceae	Funneliformis	Mixture	2.48E-09	4.7454
Mucoromycota	Glomeromycetes	Glomerales	Glomeraceae	Rhizophagus	Monoculture	5.28E-11	4.7141
Mucoromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomus	Monoculture	3.60E-11	4.6166
Mucoromycota	Glomeromycetes	Glomerales	Glomeraceae	Glomeraceae (Uncl)	Monoculture	1.68E-12	4.3298
Mucoromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomeraceae (Uncl)	Mixture	2.46E-12	4.1637
Mucoromycota	Glomeromycetes	Glomerales	Glomeraceae	Septoglomus	Mixture	5.36E-09	3.7937
Mucoromycota	Glomeromycetes	Glomerales	Claroideoglomeraceae	Claroideoglomus	Monoculture	7.88E-09	3.4925
Mucoromycota	Glomeromycetes	Paraglomerales			Monoculture	8.34E-07	4.8863
Mucoromycota	Glomeromycetes	Paraglomerales	Paraglomeraceae		Monoculture	8.34E-07	4.6246
Mucoromycota	Glomeromycetes	Paraglomerales	Paraglomerales (Uncl)		Monoculture	1.23E-06	4.5420
Mucoromycota	Glomeromycetes	Paraglomerales	Paraglomeraceae	Paraglomus	Monoculture	8.34E-07	4.6246
Mucoromycota	Glomeromycetes	Paraglomerales	Paraglomerales (Uncl)	Paraglomerales (Uncl)	Monoculture	1.23E-06	4.5420
Mucoromycota					Mixture	4.65E-16	NA
Mucoromycota	Glomeromycetes				Mixture	1.18E-15	-1.4125

Table 4.3. Differential analysis with LDA Effect Size (LEfSe) testing other fungi taxa differentially enriched in in alfalfa monoculture and mixture soils at stand ages 1 to 6 years old taken from sites near Saskatoon, SK., in August 2019.*

Phylum	Class	Order	Family	Genus	System Enriched	P - value	LDA
Basidiomycota	Agaricomycetes	Agaricales			Mixture	1.68E-12	3.3971
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Psathyrella	Mixture	1.16E-08	2.8457
Ascomycota	Dothideomycetes	Capnodiales			Monoculture	2.46E-12	3.0293
Ascomycota	Eurotiomycetes	Chaetothyriales			Monoculture	1.68E-12	3.6287
Ascomycota	Eurotiomycetes	Chaetothyriales	Trichomeriaceae	Knufia	Monoculture	7.76E-11	3.3946
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Exophiala	Monoculture	1.68E-12	3.0114
Ascomycota	Sordariomycetes	Coniochaetales			Monoculture	3.61E-12	3.3567
Basidiomycota	Tremellomycetes	Cystofilobasidiales			Monoculture	3.61E-12	2.9874
Ascomycota	Eurotiomycetes	Eurotiales			Monoculture	1.68E-12	4.1612
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	Monoculture	1.68E-12	4.1828
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Talaromyces	Monoculture	3.71E-08	2.9766
Basidiomycota	Tremellomycetes	Filobasidiales			Monoculture	3.61E-12	3.8968
Basidiomycota	Tremellomycetes	Filobasidiales	Piskurozymaceae	Solicoccozyma	Monoculture	3.61E-12	3.8997
Ascomycota	Sordariomycetes	Glomerellales			Monoculture	5.29E-12	3.9388
Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellacea	Gibellulopsis	Monoculture	1.16E-08	3.8933
Ascomycota	Leotiomycetes	Helotiales			Monoculture	1.68E-12	3.6597
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Pseudofabraea	Mixture	1.68E-12	3.4122
Ascomycota	Leotiomycetes	Helotiales	Dermateaceae	Laetinaevia	Mixture	2.46E-10	2.9346
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tetracladium	Mixture	8.06E-08	3.2095
Ascomycota	Sordariomycetes	Hypocreales			Mixture	1.68E-12	4.7614
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Gibberella	Mixture	1.68E-12	4.8033
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusicolla	Mixture	1.68E-12	3.8218
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Lasionectria	Monoculture	2.45E-11	4.0662
Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	Clonostachys	Mixture	1.68E-12	3.5999
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Neocosmospora	Mixture	3.61E-12	3.4577
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	Monoculture	3.61E-12	3.2156
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	Metarhizium	Mixture	2.46E-12	3.3684
Basidiomycota	Microbotryomycetes	Leucosporidiales			Monoculture	8.06E-08	3.7388
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiaceae	Mastigobasidium	Monoculture	2.59E-07	3.7402
Mortierellomycota	Mortierellomycetes	Mortierellales			Monoculture	1.68E-12	4.4837
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	Mortierella	Monoculture	1.68E-12	4.4882
Ascomycota	Lecanoromycetes	Ostropales			Mixture	1.68E-12	4.0951
Ascomycota	Lecanoromycetes	Ostropales	Graphidaceae	Platygramme	Mixture	1.68E-12	3.6259
Ascomycota	Dothideomycetes	Pleosporales			Mixture	1.68E-12	4.3342
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	Mixture	1.68E-12	3.5722
Ascomycota	Dothideomycetes	Pleosporales	Lophiostomataceae	Trichometasphaeria	Mixture	2.48E-09	3.7154
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Stemphylium	Monoculture	3.61E-10	3.8801
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Neosetophoma	Mixture	1.68E-12	3.6362
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Paraphoma	Mixture	3.60E-11	2.9693
Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Sporormiella	Mixture	8.34E-07	3.4149
Ascomycota	Sordariomycetes	Sordariales			Monoculture	1.68E-12	3.8042
Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae	Schizothecium	Mixture	1.68E-12	4.0648
Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Humicola	Mixture	3.61E-12	3.2686
Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Botryotrichum	Mixture	3.71E-08	3.3979
Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Chaetomium	Mixture	2.46E-12	2.8969
Ascomycota	Leotiomycetes	Thelebolales			Monoculture	1.68E-12	3.6571
Basidiomycota	Agaricomycetes	Trechisporales			Monoculture	5.36E-09	3.9301

*****Only the differentially abundant taxa from top 17 most abundant orders, 29 most abundant genera, and important genera affecting PSF according to multiple regression are shown.

Table 4.4. Differential analysis with LDA Effect Size (LEfSe) testing oomycetes taxa differentially enriched in alfalfa monoculture and mixture soils at stand ages 1 to 6 years old taken from sites near Saskatoon, SK., in August 2019.

Phylum	Class	Order	Family	Genus	System Enriched	P - value	LDA
Oomycota	Oomycetes	Lagenidiales			Monoculture	5.4E-09	3.8030
Oomycota	Oomycetes	Lagenidiales	Lagenidiaceae		Monoculture	1.7E-08	3.7885
Oomycota	Oomycetes	Lagenidiales	Lagenidiales-undef		Mixture	9.2E-03	2.7212
Oomycota	Oomycetes	Lagenidiales	Lagenidiaceae	Lagenidiaceae (Uncl)	Monoculture	2.6E-07	3.8927
Oomycota	Oomycetes	Lagenidiales	Lagenidiaceae	Lagenidium	Mixture	3.2E-04	3.1172
Oomycota	Oomycetes	Lagenidiales	Lagenidiales-undef	Paralagenidium	Mixture	9.2E-03	2.7212
Oomycota	Oomycetes	Myzocytiopsidales			Monoculture	7.8E-10	3.6637
Oomycota	Oomycetes	Myzocytiopsidales	Myzocytiopsidaceae		Monoculture	7.8E-10	3.6637
Oomycota	Oomycetes	Myzocytiopsidales	Myzocytiopsidaceae	Myzocytiopsidaceae (Uncl)	Monoculture	7.8E-10	3.7080
Oomycota	Oomycetes	Oomycetes (Uncl)			Monoculture	2.5E-11	3.6840
Oomycota	Oomycetes	Oomycetes (Uncl)	Lagenaceae		Mixture	7.8E-10	3.8660
Oomycota	Oomycetes	Oomycetes (Uncl)	Oomycetes-undef		Monoculture	5.5E-08	3.2953
Oomycota	Oomycetes	Oomycetes (Uncl)	Lagenaceae	Lagena	Mixture	7.8E-10	3.8660
Oomycota	Oomycetes	Oomycetes (Uncl)	Oomycetes-undef	Oomycetes (Uncl)	Monoculture	5.5E-08	3.2953
Oomycota	Oomycetes	Peronosporales			Monoculture	1.2E-08	3.7763
Oomycota	Oomycetes	Peronosporales	Peronosporaceae		Monoculture	1.2E-06	3.7374
Oomycota	Oomycetes	Peronosporales	Peronosporales-undef		Mixture	3.9E-03	3.3675
Oomycota	Oomycetes	Peronosporales	Peronosporaceae	Phytophthora	Mixture	2.1E-04	3.7464
Oomycota	Oomycetes	Peronosporales	Peronosporales-undef	Peronosporales (Uncl)	Mixture	3.9E-03	3.3675
Oomycota	Oomycetes	Peronosporales	Peronosporaceae	Peronospora	Mixture	3.2E-04	2.6070
Oomycota	Oomycetes	Pythiales			Mixture	1.7E-12	4.1110
Oomycota	Oomycetes	Pythiales	Pythiaceae		Mixture	1.7E-12	4.0975
Oomycota	Oomycetes	Pythiales	Pythiales-undef		Mixture	1.7E-03	2.9613
Oomycota	Oomycetes	Pythiales	Pythiaceae	Globisporangium	Mixture	8.1E-08	4.4193
Oomycota	Oomycetes	Pythiales	Pythiaceae	Pythium	Monoculture	1.7E-12	4.1659
Oomycota	Oomycetes	Pythiales	Pythiaceae	Pythiaceae (Uncl)	Monoculture	2.5E-08	3.9785
Oomycota	Oomycetes	Pythiales	Pythiales-undef	Pythiales (Uncl)	Mixture	1.7E-03	2.9613
Oomycota	Oomycetes	Saprolegniales			Monoculture	1.7E-12	4.0740
Oomycota	Oomycetes	Saprolegniales	Saprolegniaceae		Monoculture	1.7E-12	4.0931
Oomycota	Oomycetes	Saprolegniales	Saprolegniaceae	Brevilegnia	Monoculture	1.1E-10	4.0659
Oomycota	Oomycetes	Saprolegniales	Saprolegniaceae	Aphanomyces	Monoculture	1.2E-07	3.3790
Oomycota	Oomycetes	Saprolegniales	Saprolegniaceae	Protoachlya	Mixture	8.8E-06	3.1494
Oomycota					Mixture	4.6E-16	NA
Oomycota	Oomycetes				Mixture	4.6E-16	NA

