INTRASPECIES VARIATION IN MYCORRHIZAL RESPONSE OF MEDICAGO SATIVA TO *RHIZOPHAGUS IRREGULARIS* UNDER ABIOTIC STRESS

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ABSTRACT

Arbuscular mycorrhizal fungi are considered beneficial for their host plants, contributing to better growth, especially in stressful conditions. Actual plant outcomes can vary from beneficial to detrimental depending on both participant's identity and the environmental context. Understanding plant-AMF symbiosis is a key component to understanding plant functioning. Additionally, there is potential for AMF use in developing sustainable agricultural practices. There are multiple methods that seek to explain the mechanisms behind variation in AMF symbiosis, and predict outcomes, such as using resource economics, or plant root morphology. While broad differences in AMF responsiveness between plant species can be explained these ways with varying success, intraspecific differences are not well understood.

Our study aimed to target the context dependency and intraspecific variation of mycorrhizal relationships by using nine alfalfa cultivars (*Medicago sativa*) to determine how different cultivar attributes or trait expression might alter the plant-AMF, and plant-AMF-pollinator relationships in different stress contexts. We performed a greenhouse trial on alfalfa plants inoculated with *Rhizophagus irregularis*, exposing them to drought, salt, or low nutrient stress, to compare to alfalfa grown under unstressed conditions. We measured how bee visitation, flower number, seed production, biomass, N and P content changed with stress and AMF inoculation. We also measured how variation in specific root length, root tissue density, and root diameter interacted with mycorrhizal effects.

Our study showed growth conditions mattered more for determining AMF affects on growth and stress response than cultivar identity did. Biomass and nutrient concentrations were fairly consistent across cultivars in each stress treatment group, and AMF had largely neutral or negative effects on biomass across treatments. AMF Increased biomass stress responses to drought and saline soil, marginally improved nutrient uptake, but did not ultimately determine plant seed production. AMF effects did not correlate with root trait expression. Plants attained sufficient nutrients without AMF, and it is likely that the negative affects seen in inoculated plants were a result of stressed AMF acting as a drain on resources. Our study highlights the context specificity of mycorrhizal interactions with plants, and the lack of understanding of the role fungi identity and origin plays in this relationship.

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LIST OF ABREVIATIONS

AMF	Arbuscular mycorrhizal fungi
ABA	Abscisic acid
EC	Electroconductivity
LSR	Leaf:Stem ratio
MF	Mycorrhizal fungi
N	Nitrogen
P	Phosphorus
RD	Root diameter
ROS	Reactive oxygen species
RSR	Root:Shoot ratio
RTD	Root tissue density
SR	Stress response
SRL	Specific root length

LIST OF EQUATIONS

1. INTRODUCTION

Agricultural land use has intensified to meet growing demand, but many current agricultural systems are harmful to surrounding ecosystems (Emmerson et al., 2016), and are not sustainable especially considering the rapidly changing climate. Ortiz-Bobea et al. (2021) estimated climate change has slowed agricultural productivity growth by 21% since 1961, and that agricultural productivity has become more sensitive to climate extremes over time. Food production is only one of many essential ecosystem services provided by the environment for the continued health of the human population (Kremen, 2005). We can utilize knowledge about extant natural systems to integrate agriculture into existing ecosystems, creating multifunctional agroecosystems robust against climate change (Mariotte et al., 2018). For example: relationships between plants and the soil microbiome are essential for regulating nutrient availability for crops, carbon sequestration, and soil health (Faucon et al., 2017). Not all land is suitable for high volume food production; low quality land planted with forage legumes could play an important role in sustainable agriculture by integrating animal agriculture with sustainable land management through grazing (Daru & Mayulu, 2020), and improving soil quality through microbial partnerships (McCartney & Fraser, 2010).

Plant relationships with the soil microbiome are determinants of plant health and functionality (Hardoim et al., 2015). Symbiotic relationships, such as those with arbuscular mycorrhizal fungi (AMF) increase nutrient uptake, disease resistance, and alleviate plant stress (Hohmann & Messmer, 2017; Smith & Smith, 2011). Mycorrhizal symbiosis with plants has developed over millions of years (Redecker et al., 2000), but thousands of years of anthropogenic selection for agriculturally favourable traits has disrupted plant-AMF symbiosis even as we now recognize its potential to improve crop performance (Jacott et al., 2017; Porter & Sachs, 2020).

While AMF are generally considered beneficial partners for plants, research has shown that the actual growth outcomes vary with individualistic factors like genetic variation within plants and

fungi (Stahlhut et al., 2023), as well as external factors such as environmental conditions (Antunes et al., 2011). AMF can interact with most aspects of plant physiology: the regulation of nutrient uptake (Jansa et al., 2019; Simard & Durall, 2004), water use (Augé, 2001), and phytohormones (Diagne et al., 2020). Beyond that, there are also three-way relationships between AMF, plants, and their associated beneficial and detrimental insects (Barber & Gorden, 2015; Tao et al., 2016). The complexity of these interactions and relationships makes it difficult to determine what are the most important drivers of variation in symbiotic outcomes, and how those drivers can change in different contexts.

The overarching objective of this work is to connect intraspecific variations of the plant-AMF relationship caused by internal factors (i.e. plant and AMF identity) to variation caused by external factors; namely the effects of abiotic stress. Human actions have interacted with plant-AMF relationships on many levels intentionally and unintentionally (Meena et al., 2020; Su & Guo, 2007), but the consequences for fugal populations, and long term outcomes are unclear (Hart et al., 2017). Understanding these complex relationships better facilitates our ability to work with AMF populations (along with the larger soil microbiome) to improve agriculture, ecosystem services, and restoration efforts, maintaining functioning ecosystems with the minimum of disruption.

1.1. Thesis organization

This thesis is written in manuscript format with five chapters. We begin with this general introduction followed by a literature review of our current understanding of AMF, the plant-AMF symbiosis, and interactions with abiotic stress. This is followed by two research chapters: one investigating intercultivar variation in the plant-AMF relationship in alfalfa under stress, and the next on the relationship between alfalfa root trait expression and the mycorrhizal response. The objectives of the first research chapter were to determine if AMF interactions with alfalfa's stress response, and reproduction would be affected by variation among cultivars. The objective of the second chapter was to examine the role of alfalfa root traits in moderating the effects of AMF on alfalfa growth and nutrient acquisition. Finally, in the last chapter we will summarize key findings, discuss their implications, and suggest pathways for future research.

2. LITERATURE REVIEW

2.1. The soil microbiome and the origin of mycorrhizal symbiosis

Soil contains a vast microbiome that exerts influence on plant growth and functioning both positively and negatively. In turn, plants influence the ground around them both actively, e.g. by exuding carbon compounds and signaling molecules from their roots to attract beneficial microbiota (Rasmann & Turlings, 2016), and passively e.g. by introducing organic matter into soil when root tissue and litter decompose (Cotrufo et al., 2013). This reciprocal relationship between plants and soil microbes is part of a complex system of plant-soil feedback (PSF). The overall magnitude and direction of PSF is a combination of the myriad interactions between plants, microbiota, and the soil around them (Ehrenfeld et al., 2005).

Within the rhizosphere microbiome, mycorrhizal fungi (MF) are extremely important for the diversity of present-day plant life. Mycorrhizae are plant symbionts; they interface with plant roots to exchange resources. Fossil evidence places the origin of MF lineages 600 MYA, with fungi similar to extant arbuscular mycorrhizal fungi present 460 MYA (Redecker et al., 2000). These fungi's ability to take up soil nutrients was likely involved with the colonization of land by eukaryotic plant life that had yet to develop complex root systems (Heckman et al., 2001). This long history of coevolution has built an especially close relationship between the vast majority of plants and MF; only 8% of vascular plant species do not take part in mycorrhizal symbioses (e.g. those in the families Brassicaceae, Crassulaceae, Orobanchaceae, Proteaceae (Brundrett et al., 2018)).

2.2. Mycorrhizal fungi

Mycorrhizal fungi are obligate symbionts, which depend on carbon from their host plant to grow, mature, and complete their life cycle. In return these fungi supply essential nutrients like phosphorus, nitrogen, and potassium, and can improve plant defences against pathogens and herbivores (Delavaux et al., 2017). There are currently six recognized types of MF categorized

by the structural nature of the symbiosis. Fungi that cross their host's cell walls are endomycorrhizae. These MF types are: arbuscular, orchid, and ericoid. Fungi that do not cross cell walls are known as ectomycorrhizae. Arbutoid and monotropoid MF share characteristics of both endo- and ecto- types (Lewis, 2016). Arbuscular mycorrhizal fungi (AMF) are the oldest, most common of the mycorrhizae; about 80% of plant-mycorrhizal relationships are with AMF (Smith & Read, 2008).

To initiate symbiosis, plants secrete strigolactones into the soil that stimulate hyphal branching in AMF while also signalling the direction of the plant root (Akiyama et al., 2005). Germinated fungal spores transmit oligosaccharide myc factors which induce pro-symbiosis gene expression in plants (Kosuta et al., 2003; MacLean et al., 2017). When hyphae contact the root epidermis the root cell reshapes to allow entry (Genre et al., 2005). Fungal hyphae traverse the plant cell wall, forming branching "trees" where they exchange resources with the plant. Once situated, AMF extend hyphae out into the surrounding soil, absorbing essential nutrients from a greater range and soil volume than plant roots alone have access to.

These processes that facilitate and maintain symbiosis are under genetic control. Genes for differentiating beneficial from harmful microbes, and symbiosis initiation have been highly conserved in plants (Delaux et al., 2013). The suite of genes involved in maintaining symbiosis are dependant on plant genotype (Mateus et al., 2019). Plants do not solely control symbiosis; plant-AMF symbiosis is facilitated by both parties, but the role of fungal genetic variation in symbiosis has been challenging to study. AMF can exist as homo- or heterokaryotic (Mathieu, 2021) with each individual fungi carrying multiple hundreds or thousands of nuclei in coenocytic hyphae (Jany & Pawlowska, 2010). The passage of these nuclei to daughter spores is not uniform (Cornell et al., 2022) allowing rapid genetic change to occur over generations (Ehinger et al., 2012). Further research into AMF genetics is required before we can understand how much fungal genotype contributes to variation in the plant-AMF symbiosis.

2.3. Mycorrhizal assisted nutrient acquisition

Phosphorus and nitrogen are limiting factors for plant growth (Elser et al., 2007). Phosphorus enters the environment via mineral weathering or slow breakdown of organic material. It quickly oxidizes or binds to soil particles resulting in a heterogeneous distribution of P pools with limited diffusion range (Bolan, 1991). Already low plant P availability is worsened when natural

mechanisms for replenishment can not keep up with demand (Vitousek et al., 2010). Nitrogen is found in soil as nitrate (NO_3^-), ammonium (NH_4^+), or in organic form as amino acids (Näsholm et al., 2009; Williams & Miller, 2001). Nitrate is more readily available but is more energetically expensive for plants to take up and use compared to ammonium (Forde & Clarkson, 1999). The availability of organic N is mediated by soil microbial activity and depends on soil pH and amount of organic matter (Näsholm et al., 2009).

Mycorrhizal fungi assist plants in acquiring P and N along with other nutrients (Smith & Read, 2008). Fungal hyphae are well suited to take up nutrients; hyphae are smaller, they can access nutrient patches that may be too transient to attract roots (Hodge et al., 2009). Additionally, AMF hyphae have higher affinity for ammonium than plant roots (Pérez-Tienda et al., 2012). AMF increase availability of organic P in soil by recruiting and translocating P solubilizing bacteria to organic P patches (Jiang et al., 2021). Improved P uptake via AMF has been shown to increase biological N fixation in legumes (Püschel et al., 2017), as well as stimulate N uptake from organic sources (Jansa et al., 2019). In return, AMF gain photosynthetically derived carbon from their plant hosts. The amount of the plant's carbon supply allocated to AMF is estimated to be below 10% on average (Řezáčová et al., 2017), representing a third of the plant's carbon allocated below ground. Although this represents a significant carbon cost, plants benefitting from AMF can capture more carbon than plants without beneficial mycorrhizal associations, therefore, it is advantageous for plants to receive P and N from AMF that can scavenge for distant resources, assuming that light supply is sufficient.

2.4. Tripartite interactions

The effects of AMF on plant physiology can extend to influence plant-pollinator relationships, the complexities of which have developed over a long period of coevolution. Approximately 87% of flowering plants rely on animals for reproduction (Ollerton et al., 2011), overlapping extensively with the types of plants capable of forming mycorrhizal symbiosis.

Reproductive success depends on pollinator quality and visitation frequency, so plants have developed various strategies to attract the most beneficial pollinators to their flowers. These include: flower colour, shapes, size, nectar rewards, and various volatile organic compounds (Raguso, 2004; Schiestl & Johnson, 2013). Pollinator attraction is mediated by floral displays as they initially tend to be drawn to larger or more numerous flowers, but can learn over time which

displays have higher rewards (Makino & Sakai, 2007). Nectar and pollen reward visitors by supplying sugars and amino acids (Lu et al., 2015), the composition of these rewards varies by plant species, and nutrient status (Ceulemans et al., 2017; Hanley et al., 2008).

The effects of plant-AMF mutualism on plant-pollinator interactions depends on the degree that AMF can change the key signals that mediate pollination. Changes to pollinator visitation rate and community composition have been shown to occur in AMF manipulation studies, but the mechanisms are not clear. Visitation to AMF associated plants could be increased by higher resource allocation to reproductive stems as seen in *Chamerion angustifolium* (Wolfe et al., 2005), but many other factors are involved as well. Gange and Smith (2005) found higher pollinator visitation in three species (*Centaurea cyanus, Tagetes erecta, and Tagetes patula*), but the effect of AMF on signals such as total number of flowers, flower size, nectar sugar content were not consistent between the three. The species of AMF may also be important; Barber et al. (2013) found that different pollinator taxa preferred *Cucumis sativus* inoculated by different AMF strains. For instance, *Bombus spp.* increased visitation to individuals inoculated by *Rhizophagus irregularis*, although they could not connect this preference to a specific change in floral traits. Adding or supressing AMF in a community can change the flowering species community composition, resulting in an altered pollinator community. Cahill et al. (2008) found *Bombus spp.* dominating the cadre of pollinators for a community of 23 flowering plant species, but when AMF were supressed pollinator community makeup was seen to shift towards smaller bees and flies due to *Cerastium arvense* dominating the floral display. The abundance of *C. arvense* was particularly attractive to smaller pollinators and additionally, may have obscured floral cues of other species to attract *Bombus spp.*

2.5. Mitigation of abiotic stress

2.5.1. Physiological effects of stress on plants

Abiotic stresses (such as hotter seasons, increased drought frequency, floods, and soil salinization) disrupt processes necessary for plant growth, and are increasing in frequency and intensity with climate change (Bonsal et al., 2013). A plant's responses to stress are regulated by phytohormones such as abscisic acid (ABA) which is especially important in water shortages as it signals to close stomata to reduce water loss, among other important functions (Hossain et al., 2011). Reduced stomatal conductance caused by lack of water disrupts photosynthesis, unbalancing the production and scavenging of reactive oxygen species during photorespiration

(Gill and Tuteja 2010). Reactive oxygen species (ROS) cause oxidative damage to multiple cellular structures that can lead to cell death (Choudhary et al., 2017). Even prolonged mild drought is detrimental as plants have reduced capacity to take up nutrients in dryer soils and the lack of water leads to cells losing pressure and function (Bahadur et al., 2019). Saline soils bring many of the same challenges as drought stress because salt lowers soil's osmotic potential. In addition to water balance difficulties, salts can cause ion toxicity. Sodium transport from root to shoot is largely unidirectional leading to build up in shoot tissues (Tester & Davenport, 2003) where Na^+ displaces K^+, disrupting cellular processes and protein synthesis (Bhandal & Malik, 1988). Therefore, the ability to exclude salt from entering root tissue, a low rate of salt transfer from root to shoot, and maintenance of a high cytosolic K:Na ratios are highly correlated with salt tolerance in plants (Munns et al., 2006).