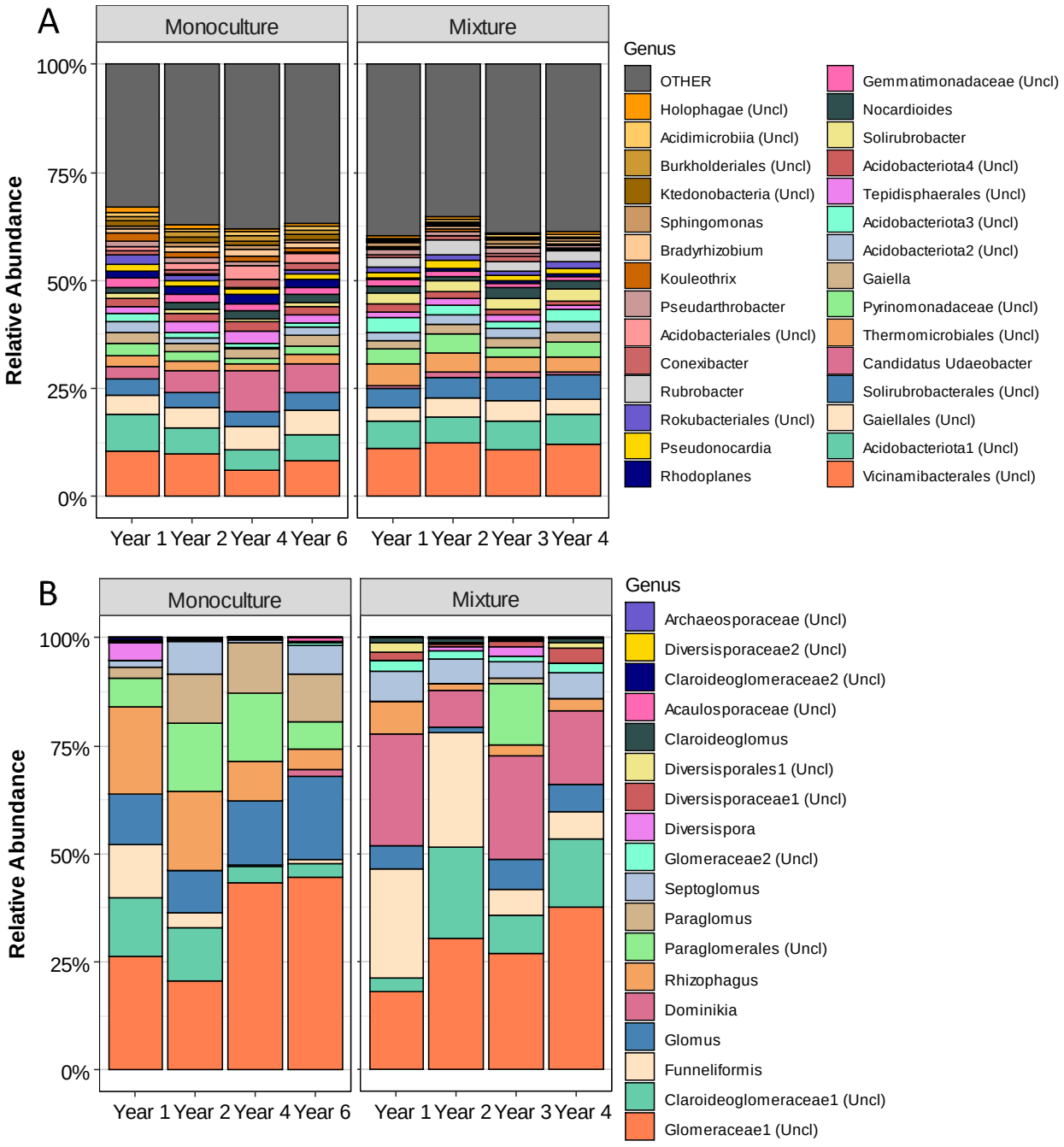

Figure 4.3 a-d. Relative taxonomic composition of soil bacteria **(a)**, arbuscular mycorrhizal fungi **(b)** and other fungi **(c)**, and **(d)** oomycetes based on the genus-level relative abundance of ASVs identified in alfalfa monoculture and mixture soils at stand ages 1 to 6 years old taken from sites near Saskatoon, SK., in August 2019. Extremely low-abundance taxa are summarized as "OTHER". (Uncl) = Unclassified.

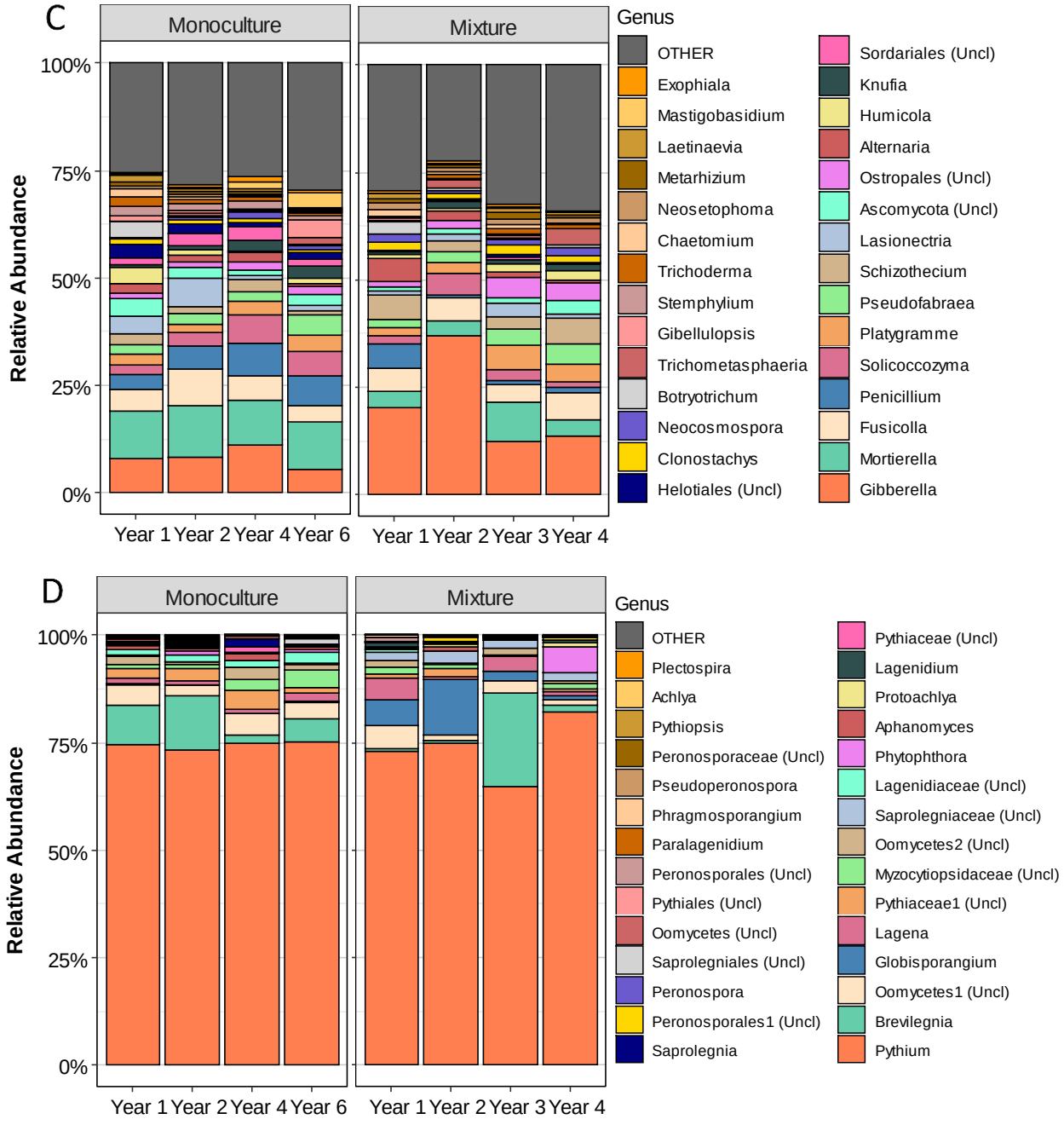

Figure 4.3 a-d. Continued

4.5.2. Relationships between functional soil microbial communities and intra- and inter-specific plant-soil feedbacks

Many soil microbial taxa, most of which were differentially enriched in mixture or monoculture soils, were associated with the PSF of the varieties (Fig. 4.4a; Table 4.5). Many genera belonging to different bacterial and fungal orders correlated with the PSF of the varieties 2010 and Foothold with 2010 responding positively to only *Haliangium* sp. Other taxa including potentially beneficial microbes were exclusively associated with PSF of Foothold either positively or negatively (Fig. 4.4a; Table 4.5). For example, potential mutualists (*Devosia* sp. and *Funneliformis* sp.) and plant-growth promoting microbes (*Micromonospora* sp.) that were differentially enriched in mixture correlated positively with the PSF of Foothold, while *Metapochonia* sp. and *Haliangium* sp. that were differentially enriched in monoculture correlated negatively. However, PSFs of varieties Spyder and 3010 were not predicted by any soil microbes, and no potentially pathogenic microbe correlated with the PSFs of the varieties (Fig. 4.4a; Table 4.5). Analysis of selected models showed that while *Haliangium* sp. significantly ($P < 0.008$) predicted PSF of 2010, only 3 taxa including *Metapochonia* sp. significantly ($P < 0.039$) predicted the PSF of Foothold (Table 4.6).

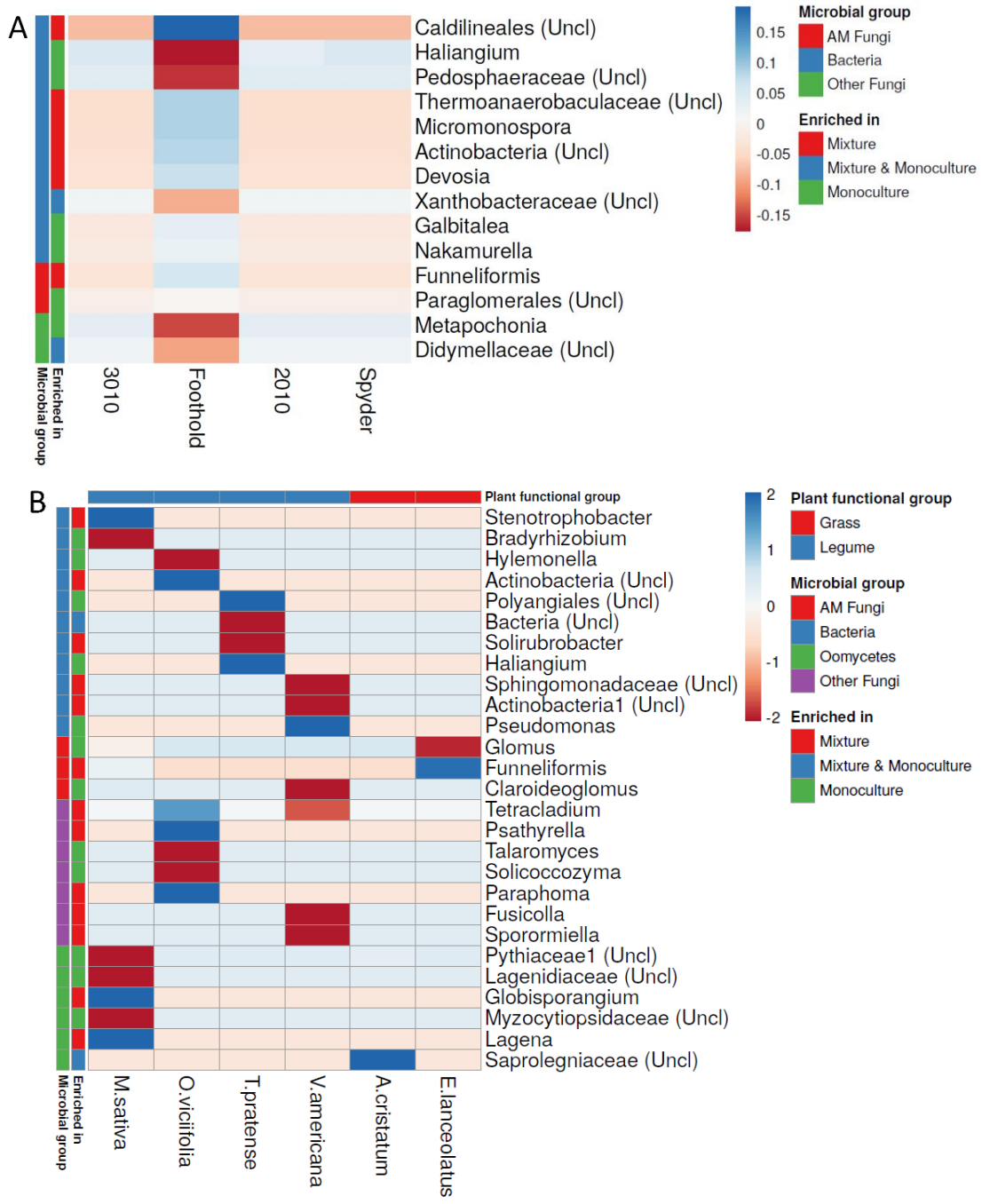

Figure 4.4. Correlation between plant-soil feedbacks of alfalfa varieties **(a)**, forage species **(b)** and soil microbial ASVs at genera level calculated from least absolute shrinkage and selection operator (lasso) multiple regression models with plant-soil feedbacks values calculated as the ln ratio of plant biomass in soils previously conditioned by alfalfa-associated soil inoculum vs sterile soils. 'Enriched in' denotes LEfSe differential analysis of taxa enriched in mixture, monoculture, or not differentially enriched (mixture & monoculture). (Uncl) = Unclassified.

Table 4.5. Multiple regression analysis testing the correlation between the abundance of genera-level soil microbial ASVs and plant-soil feedbacks for alfalfa varieties.

Group	Variety	Taxa	Coefficient	Taxa Importance	R-squared
Bacteria	2010	Haliangium	-0.0017	100	0.0579
	Foothold	Caldilineales (Uncl)	0.0328	100	0.3123
	Foothold	Haliangium	-0.0235	72	
	Foothold	Pedosphaeraceae (Uncl)	-0.0185	56	
	Foothold	Thermoanaerobaculaceae (Uncl)	0.0074	23	
	Foothold	Micromonospora	0.0073	22	
	Foothold	Actinobacteria (Uncl)	0.0071	22	
	Foothold	Devosia	0.0056	17	
	Foothold	Xanthobacteraceae (Uncl)	-0.0051	15	
	Foothold	Galbitalea	0.0017	5	
	Foothold	Nakamurella	0.0012	4	
Fungi	Foothold	Metapochonia	-0.0163	100	0.6400
	Foothold	Didymellaceae (Uncl)	-0.0060	37	
AMF	Foothold	Funneliformis	0.0041	100	0.7403
	Foothold	Paraglomerales (Uncl)	-0.0001	2	

Table 4.6. Results of mixed-effects models testing the effects of the relative abundance of important taxa on plant-soil feedbacks of varieties.

Variety	Taxa	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
2010	Haliangium	5.91E-03	5.91E-03	1	42	7.5726	**0.0087**
Foothold	Caldilineales (Uncl)	4.83E-07	4.83E-07	1	47	0.5364	0.4676
	Haliangium	7.09E-07	7.09E-07	1	47	0.7879	0.3793
	Pedosphaeraceae (Uncl)	3.68E-06	3.68E-06	1	47	4.0902	**0.0488**
	Thermoanaerobaculaceae (Uncl)	1.00E-07	1.00E-07	1	47	0.1115	0.7399
	Micromonospora	1.51E-06	1.51E-06	1	47	1.6785	0.2015
	Actinobacteria1 (Uncl)	1.29E-06	1.29E-06	1	47	1.4362	0.2368
	Devosia	5.39E-07	5.39E-07	1	47	0.5985	0.4430
	Xanthobacteraceae (Uncl)	1.07E-08	1.07E-08	1	47	0.0119	0.9135
	Galbitalea	1.48E-06	1.48E-06	1	47	1.6437	0.2061
	Nakamurella	3.87E-06	3.87E-06	1	47	4.2993	**0.0436**
	Metapochonia	4.04E-06	4.04E-06	1	47	4.4914	**0.0394**
	Didymellaceae (Uncl)	1.71E-06	1.71E-06	1	47	1.8989	0.1747
	Funneliformis	6.57E-08	6.57E-08	1	47	0.073	0.7882
	Paraglomerales (Uncl)	1.17E-07	1.17E-07	1	47	0.1304	0.7196

Many soil microbial taxa, most of which were differentially enriched in mixture or monoculture soils, were associated with the PSF of the species (Fig. 4.4b; Table 4.7). Microbial taxa belonging to different bacterial, fungal, oomycetes orders correlated with PSF among the species (Fig. 4.4b; Table 4.7). Many of these microbial taxa are either potential mutualists, saprotrophs, plant-growth promoting microbes, or pathogens and are differentially enriched in mixture and monoculture soils (Fig. 4.4b).

Each forage species responded to different microbial taxa, except for where *O. viciifolia*, a tame legume, and *V. americana*, a native legume, correlated with *Tetracladium* sp. in opposite directions (*O. viciifolia* positive and *V. americana* negative; Fig. 4.4b). While some of the potentially beneficial microbes (e.g. *Pseudomonas* sp. and *Funneliformis* sp), which were enriched in monoculture and mixture soils, respectively correlated positively with PSF, others (e.g. *Glomus* sp. and *Bradyrhizobium* sp.) enriched in monoculture soils correlated negatively, depending on species identity. Some potentially pathogenic microbes (e.g. unclassified species of Pythiceae and Lagenidiaceae) enriched in monoculture correlated negatively with PSF of *M. sativa*, while others including *Lagena* sp. and *Globisporangium* sp. enriched in mixture soils correlated positively. Negative associations with microbial taxa, however, were more prevalent in native species than tame species (Fig. 4.4b; Table 4.7).

Each of the forage species responding to individual soil microbial genus showed a unique pattern in their associations (Fig. 4.4b; Table 4.7). For example, *E. lanceolatus*, a native grass, responded positively to one group of AMF and negatively to another. *A. cristatum*, a tame grass, only responded (positively) to one genera containing potential pathogens. *V. americana*, a native legume, responded negatively to most microbes, including *Sporormiella* sp. and several putative beneficials, with the only positive association being with *Pseudomonas* sp. *T. pratense*, a tame legume, responded positively to soil microbes (e.g. *Haliangium* sp.) that were enriched in monoculture soils and negatively to those enriched in mixture soils. In contrast, response of *O. viciifolia*, a tame legume, was negative to microbial groups that were enriched in monoculture and positive to those enriched in mixture soils, matching the change in direction of PSF for this species (Fig. 4.4b). Re-analysis with mixed effects models indicated that most of these relationships were non-significant (Table 4.8).

Table 4.7. Multiple regression analysis testing the correlation between the abundance of genera-level soil microbial ASVs and plant-soil feedbacks for forage species.

Group	Species	Taxa	Coefficient	Taxa Importance	R-squared
Bacteria	*M. sativa*	Stenotrophobacter	0.0046	100	0.4753
	M. sativa	Bradyrhizobium	-0.0046	99	
	O. viciifolia	Hylemonella	-0.0194	100	0.9683
	O. viciifolia	Actinobacteria1 (Uncl)	0.0002	1	
	T. pratense	Polyangiales (Uncl)	0.0186	100	0.9292
	T. pratense	Bacteria (Uncl)	-0.0153	82	
	T. pratense	Solirubrobacter	-0.0034	18	
	T. pratense	Haliangium	0.0005	3	
	V. americana	Sphingomonadaceae (Uncl)	-0.0286	100	0.2736
	V. americana	Actinobacteria2 (Uncl)	-0.0227	79	
	V. americana	Pseudomonas	0.0013	5	
Fungi	*O. viciifolia*	Tetracladium	0.0152	100	0.3282
	O. viciifolia	Psathyrella	0.0055	36	
	O. viciifolia	Talaromyces	-0.0042	28	
	O. viciifolia	Solicoccozyma	-0.0013	9	
	O. viciifolia	Paraphoma	0.0012	8	
	V. americana	Fusicolla	-0.0839	100	0.0002
	V. americana	Tetracladium	-0.0180	21	
	V. americana	Sporormiella	-0.0019	2	
AMF	*M. sativa*	Glomus	-0.0070	100	0.0201
	M. sativa	Funneliformis	0.0027	39	
	M. sativa	Claroideoglomus	0.0001	2	
	V. americana	Claroideoglomus	-0.0514	100	0.0390
	E. lancelatus	Glomus	-0.0250	100	0.0064
	E. lancelatus	Funneliformis	0.0087	35	
Oomycetes	*M. sativa*	Pythiaceae1 (Uncl)	-0.0173	100	0.3229
	M. sativa	Lagenidiaceae (Uncl)	-0.0111	64	
	M. sativa	Globisporangium	0.0097	56	
	M. sativa	Myzocytiopsidaceae (Uncl)	-0.0054	31	
	M. sativa	Lagena	0.0029	17	
	A. cristatum	Saprolegniaceae (Uncl)	0.0045	100	0.0782
	A. cristatum	Pythiaceae1 (Uncl)	-0.0007	16	

Table 4.8. Results of mixed-effects models testing the effects of the relative abundance of important taxa on plant-soil feedbacks of species.