2.5.2. AMF reduce the effects of drought and salt stress

There are protective effects of AMF on plants experiencing water deficits widespread beyond improving nutritional uptake (Bahadur et al., 2019). AMF partnerships increase water availability which mitigates drought before stress takes effect through a combination of direct water transfer (Faber et al., 1991), increasing soil hydraulic abilities by aggregating soil particles, and wicking water from soil pores and other inaccessible areas to root accessible zones (Allen, 2007; Bitterlich et al., 2018; Daynes et al., 2013). Some questions remain on how hyphae might transport relatively large volumes of water at physiologically relevant speeds (Koide, 1993; Smith et al., 2010); in response, Ruth et al. (2011) demonstrated that 20% of total water uptake over several drought cycles was transferred directly or indirectly by AMF from separate chamber accessible only to hyphae. More recently Püschel et al. (2020) recorded deuterium labelled water (DH_2O) supplied to plants by hyphae vs roots in a similar set up. They found that mycorrhizal plants had much improved water content than nonmycorrhizal ones, incorporating twofold more DH_2O, but only 0.15-7.5% of total consumed water could have come from the AMF chamber indicating that AMF's influence over root size, surface area, and soil hydraulic properties was more important than direct water uptake.

AMF act on plant systems to alleviate drought effects. For instance, AMF affect phytohormone levels leading to more drought resistant profiles. AMF inoculated lettuce were larger than uninoculated controls in a drought stress trial correlating with the accumulation of more than double the ABA compared to nonmycorrhizal lettuce when moderately and severely drought

stressed. This allowed stomatal conductance to remain closer to unstressed levels (Ruiz-Lozano et al., 2016). Antioxidant production (e.g. catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX)) is promoted by AMF under drought conditions which assists plants in preventing oxidative damage. Antioxidant levels in plants can be affected locally and systemically: Maize grown with AMF in a split plot study maintained APX levels only in the inoculated half of the root system when both halves were drought stressed (Bárzana et al., 2015), but leaves of AMF inoculated rose geraniums saw reduced ROS accumulation under drought due to increased CAT and APX concentration in their tissue (Amiri et al., 2015). Finally, AMF increased osmolytes in their hosts. AMF increased proline and soluble sugar content was associated with improved leaf water content and PSII function of drought stressed Macadamia (Yooyongwech et al., 2013).

In addition to the physiological benefits of associating with AMF for osmotic stress, AMF also improve salt tolerance in many plants by protecting against ion toxicity. A meta-analysis of 249 studies found a 58% increase in K:Na ratio of plants associated with AMF and a 18% decreased shoot Na^+ concentration (Augé et al., 2014). This can be achieved because AMF selectively take up and transfer K from soil but not Na (Hammer et al., 2011; Talaat & Shawky, 2011), along with reducing the transfer of Na from roots to shoot (Evelin et al., 2019).

2.6. Variation in the plant-AMF relationship

Symbiosis is near ubiquitous, but not universally beneficial; the response fungi elicit in their plant hosts ranges from supressed to enhanced growth. Plant growth response variation is well documented across and within many plant species. For example: Eo and Eom (2009) showed only four of nine tested crops had a positive growth response to an AMF inoculant sourced from bulk agricultural soil. *Sorghum bicolor* had no significant AMF response in that trial, though they do not specify the cultivar used. Watts-Williams et al. (2019) studied the AMF response of 18 sorghum accessions to inoculation with one of four separate AMF species: *Glomus versiforme, Rhizophagus irregularis, Claroideoglomus claroideum, or Gigaspora gigantea,* discovering a wide array of plant growth responses, with eight accessions showing no response to any AMF species. These two papers also exemplify how most research describes permutations of plant-AMF symbiosis from the plant success perspective, leaving gaps in our understanding of

how mycorrhiza might be affected by different plant hosts, and what mycorrhizal characteristics might modulate growth responses (Bennett and Groten 2022).

The species, strain, and combinations of AMF all can alter host growth responses. Much research is done with one (or a combination of) of *R. irregularis, R. intraradices, Funneliformis mosseae,* or *Gigaspora margarita* (Berruti et al., 2016). *Rhizophagus irregularis* is by far the most widely utilized strain, and is the most prevalent in commercially available AMF inoculants (Salomon et al., 2022). The natural environment, however, supports a far more diverse selection of AMF species. Plants can connect to multiple AMF at once, and AMF can connect to many plants at once, forming common networks (Simard & Durall, 2004). Both plants and AMF have been shown to select partners in one-on-one studies, preferentially allocating resources to achieve mutualism or commensalism (Werner & Kiers, 2015) but exchange becomes more complex in multi-partner networks. Walder et al., (2012) found that when grown together, *G. mosseae* delivered double the amount of P to flax as *G. intraradices* did, but both fungi delivered the same amount of P to sorghum. All while sorghum delivered the majority of carbon to the network.

It is difficult to link specific plant or fungal identities and traits to specific growth outcomes. Fungal morphology and growth traits like hyphal volume and spore production, are highly conserved at the family level, and symbiotic mechanisms are highly conserved in plants as discussed earlier. None the less, evidence from 456 unique plant-AMF pairs in the mycoDB suggest that recent divergences in AMF genera and speciation in plants are a larger source of growth response variation than earlier phylogenetic splits (Hoeksema et al., 2018). Genetic diversity within an AMF species can affect plant growth outcomes. AMF show tremendous genetic diversity on an individual level as described above. Koch et al. (2017) observed that 60-70% of plant biomass variation in a study on three host species inoculated with 56 AM fungal isolates was explained by isolate identity regardless of AMF species.

Therefore, the plant-AMF relationship is highly context dependant. We know that AMF interact with abiotic stress responses but do not know how to predict the outcomes, or how responses are determined by environment because conclusions about the main symbiotic drivers that have been studied in isolation can not be generalized. Nor do we know which plant functional traits are especially important for determining symbiotic outcomes.

3. THE ABIOTIC STRESS RESPONSES OF ALFALFA CULTIVARS CAN BE DIFFERENTIALLY INFLUENCED BY AMF

3.1. Preface

In most cases AMF are thought to reduce stress, however, actual plant stress responses are difficult to predict due to variation in the plant-AMF relationships caused by genetic variation between plant cultivars, as well as interactions with different external stresses. Research into utilizing AMF and better understanding plant-AMF relationships depends on understanding this relationship variation. As such, in this chapter we set out to measure how much variation in stress responses existed between alfalfa cultivars exposed to common stressors when grown with or without AMF, as well as the effects of stress and AMF on pollination, flower, and seed production with the goal of connecting cultivar attributes to growth outcomes.

3.2. Abstract

Arbuscular mycorrhizal fungi are important soil microbes that have the potential to improve plant fitness and success. Partnerships with mycorrhizae have been shown to improve plant resilience to stress by increasing access to and uptake of essential nutrients and water, as well as regulating the plant stress response. The magnitude and direction of AMF effects depends on multiple factors including plant identity and environmental context. Though plant-AMF relationships are common, we do not have a clear understanding of why AMF effects can differ widely, even within a single plant species. The objective of this chapter was to investigate how AMF interact with the abiotic stress response of alfalfa, and if this relationship depends on cultivar identity. We measured the effects of low nutrients, drought, and saline soil on biomass, pollinator visitation and reproductive capabilities of alfalfa cultivars grown with or without AMF compared with unstressed alfalfa in a fully factorial study design. We found that the effects of AMF depended on stress type and cultivar, as hypothesized. Inoculation uniformly worsened drought stress and selectively worsened salt stress effects on alfalfa's aboveground biomass among cultivars. Both AMF and stress type had variable effects on alfalfa flower number depending on the cultivar, but only drought treated plants differed in seed production when inoculated. We did not find a clear pattern distinguishing why certain stressed cultivars were more affected by AMF than others, possibly because many cultivars did not have distinct growth parameters or stress responses from each other, however, our results show that AMF does alter plant responses to stress, contingent on stress type.

3.3. Introduction

Plant-AMF symbioses can be beneficial to plants growing in less than ideal environments by providing nutrients, maintaining water use efficiency, and stabilizing soil structure (Smith et al., 2010). Understanding plant-AMF relationships, and their interactions with the environment and other organisms can let us utilize an effective soil microbiome to manage and restore land as well as improve sustainable crop management practices (Ray et al., 2020). The past decades of research have revealed that the mycorrhizal symbiosis is complex. Resource exchange is an important factor in many frameworks that model plant-AMF systems (e.g. mutualist to parasitism spectrum in Johnson et al. (2015)), but it is clear that we can not ascribe plant responses purely to a phosphorus-carbon exchange paradigm (Delavaux et al., 2017). Plant-AMF

relationships are highly context dependant at the community and individual level, with the responses of host plants depending on environmental conditions (Begum et al., 2019) as well as varying among species and cultivars (Chaudhary et al., 2016).

Mycorrhizal responses often vary based on environmental conditions and the local soil quality. Environmental stressors can alter the response of plants to AMF. Mycorrhizae may be selectively beneficial depending on stressor type. AMF that successfully mitigate stress may not benefit unstressed plants (Porcel & Ruiz-lozano, 2004). Additionally, the potential benefit of a given symbiont may not be evident in a high-quality environment (Thrall et al., 2007). Susceptibility of AMF to biotic or abiotic stressors is becoming more well documented (Branco et al., 2022). AMF appear to have variable tolerance to disturbances (van der Heyde et al., 2017), but the effects of stressed AMF on plants is unclear.

Plants adapted to local soil can also benefit more from AMF, even when the fungi are non-local. AMF adapted to phosphorus limited soil produced more arbuscules in their home soil when hosted by local plants, and up to 87% more extraradical mycelia when colonizing local or exotic plants compared to when the AMF were grown in phosphorus-enriched, exotic soil. The increased fungal growth correlated with increased uptake of nutrients for the plants, and often higher shoot growth in these P limited soils (Johnson et al., 2010). Additionally, Rúa et al. (2016) showed that local plants had a positive biomass response to growing with sympatric fungi compared to when plant, fungi, and soil were allopatric.

Differences in AMF responses among plant species and cultivars are caused by more than a lack of adaption between plant and fungi (e.g. Klironomos, 2003; Tawaraya, 2003; Wilson & Hartnett, 1998). We find examples of inter- and intraspecific variation in many aspects of plant life such as biomass accumulation, nutrient uptake, competitive ability, or reproduction of plants inoculated with AMF in both natural and agricultural situations (Bryla & Koide, 1990; de Souza Campos et al., 2021; Stanescu & Maherali, 2017), however why certain plant species or cultivars perform better or worse is not entirely clear. The range of AMF responses occurs partially as an effect of natural individual variation in plants, but artificial selection can disrupt mycorrhizal symbiosis as well as the mechanisms of natural selection, genetic drift, etc.

Crop breeding trade offs occur when selection for agriculturally favoured traits like high yield or fast growth overwhelms or is in opposition to selection for pro-symbiosis traits. Also, relaxed

selection on traits important to successful symbiosis allows the proliferation of anti-symbiosis traits (Porter & Sachs, 2020). For example, years of cotton breeding with high nutrient input produced plants reliant on high P fertilizer instead of being able to benefit from AMF (Wang et al., 2023). Research into plant selection with a focus on plant-microbe symbiosis is relatively new compared to mycology and agricultural breeding as a whole. Arbuscular mycorrhiza were not thought to be potentially beneficial until the mid 1900's (Koide & Mosse, 2004); interest in their potential grew quickly (Ruehle & Marx, 1979), but despite many calls to integrate knowledge of AMF into plant breeding since (e.g. Hooker & Black, 1995; Ryan & Graham, 2002), there are still many gaps to fill to understand symbiotic variation in domesticated plants.

To properly utilize AMF, it is important to understand all the interactions occurring in the greater environmental context of the plant-AMF relationship. Studies of mycorrhizal effects tend to target one or two cultivars and are often focused on unstressed plants or a single stress treatment. Our objective was to target the context dependency and intraspecific variation of mycorrhizal effects using alfalfa (*Medicago sativa*), a common and economically important forage legume as the study species. Alfalfa is generally responsive to AMF; inoculated alfalfa have shown increased resistance to abiotic (Shi-chu et al., 2019) and biotic stressors,(Li et al., 2019), as well as improved P uptake (Püschel et al., 2017), but responses are not always positive: Püschel et al. found no change in alfalfa biomass even with improved P uptake. Additionally alfalfa showed reduced vigour and seedling growth when grown with *Glomus monosporus* (O'Bannon et al., 1980). Alfalfa yield has not increased over the years as much as other crops (Annicchiarico et al., 2015; Lamb et al., 2006). There are an abundance of alfalfa cultivars in existence as breeders attempt to adapt alfalfa to local growing conditions and needs, but it may be important to incorporate new research on AMF and the greater soil microbiome into the selection process going forward (Cobb et al., 2021).

In this study we investigated how nine modern alfalfa cultivars differed in their response to three environmental stresses (drought, nutrient limitation, and high salinity) and inoculation with *Rhizophagus irregularis*. We selected cultivars that we expect to vary in stress resistance, growth and physiology in ways that may interact with their ability to gain benefit from mycorrhizal symbiosis.

We hypothesize:

(1) AMF will improve alfalfa responses to experimental stresses.
(2) The effect of AMF on stress responses will vary between alfalfa cultivars depending on the stress type.
(3) AMF will increase alfalfa seed production by improving nutrient uptake, flower production, and pollinator visitation.

3.4. Methods

3.4.1. Experimental design and set up

We selected alfalfa cultivars from the three main commercial seed suppliers for western Canada: BrettYoung (Manitoba), DLF-Pickseed (Saskatchewan), and Northstar seeds (Manitoba), with the aim to maximize the trait diversity based on trait descriptions, fall dormancy, and winter hardiness ratings as reported in the seed catalogs from each respective seed supplier in 2020 (Table 3.1). One additional salt-tolerant cultivar (AC-Bridgeview) was sourced from Agriculture and Agri-food Canada. Most cultivars suitable for Canadian agriculture have a fall dormancy rating below 4. This means that the plants slow growth, metabolic activity, and increase carbohydrate storage near the end of the growing season. Fall dormancy is linked to better winter hardiness, though there are additional mechanisms that support winter survival (Claessens et al., 2022). Some descriptions of cultivar characteristics overlap between cultivars and suppliers, and these characteristics were not independently verified, but we believe this list is a good representation of the alfalfa types available in 2020.

Table 3.1. The alfalfa cultivars selected for this experiment with their attributes as listed on producer literature in 2020.

Cultivar	Producer	Characteristics	Fall dormancy	Winter hardiness
2010	BrettYoung	• Branched roots • Suited to dry soil	2	2
3010	BrettYoung	• Disease resistant • Deep crown	2	2
AC-Bridgeview	Agriculture and Agri-Food Canada	• Salt tolerant • Deep crown	NA	NA
Assalt	DLF pickseed	• Tolerant to high pH • High multifoliate expression • Disease resistant	4	1.5
Foothold	BrettYoung	• Spreader root type • High leaf/stem ratio • High multifoliate expression • High yield	2	1.7
Perfection	Northstar seed	• Fast growth • High digestibility • High dry matter production	4	2
Rugged	Northstar seed	• Deep set crown • Stress resistant • Salt tolerant	3	2
TH2	Northstar seed	• High multifoliate expression • Disease resistant	3	3
Vision	DLF pickseed	• High multifoliate expression • Tall • Fast regrowth	4	1.5

Growth conditions and locations

We completed two different growth trials. The first main trial took place in the University of Saskatchewan Agriculture greenhouses (45 Innovation Blvd., Saskatoon, SK). The second one was a scaled down version in a University of Saskatchewan phytotron growth chamber. We performed this second, smaller trial to check the consistency of the growth results due to unforeseen additional stresses caused by insect infestations and pesticide applications in the greenhouse.

Greenhouse trial:

This experiment consisted of a fully factorial combination of two AMF treatments (with or without AMF inoculation), on nine cultivars subjected to three stress treatments (plus one unstressed control), replicated five times (n=360). Planting began on June 22nd, 2020. Planting was staggered over the next three weeks, with two full replicates planted per week. All plants were grown for four months to complete a growth cycle from seedling to seed pod production. Harvest began on October 23rd, staggered so that each replicate had an equal growth period, with the final replicate harvested on November 22nd, 2020. Daylength and temperature fluctuated over the season; natural sunlight was supplemented with sodium halogen lamps keeping a minimum of 15h daylight. Daytime maximum temperature was 26 °C throughout most of the growth period except August that peaked at an average of 30°C, the maximum temperature recorded in a day was 33 °C. Minimum nighttime temperature was 18°C throughout. These temperatures are within normal Saskatchewan climate parameters.

We mixed soil from equal parts (by volume) screened topsoil, sand, and sphagnum peat moss. The soil was moistened then baked at 150°C for 4 hours in a drying oven, twice, ensuring that the internal temperature reached at least 120 °C to sterilize. The commercially sourced seeds had a manufacturer-applied coating. These coats typically contain layers of rhizobia, fungicide, and fertilizers, but the precise makeup of the seed coating is proprietary. We removed this coating to isolate the effects of our selected AMF inoculant from any enhancement from the coatings, or damage from the fungicides. To dissolve this seed coating, we soaked the seeds in water, then 70% EtOH for one minute each, then sterilized the seeds in 5% hypochlorite for five minutes. We rinsed the seeds well then coated with *Sinorhizobium meliloti* inoculant (Exceed® alfalfa and true clover inoculant , Visjon Biologics) immediately prior to planting.