Species	Taxa	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
M. sativa	Stenotrophobacter	2.80E-08	2.80E-08	1	52	3.8177	**0.0561**
	Bradyrhizobium	2.73E-08	2.73E-08	1	52	3.7255	**0.0591**
	Pythiaceae1 (Uncl)	6.26E-09	6.26E-09	1	52	0.8524	0.3601
	Lagenidiaceae (Uncl)	2.29E-09	2.29E-09	1	52	0.3115	0.5792
	Globisporangium	2.67E-11	2.67E-11	1	52	0.0036	0.9522
	Myzocytiopsidaceae (Uncl)	3.63E-09	3.63E-09	1	52	0.4943	0.4852
	Lagena	1.33E-08	1.33E-08	1	52	1.8119	0.1841
	Glomus	3.81E-09	3.81E-09	1	52	0.5191	0.4744
	Funneliformis	8.90E-11	8.90E-11	1	52	0.0121	0.9127
	Claroideoglomus	6.50E-09	6.50E-09	1	52	0.8854	0.3511
O. viciifolia	Hylemonella	2.78E-04	2.78E-04	1	47	1.7133	0.1969
	Actinobacteria1 (Uncl)	2.86E-04	2.86E-04	1	46	1.7623	0.1909
	Tetracladium	1.90E-04	1.90E-04	1	51	1.1698	0.2845
	Psathyrella	2.61E-04	2.61E-04	1	40	1.6076	0.2122
	Talaromyces	9.39E-04	9.39E-04	1	1	5.7903	0.3248
	Solicoccozyma	1.28E-03	1.28E-03	1	13	7.8921	**0.0151**
	Paraphoma	6.36E-05	6.36E-05	1	33	0.3922	0.5355
T. pratense	Polyangiales (Uncl)	2.42E-02	2.42E-02	1	39	8.0978	**0.0070**
	Unclassified	5.03E-03	5.03E-03	1	43	1.6853	0.2011
	Solirubrobacter	5.77E-03	5.77E-03	1	48	1.9307	0.1711
	Haliangium	1.22E-03	1.22E-03	1	53	0.4091	0.5252
V. americana	Sphingomonadaceae (Uncl)	2.39E-02	2.39E-02	1	58	0.764	0.3857
	Actinobacteria1 (Uncl)	4.61E-03	4.61E-03	1	58	0.1476	0.7022
	Pseudomonas	3.03E-02	3.03E-02	1	58	0.9689	0.3291
	Fusicolla	1.16E+00	1.16E+00	1	58	37.1291	**<0.0001**
	Tetracladium	1.07E-02	1.07E-02	1	58	0.3419	0.5610
	Sporormiella	1.29E-02	1.29E-02	1	58	0.412	0.5235
	Claroideoglomus	2.67E-01	2.67E-01	1	58	8.5543	**0.0049**
A. cristatum	Saprolegniaceae (Uncl)	6.96E-02	6.96E-02	1	62	1.8834	0.1749
	Pythiaceae1 (Uncl)	4.19E-02	4.19E-02	1	62	1.1335	**0.2912**
E. lanceolatus	Glomus	1.07E-01	1.07E-01	1	60	2.3661	0.1293
	Funneliformis	7.37E-02	7.37E-02	1	60	1.6254	0.2072

4.6. Discussion

Plant communities are known to modulate soil microbiota (Bartelt-Ryser et al., 2005; Beugnon et al., 2021), and the changes induced can in turn affect plant growth (Hannula et al., 2020). The present study highlights the importance of plant diversity and plant identity in microbe-mediated plant-soil feedback. This study demonstrated that plant diversity alters the composition of soil microbial communities, and these changes in the soil microbiome influence the growth of

subsequent plants. These effects, however, were dependent on both plant variety and species identity, indicating that selection of crop variety and species can improve productivity by buffering the effects of detrimental soil microbes and intensifying the role of beneficial microbes.

Several studies have shown that microbiome composition and diversity shift with plant diversity (Beugnon et al., 2021; Hannula et al., 2020), and stand age (Orr et al., 2015; Wagner et al., 2016). Here, this experiment showed that plant diversity and stand age are associated with changes in the composition of soil microbial communities. The proportion of variation in communities explained by plant diversity alone is around 18% for oomycetes, 17% for AM fungi, and 7% for other fungi, while stand age explained lesser percentages across the microbial groups (< 6%). This indicates that plant diversity plays an important role in shaping these microbial communities of alfalfa production systems and that the communities shift over time. The diversity of AMF increased in mixture relative to monoculture soils, while oomycetes diversity decreased in mixture soils as stands aged. This suggests that greater plant diversity favors the proliferation of diverse AMF and reduces potential pathogens over time, thus reducing the strength of negative PSF. This is consistent with previous observations on plant diversity influencing PSF via changes in belowground soil microbial diversity (Hannula et al., 2020; Mao et al., 2021; Schmid et al., 2020). In contrast to my hypothesis, stand age and plant diversity during soil conditioning did not affect the diversity and community composition of soil bacteria. Shift in bacterial communities have been associated with the response of the dominant taxa to environmental cues (Hannula et al., 2019), suggesting that soil physicochemical factors such as pH, water availability, nutrients, and aggregate stability might be important in shaping the composition of this group in alfalfa stands (Mariotte et al., 2018; Gornish et al., 2020; Jangid et al., 2008).

Many of the microbial groups affected by plant diversity also influenced PSF. The orders Rhizobiales, Burkholderiales and Frankiales were enriched in alfalfa monoculture soils. Previous studies showed that many members belonging to these groups have positive effects on plant growth (Barea et al., 2005; Batista & Singh, 2021; Wang et al., 2021). This is consistent with *Nakamurella* sp., (from Burkholderiales), and *Devosia* sp. (from Rhizobiales) relating positively with PSF of Foothold. Mixture soils, on the other hand, had greater abundance of other orders including Bacillales, Glomerales, Hypocreales and Pythiales, many members of which influence plant growth through disease suppression, phytohormones production, nutrient uptake, and pathogenicity (Batista & Singh, 2021; Domínguez-Begines et al., 2021). However, these

functional traits are not conserved at the order taxonomic level (Martiny et al., 2015). For instance, the order Burkholderiales, which contains many beneficials, was negatively related to plant growth, due to the pathogenicity of some members (Hannula, Ma, et al., 2020b). Similar to *Hylemonella* sp., a denitrifying bacteria genus within Burkholderiales (Li et al., 2019), relating negatively to PSF of *O. viciifolia* (Fig. 4.5b). Nonetheless, this study provides further evidence that plant diversity influences the composition of soil microbial communities and, consequently, plant growth.

At finer taxonomic resolution many microbial genera were also strongly influenced by plant diversity. Alfalfa monoculture was enriched with mutualists (e.g. *Rhizophagus* sp., *Glomus* sp., *Bradyrhizobium* sp.), plant-growth promoting microbes (e.g. *Haliangium* sp., *Mortierella* sp.), and pathogens (e.g. *Pythium* sp. and *Aphanomyces* sp.) relative to mixtures. Conversely, mixtures were enriched with other mutualists (e.g. *Dominikia* sp., *Funneliformis* sp.), plant-growth promoting microbes (e.g. *Micromonospora* sp., *Fusicolla* sp., *Microvirga* sp.), and pathogens (e.g. *Gibberella* sp., *Alternaria* sp., and *Phytophthora* sp.). Among the pathogenic genera specific to alfalfa (Annicchiarico et al., 2015; Munkvold et al., 2001), only *Aphanomyces* sp. was enriched in monoculture, while *Phytophthora* sp. and *Collectrotrichum* sp. were enriched in mixture soils. It is evident that some beneficial and pathogenic genera were more abundant in different cropping systems, whereas other beneficial and pathogenic genera were rarer or more cosmopolitan, leading to functional similarity of these cropping systems. The causes of this similarity in the distribution of potentially beneficial and pathogenic microbes in mixture and monoculture is not clear, however. This result suggests that despite the effect of plant diversity on the relative abundance of soil microbial groups, subsequent plants are predisposed to some beneficial and pathogenic soil microbes than others in monoculture and mixture stands.

Additionally, some saprotrophic microbes including *Psathyrella* sp. known to decompose litter (Capelari & Zadrazil, 1997), and *Stenotrophobacter* sp. involved in the degradation of organic matter (Yan et al., 2018) were enriched in mixture relative to monoculture and related positively with PSF of *M. sativa* and *O. viciifolia*, respectively. Saprotrophs can induce positive PSF by decomposing litter, thereby increasing the availability of nutrients to plants (Bennett & Klironomos, 2019). This result implies that increased plant diversity favors the abundance of certain saprotrophs that contribute to positive PSF in alfalfa-associated mixture stands.

Among the varieties, the relationship between different soil microbial taxa and intraspecific PSF was variety dependent. While relationships between soil microbes and PSF were not detected for some varieties, other varieties, especially Foothold, related to a number of soil microbes in different directions. This is in line with previous observations on cultivar-specific differences in plant-microbe interactions (An et al., 2011; Ulbrich et al., 2021). Many potentially beneficial soil microbes, including plant-growth promoting microbes and AMF known to promote growth in crop plants through biological-nitrogen fixation, phosphorus uptake, and disease protection (Edwards et al., 2019; Säle et al., 2021; Wang et al., 2021) were enriched in mixture soils and related positively to PSF of Foothold. Conversely, many soil microbial taxa that related negatively to the PSF were enriched in monoculture soils. The enrichment of cultivar-specific beneficial microbes in mixture soils, and detrimental microbes in monoculture soils contribute to the more negative intraspecific PSF observed in monoculture stands relative to mixture (Edwards et al., 2019; Mao et al., 2021). These interactions were not detected here, however, potentially because microbial consortiums promote plant growth thereby neutralizing the effects of some individual taxa, consistent with previous observation (Hannula et al., 2020). Another possible explanation is that the genus level is still too broad (Martiny et al., 2015) or that the important microbes were not sequenced if, for example, nematodes drive PSF (Dias et al., 2018). It is not entirely clear why the PSF of variety Foothold had stronger relationships with soil microbial taxa, however, this variety could have a trait (other than the ones studied in Chapter 3) that enhances its interaction with soil biota, leading to the strong PSF effects. Nevertheless, this result indicates that some varieties are suitable for alfalfa stand rejuvenation than others due to differences in growth response to soil microbial communities.

The relationship between different soil microbial genera and interspecific PSF depended on species identity. Although PSF of legumes correlated with more microbial genera than the grasses, these relationships were mostly negative and specific to individual species. This is consistent with previous observations that plant interactions with soil microbial groups vary among plant species and functional groups (Bezemer et al., 2006; Samaddar et al., 2021). Other soil microorganisms like nematodes can also promote negative PSF (Dias et al., 2018), especially in grasses (Atul-Nayyar et al., 2008), but these groups were not assessed in this study. This result indicates that the impact of the microbial legacies of alfalfa production on stand productivity depends on the species

being seeded and that the growth responses to these microbes are more positive for legumes and negative for grasses.

Previous studies have shown that native species are more likely to encounter their natural enemies in their native environment than non-natives species (Agrawal et al., 2005; Kendig et al., 2020). This hypothesis likely holds true for the native species in this study as the only negative relationship between grasses and soil microbes was found in the native species (*E. lanceolatus*). Similarly, the relationships with the soil microbes were more negative for the native legume (*V. americana*) than the non-native legumes. Furthermore, *Tetracladium* sp., which contains pathogenic species (Wang et al., 2020), positively related to PSF of a tame legume (*O. viciifolia*), native legume related negatively. It is not clear how this genus may drive positive PSF for *O. viciifolia*; however, these plant species may be responding to different members of this genus. The difference between the native and non-native legumes has been attributed to pathogen specialization on native plants due to greater amount of evolutionary history with resident soil community (Wippel et al., 2021). Interactions with soil microbes were more negative than positive for native species, although positive interactions still existed. For instance, the relationship between *E. lanceolatus* and AMF was negative with *Glomus* sp. and positive for *Funneliformis* sp. Similarly, *V. americana* had negative relationships with most microbes, including beneficial microbes, but still had a positive relationship with *Pseudomonas* sp. The positive relationships suggest that these two genera are more compatible with the specific native crops than other microbial taxa.

Many potentially beneficial microbial genera were positively related to PSF of a specific variety or species, while others related negatively. Previous studies have shown that mutualists such as AMF (Mao et al., 2021) and other beneficial soil microbes promote positive PSF (Bennett & Klironomos, 2019; Crawford et al., 2019). In contrast to my prediction, some nontarget pathogens including *Lagena* sp. and *Globiosporangium* sp. related positively to PSF of *M. sativa* and *A. cristatum*, which is in line with previous observations that nontarget pathogens can positively influence plant growth, possibly by outcompeting host specific pathogens in the soil (Cortois et al., 2016; Hannula et al., 2020). Negative PSF, on the other hand, has frequently been shown to be promoted by pathogenic soil microbes (Huang et al., 2013; Jiang et al., 2020; Klironomos, 2002) including oomycetes (Domínguez-Begines et al., 2021). This is consistent with plant pathogens (e.g. oomycete taxa) relating negatively to the PSF of *M. sativa* in the current study. Surprisingly,

some potential mutualists including *Glomus* sp. and *Bradyrhizobium* sp. related negatively to the PSF of specific species. This is in line with previous observations that some mutualists are more competitive but less beneficial to specific hosts than others, leading to negative PSF (Mao et al., 2021; Strom et al., 2020).

Growth depression by AMF or other beneficials can be attributed to a number of factors including greater demand for host photosynthates, and host compatibility and physiological stage (Jacott et al., 2017). Moreover, the presence of pathogens, nematodes and other parasites in the environment can also reduce AMF benefits by decreasing their efficiency to supply nutrients (Atul-Nayyar et al., 2008; Dias et al., 2018). Furthermore, *Bradyrhizobium* sp. are poor symbionts of *M. sativa* (Alías-Villegas et al., 2015). These types of relationships may result in a wasteful energy cost when plant does not get return on carbon investment, and thus negative PSF (Revillini et al., 2016). Interestingly, many of the soil microbes that related positively to PSF were more enriched in mixture than monoculture, while many of those that correlated negatively had greater abundance in monoculture than mixture. Conversely, many of the soil microbes that related positively to PSF of *T. pratense* and *V. americana* were more enriched in monoculture than mixture, while many of those that correlated negatively with them had greater abundance in mixture than monoculture. There is evidence that plant diversity influences the relative abundance of deleterious and beneficial soil microbes (Shi et al., 2021; Vukicevich et al., 2016). Collectively, this study suggests that plant diversity changes the relative abundance of beneficial and pathogenic soil microbes and that some crop species will benefit from positive PSF in mixture soils while others will have positive PSF in monoculture soils depending on their specific interactions with these soil microbes.

4.7. Conclusion

Soil microbial communities are important agricultural resources for sustainable crop production. Microbial communities conditioned by diverse crops support sustainable crop production by increasing diversity of beneficial microbes and suppressing soil-borne pathogens. Monoculture systems can lead to greater productivity decline through the accumulation deleterious soil microbes that promote negative feedbacks. However, whether conditioned in mixture or monoculture soils, the effects of soil microbial communities on plant growth depend on the variety, crop species identity, and the shared evolutionary history between the plant and the resident microbiota. Selection of varieties or crop species based on their interactions with soil microbial

taxa present in a system can therefore enhance positive PSF and mitigate negative PSF. The differences in microbial community composition between mixture and monoculture indicate that *in situ* microbiome manipulation can be performed through cropping system approaches. Future research should focus on identifying environmental factors shaping the co-occurrence network of core microbiomes driving PSF of specific crops, and how cultivars can be enhanced to recruit functionally beneficial soil microbes for growth promotion and protection against pathogens. Understanding microbial composition and the nature of their interactions with different crop varieties and species under different cropping systems is critical in the mitigation of productivity decline in agroecosystems.

5. GENERAL DISCUSSION AND FUTURE RESEARCH

Livestock production continues to contend with a number of critical issues, including the need to increase forage productivity while minimizing the negative impacts on soil health and environmental sustainability (Annicchiarico et al., 2015; Li et al., 2019; Kilcher & Heinrichs, 1996). There is need to conduct research not only to understand the development of negative microbial legacies, but also to identity mechanisms by which crop species and varieties can resist deleterious soil organisms and benefit from beneficial soil microbes (Edwards et al., 2019; Mao et al., 2021). These objectives are complicated by the complex interactions among forage crops, soil microbial communities and management practices. Applying the principles of plant ecology to crop production, however, offers unique opportunities to meet these challenges by improving crop productivity with relatively few pitfalls (Mariotte et al., 2018). The resources provided in this study is critical to sustainable production of forage crops to meet the increasing food demands of the 21st century.

Alfalfa (*M. sativa*) production is a perennial system that supports the continuous supply of animal-based food and increasing nitrogen availability for subsequent crops, but it faces its own set of limitations including microbe-mediated productivity decline (Annicchiarico et al., 2015). The experiments described herein were conducted to better understand effects of field conditions of alfalfa production in Saskatchewan on soil microbial legacies. We further assessed the forage potentials of four alfalfa varieties (2010, Foothold, 3010, and Spyder) and five forage species including native and tame grasses and legumes (*O. viciifolia, V. americana, T. pratense, E. lanceolatus,* and *A. cristatum*) in response to the soil microbial legacy effects using plant-soil feedback framework.

Plants differ in how they adjust their root expression when encountering certain soil microbial groups (Gorim & Vandenberg, 2017; Hendriks et al., 2015). Many studies have linked root trait expression to variation in PSF, but they neither considered plant diversity effects on microbial legacies nor forage potentials of their plants (Cortois et al., 2016; Hendriks et al., 2015; Spitzer et al., 2021). Consequently, it is not clear how root trait-induced variation in PSF can contribute to the mitigation of productivity decline in perennial systems (Annicchiarico et al., 2015; Mariotte et al., 2018). In this study, negative soil biota effects were generally larger for the variety Foothold, while 3010 benefited more from soil biota of alfalfa production. The growth suppression of alfalfa

varieties in monoculture soils, and the growth promotion of variety 3010 in mixture soils, indicates that these alfalfa varieties may not perform well when seeded into former alfalfa monocultures but that the growth of some varieties will increase in mixed grass-alfalfa stands. Studies have shown that accumulation of species-specific enemies in the soil suppresses plant growth (Edwards et al., 2019). Inter-variety differences in disease resistance, symbiosis, and root trait expression, can influence PSF (Mao et al., 2021), and thus suitability to restore stand productivity. When testing the mechanism of growth response variation among the varieties to soil microbial legacies, we found that the variety that performed best had the lowest root tissue density. This implies that this trait is important in variety selection to mitigation productivity decline (Bergmann et al., 2020; Kramer-Walter et al., 2016).

Root traits significantly varied among the forage species, and thus influenced microbial legacy effects on their growth. This variation is critical to determining whether a forage species will grow better when seeded into alfalfa mixture or monoculture stands. Given the interspecific variation in PSF, ensuring that alfalfa and *O. viciifolia* are seeded into alfalfa-associated mixture stands, and *T. pratense* into alfalfa monoculture stands or stands with high alfalfa density is important. This will not only mitigate stand productivity decline, but will also enhance positive plant-microbes interactions to improve agronomic potential (Mariotte et al., 2018). When testing for the mechanism by which forage species may perform better to mitigate productivity decline in alfalfa stands, we found that greater root nodulation and AMF colonization rates, average root diameter, root-biomass ratio, lower root tissue density and specific root length were significant. Consequently, future research in forage breeding should focus on the genetic basis of these traits, and evaluate their expression in response to soil biota under field conditions. This will facilitate trait-based selection of crops that can resist biotic stress in perennial systems including alfalfa production.

Field conditions have been shown to influence how plants condition soil microbial communities (Ma et al., 2016; Putten et al., 2016). However, there is no clear evidence on how interactions of previous plant community and abiotic soil components with soil biota may help in the selection of suitable varieties and forage species in perennial systems (Hannula et al., 2020; Samaddar et al., 2021). In this study, we showed that stand age, soil texture, soil nutrients, weed abundance, plant diversity and species richness, and traits of focal crops are important factors predicting how soil biota would affect the growth of subsequent crops. For instance, higher fiber content (ADF and

NDF) of standing alfalfa contributed to positive soil biota effects on the growth of variety 3010 and all the forage species in monoculture. Higher nitrogen content (i.e. crude protein) in standing alfalfa also induced positive soil biota effects on the forage species in mixture. In contrast, fiber content contributed to negative soil biota effects on the forage species in mixture. These effects of plant traits on the composition of soil biota could be induced through litter decomposability or tradeoffs in allocation of resources to belowground (Ke et al., 2015; Revillini et al., 2016; Yan et al., 2018). Consequently, negative microbial legacies can be reduced when forage species or varieties like 3010 are seeded into pure alfalfa stands with higher fiber content, and when forages are seeded into alfalfa-grass mixture with higher alfalfa nitrogen, rather than fiber content. Therefore, sampling of previous stands for forage quality assessment can help in the selection process of subsequent varieties or forage species for stand rejuvenation.

The ability of plants to condition and respond differently to soil microbial communities through PSFs can enhance *in situ* manipulation of soil microbiome to mitigate the negative impacts of soil microbes and stimulate their beneficial effects. We showed that variation in root traits, and thus growth responses among alfalfa varieties and forage species, result from their specific interactions with different beneficial and deleterious soil microbes. The relative abundance of these microbial groups was affected by seeded plant diversity and, to a smaller extent, stand age, supporting conclusions from previous studies (Bartelt-Ryser et al., 2005; Beugnon et al., 2021; Hannula et al., 2020). Some AMF, plant-growth promoting rhizobacteria, saprotrophs and pathogenic soil microbes were significantly enriched in monoculture, while many others were enriched in mixture soils. Many of the microbial groups that were more abundant in monoculture related negatively with the plants, compared to those that were more enriched in mixture soils. However, we provide new evidence that regardless of whether the microbial group in a system is potentially beneficial or pathogenic, their effects on plant growth would depend on the identity of the crop variety and species that are being seeded into the system. This result supports previous observations on host preferences in plant-microbe interactions (Gornish et al., 2020; Hannula et al., 2020; Wippel et al., 2021).

In addition to plant identity and changes in root trait expressions, we also showed that plant origin (i.e. native or tame) can influence growth responses of feedback plants. Studies have shown that tame species perform better than native species in native soils due to the presence natural enemies (Hannula et al., 2020; Parker & Gilbert, 2007). Contrary to this assumption, growth response was

not consistently different between the native and tame forages, however, we found conditions that can enhance the success of native species, even in their native soils. Growth of native species, but not tame species, reduced with increasing weed abundance during soil conditioning in alfalfa monoculture soils. This result supports previous research findings on the role of weeds in the accumulation of deleterious soil microbes (Flory & Clay, 2013).