Three alfalfa seeds were planted for each sample in half gallon pots filled with 2L of the soil mix. Half the pots were inoculated with 0.04g of AGTIV® forages powder (PremierTech ; 8000 spores/g of *Rhizophagus irregularis*), approximately 320 spores per pot, as per the recommended rate of application from the manufacturer. We planted inoculated alfalfa on alternate days to uninoculated alfalfa to minimize the risk of cross contamination in addition to sterilizing tools with 70% etOH between planting sessions. Pots were thinned to two seedlings a week after they sprouted.

We applied three stress treatments: drought, low nutrients, and high salinity, beginning 40 days after seedlings sprouted to allow them to become well established before stress treatments began. After initial establishment, all plants except the drought treatment group were watered to saturation three times a week, the watering interval increased to every other day as the plants increased in size, and the daily greenhouse temperature increased. All plants except the low nutrient treated plants were fertilized weekly with 250mL of half strength Hoagland's solution (composition: 0.5mM KH_2PO_4, 2.5mM KNO_3, 2.5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1mM $MgSO_4$, 0.066mM FeEDTA, 23µM H_3BO_3, 4.5µM $MnCl_2$, 0.16µM $CuSO_4$, 0.04µM Na_2MoO_4, 0.38µM $ZnSO_3$ and DI water). Drought was simulated using a reduced watering frequency regime. Plants in the drought treatment were watered to saturation as with unstressed plants, but only once a week increasing to every five days as the plants grew. Similarly, plants subjected to nutrient restriction had a reduced fertilization schedule compared to unstressed controls. They were fertilized every three weeks with 250mL of half strength Hoagland's solution or 250mL of water on weeks they were not fertilized to keep water intake consistent. We applied NaCl to plants in the high salinity treatment group dissolved in the weekly fertilizer. The salt treatment began at 20mM NaCl, increasing by 40mM each week (to prevent shock) to a final to a final concentration of 140mM.

Pest control:

Thrips, aphid, and spider mite infestations occurred periodically during the trial. We used *Amblyseius cucumeris* mites (Biobest® ABS-Mini sachet) throughout the growing period to control thrips. Near the end of the growing period (September 28th and October 2nd) all plants were sprayed with a *Beauveria bassiana* based biological insecticide (Botanitguard, BioWorks®), and a pymetrozine based insecticide (Endeavor, Syngenta®) to control an aphid and spidermite outbreak. No plants were lost to insect infestation, but there was some dieback. There was no clear pattern of insect damage between treatments, so the dead shoot biomass was clipped, but not included in the final biomass at harvest.

Growth chamber trial:

We reduced the number of cultivars to three (Foothold, Rugged, TH2), picking cultivars that seemed to have unique growth responses in preliminary data analysis. We reduced stress treatments to two (drought, high salinity), removing the low nutrient stress from the trial as

preliminary results from the greenhouse suggested that fertilization had not been reduced enough to adequately stress the plants. This trial was conducted in the same way as the first except that plants per pot were reduced to one and the growth period was reduced to three months. Harvesting and biomass data collection proceeded as with the greenhouse trial. No aphids or spidermites were seen in this trial. Thrips were kept in check with *A. cucumeris* mites, and sticky cards placed in the pots.

3.4.2. Data collection

Pre-harvest:

During the growth period we measured plant height, and the number of non-tripped, unwilted flowering stems present on each plant weekly for seven weeks 10 days after treatments began. In October we performed an observational study of bumble bee (*Bombus impatiens*) visitation rates over four days (October 11th, 15th,17th and 21st , 2020). The bees were acquired from Biobest sustainable crop management. Bees were acclimated to the greenhouse environment in a cardboard habitat box provided by Biobest for two days prior to use, during this time they had access to a liquid food source. For each observation trial we selected inoculated and uninoculated pairs of alfalfa in the same cultivar and treatment group. We picked as many pairs as were actively flowering at the time of the trial (3-9 pairs), and plants were not reused once used in a trial. Groups of inoculated or uninoculated alfalfa were set about 2 meters apart on a metal work bench in the greenhouse chamber. We grouped inoculation treatments together regardless of stress treatment to maximize any potential patch level signal related to inoculation. To initiate a trial, bees were allowed to exit their box and were given free access to the alfalfa, simulating a natural environment. We observed and recorded each visit (defined here as landing on a flower for longer than 5 seconds) over the course of two hours. At the end of the observation period the opening on the habitat box was swapped so bees could return home but not exit again until the next trial. Because we did not track individual bees or keep them naive of their surroundings, we can not assume that bee choice was not affected by pretrial learning. After the end of the final trial, bees remained in the greenhouse chamber and were allowed to enter or exit their habitat at will to facilitate alfalfa pollination. Seed pods had been observed on some plants prior to this point in the experiment but had not been checked for seeds; it is possible that pollination was also occurring by other means.

Post-harvest:

All plants were harvested after four months of growth. At the time of harvest, we removed and dried seed pods for later seed extraction. Alfalfa shoot mass was clipped, then dried for 48h at ~70°C. After drying, leaves and stems were separated and weighed. All roots were washed, dried for 48h at ~70° C, then weighed, except for small subsamples (approximately 1g) for AMF colonization which we stored in 70% EtOH prior to processing.

To measure the amount of root colonization we used a root staining method modified from Vierheilig et al. (1998): roots were heated to 90°C in beaker of 10% w/v KOH solution for 30 minutes, followed by 2% v/v HCl solution for a further 15 minutes. The heat was reduced to 80°C, then the roots were stained in a dye composed of 5% v/v black ink and 5% v/v acetic acid for 15 minutes. Roots were well rinsed in DI water between each step. Finally, the roots were stored in a mixture of equal parts glycerol, 5% acetic acid, and DI water for a minimum of two days to remove excess ink. We mounted 25cm of root tissue on glass slides with Permount™ (Fisher scientific) for colonization analysis. We measured the amount of root colonization by picking 100 random intercepts along the root length and noting if any hyphae, arbuscules, or vesicles were present (modified from McGonigle et al., 1990).

We quantified the nitrogen, phosphorous, and sodium content in leaves from six of the nine cultivars (3010, AC-Bridgeview, Foothold, Rugged, TH2, Vision) across all treatment combinations (n=232). We excluded Assalt, Perfection, and 2010 as they had similar growth responses to other cultivars according to preliminary results. Dried leaf tissue was finely ground then 0.15 ± 0.01g was digested in sulphuric acid as in Lindner (1944). Nitrogen and phosphorous concentrations were measured colorimetrically with an AA3 Segmented flow analyzer (SEAL analytical). Sodium levels in the same samples were measured by atomic absorption spectrometry with a 200 Series AA systems analyzer (Agilent).

We measured the final electroconductivity (EC) of soil collected after plants had been harvested to see the extent of soil salinization and determine if AMF affected soil EC. Soil from each AMF and treatment group combination within a replicate was pooled together (n=40). We mixed 250mL of each soil sample with 500mL of DI water, stirring for three minutes. The aqueous solution was then filtered off for electroconductivity measurement.

3.4.3. Statistical analysis

Prior to analysis, 11 plants were removed due to labeling errors leaving 349 individuals. All analysis was done in R version 3.6.3 with mixed models conducted with the package lme4 (Bates et al., 2015) unless otherwise noted. We checked model homoscedasticity via Levene's test in the Car package (Fox & Weisberg, 2019). Model residuals were checked visually using the package DHARMa (Hartig, 2022), and using a Shapiro-Wilks test of normality in base R. We ran an ANOVA for each model using the lmerTest package (Kuznetsova et al., 2017) unless otherwise noted. When predictors were found to have significant or marginally significant ($p<0.1$) effects in the above models we used emmeans or emtrends (depending on if continuous variables were significant or not) to perform post hoc, pairwise tests with the Tukey method for adjusting p values (Lenth, 2022). Figures for each model were created using Interact plot from the interactions package (Long, 2019) or afex plot from the afex package (Singman et al., 2021).

We calculated the biomass and nutrient stress response for each plant in the three stress treatments as the biomass or nutrient concentration of a stressed plant divided by the corresponding value in an unstressed plant within the same replicate block (Eq. 3.1) for both the greenhouse and growth chamber trials.

$$\text{stress response} = \frac{\text{stressed value}}{\text{unstressed value}} \qquad (3.1)$$

We determined the relationships between biomass stress response and mycorrhizal inoculation in different treatment conditions by creating six separate mixed models, each testing the interaction of cultivar and AMF inoculation on either shoot or root stress in one of the three stress treatments. Each model had replicate as a random effect to control for temporal differences in planting and harvest time. We used this same model set up for testing biomass stress in the growth chamber trial, and N and P stress response models, with the addition of random effect to control for separate acid digestion batches. All stress responses were log transformed to satisfy to requirement for normally distributed residuals.

The rate at which alfalfa increased in height was linear across the first 5 weeks of measurements before plateauing across weeks 6 and 7. Therefore we modeled height as a response to time across the first 5 weeks to determine how AMF affected growth over time, and how cultivars

may differ in growth rate using a separate model for each treatment condition, with an individual designation as a random factor to account for repeated measures.

We modeled the number of flowers at three time points in relation to peak flowering time: early, mid peak and late peak. These time points are two weeks apart which minimized the number of flowers that may be double counted from time point to time point. Flower number was modeled using four Poisson generalized linear models (one per treatment) with AMF, cultivar, and time point as interacting fixed effects and block and plant ID (to account for repeated measures) as random effects.

Bumble bee visitation rate was calculated as visits per flowering stem per plant rather than the raw number of visits to control for the increased attractiveness of a higher number of flowers. We used the interaction of AMF and stress treatment here because we did not sample enough cultivars to test our main question regarding the interaction of cultivar and AMF. Visitation rate was square root transformed.

Out of 349 plants, 194 produced seeds. Foothold did not produce any seeds in the low nutrient or drought treatments, nor did Asalt plants in the unstressed, uninoculated treatment. With just over 55% of the plants setting seed, there was not enough statistical power to test if cultivar and AMF interacted to affect the likelihood of producing seed. The uneven distribution of seed producing plants between treatment groups caused convergence errors. We attempted to test the effect of AMF or cultivar on the likelihood of seed production by constructing three binomial generalized mixed models per stress treatment group using either AMF, cultivar, or both as predictor variables, with a binary response variable to represent if a plant set seed or not. We compared the three models to a null model via likelihood ratio test. The coefficient of each parameter in all tested models were equal to zero, and none were statistically different to an intercept only model. Therefore, we only modeled the total mass of seeds produced by plants that set seed. We calculated the mass of produced seeds per total gram of plant mass to control for higher seed production in larger plants, the resulting seed mass was square root transformed to conform with assumptions of normality. Seed mass was modeled with four linear mixed models (one per treatment). Asalt was excluded from the unstressed model and Foothold was excluded from the drought stress model because their lack of seed production prevented models from converging.

3.5. Results

3.5.1. Stress response of alfalfa cultivars

Mean colonization was above zero for all inoculated cultivars in all treatment groups (Fig. 3.1). There were no indications of colonization in uninoculated plants. Total colonization differed between cultivars depending on stress treatment ($\chi 2 = 493$, $p = 2^{-16}$).

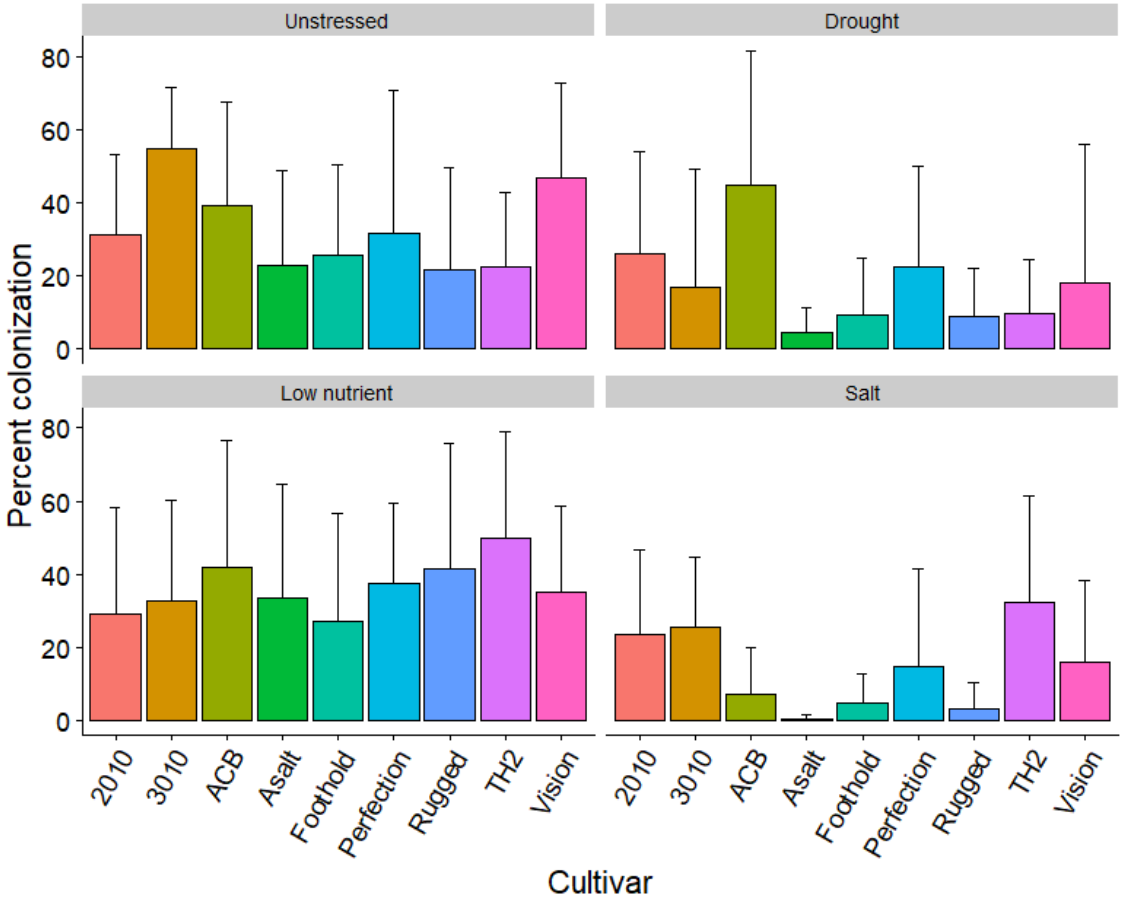

Figure 3.1. The mean percent colonization of each cultivar in each treatment group. Error bars represent the standard deviation of the mean across five replicates.

Cultivar level variations in stress response were less explicit than we expected and not all alfalfa response parameters responded to all stress treatments. The low nutrient treatment, for example, did not induce a shoot or root biomass, nitrogen (N), or phosphorus (P) stress response in any cultivar (Tables 3.2, 3.3). Due to this lack off effect, results from this treatment will not be presented. Drought stress had a greater effect: all drought-treated cultivars shared an equally

negative biomass stress response (i.e. stressed plants were smaller: stress response ratio of 0.77± 0.05 in shoot and 0.66± 0.05 in root), but N and P levels did not respond to drought in any cultivar. The salt stress response of shoots did differ between cultivars: AC-Bridgeview, Perfection, and TH2 responded negatively, unlike the other six cultivars that had more neutral stress responses. Salt treatment incurred a negative N stress response across all cultivars (0.69± 0.04), but P concentration did not respond to salt stress.

3.5.2. Mycorrhizal modulation of alfalfa stress response

Counter to our first hypothesis, when AMF inoculation interacted with alfalfa stress responses it increased stress. Inoculated alfalfa were more stressed by drought treatments than uninoculated alfalfa when measuring stress in shoot biomass, N, and P ($p=0.024$, 0.013, 0.025 respectively, Fig 3.2, 3.3, 3.4). For mean biomass and nutrient concentrations of all cultivars see appendix A and B). Our second hypothesis depended on the assumption that the alfalfa cultivars would differ in their inherent stress responses or their ability to synergize with AMF. Under drought all cultivars were equally affected by the stress, and AMF, with no interactions in any parameter (Tables 3.2, 3.3), however there was a significant interaction of cultivar and AMF on shoot stress response in the salt treatment (Table 3.2). Two of the nine cultivars (Asalt and Foothold) had a negative stress response when grown with AMF but were not stressed when grown alone ($p=0.004$, and 0.030 respectively), no other cultivars experienced a change in stress response when inoculated. These cultivar level differences were not seen when testing the effects of stress and AMF on nutrients: across all tested cultivars the N and P salt stress responses were worsened in the AMF group ($p=0.058$, and 0.007 respectively). Rather than inoculation inducing a negative N response in drought treated plants and a negative P response in salt stressed plants (Figures 3.3, 3.4), however, the change in the response ratio is being driven by marginal increases in nutrients in the unstressed AMF inoculated plants.

Table 3.2. Summary of the ANOVA results for testing the effects of stress treatments and AMF inoculation treatments on alfalfa cultivar's biomass. Results separated by a double line are from separate models.

	Treatment	**Model terms**	**DF**	**F**	**p**
Shoot	Drought	**AMF**	**1**	**5.363**	**0.023**
		Cultivar	8	0.914	0.511
		AMF: Cultivar	8	0.863	0.552
	Low nutrients	AMF	1	0.910	0.343
		Cultivar	8	0.413	0.909
		AMF:Cultivar	8	0.258	0.977
	Salt	AMF	1	1.396	0.242
		Cultivar	8	1.543	0.160
		AMF:Cultivar	**8**	**2.184**	**0.040**
Root	Drought	AMF	1	0.919	0.341
		Cultivar	8	0.932	0.496
		AMF:Cultivar	8	1.487	0.178
	Low nutrients	AMF	1	2.666	0.107
		Cultivar	8	1.264	0.277
		AMF:Cultivar	8	1.253	0.283
	Salt	AMF	1	0.265	0.609
		Cultivar	8	1.048	0.410
		AMF:Cultivar	8	0.854	0.559

Table 3.3. ANOVA summary table of for models of the stress response in nitrogen and phosphorus concentrations. Results separated by a double line are from separate models.

Nutrient	Treatment	Model terms	DF	F	p
Nitrogen	Drought	**AMF**	**1**	**6.828**	**0.012**
		Cultivar	5	0.597	0.703
		AMF:Cultivar	5	0.455	0.808
	Low nutrients	AMF	1	0.059	0.810
		Cultivar	5	0.944	0.463
		AMF:Cultivar	5	0.471	0.796
	Salt	**AMF**	**1**	**3.799**	**0.058**
		Cultivar	5	0.324	0.896
		AMF:Cultivar	5	0.612	0.692
Phosphorus	Drought	**AMF**	**1**	**5.493**	**0.024**
		Cultivar	5	0.168	0.973
		AMF:Cultivar	5	0.289	0.916
	Low nutrients	AMF	1	0.062	0.804
		Cultivar	5	0.950	0.459
		AMF:Cultivar	5	1.524	0.202
	Salt	**AMF**	**1**	**7.962**	**0.007**
		Cultivar	5	0.151	0.979
		AMF:Cultivar	5	0.424	0.829

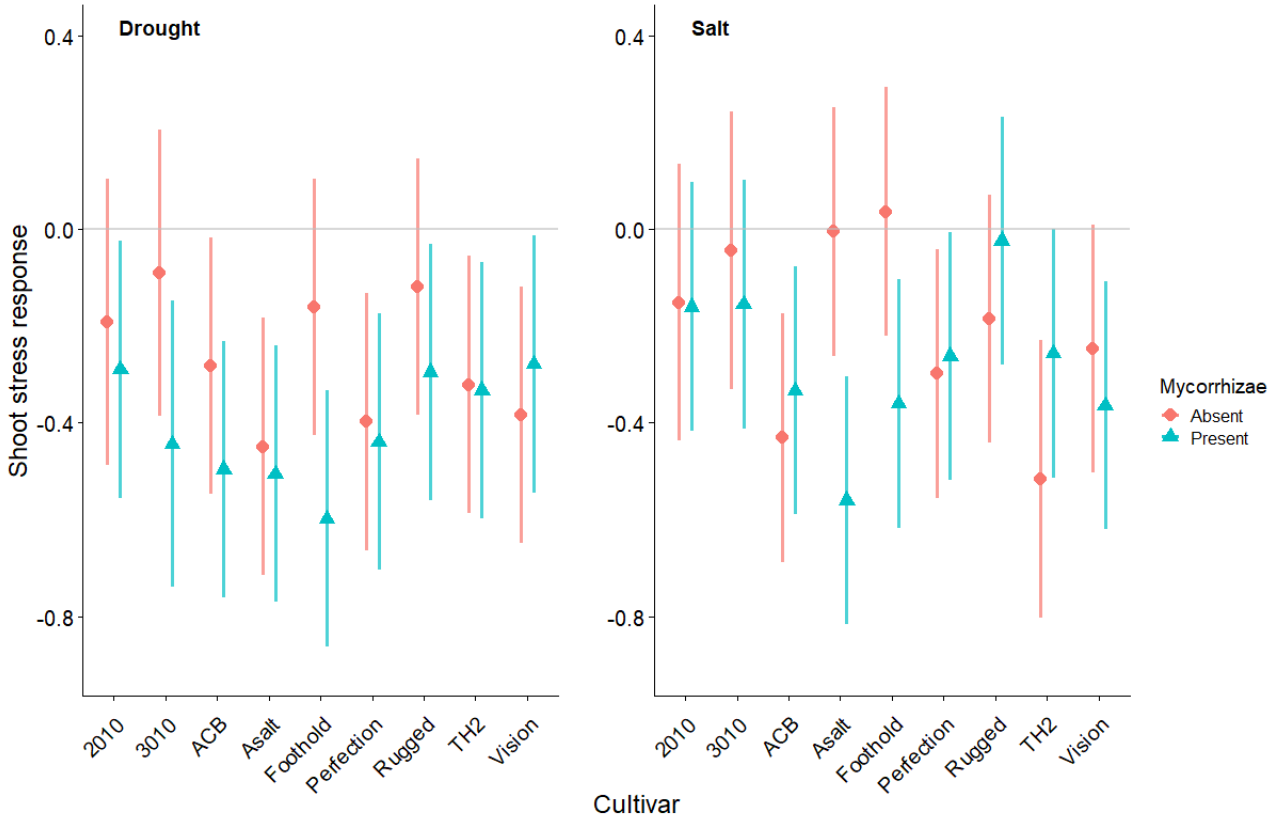

Figure 3.2. The stress response of alfalfa shoot biomass under drought (left) or salt stress (right). Stress responses below zero (the gray line) indicate that plants in the stressed treatment were smaller than their control counterparts. Pink circles represent the mean stress responses of uninoculated alfalfa, whereas blue triangles represent the mean stress response of AMF inoculated alfalfa. Bars represent the 95% confidence intervals.

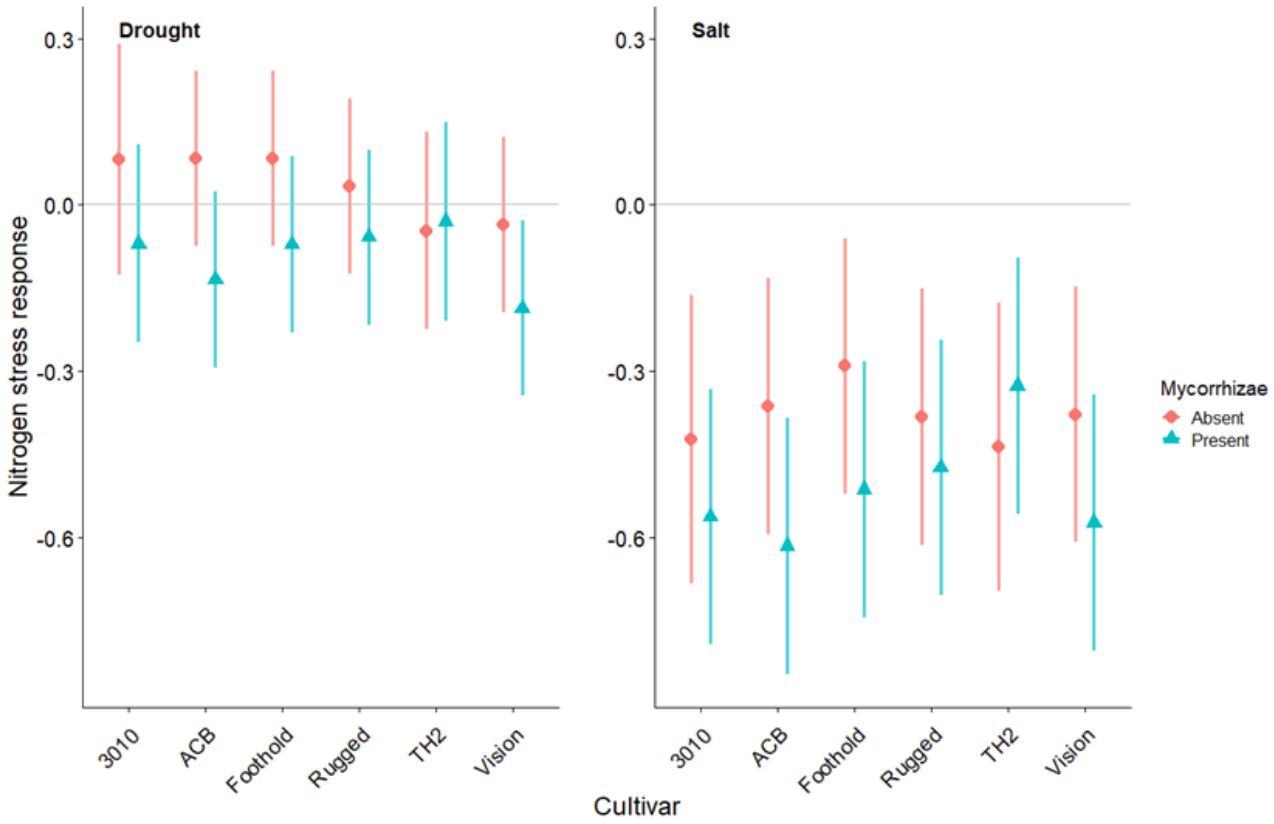

Figure 3.3. The nitrogen stress response in alfalfa leaves under drought (left) or salt stress (right). Stress responses below zero (the gray line) indicate that plants in the stressed treatment had lower N levels than their control counterparts. Pink circles represent the mean stress responses of uninoculated alfalfa, whereas blue triangles represent the mean stress response of AMF inoculated alfalfa. Bars represent the 95% confidence intervals.

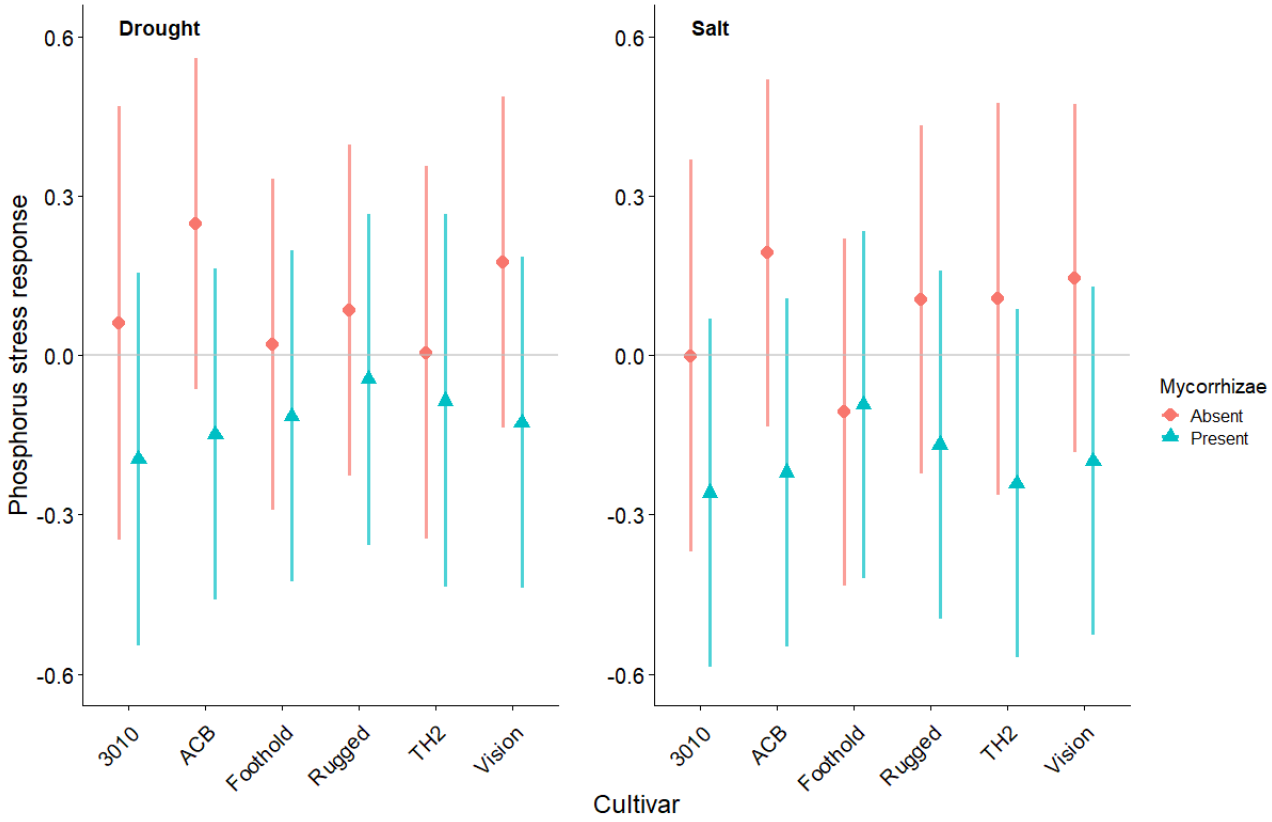

Figure 3.4. The Phosphorus stress response in alfalfa leaves under drought (left) or salt stress (right). Stress responses below zero (the gray line) indicate that plants in the stressed treatment had lower P levels than their control counterparts. Pink circles represent the mean stress responses of uninoculated alfalfa, whereas blue triangles represent the mean stress response of AMF inoculated alfalfa. Bars represent the 95% confidence intervals.

3.5.3. Salt accumulation in plant tissue and soil

Sodium accumulation in leaf tissue did not fully explain the observed differences in stress responses between cultivars or the interactions with AMF. Cultivars differed marginally in their accumulation of sodium (Table 3.4, $F_{(5,42)}$=2.21, p=0.071), but there was no effect of AMF on sodium content ($F_{(1,42)}$=0.002, p=0.967). Interestingly we found that sodium content negatively correlated with shoot stress response only in inoculated plants (p=0.001), there was no relationship between sodium and stress response in uninoculated plants (p=0.111), and these trends did not differ among cultivars.

AMF inoculation changed the final amount of salt in the soil of salt stressed plants ($F_{(1,15)}$=4.85,p=0.044). In the salinity treatment soil EC was lowered by ~3 dS from 9.47±2.32 dS

in uninoculated pots to 6.36 ±1.79 dS in inoculated pots (p=0.007). There was no difference between inoculation treatments of soil EC in the unstressed treatment (p=0.780).

Table 3.4. The sodium concentration in the leaf tissue of six alfalfa cultivars subjected to salt stress.

Cultivar	Mycorrhiza	Na (mg/g)
3010	Absent	9.16±5.1
	Present	16.46±7.8
ACB	Absent	16.31±7.7
	Present	13.09±6.2
Foothold	Absent	16.4±7.7
	Present	21.57±10.1
Rugged	Absent	11.11±5.3
	Present	10.8±5.1
TH2	Absent	18.14±9.9
	Present	16.43±7.8
Vision	Absent	17.57±8.3
	Present	10.82±5.1

3.5.4. Growth rate of alfalfa

Alfalfa shoot growth was inhibited by the presence of mycorrhizae when the plants were drought stressed. The mean height of alfalfa was lower in inoculated drought-stressed plants than in uninoculated ones in four of the nine cultivars (Fig. 3.6), but there was no difference in mean height between inoculation treatments in the salt stressed plants (Fig. 3.7). Drought stress could affect plants faster than salt stress as water supply was reduced all at once but salt levels were gradually increased over time, so it is not surprising to see this contrast. The slope of growth over time was largely unaffected by AMF in both stress treatments (Table 3.5) except in TH2 where inoculation increased growth rate under drought (p=0.004, Fig. 3.6) AMF interacted with growth rate among cultivars when plants were unstressed (Table 3.5): growth was faster when inoculated for 2010, 3010, AC-Bridgeview, Asalt, and TH2 despite no mean difference in height between inoculated and uninoculated plants (Fig. 3.5). These same cultivars (except TH2) had reduced mean heights under drought.