Consequently, we observed that interactions of the native species with different soil microbial groups including AMF were mostly negative, compared to tame species. For instance, *V. americana*, a native legume, had negative interactions with many soil microbes including plant-growth promoting bacteria and AMF in mixture, while it related positively with *Pseudomonas* sp. in monoculture. Native grass species *E. lanceolatus*, on the other hand, related positively with one AMF taxa in mixture and negatively with another in monoculture. This result is consistent with previous observations that plant growth benefits of mutualists depend on plant identity and microbial species (Burleigh et al., 2002; Revillini et al., 2016; Säle et al., 2021). These results imply that, seeding *V. americana* into alfalfa monoculture stands with adequate weed control, and *E. lanceolatus* into alfalfa mixed stands, will reduce the negative biota effects of native soils.

Soil characteristics also influenced some of the effects of conditioned soil microbial communities on subsequent crops. For instance, higher soil phosphorus during soil conditioning contributed to the reduction in negative soil biota effects on variety 3010 in monoculture, while the opposite was true for other varieties. Elevated soil nutrients are known to reduce plant growth benefits of mutualists and promote suitable environment for detrimental soil microbes to thrive (Revillini et al., 2016), consistent with the growth suppression experience by all the varieties except 3010. Selection of varieties with qualities similar to 3010 may help rejuvenation of alfalfa and other leguminous stands, especially where soil phosphorus content is high.

Additionally, greater sand and clay contents led to the development of more detrimental soil biota in monoculture, while greater silt content promoted the development of soil biota that are favorable to the varieties in mixture. This result supports conclusions from other studies that soil texture affects the assembly of soil microbial communities (Ma et al., 2016; Obayomi et al., 2021). From a practical perspective, our result provides novel evidence that alfalfa stand productivity decline may be reduced by avoiding establishment of alfalfa monocultures on clayey soils but rather on

silt loam. Further research is needed to identify soil factors promoting specific microbial groups that are beneficial to different varieties and forage species in perennial agroecosystems.

Combined, our results suggest that the challenges facing alfalfa production can be addressed through management practices that promotes the abundance of beneficial microbes, and trait-based selection of varieties and species being seeded into existing stands. Plant-soil feedback studies have long focused on wild plants in their natural habitats, increasing our understanding of how plants coexist and self-regulate ecosystem services in natural systems (Klironomos, 2002; Mariotte et al., 2018). However, a holistic framework to develop this concepts into principles that can alleviate productivity decline in agricultural systems is lacking. Until relatively recently, studies were conducted to address the needs of integrating PSF framework into agricultural systems (Mariotte et al., 2018). Although these are becoming common (Edwards et al., 2019; Hendriks et al., 2013; Mao et al., 2021), no study has considered the implication of feedbacks for different crop varieties and species in perennial systems. Such advances, as demonstrated in this project, will increase productivity of perennial systems and encourage sustainable production of crops with reduced negative impact on soil health.

The development of crops suited to selectively support beneficial interactions with soil biota that are likely enriched in a system and adjust expression of root traits for optimal growth and protection against pathogens will be another hurdle in coming years. Further work will be required to isolate the role of individual soil microbial groups associated with specific cultivar or species and whether these microbial groups influence the co-occurrence network of the microbiome affecting PSF. This process will also require understanding of functional metabolomics associated with specific interactions between a crop and keystone soil microbial taxa. This will improve our ability to select cultivars that can identify, attract and enhance the proliferation of their beneficial microbes while suppressing their specific pathogens. Some traits, including specific root length, root nodulation, arbuscular mycorrhizal colonization, average root diameter, higher allocation of resources to root system, are related to crop interactions with soil biota, and will therefore be a valuable tool in crop resistance to soil biotic stress. Evaluating the genotype by environment interactions of these traits under field conditions in ‘own’ and ‘away’ soils will be critical to achieving these aims.

Modern agriculture continues to encounter numerous bottlenecks. By manipulating the soil microbial resources at our disposal to enhance crop productivity through management practices and trait-based selection, we will be able to grow crops continuously in a more productive way with little input. The advances in agroecological approaches, rapid phenotyping methods, machine learning, and metagenomics resources will play a pivotal role in feeding the growing world population. Collectively, the findings of this project imply that simply seeding a new forage species into existing perennial stands does not guarantee that the soil environment will benefit that species due to the potential to share pathogens with the focal crops. For alfalfa stands, some alfalfa varieties and legume species are promising for rejuvenation, but their success, however, depends on alfalfa density in the previous stand.

6. LITERATURE CITED

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APPENDIX

Appendix A. Site description and absolute values of field variables in alfalfa monoculture and mixture stands. Samples taken near Saskatoon, SK., Western Canada in August, 2019.

Site number	Site name	Plant diversity	Stand age	Sample	Clay (%)	Silt (%)	Sand (%)	Soil carbon (%)	Soil phosphorus (ppm)	Soil nitrogen (ppm)	Alfalfa nitrogen (%)	ADF (%)¶	NDF (%)‡	Legume abundance	Weed abundance	Grass abundance	Productivity (g)
1	Saelhof	Mixture	1	1	30	44	26	2.84	1.89	11.53	18.905	25.757	33.993	0.423	0.081	0.495	379.800
1	Saelhof	Mixture	1	2	34	32	34	4.19	2.04	6.51	17.636	27.214	36.019	0.472	0.009	0.524	384.500
1	Saelhof	Mixture	1	3	38	28	34	3.89	1.99	9.29	18.133	27.185	36.484	0.432	0.114	0.476	469.100
2	Olson	Mixture	3	1	26	44	30	2.83	2.23	8.08	22.294	22.208	30.189	0.810	0.000	0.190	279.100
2	Olson	Mixture	3	2	38	44	18	2.91	1.57	10.88	20.325	20.846	28.442	0.330	0.090	0.580	3.600
*2	Olson	Mixture	3	3													
3	Crawford	Mixture	1	1	42	40	18	3.23	2.27	10.77	20.968	24.772	32.027	0.360	0.432	0.216	387.600
*3	Crawford	Mixture	1	2													
3	Crawford	Mixture	1	3	46	36	18	3.96	1.89	7.96	18.743	28.693	39.675	0.636	0.254	0.288	189.100
4	McBride	Mixture	4	1	26	28	46	2.06	1.89	8.99	19.198	24.672	36.308	0.333	0.333	0.333	384.700
4	McBride	Mixture	4	2	26	32	42	3.14	2.10	10.43	15.03	33.042	44.099	0.255	0.235	0.520	483.500
4	McBride	Mixture	4	3	22	32	46	2.64	1.89	8.82	20.569	26.255	36.516	0.380	0.469	0.150	222.900
5	Woodcock SW	Mixture	1	1	30	40	30	2.14	1.60	8.15	18.471	29.760	40.575	0.556	0.424	0.090	416.600
5	Woodcock SW	Mixture	1	2	14	20	66	1.69	2.12	6.40	18.823	24.125	31.913	0.714	0.155	0.143	282.400
5	Woodcock SW	Mixture	1	3	38	28	34	2.06	1.88	6.51	19.135	25.109	35.603	0.370	0.346	0.370	216.600
6	Woodcock SE	Mixture	3	1	34	40	26	2.46	1.59	9.37	18.488	29.602	39.035	0.652	0.022	0.337	505.700
6	Woodcock SE	Mixture	3	2	22	44	34	2.91	2.15	9.26	16.469	27.896	39.337	0.556	0.000	0.444	733.100
6	Woodcock SE	Mixture	3	3	46	44	10	2.92	1.74	6.83	16.116	24.664	36.286	0.750	0.000	0.250	233.900
7	DanMarchildon 1	Monoculture	1	1	58	40	2	2.08	2.44	6.54	19.052	27.787	37.861	0.975	0.025	0.000	258.200
7	DanMarchildon 1	Monoculture	1	2	54	32	14	2.57	2.47	12.72	17.948	29.652	38.484	1.000	0.000	0.000	364.400
7	DanMarchildon 1	Monoculture	1	3	38	40	22	2.99	3.61	11.97	16.265	33.643	43.426	0.950	0.050	0.000	429.800
8	DanMarchildon6	Monoculture	6	1	34	40	26	5.11	1.98	23.10	16.151	34.329	44.042	0.669	0.331	0.000	607.100
8	DanMarchildon6	Monoculture	6	2	42	36	22	3.45	1.50	12.04	19.059	33.403	44.438	0.864	0.136	0.000	402.100
8	DanMarchildon6	Monoculture	6	3	46	40	14	3.51	2.10	10.08	15.967	36.108	47.530	0.905	0.095	0.000	616.900
9	Dan Marchildon 2	Monoculture	2	1	34	48	18	5.67	3.59	15.11	15.794	32.687	43.093	0.990	0.010	0.000	815.700
9	Dan Marchildon 2	Monoculture	2	2	54	28	18	1.57	1.79	6.41	11.971	42.437	54.202	0.951	0.049	0.000	488.100
9	Dan Marchildon 2	Monoculture	2	3	50	32	18	1.13	1.72	4.30	20.674	26.576	35.777	0.951	0.049	0.000	572.400
10	Dennis Marchildon 6	Monoculture	6	1	42	40	18	3.87	3.71	11.44	17.603	25.001	35.303	0.968	0.032	0.000	303.500
10	Dennis Marchildon 6	Monoculture	6	2	54	32	14	2.47	1.70	8.31	16.609	30.286	41.777	0.915	0.085	0.012	334.100
10	Dennis Marchildon 6	Monoculture	6	3	42	36	22	2.74	1.59	8.78	17.765	22.810	33.487	0.875	0.125	0.000	247.400
11	Weighill Field 1 Yr1	Monoculture	1	1	54	36	10	2.83	2.16	9.74	18.666	31.572	41.111	1.000	0.000	0.000	918.000
11	Weighill Field 1 Yr1	Monoculture	1	2	42	44	14	2.55	1.63	7.96	15.457	33.951	43.938	1.000	0.000	0.000	313.500
11	Weighill Field 1 Yr1	Monoculture	1	3	14	20	66	1.21	2.73	9.51	17.583	32.147	41.883	1.000	0.000	0.000	341.200
12	Weighill Field 1 yr 2	Monoculture	2	1	46	44	10	2.69	2.33	10.02	19.08	31.498	41.789	0.990	0.010	0.000	731.400
12	Weighill Field 1 yr 2	Monoculture	2	2	42	40	18	3.91	2.70	12.25	20.931	28.136	38.049	1.000	0.000	0.000	835.900
12	Weighill Field 1 yr 2	Monoculture	2	3	22	40	38	2.61	2.74	7.71	15.796	27.800	37.952	0.824	0.176	0.000	564.900
13	Weighill Field 2 Yr1	Monoculture	1	1	42	44	14	2.78	1.96	9.30	17.24	34.289	45.050	0.962	0.038	0.000	176.900
13	Weighill Field 2 Yr1	Monoculture	1	2	42	44	14	2.73	2.11	7.44	18.184	29.193	39.090	0.978	0.022	0.000	605.200
13	Weighill Field 2 Yr1	Monoculture	1	3	34	48	18	3.32	1.78	11.59	15.721	30.438	41.354	0.989	0.000	0.011	583.300
14	Weighill Field 2 Yr 2	Monoculture	2	1	46	36	18	3.19	2.18	10.00	19.87	43.332	54.356	0.979	0.021	0.021	282.500
14	Weighill Field 2 Yr 2	Monoculture	2	2	58	28	14	3.62	2.75	11.72	19.674	35.801	46.434	1.000	0.000	0.000	596.600
14	Weighill Field 2 Yr 2	Monoculture	2	3	34	32	34	8.52	3.67	26.61	18.01	33.040	42.933	0.933	0.067	0.000	504.600
15	Stewart 2	Monoculture	2	1	30	36	34	3.07	2.57	10.77	18.653	29.710	39.382	0.704	0.296	0.000	138.400
15	Stewart 2	Monoculture	2	2	18	52	30	3.07	1.80	9.18	17.748	29.044	38.989	0.942	0.058	0.000	614.900
15	Stewart 2	Monoculture	2	3	26	48	26	3.23	1.87	10.37	17.46	27.689	38.441	0.556	0.444	0.000	325.500
16	Stewart 6	Monoculture	6	1	22	48	30	3.24	2.09	8.96	13.155	39.089	53.091	0.667	0.333	0.000	333.400
16	Stewart 6	Monoculture	6	2	22	28	50	2.98	1.95	9.87	16.517	29.864	41.620	0.845	0.155	0.000	244.700
16	Stewart 6	Monoculture	6	3	22	32	46	3.61	2.68	11.50	14.762	29.757	42.350	0.764	0.236	0.069	235.600
17	LaBras Yr 4 Field 1	Monoculture	4	1	34	36	30	2.61	1.91	8.32	15.708	28.885	39.689	0.921	0.079	0.000	415.200
17	LaBras Yr 4 Field 1	Monoculture	4	2	42	40	18	3.04	2.32	9.75	19.31	22.405	31.789	0.862	0.138	0.000	399.400
17	LaBras Yr 4 Field 1	Monoculture	4	3	34	36	30	3.45	2.44	11.60	18.339	22.568	31.147	1.000	0.000	0.000	318.400
18	LaBras yr 4 Field 2	Monoculture	4	1	26	48	26	2.82	2.61	9.42	14.487	33.416	44.425	0.769	0.183	0.067	377.500
18	LaBras yr 4 Field 2	Monoculture	4	2	42	40	18	1.76	2.24	6.78	18.552	28.487	39.276	0.906	0.094	0.010	434.600
18	LaBras yr 4 Field 2	Monoculture	4	3	38	36	26	2.38	1.84	8.96	16.674	22.514	31.481	0.694	0.306	0.000	326.400
19	Beddome	Mixture	3	1	42	32	26	1.90	1.96	6.83	16.46	31.490	43.632	0.444	0.347	0.210	385.400
19	Beddome	Mixture	3	2	46	40	14	4.60	2.35	14.41	14.512	33.578	46.591	0.570	0.184	0.246	521.100
19	Beddome	Mixture	3	3	34	44	22	2.83	1.44	5.67	16.843	26.963	37.961	0.427	0.049	0.561	273.100
20	John Huber	Mixture	2	1	26	28	46	3.09	2.99	9.10	14.929	25.095	37.381	0.732	0.000	0.268	340.900
*20	John Huber	Mixture	2	2													
*20	John Huber	Mixture	2	3													
21	Paul Huber	Mixture	4	1	34	40	26	5.94	2.98	14.22	17.437	21.530	33.732	0.263	0.000	0.737	351.400
*21	Paul Huber	Mixture	4	2													
*21	Paul Huber	Mixture	4	3													
22	Dan Andreas	Mixture	4	1	22	32	46	4.25	1.75	13.94	16.849	18.317	28.960	0.459	0.083	0.459	1230.100
22	Dan Andreas	Mixture	4	2	30	36	34	4.29	1.95	7.87	13.153	28.194	42.302	0.613	0.009	0.377	453.500
22	Dan Andreas	Mixture	4	3	14	32	54	2.68	2.23	10.27	13.96	30.459	46.918	0.729	0.010	0.260	501.300
23	Neil MacRae	Mixture	2	1	26	44	30	4.70	2.44	13.77				0.130	0.261	0.626	729.200
23	Neil MacRae	Mixture	2	2	26	36	38	3.19	2.37	8.99	17.006	25.476	34.955	0.500	0.089	0.411	366.200
23	Neil MacRae	Mixture	2	3	42	40	18	3.00	2.08	11.38	16.863	24.280	33.471	0.420	0.277	0.328	334.300
24	Biligetu	Mixture	2	1	22	32	46	2.14	2.33	7.02	13.4569	28.808	54.048	0.275	0.011	0.714	554.473
24	Biligetu	Mixture	2	2	58	32	10	2.29	1.98	8.19	11.7945	29.697	58.291	0.341	0.034	0.625	516.962
24	Biligetu	Mixture	2	3	58	28	14	1.68	2.32	6.93	20.7751	30.120	54.632	0.388	0.000	0.612	380.577