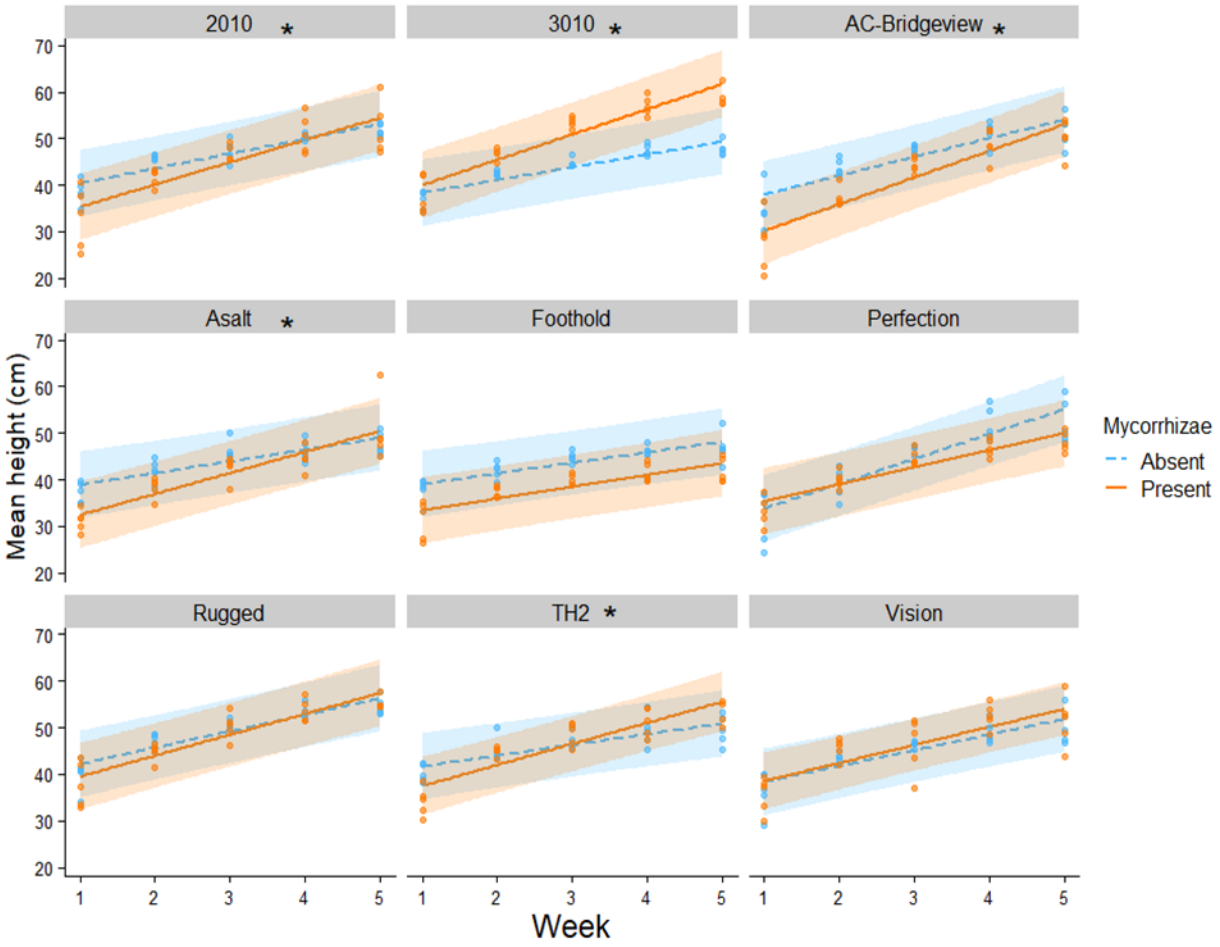

Figure 3.5. Average height of unstressed alfalfa cultivars over five weeks of treatments. Week 1 occurred 10 days post treatment initiation. Inoculated plants are shown with a solid orange line, uninoculated plants are shown with a dashed blue line, with 95% CI around both lines. Dots indicate partial residuals. Cultivars where AMF affected the slope of growth over time are marked with * and have p values as follows: 2010, p=0.030, 3010, p=0.001, AC-Bridgeview, p=0.021, Asalt, p=0.007, TH2, p=0.003.

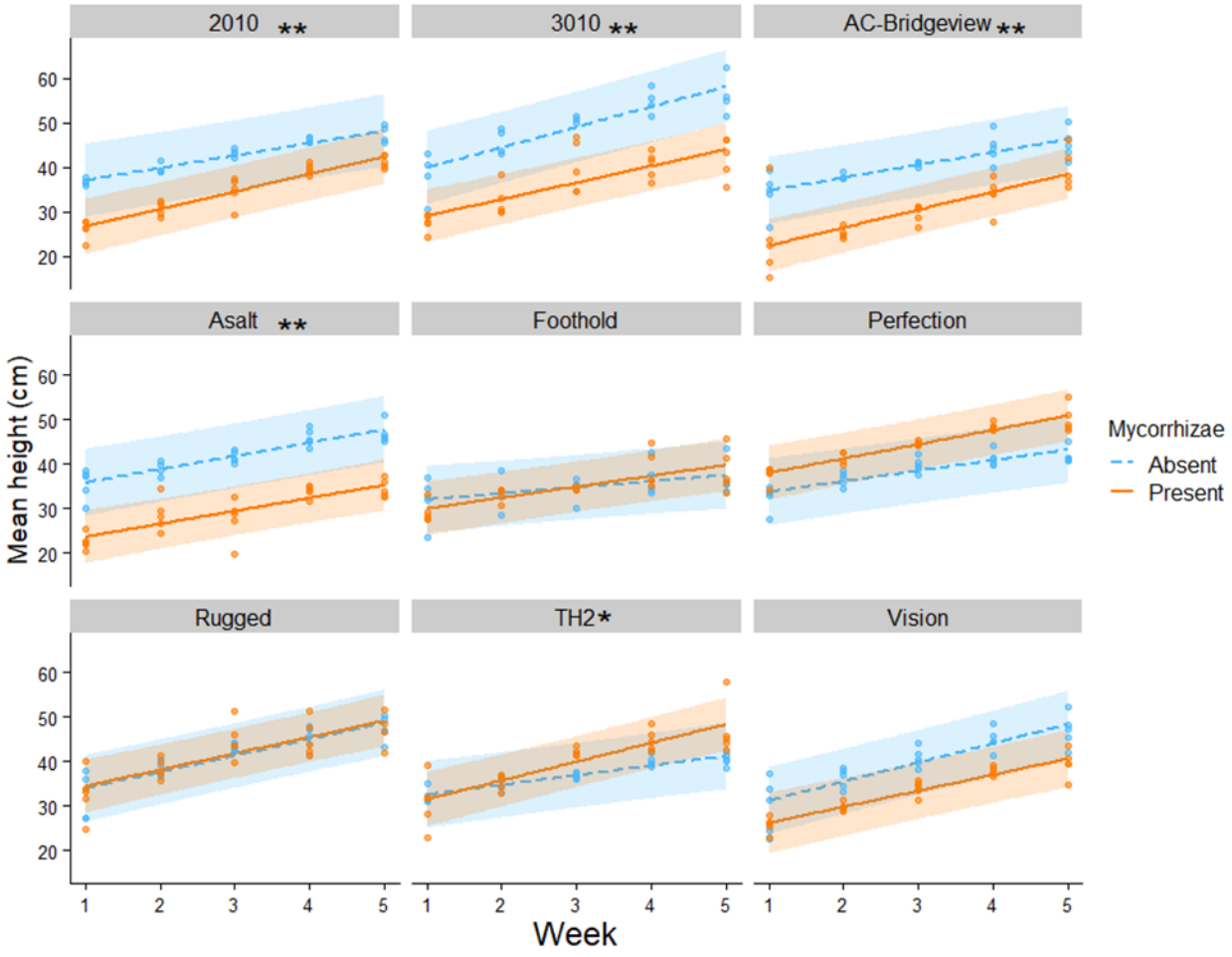

Figure 3.6. Average height of drought stressed alfalfa cultivars over five weeks of treatments. Week 1 occurred 10 days post treatment initiation. Inoculated plants are shown with a solid orange line, uninoculated plants are shown with a dashed blue line, with 95% CI around both lines. Dots indicate partial residuals. Cultivars where AMF affected the slope of growth over time are marked with *, Cultivars where AMF affected mean height marked with ** with p values as follows: 2010, p=0.025, 3010, p=0.016, AC-Bridgeview, p=0.003, Asalt, p=0.003.

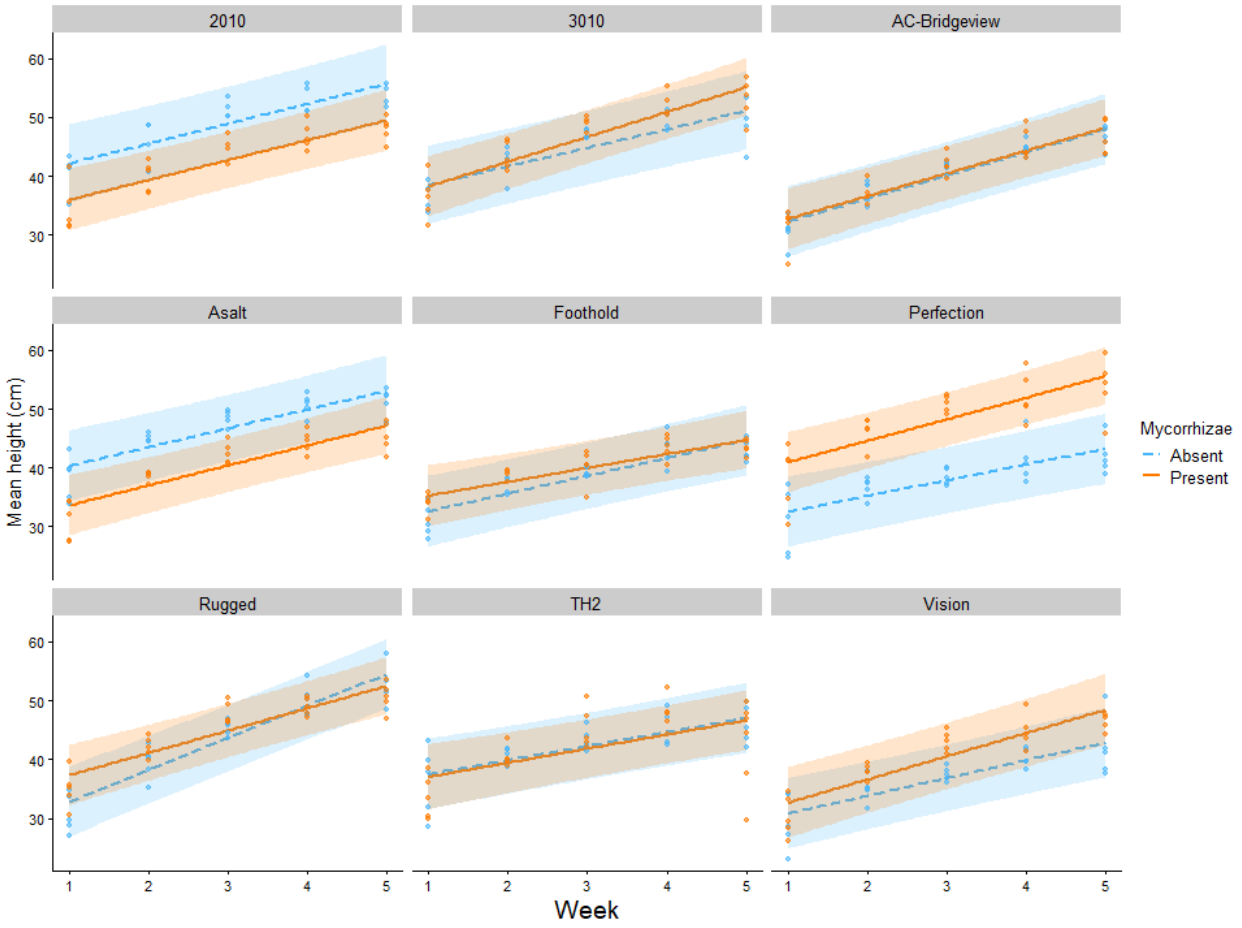

Figure 3.7. Average height of salt stressed alfalfa cultivars over five weeks of treatments. Week 1 occurred 10 days post treatment initiation. Inoculated plants are shown with a solid orange line, uninoculated plants are shown with a dashed blue line, with 95% CI around both lines. Dots indicate partial residuals. AMF had no effect on mean height, or the slope of growth rate over time.

Table 3.5. Summary of ANOVA table for models of alfalfa height in the first five weeks of treatments. Results separated by a double line are from separate models.

Treatment	Model terms	DF	F	p
Unstressed	**AMF**	**1**	**6.011**	**0.016**
	Cultivar	8	0.992	0.447
	Week	**1**	**952.099**	**< 2.2E^{-16}**
	AMF:Cultivar	8	0.654	0.731
	AMF:Week	**1**	**21.003**	**6.50E^{-6}**
	Cultivar:Week	**8**	**3.700**	**3.71E^{-4}**
	AMF:Cultivar:Week	**8**	**3.196**	**0.002**
Drought	AMF	1	12.700	0.001
	Cultivar	8	1.397	0.202
	Week	1	732.468	< 2.2E^{-16}
	AMF:Cultivar	8	1.622	0.123
	AMF:Week	**1**	**4.692**	**0.031**
	Cultivar:Week	**8**	**3.367**	**0.001**
	AMF:Cultivar:Week	8	1.728	0.091
Salt	AMF	1	0.063	0.803
	Cultivar	8	1.675	0.108
	Week	**1**	**762.371**	**< 2.2E^{-16}**
	AMF:Cultivar	8	1.289	0.253
	AMF:Week	1	0.150	0.699
	Cultivar:Week	**8**	**3.148**	**0.002**
	AMF:Cultivar:Week	8	1.368	0.210

3.5.5. Growth chamber check trial

Stress responses were slightly different between trials for drought-stressed plants. There was a marginal interaction between location, inoculation, and cultivar in the shoot and root stress responses (Table 3.6). In the growth chamber trial both inoculated and uninoculated alfalfa were more drought stressed than greenhouse-grown alfalfa. Unlike what we observed in the greenhouse plants, there was no additional stress effect of AMF on the shoots or roots in the growth chamber trial. Finally, uninoculated TH2 had a neutral root stress response in the greenhouse but negative root-stress response in the growth chamber(Fig. 3.8).

As in greenhouse plants, there was an AMF:cultivar interaction in salt stressed alfalfa that did not depend on study location (Table 3.6). Across both studies mycorrhizal inoculation had a (marginally) negative effect on Foothold's stress response ($p=0.057$), and marginally improved TH2's stress response ($p=0.055$, Fig. 3.9).

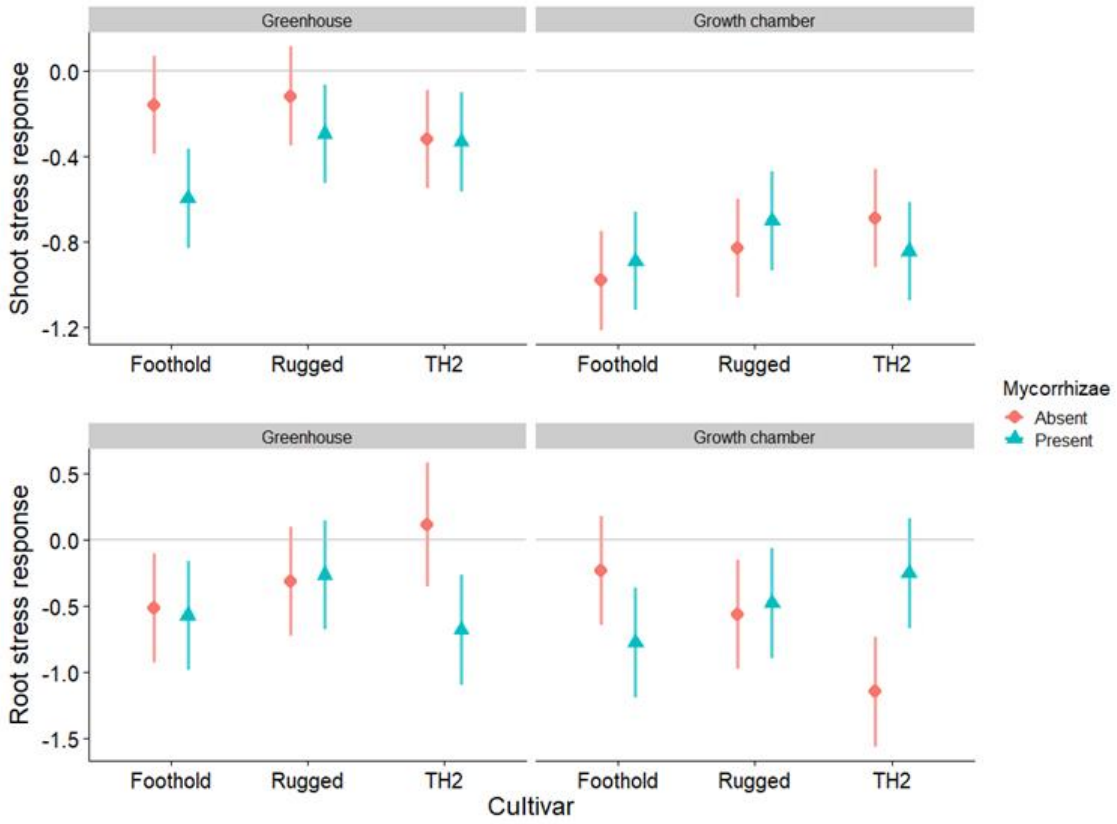

Figure 3.8. The Drought stress response of shoot (top) and root (bottom) biomass of alfalfa grown in the greenhouse (left) or growth chamber (right). Stress responses below zero (the gray line) indicate that plants in the stressed treatment had lower biomass than their control counterparts. Pink circles represent the mean stress responses of uninoculated alfalfa, whereas blue triangles represent the mean stress response of AMF inoculated alfalfa. Bars represent the 95% confidence intervals.

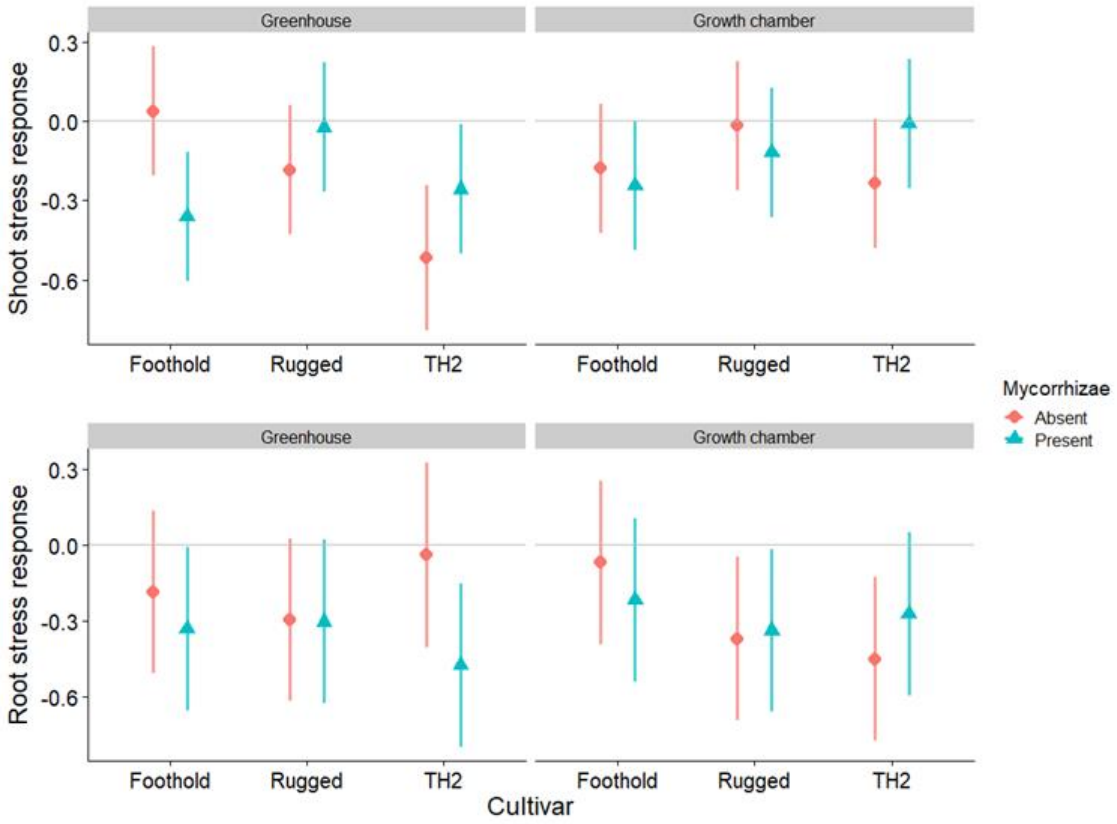

Figure 3.9. The salt stress response of shoot (top) and root (bottom) biomass of alfalfa grown in the greenhouse (left) or growth chamber (right). Stress responses below zero (the gray line) indicate that plants in the stressed treatment had lower biomass than their control counterparts. Pink circles represent the mean stress responses of uninoculated alfalfa, whereas blue triangles represent the mean stress response of AMF inoculated alfalfa. Bars represent the 95% confidence intervals.

Table 3.6. ANOVA summary table comparing the effect of AMF on stress response between the two different study locations. Results separated by a double line are from separate models.

	Stress	**Model terms**	**DF**	**F**	**p**
Shoot	Drought	AMF	1	2.246	0.141
		Cultivar	**2**	**2.587**	**0.087**
		Location	**1**	**69.28**	**1.40E^{-10}**
		AMF:Cultivar	2	0.482	0.621
		AMF:Location	**1**	**3.416**	**0.071**
		Cultivar:Location	2	0.399	0.673
		AMF:Cultivar:Location	**2**	**2.493**	**0.094**
Shoot	Salt	AMF	1	0.037	0.848
		Cultivar	2	1.993	0.149
		Location	1	1.459	0.234
		AMF:Cultivar	**2**	**3.892**	**0.028**
		AMF:Location	1	0.008	0.931
		Cultivar:Location	2	1.786	0.180
		AMF:Cultivar:Location	2	1.610	0.212
Root	Drought	AMF	1	0.263	0.611
		Cultivar	2	0.367	0.695
		Location	**1**	**2.931**	**0.094**
		AMF:Cultivar	2	1.020	0.369
		AMF:Location	**1**	**2.990**	**0.091**
		Cultivar:Location	2	1.238	0.300
		AMF:Cultivar:Location	**2**	**7.543**	**0.002**
Root	Salt	AMF	1	0.869	0.356
		Cultivar	2	0.725	0.490
		Location	1	0.024	0.877
		AMF:Cultivar	2	0.292	0.748
		AMF:Location	1	1.336	0.254
		Cultivar:Location	2	0.510	0.604
		AMF:Cultivar:Location	2	1.107	0.339

3.5.6. Alfalfa flowering, pollination, and seed production.

We observed the number of flowering stems produced at three time points while the alfalfa were flowering: early flowering (~2 weeks after the first flowers appeared), mid peak (2 weeks later) and late peak (2 weeks after mid). We found significant interactions between AMF, cultivar, and flowering period in unstressed, drought and salt stressed groups (Table 3.7). The effect AMF had on flower number was inconsistent within and between treatments, and within or between time periods. Almost all cultivars were affected in some way by AMF under salt stress (Fig. 3.12), but almost none were in the drought treatment (Fig. 3.11), although inoculated 2010 plants had yet to flower at the early time point. The lack of any trend is probably a symptom of variable stress response in salt treated plants more than any direct AMF effect. As for drought plants, it is possible that drought was severe enough to supress flowering past all noticeable effects of AMF. AMF seem to have both enhanced and supressed flowering under unstressed conditions most visibly in the mid and late flowering period (Fig. 3.10).

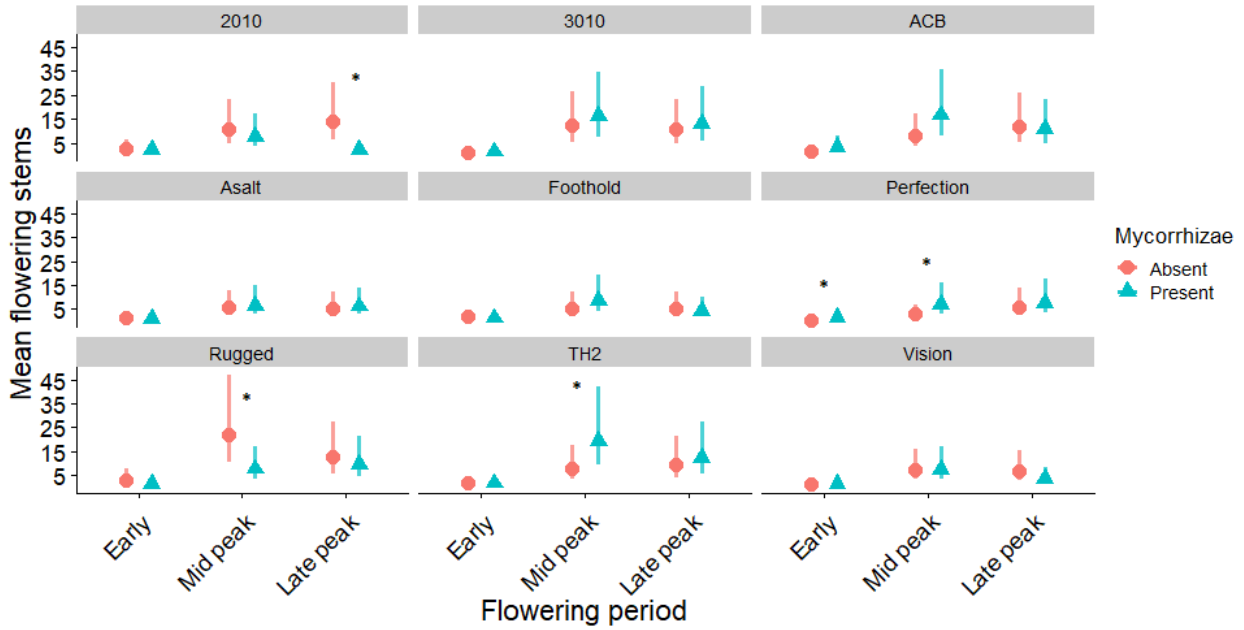

Figure 3.10. The mean number of flowering stems on unstressed alfalfa. Pink circles represent the mean number of flowering stems on uninoculated alfalfa, whereas blue triangles represent the mean number on AMF inoculated alfalfa. Bars represent the 95% confidence intervals. Asterix mark the time periods where there were differences between inoculated and uninoculated plants with the following p values: 2010 p=0.002, Perfection early p=0.065, mid p=0.090, Rugged p=0.031, TH2 p=0.068.

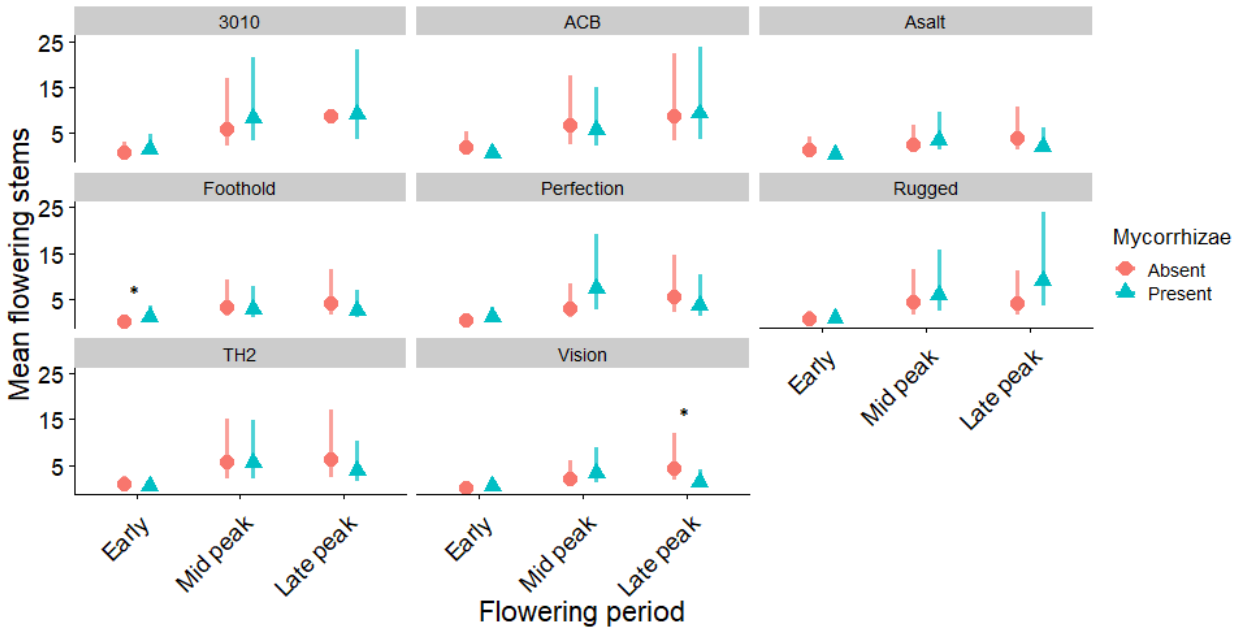

Figure 3.11. The mean number of flowering stems on drought stressed alfalfa. Pink circles represent the mean number of flowering stems on uninoculated alfalfa, whereas blue triangles represent the mean number on AMF inoculated alfalfa. Bars represent the 95% confidence intervals. Asterix mark the time periods where there were differences between inoculated and uninoculated plants with the following p values: Foothold p=0.065, Vision p=0.055.

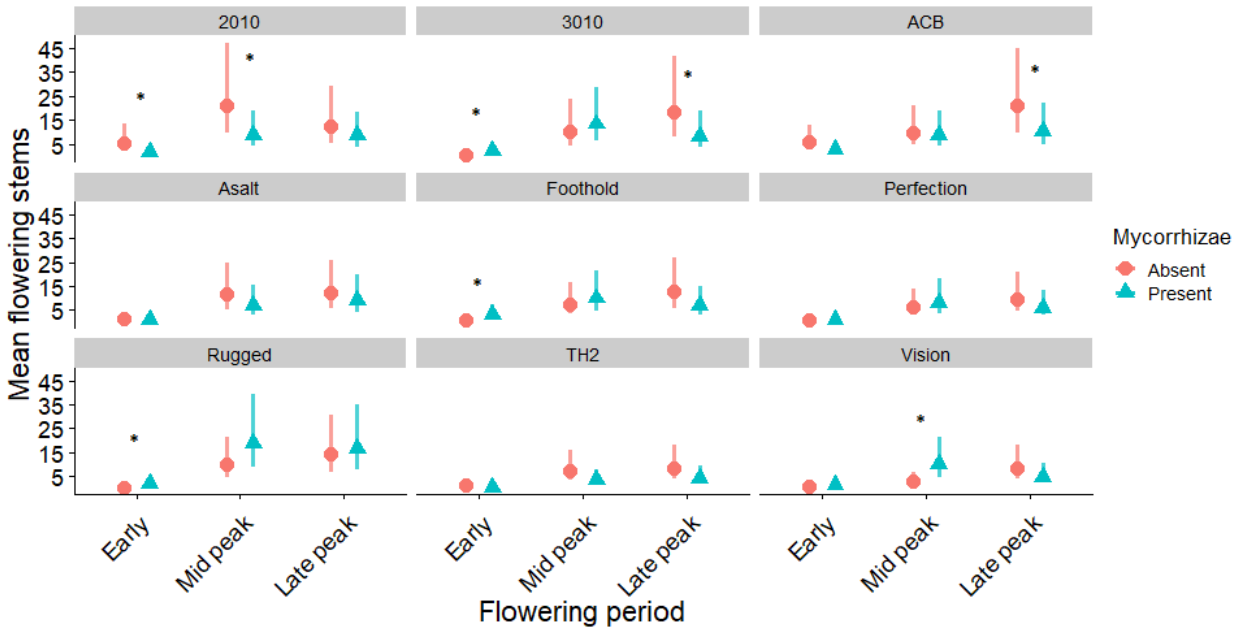

Figure 3.12. The mean number of flowering stems on salt stressed alfalfa. Pink circles represent the mean number of flowering stems on uninoculated alfalfa, whereas blue triangles represent the mean number on AMF inoculated alfalfa. Bars represent the 95% confidence intervals. Asterix mark the time periods where there were differences between inoculated and uninoculated plants with the following p values: 2010 early p=0.019, mid p=0.051, 3010 early p=0.032, late p=0.088, AC-Bridgeview p=0.092, Foothold p=0.070, Rugged p=0.041, Vision p=0.008.

Table 3.7. Summary of ANOVA tables for models testing the effects of AMF, cultivar, and flowering time point on the number of flowering stems produced in each treatment group. Entries separated by a double line are from separate models.