¶ Acid detergent fiber in alfalfa; ‡ Neutral detergent fiber in alfalfa; *missing samples due to labeling error.

Appendix B. Results of the principal components analysis (PCA) for the abundance of plant species functional groups, soil texture class, forage quality, and soil nutrients from alfalfa monoculture and mixture stands.

	PC1	**PC2**
Eigenvalues	1.830	1.166
Proportion of variance	0.610	0.389
Cumulative proportion	0.610	0.998
Legumes	-0.916	-0.397
Weeds		0.997
Grasses	0.992	-0.114
Eigenvalues	1.811	1.189
Proportion of variance	0.604	0.396
Cumulative proportion	0.604	1.000
% Clay	0.996	
% Silt		0.997
% Sand	-0.901	-0.433
Eigenvalues	1.765	1.090
Proportion of variance	0.588	0.363
Cumulative proportion	0.588	0.952
% Nitrogen	-0.212	0.975
% ADF	0.961	-0.129
% NDF	0.893	-0.351
Eigenvalues	2.166	0.646
Proportion of variance	0.722	0.215
Cumulative proportion	0.722	0.937
Soil carbon (%)	0.619	-0.339
Soil total phosphorus	0.484	0.875
Soil total nitrogen	0.618	-0.345

Appendix C. Results of the analysis of reduced model testing variety by important predictor effects on intraspecific plant-soil feedbacks for establishing alfalfa varieties in soils previously conditioned in monoculture (n = 139), and mixed (n = 99) stands using mixed-effects model.

Predictors in Monoculture stands	**Sum Sq**	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr (>F)***
Variety	0.9999	0.3333	3	139	7.8462	**<0.0001**
Stand age	0.1886	0.0943	2	139	2.2207	0.1123
Fiber content PC	0.2449	0.2449	1	139	5.7647	**0.0176**
Soil phosphorus PC	0.1992	0.1992	1	139	4.6909	**0.0320**
Variety × Fiber content PC	0.4867	0.1622	3	139	3.8190	**0.0114**
Variety × phosphorus PC	0.3299	0.1099	3	139	2.5889	0.0554
Predictors in Mixed stands						
Variety	1.1135	0.3712	3	99	13.2284	**<0.0001**
Soil clay:sand PC	0.1183	0.1183	1	99	4.2151	**0.0427**
Soil silt PC	0.1158	0.1158	1	99	4.1278	**0.0448**

**Boldface type indicates P-values < 0.05*

Appendix D. Results of the reduced model testing the relationship between important predictors and interspecific plant-soil feedback of forage species in alfalfa-associated monoculture (n = 210), and mixture (n = 165) soils.

Predictors in Monoculture	**Sum Sq**	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr (>F)**
Forage species	2.4558	0.4911	5	175	15.7422	**<0.0001**
Stand age	0.2197	0.1098	2	35	3.5222	**0.0401**
Fiber content PC	0.3091	0.3091	1	40	9.9086	**0.003**
Weed abundance PC	0.1597	0.1597	1	36	5.1189	**0.0296**
Plant species richness	0.0966	0.0966	1	34	3.0973	0.0871
Forage species × weed abundance PC	0.5897	0.1179	5	175	3.7804	**0.0028**
Predictors in Mixture						
Forage species	2.0275	0.4055	5	153	12.612	**<0.0001**
Legume:grass abundance PC	0.0826	0.0826	1	41	2.57	0.1164
Plant species richness	0.233	0.233	1	34	7.2487	**0.0108**
Alfalfa nitrogen PC	0.1455	0.1455	1	47	4.5272	**0.0386**
Fiber content PC	0.1016	0.1016	1	37	3.1625	0.0835

**Treatments with boldface type indicates P-values < 0.05*

Appendix E. Results of the mixed-effects models (each row) testing variety differences in trait expression of arbuscular mycorrhizal fungi rate, nodulation rate, root-shoot ratio, root diameter, specific root length, and root tissue density.

Response variables	Fixed effect	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
AMF	Variety	7.1054	2.3685	3	178	0.5962	0.6180
Nodulation	Variety	5.4473	1.8157	3	231	1.2371	0.2970
Root-shoot ratio	Variety	0.1296	0.0432	3	228	1.1492	0.3301
Root diameter	Vareity	0.0049	0.0016	3	231	0.4579	0.7119
SRL	Variety	1483471	494490	3	173	0.4435	0.7222
RTD	Variety	0.2349	0.0783	3	180	3.5996	**0.0147**

Appendix F. Results of the mixed-effects models testing the effects of stand age and plant diversity on arbuscular mycorrhizal fungi (AMF) colonization, nodulation, root-shoot ratio, root diameter, specific root length (SRL), and root tissue density (RTD) of feedback varieties.

	AMF		Nodulation		Root-shoot ratio		Root diameter		SRL		RTD	
	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)
Variety	0.778	0.508	0.863	0.461	1.430	0.235	1.046	0.373	0.653	0.582	0.121	0.948
Plant diversity	0.027	0.870	0.037	0.849	4.690	**0.031**	0.184	0.668	0.880	0.352	0.205	0.653
Stand age	0.490	0.615	0.906	0.406	1.312	0.271	0.549	0.578	0.763	0.471	0.666	0.517
Variety × plant diversity	0.224	0.880	0.225	0.879	1.342	0.262	1.329	0.266	0.388	0.762	0.454	0.715
Variety × stand age	1.420	0.209	0.843	0.538	0.406	0.875	1.333	0.243	1.336	0.244	0.314	0.929
Plant diversity × stand age	0.604	0.550	0.351	0.705	0.939	0.393	0.654	0.521	0.403	0.670	0.089	0.915
Variety × plant diversity × stand age	1.126	0.349	0.918	0.483	0.589	0.739	1.056	0.390	0.702	0.649	0.265	0.952

Appendix G. Results of the mixed-effects models (each row) testing species differences in trait expression of arbuscular mycorrhizal fungi rate, nodulation rate, root-shoot ratio, root diameter, specific root length, and root tissue density.