	Model terms	**DF**	**Chisq**	**p**
Unstressed	AMF	1	0.0462	0.830
	Cultivar	8	10.365	0.241
	Time point	**2**	**38.423**	**4.54E^{-9}**
	AMF:Cultivar	8	7.991	0.435
	AMF: Time point	**2**	**17.309**	**1.743E^{-4}**
	Cultivar: Time point	**16**	**38.617**	**0.001**
	AMF:Cultivar: Time point	**16**	**54.127**	**4.94E^{-6}**
Drought	AMF	1	0.674	0.412
	Cultivar	**8**	**13.017**	**0.072**
	Time point	**2**	**25.789**	**2.51E^{-6}**
	AMF:Cultivar	8	11.511	0.118
	AMF: Time point	2	1.691	0.429
	Cultivar: Time point	**16**	**25.670**	**0.029**
	AMF:Cultivar: Time point	**16**	**42.606**	**9.90E^{-4}**
Salt	**AMF**	**1**	**5.466**	**0.038**
	Cultivar	**8**	**38.961**	**0.001**
	Time point	**2**	**32.184**	**0.002**
	AMF:Cultivar	**8**	**23.862**	**0.003**
	AMF: Time point	2	5.362	0.275
	Cultivar: Time point	**16**	**73.655**	**2.26E^{-9}**
	AMF:Cultivar: Time point	**16**	**64.194**	**1.013E^{-7}**

There was no interaction between stress treatment and AMF inoculation on bumblebee visits to alfalfa per flower group ($F_{(2,58)}$=2.06, p=0.136) nor main effects of either one (Fig. 3.13). The overall visitation rate was 0.96 ± 0.14 visits per flowering stem in a two-hour window. There were only four plants in the drought group because of the low number of plants flowering at the time of pollination, but excluding the drought treatment from the model did not change the results. This analysis can not account for pollination success , or the possibility repeat visits.

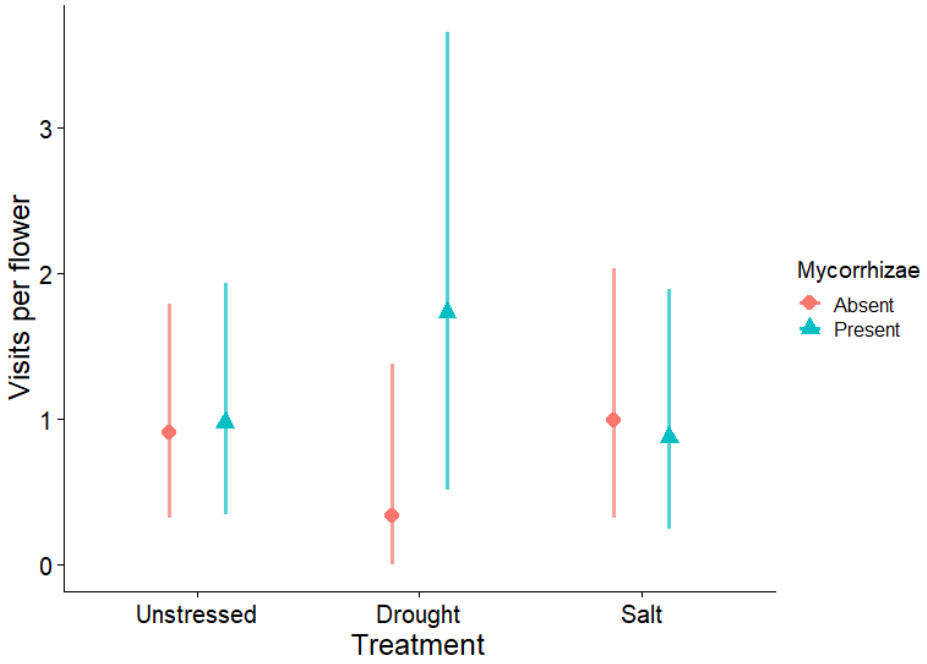

Figure 3.13. The mean number of bumblebee visits to alfalfa flowers in a 2-hour window. Pink circles represent the mean number of visits to uninoculated alfalfa, whereas blue triangles represent the mean number of visits to inoculated alfalfa. Bars represent the 95% confidence intervals.

Almost all of the 349 plants produced flowers, but only 194 (55%) went on to produce seeds. Foothold did not produce any seeds in the drought treatment, nor Asalt plants in the unstressed, uninoculated treatment group, even though both cultivars produced flowers, and were not lacking nutrients.There was a marginal interaction between AMF and cultivar affecting seed mass produced by drought stressed plants (Table 3.8), but not in salt stressed or unstressed plants (only drought figures are shown here, Fig. 3.14). Drought stressed Rugged and Vision produced more seeds when uninoculated than inoculated (p=0.082 and 0.002 respectively), but Asalt produced more seeds when inoculated (p=0.075).

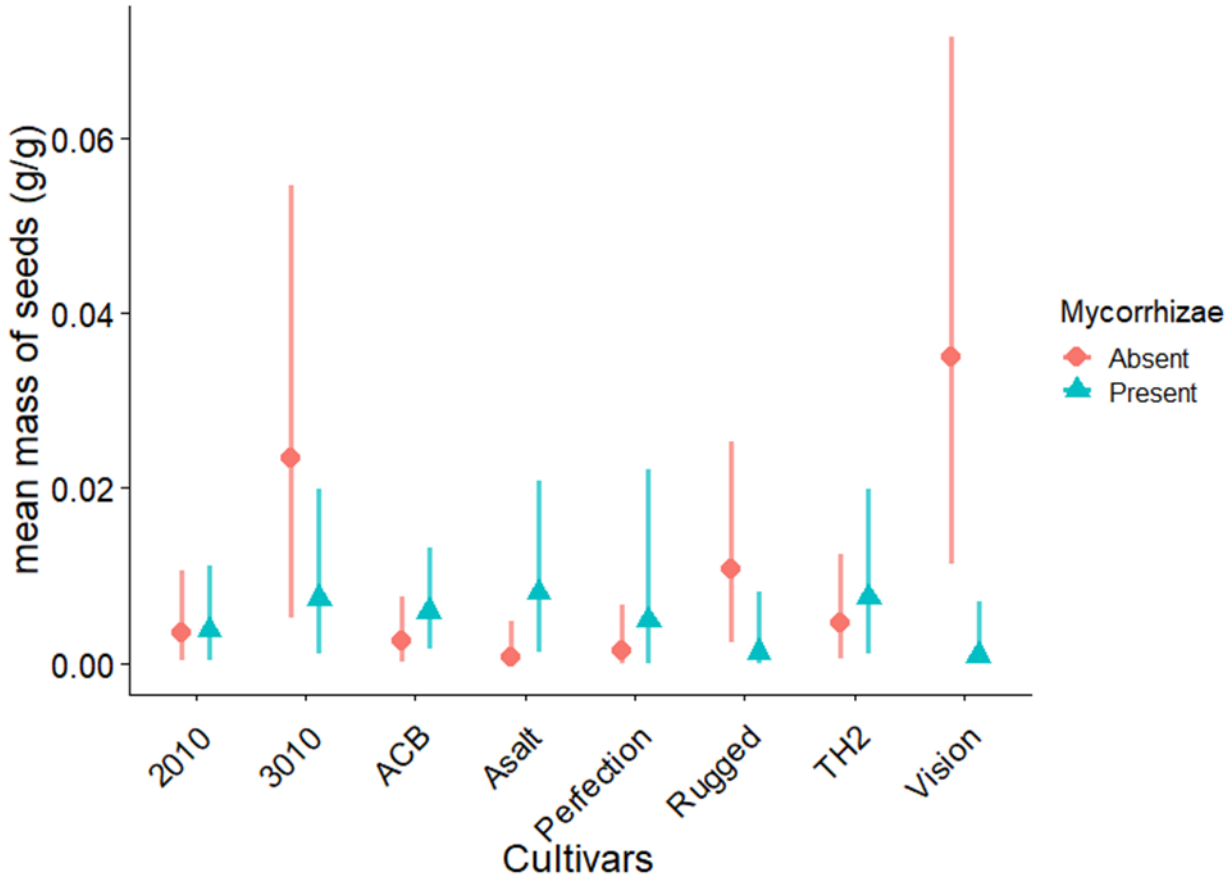

Figure 3.14. Mass of seeds produced per gram of plant biomass by drought stressed alfalfa. Inoculated foothold plants produced no seeds in this treatment so are not shown here. Pink circles represent the mean number of flowering stems on uninoculated alfalfa, whereas blue triangles represent the mean number on AMF inoculated alfalfa. Bars represent the 95% confidence intervals.

Table 3.8. Summary of ANOVA table for models testing the affect of stress treatment and AMF on the mean mass of seeds produced by alfalfa. Results separated by a double line are from separate models.

Treatment	Model terms	DF	F	p
Unstressed	AMF	1	1.270	0.268
	Cultivar	7	0.861	0.547
	AMF:Cultivar	7	0.258	0.966
Drought	AMF	1	2.202	0.152
	Cultivar	7	1.490	0.222
	AMF:Cultivar	7	3.622	0.010
Salt	AMF	1	2.494	0.123
	Cultivar	8	0.329	0.949
	AMF:Cultivar	8	0.809	0.600

3.6. Discussion

We examined the context dependency of AMF's affects on alfalfa cultivars under stress over the course of one growth cycle and found that, counter to our hypotheses, inoculation provided no growth or reproductive benefits. Inoculation did not protect against the effects of stress on biomass, or nutrients. Negative growth responses to AMF are not unheard of (Li et al., 2008), but it was surprising to see almost a total lack of positive effect, even in unstressed alfalfa. AMF uniformly worsened the effects of drought stress, and selectively worsened the effects of salt stress on aboveground biomass accumulation. Therefore, differences among cultivars did depend on stress type, as in our second hypothesis, but responses were not associated with the inherent stress resistance of a cultivar.

Our intention was to test three stress treatments, however our "low nutrient" treated plants did not differ from the unstressed control plants in any of the metrics covered in this chapter. Drought and salt treated plants received the same amount of fertilization as unstressed plants, we must then recognize that abundant nutrient supply is one possible explanation for the lack of mycorrhizal benefit across all treatment groups. At least one study has found AMF to decrease alfalfa biomass at high (100mg/kg) P fertilization rate, but not at a moderate rate (30mg/kg) (Liu et al., 2020), though this was a study focused on soil remediation using *Glomus aggregatum*, *G. versiforme* and *G. tortuosum*.

In the primary greenhouse trial alfalfa response to mycorrhizal inoculation and salt stress depended on cultivar. At the outset, we expected that differences in stress tolerance between cultivars would explain variation in AMF effects, such as modulating salt uptake by the plants. Alfalfa cultivars varied in their salt susceptibility when uninoculated, but we did not see a relationship between their measured susceptibility and the resulting stress effects when inoculated. This could mean that cultivars differed in their salt tolerance mechanisms. One salt tolerance mechanisms is preventing the build up of ions in the shoot tissue, for example, a salt tolerant alfalfa cultivar had upregulated gene expression on Na^+ transporters and Na^+ / H^+ exchangers on vacuoles to maintain ionic homeostasis when salt shocked (Lei et al., 2018). We found differences between cultivar's leaf sodium levels, but they did not necessarily reflect the overall stress response of the plant. additionally, there was a negative relationship between

sodium concentration and shoot stress in inoculated plants even though AMF did not affect salt accumulation.

The soil electroconductivity in salt stressed pots was lower by 33% on average when AMF were present. Other studies have found similar reductions in soil EC (27-51%, Li et al. 2012), they postulate that the increased root growth they observed in mycorrhizal plants absorbed more salts. We found no difference in root size between inoculated and uninoculated salt stressed plants, but it is still possible that AMF stimulated increased ion uptake into the root system where they were then sequestered. Additionally, many more ions than we accounted for contribute to soil EC, so even though there were no significant differences in plant leaf sodium content, or stress response between inoculation treatments, the inoculated plants could still have been sequestering more ions, especially in the root system where we did not measure salinity. The EC of both AMF and non-AMF soils (9.48±2.3 to 6.37±1.8 dS respectively) were still between weakly and moderately saline (Aspinall et al., 1982).

There was no variation in our tested alfalfa cultivars' stress response to drought. Variability of drought tolerance in alfalfa cultivars is well documented globally (Anower et al., 2017; Maghsoodi & Razmjoo, 2015; C. Zhang et al., 2019), but a study of Canadian forage yield trends found that newer cultivars have lower yield under rain-fed conditions compared to irrigated locations. When relying on rainfall, early spring rain was the most important factor for determining yield, indicating that recent breeding trends focused on increased yield under water abundant conditions may have negatively impacted drought tolerance overall in these newer cultivars (Ren et al., 2021). We also saw a uniformly negative effect of AMF on drought stress response, which is very uncommon. Indeed a meta analysis on the affects of AMF on drought-stressed plants found almost universally positive effects on 29 measures of plant growth and success, although legumes had the least positive response in aboveground growth, and *R. irregularis* (this study uses its former name: *G. intraradices*) had the least positive effect on aboveground growth out of five tested species(Jayne & Quigley, 2014).

Comparing greenhouse and growth chamber trials:

When repeating the experiment, we found that drought stressed plants displayed worse stress in terms of shoot mass regardless of inoculation status, and AMF no longer increased stress. The growth chamber we used for this trial had a much more controlled climate with constant air

circulation leading to overall dryer conditions than the greenhouse of the first trial. We did not collect colonization data from the growth chamber plants, so the lack of an AMF effect on biomass stress here could be an indication that the AMF themselves were unable to grow properly, so were not exerting additional stress on the alfalfa. There was an unexplained change in the root stress response of uninoculated drought stressed plants: TH2's stress response was much worse in the growth chamber than in the greenhouse raised plants. This was the only place we found a statistical difference in cultivar biomass amongst drought stressed plants. TH2 may have been slightly more susceptible to drought and crossed some stress threshold here.

The interaction between salt stress and AMF did not differ between the two trials. When looking across both trials there was a marginal improvement of TH2's stress response in inoculated plants not seen in in the greenhouse trial alone. The variation within cultivars was high, this indicates that more replicates in a single trial were needed overall to get more robust or replicable results.

Interactions of AMF with alfalfa growth:

To capture flowering and seeding we allowed the alfalfa to grow for four months. This length of time may have diminished our ability to detect stress responses if unstressed alfalfa growth was restricted by pot size. We saw alfalfa height plateau about two months into the growing period, with almost no effect of AMF on growth rate. Of course, height does not correlate perfectly with total shoot mass as alfalfa tends to increase in breadth, adding more stems as they age. Murphy et al. (2013) found that root mass increases with pot size when there is high nutrient availability as there was in our study. When looking at the range of root mass, mass in the unstressed treatment was normally distributed indicating that the roots were not approaching a pot maximum. Long growth trials are less common in AMF studies, so Information on the later stage of the plant-AMF relationship could be more applicable to AMF effects on perennial plants, including forage crops.

Several drought-stressed alfalfa cultivars were shorter on average over the recorded growing period when inoculated compared to when uninoculated. The corresponding unstressed cultivars had higher growth rates when inoculated than uninoculated. This suggests that unstressed alfalfa could be recovering from growth depression at the start of the growth period before height was recorded, or faster growth was a mycorrhizal benefit that was reflected in height but not the final

biomass. Salt stressed cultivar's growth and mean height were unaffected by AMF, even in cultivars that were more stressed when inoculated. The more gradual onset of salt stress could be responsible for the lack of response here.

Flowering and seed yield:

The number of flowering stems was dependant on cultivar ID, mycorrhizal inoculation, and time. The interaction of these three factors also differed across the stressed and unstressed treatments. We found no clear pattern connecting AMF or stress response to promotion or suppression of flower number in any treatment, for instance, under salt stress AMF supressed flowering in some cultivars that had a high flower number when uninoculated, but also enhanced flower production in other cultivars. AMF supressed flower number in 2010 even though neither inoculated or uninoculated plants were particularly salt stressed. Interactions between AMF and cultivar could occur at any time point in the flowering window, possibly because stress can shift flowering initiation thereby shifting peak flower production forwards or backwards in time relative to other cultivars.