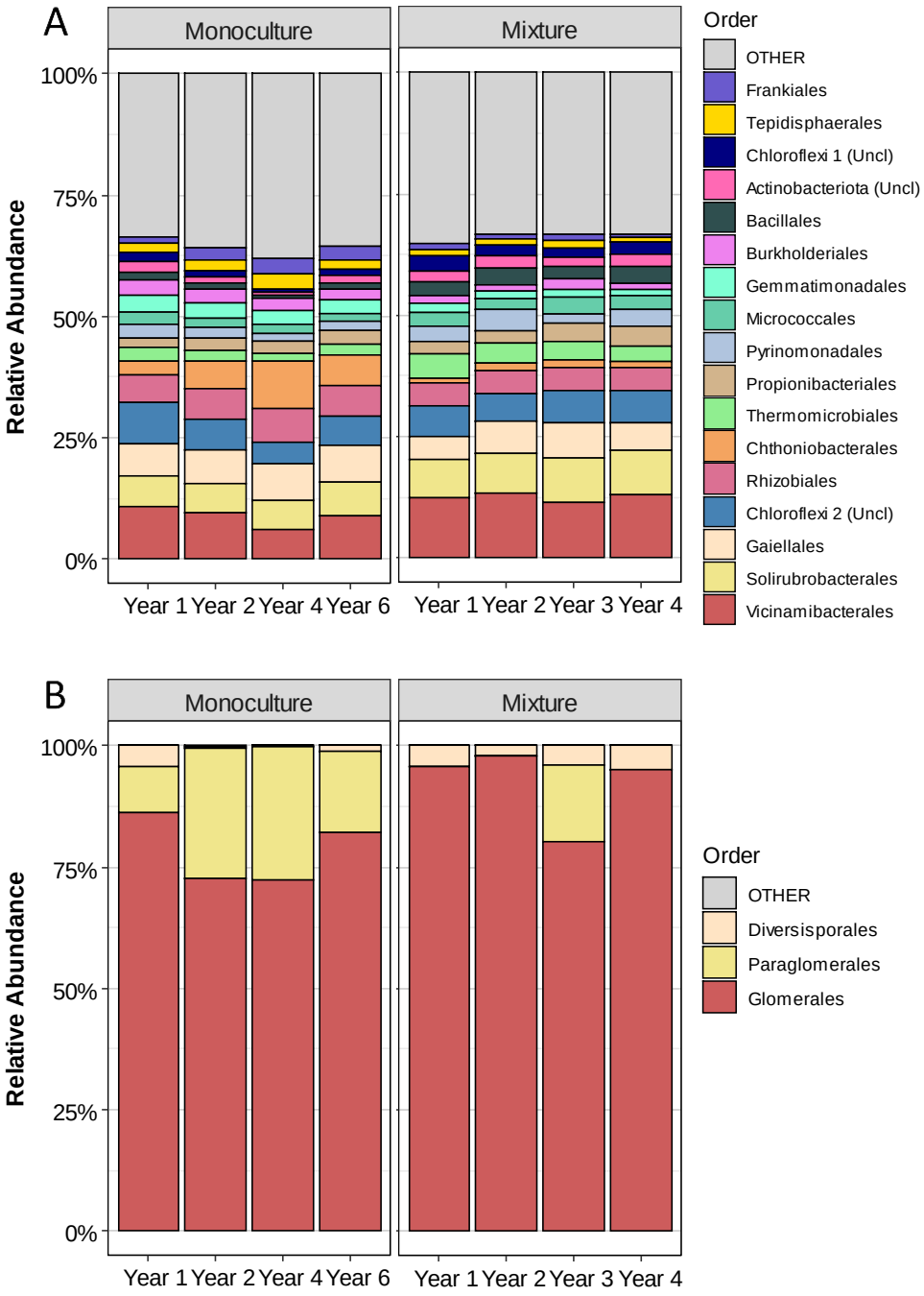
Response variables	Fixed effect	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
AMF	Species	126.71	25.34	5	341	4.84	**<0.001**
Nodulation	Species	631.12	210.37	3	223	49.67	**<0.001**
Root-shoot ratio	Species	17.67	3.53	5	299	61.24	**<0.001**
Root diameter	Species	5.97	1.19	5	306	110.17	**<0.001**
SRL	Species	105124	21025	5	304	88.53	**<0.001**
RTD	Species	0.54	0.11	5	304	7.58	**<0.001**

Appendix H. Results of the mixed-effects models testing the effects of stand age and plant diversity on arbuscular mycorrhizal fungi (AMF), colonization, nodulation, root-shoot ratio, root diameter, specific root length (SRL), and root tissue density (RTD) of feedback species.

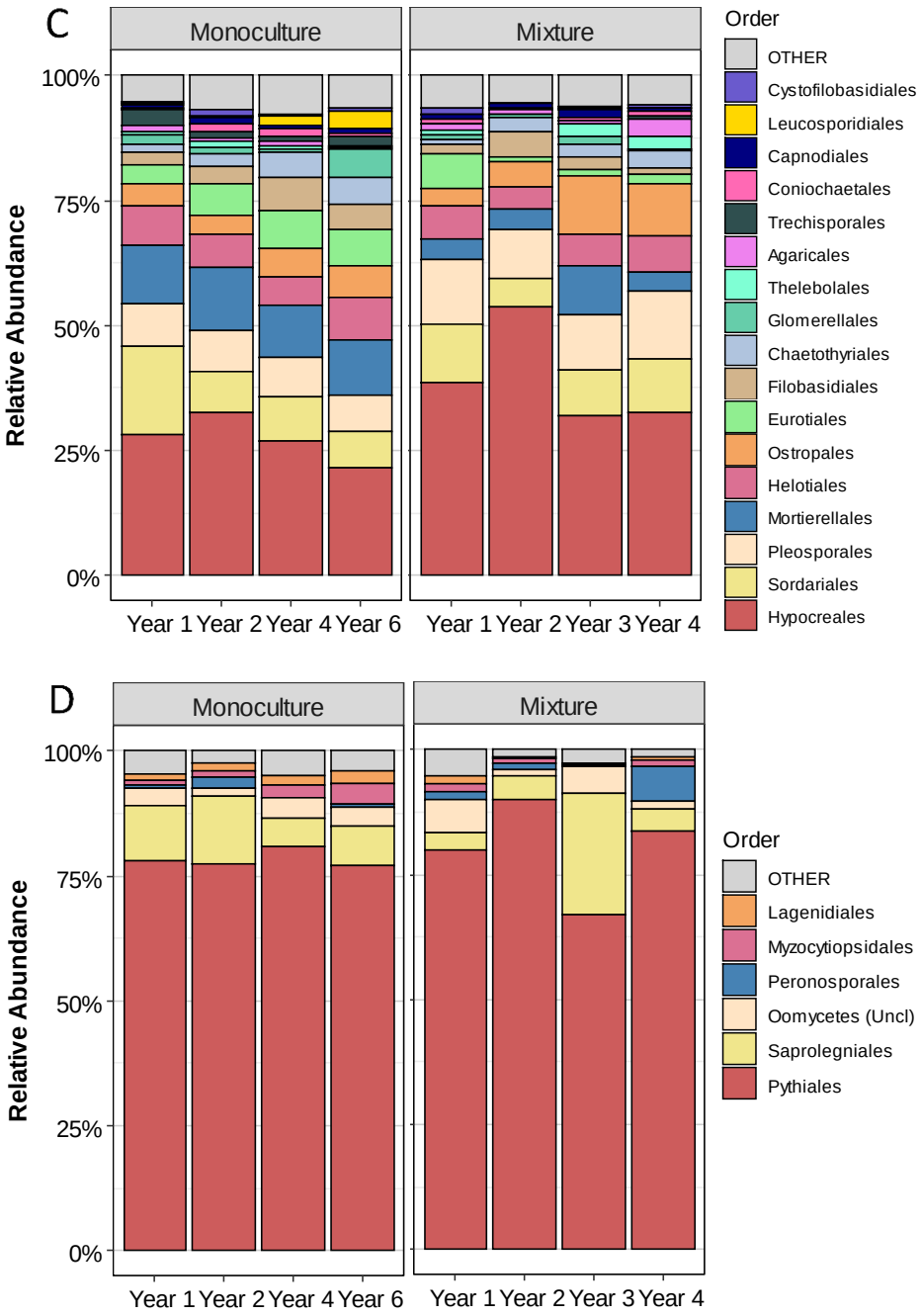
	AMF		Nodulation		Root-shoot ratio		Root diameter		SRL		RTD	
	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)
Species	2.396	**0.037**	13.525	**<0.001**	37.252	**<0.001**	52.812	**<0.001**	**32.745**	**<0.001**	2.469	**0.033**
Plant diversity	1.331	0.258	11.376	**0.001**	0.061	0.806	0.359	0.551	2.954	0.090	1.860	0.177
Stand age	1.012	0.378	0.804	0.452	1.129	0.329	0.115	0.892	0.572	0.567	0.950	0.391
Species × plant diversity	0.972	0.435	10.490	**<0.001**	2.362	**0.040**	2.301	**0.045**	1.795	0.114	1.000	0.418
Species × stand age	0.290	0.983	0.279	0.947	1.727	0.074	1.033	0.415	0.657	0.764	3.407	**<0.001**
Plant diversity × stand age	0.552	0.583	1.307	0.277	3.465	**0.037**	0.571	0.568	1.060	0.352	1.798	0.173
Species × plant diversity × stand age	0.949	0.488	0.464	0.835	2.304	**0.013**	0.643	0.776	0.917	0.517	1.515	0.133

Appendix I. Mixed-effects models, and PERMANOVA analysis testing the effects of stand age and plant diversity, and community composition of the soil microbial communities, respectively, in alfalfa monoculture and mixture soils at stand ages 1 to 6 year taken from sites near Saskatoon, SK., in August 2019.

Group	Sampling treatment	Shannon diversity		PERMANOVA		
		F value	P value	R-squared	F value	P value
Bacteria	Stand age	1.4127	0.2673	0.0374	1.2227	0.2032
	Plant diversity	1.1015	0.3065	0.0112	0.7349	0.9402
	Stand age × plant diversity	0.5962	0.5606	0.0335	1.0934	0.4531
Fungi	Stand age	0.8288	0.4521	0.0391	1.3699	**0.0003**
	Plant diversity	0.0531	0.8203	0.0662	4.6368	**0.0001**
	Stand age × plant diversity	0.3423	0.7145	0.0377	1.3204	**0.0035**
AMF	Stand age	2.5831	0.1024	0.0571	2.3402	**0.0002**
	Plant diversity	4.7871	**0.0414**	0.1702	13.9455	**0.0001**
	Stand age × plant diversity	0.7912	0.4680	0.0403	1.6527	**0.0287**
Oomycetes	Stand age	3.2461	**0.0459**	0.0385	1.5504	**0.0193**
	Plant diversity	0.2374	0.6279	0.1765	14.2250	**0.0001**
	Stand age × plant diversity	5.0116	**0.0097**	0.0406	1.6341	**0.0052**



Appendix J (a-d). Relative taxonomic composition of soil bacteria **(a)**, arbuscular mycorrhizal fungi **(b)** and other fungi **(c),** and oomycetes **(d)** based on the order-level relative abundance of ASVs identified in alfalfa monoculture and mixture soils at stand ages 1 to 6 years old taken from sites near Saskatoon, SK., in August 2019. Extremely low-abundance taxa are summarized as "OTHER". (Uncl) = Unclassified.



Appendix J (a-d). Continued