Only 55% of the plants produced seeds during the growth period. We had expected an increase in seed yield based on the tendency of AMF to increase nutrient uptake in plants. Contrary to our third hypothesis, however, even though unstressed plants did not exhibit nutrient stress when inoculated this did not increase seed yield. Higher inflorescence number did not result in higher seed yield in any treatment or cultivar either. Some uninoculated drought-stressed cultivars had a higher seed yield than when inoculated even with relatively low inflorescence numbers overall. Seed yield is highly correlated with the number of pods per inflorescence in alfalfa (Bolaños-Aguilar et al., 2002) so only counting inflorescences rather than individual flowers might not give a fine enough level of detail to ascribe seed yield variation to altered flowering. We did not observe any differences in visitation rates that could explain altered seed yield or the lack of seed production in some cultivars. Just observing visitation does not measure if treatments changed pollination success, or if there were changes in floral rewards that the bees did not respond to. A follow up study combining visitation, measuring nectar volume or composition, and seed yield per inflorescence could better assess if there were pollination deficiencies.

The role of AMF identity and origin in symbiosis:

Plant-AMF symbiosis depends not only on the host plant but on the identity of the fungal symbiont. *Rhizophagus irregularis* inoculant is common, but there are not many studies specifically on alfalfa's response to *R. irregularis.* He et al. (2017) found *R. irregularis* increased regrowth and nutrient content of alfalfa cultivar Golden Empress in drought (35% field water capacity) and well watered (70% field water capacity) conditions. These plants were established for three months under normal greenhouse conditions (mean day/ night temperature of 27/18 °C, with a constant soil moisture level , then cut back before stress treatments were applied, a major difference from our study. Zhang et al. (2018) also found increased alfalfa biomass (cultivar unspecified) and P uptake in arsenic contaminated soil in alfalfa inoculated with *R. irregularis*. In contrast, Püschel et al. (2017) found no effect of *R. irregularis* on alfalfa cultivar Vlasta's biomass across a P supply gradient, but they did find an increase in both P uptake and N fixation in inoculated plants. These three studies used soil, trap plant roots, hyphae, and spores as inoculum whereas we used a product cultured in vitro with a proprietary aseptic technique (Khasa et al., 2009). It is not known how decades of propagation in vitro may alter the compatibility of fungi with plant hosts. In one example of indigenous vs in vitro cultured AMF *Hedysarum coronarium* (also a forage legume) inoculated with a mixture of AMF trapped from local soil (including *R. irregularis*) were 1.9-fold larger than plants inoculated with a commercial *R. irregularis* product, though both AMF treatments equally improved N and P uptake (Labidi et al., 2015). We can not conclude that the production method was responsible for our results contrasting theirs, AMF mixtures do tend to be more beneficial than single isolate inoculants, but it is worth investigating further as commercial inoculants become more popular.

Conclusion:

We found that alfalfa's stress response could be moderated by AMF, but not in ways we predicted. The alfalfa cultivars we used had responses that were more similar to each other than expected. A lack of clear distinction between cultivars made it difficult to determine possible mechanisms of context dependency here. The limited positive AMF responses did not point to cultivar traits or growth conditions that promote symbiosis. We did not design this study to evaluate the effectiveness of *R. irregularis* in particular, but it may not be very synergistic with any of our tested alfalfa cultivars to begin with as indicated by the limited positive responses even under unstressed conditions. Combining a low suitability fungus with high nutrient

availability created an environment that was not conducive to mutualistic symbiosis. Some of the cultivars did not display biomass responses to stress but did have altered flowering and seed production induced by interactions of stress and AMF. This shows that there were some physiological effects of AMF that were not captured in the stress response but may be teased out with further investigation.

4. RELATIONSHIPS BETWEEN ALFALFA'S FINE ROOT TRAITS AND AMF

4.1. Preface

Root physiology is often used to understand how plants function and interact with their world. Here we probe the supposition that plant responses to AMF can be explained by root trait expression. Additionally, root trait expression can change depending on environmental stressors, which may be an important piece to understanding variation in the plant-AMF relationship caused by external factors. Our research in the previous chapter showed that alfalfa responses to AMF overlapped between cultivars, so the purpose of this chapter was to connect actual alfalfa trait expression to their mycorrhizal growth responses.

4.2. Abstract

Plant root physiology can help us understand whole plant functions and responses. Roots are sensory organs as well as tools for resource acquisition; they respond rapidly to changing conditions. Root trait expression can be indicative of how plants interface with beneficial symbionts like AMF and respond to stressful stimuli. AMF benefit plants by increasing the availability of nutrients, but it is not well understood why "mycorrhizal benefits" vary in magnitude and direction between and within plant species. Some frameworks predict that plants with coarser root systems outsource nutrient acquisition to mycorrhizal partners, while finer rooted plants are more self sufficient. In this study we test the hypothesis: The effect of AMF on alfalfa biomass and nutrient concentration will change across the spectrum of root trait expression. As root and mycorrhizae are both influenced by stress, we also hypothesize that the slope of the root trait - AMF response relationship will change depending on the stress treatment. We measured specific root length, root tissue density, and root diameter of nine alfalfa cultivars exposed to one of three stress treatments (salt, drought, or low nutrients) in comparison to unstressed plants, and examined changes to biomass production, resource partitioning, nitrogen, and phosphorus concentration. AMF affected biomass and root trait expression, but there was no relationship between root trait expression and the effects of AMF on biomass. Nutrient concentrations were increased when SRL increased across treatment groups, and in inoculated plants we found that AMF negated RTD and RD relationships with nutrients in some treatments , flattening the slope to zero.

4.3. Introduction

Most vascular plants take part in highly context dependant relationships with arbuscular mycorrhizal fungi (Brundrett et al., 2018). Plant characteristics, nutrient availability, and AMF identity can all influence these relationships (Bennett & Groten, 2022; Hoeksema et al., 2010; Johnson et al., 2015). Root system morphology and physiology is important in understanding plant function (Bardgett et al., 2014), and root architecture, for example, can be indicative of how reliant a plant is on mycorrhizal symbionts. Plants with simpler root systems are thought to receive more benefit from symbionts than those with elaborate self-sufficient roots (Fitter, 2004) because a high density of long, thin roots are better suited to nutrient absorption from the soil than stubbier more simplistic systems that have less surface area (Eissenstat, 1992). Therefore,

plants with coarser root systems are thought to outsource nutrient acquisition to mycorrhizal partners (Hetrick, 1991).

Geoff Baylis linked root structure to dependence on mycorrhizae in early plant evolutionary history. While acknowledging that modern root systems were more varied and complex, he stated:

> "The length and frequency of the root hairs is clearly the best single index of a plant's capacity for non-mycotrophic growth." (Baylis, 1975)

This idea has been influential despite few examples explicitly testing the effect of root structure on mycorrhizal responsiveness early on (Veresoglou & Rillig, 2014). This hypothesis has been generalized and expanded on; many root traits in addition to root hairs have been evaluated for their potential influence on mycorrhizal growth response in an attempt to achieve greater specificity with more predictive power (Smith & Smith, 2011). More recent literature varies broadly in what root traits are considered, scaling from whole root systems to 1st order roots, and the scope varies from within a single species to among plant families. For instance, Yang et al. (2015) categorized whole root systems from 943 publications as either fibrous (monocotyledons, some herbaceous dicotyledons) or tap rooted (woody dicotyledon, and some herbaceous dicotyledons) in their meta-analysis, concluding that tap rooted species have a higher AMF growth response than fibrous rooted species. Comparatively, Maherali, (2014) tested the effect fine root traits (specific root length, root diameter, root hair length and root hair density) had on mycorrhizal response in a meta analysis of 12 papers but did not find conclusive relationships between these traits and the overall plant growth response.

The availability of trait databases has made large scale analyses of fine root traits easier (FRED, GRooT etc. See Iversen et al., 2017 and Guerrero-Ramírez et al., 2021). Using these data, we have refined our understanding of the mycorrhizae-root relationship dynamics by placing them into the greater context of underground resource economics. Using a resources economics model, root trait expression of a given plant should fall on a gradient between "fast" foraging with quick turnover to "slow" high investment tissues with a longer life span. In this framework root diameter trades off with specific root length and should be positively correlated with root tissue density (Kong et al., 2016). However, both increases in specific root length and utilizing AMF for foraging can equally provision plant roots, so the presence of AMF needs to be accounted for

in the resource economics model (McCormack & Iversen, 2019). Bergmann et al. (2020) connect AMF to resource economics in defining the AMF collaboration gradient as a dimension of the root economic space. Broadly, self sufficient plants cluster at the end of the gradient defined by high specific root length, and more collaborative plants on the end defined by higher root diameters. These guidelines are useful for large scale systems, but they say little about the more subtle inter or intra-species variations that can exist due to root trait plasticity, interactions between different mycorrhizal species, or how environmental context could affect mycorrhizal relationships (Atkinson et al., 2003; Marro et al., 2022)

Roots are a major sensory organ in plants; they are often the first site of a plant's stress response. Plants can respond to stress by altering their growth to avoid stressors or reduce stress effects. For instance, under mild water restriction plants may develop a more extensive root system; roots will elongate and angle down in search of moister soil (Comas et al., 2013). Just as roots will seek out water through hydrotropism, roots exhibit halotropism by growing away from saline patches (Galvan-Ampudia et al., 2013). Plants may also utilize root trait plasticity to mitigate stress. Rice cultivars with genetic predisposition for higher root length and branching plasticity were better able to handle drought (Kano et al., 2011). Higher stress can alter and limit root growth: root tissue density, volume, specific length, branching, and root length ratio were all altered in maize by drought, heat, or combinations of the two (Vescio et al., 2021). Under salt stress root elongation was reduced or prevented due to cell death in key meristematic tissue in Arabidopsis (West et al., 2004).

Plant associated microbes in the rhizosphere are heavily influenced by their plant hosts and the surrounding environment. As symbionts, stress can affect either plant or AMF directly, or indirectly through their partners response (Finlay et al., 2008). Mycorrhizal responses to stress are not well understood, but hyphal growth, sporulation, and colonization have all shown negative responses to saline soil and other stresses (Santander et al., 2019). As seen in the previous chapter, stress can interact with AMF effects on their host plants. AMF interact with phytohormones such as ABA a signalling molecule that is key in stress responses and mediates root cell elongation and direction in aid of hydrotropism (Dinneny, 2019). Root growth responses to AMF colonization are also subject to the genetic variation of the host plant (Wang et al., 2011).

In this chapter we focus on the relationship between alfalfa root traits and the plant's mycorrhizal response to see if variation in fine root trait expression is associated with mycorrhizal growth benefits or deficits. We use three stress treatments (salt, drought, low nutrients) in comparison to unstressed plants to determine if different stress contexts alter the relationship between root traits and mycorrhizal response. We examined how these relationships affect biomass production, resource partitioning, nitrogen, and phosphorus concentration. We hypothesize that:

(1) There will be a relationship between the magnitude of AMF's effect on alfalfa biomass, root:shoot ratio, leaf:stem ratio, or nutrient concentration with alfalfa's specific root length, root tissue density, or root diameter.
(2) The slopes and signs (positive or negative) of these relationships will change depending on the stress treatment.

4.4. Methods

We looked at trait expression at harvest across nine alfalfa cultivars to get a wider spectrum of possible root phenotypes. We chose to use 1st order roots in this study because they are highly plastic, and we were interested in the variability of the plant-mycorrhiza relationship within a single species, so using whole system architecture would not have produced enough variability. The cultivars of alfalfa we chose included tap rooted and creeping rooted types, but the plasticity of fine root expression allows us to look along a gradient of traits rather than a dichotomy, and these traits are commonly used and catalogued in the aforementioned databases making it easier to compare results (Vierheilig et al., 1998) roots were heated to 90°C in beaker of 10% w/v KOH solution for 30 minutes, followed by 2% v/v HCl solution for a further 15 minutes. The heat was reduced to 80°C, then the roots were stained in a dye comprised of 5% v/v black ink (Sheaffer) and 5% v/v acetic acid for 15 minutes. Roots were well rinsed in DI water between each step. Finally, the roots were stored in a mixture of equal parts glycerol, 5% acetic acid, and DI water for a minimum of two days to remove excess ink. We mounted 25cm of root tissue on glass slides with Permount™ for colonization analysis. We measured the amount of root colonization by picking 100 random intercepts along the root length and noting if any hyphae, arbuscules, or vesicles were present (McGonigle et al., 1990).

To measure other fine root traits, we haphazardly collected 1st order roots from the dried alfalfa root systems of the previous study, picking unbroken lengths of at least 40mm. Root samples

were re-hydrated by soaking in water for about five minutes before being scanned. We immersed hydrated root fragments into a 21x29cm clear acrylic tray of water, ensuring no fragments overlapped. The capacity for well placed fragments was approximately 0.02g of roots. The whole tray was then scanned in a flat bed scanner (Epson Perfection V800). Scanned images were analysed using WinRHIZO™ to obtain estimates of average root diameter, total root length, and total root volume for the samples. We re-dried then weighed the scanned root samples to calculate specific root length (SRL, eq. 4.1) and root tissue density (RTD, eq. 4. 2).

$$SRL=\frac{\text{length(cm)}}{\text{dry mass(g)}} \qquad (4.1)$$

$$RTD=\frac{\text{dry mass(g)}}{\text{volume(cm3)}} \qquad (4.2)$$

4.4.1. Statistical analysis

In chapter 3 where stress response was our focus, the low nutrient treatment did not differ from the unstressed treatment in any measure. However, the fine root traits of the low nutrient treatment did show some differences from the unstressed treatment, so they are included here.

All analysis was done in R version 3.6.3 with mixed models using the package lme4 (Bates et al., 2015) unless otherwise noted. When predictors were found to have significant ($p<0.05$) or marginally significant ($p<0.1$) effects we used emmeans or emtrends to perform post hoc, pairwise tests with the Tukey method for adjusting p values (Lenth, 2022).

Model selection:

We constructed models testing the effects of mycorrhizal inoculation in conjunction with the three root traits we measured on alfalfa biomass, and nutrient assimilation. Root tissue density and root diameter were individually highly correlated with specific root length but not with each other, so we modeled them separately from SRL to avoid collinearity. Models with both RTD and RD were overfitted though, so we went through a process of model selection to determine the parsimonious model for eliminating one from the following maximal model:

response~RD*AMF*treatment+RTD*AMF*treatment+(1|cultivar)+(1|block)

We compared AIC scores of progressively reduced models while retaining both the random factors. Each model used cultivar identity and block as random effects to account for the possible variation between cultivars and the temporal separation of the replicates' planting.

Variance of root trait expression:

Our primary question regards how root traits influence mycorrhizal response, but inoculation does have the capacity to affect root trait expression as well. We needed to measure the amount of trait variation within a treatment to see how AMF or stress affected plasticity or expression, in what direction, and if that was a strategy for improving relations with AMF or a side effect of stress. We quantified the effects of AMF and stress treatments on root trait expression by calculating the coefficient of variation (CV, eq. 4.3) for each trait across all cultivars but within treatment groups (Schlichting & Levin, 1986), and modeling mean trait values with inoculation and treatment as predictor variables.

$$CV=\frac{SD}{mean} \qquad (4.3)$$

Each root trait was modeled separately here because each has the capacity to change independent of the other even if they are normally highly correlated (Bergmann *et al.*, 2020).

AMF colonization:

Evidence of AMF colonization was not found in uninoculated plants so only inoculated alfalfa was included in this analysis. We used two negative binomial models with the number of colonized segments as the dependant variable, one with SRL as the predictor, and one with RD and RTD (models 1 and 2):

Model 1: Total colonization~SRL*treatment+(1|cultivar)+(1|block))

Model 2: Total colonization~RTD*treatment+RD*treatment+(1|cultivar)+(1|block))

Poisson models were trialed but found to be a poor fit. In this instance we used glmer.nb also from lme4 package, checking for overdispersion with the DHARMa package. We used the afex package to run ANOVA on these models, rather than lmerTest as lmerTest does not provide F-tests for these models.

Plant biomass and allocation:

We determined root trait and mycorrhizal inoculation effects on biomass production in different treatment conditions by creating four separate mixed models. These tested the interaction of SRL, RTD, or RD with treatment type and AMF inoculation as predictor variables, and either

shoot or root biomass as the response variable (models 3-6). We chose biomass as a response variable because the mass of inoculated and uninoculated plants were not well correlated, thus calculating the mycorrhizal response, as would be typically done, introduced more noise into the results. Shoot biomass was square root transformed to conform with the assumption of normally distributed residuals.

Model 3: sqrt(shoot mass)~SRL*AMF*Treatment+(1|cultivar)+(1|block)

Model 4: root mass~SRL*AMF*Treatment+(1|cultivar)+(1|block)

Model 5: sqrt(shoot mass)~RTD*AMF*Treatment+(1|cultivar)+(1|block)

Model 6: root mass~RD*AMF*Treatment+(1|cultivar)+(1|block)

We determined changes in allocation to root and shoot biomass by modeling root-shoot ratio (RSR, eq. 4.4) and leaf-stem ratio (LSR, eq. 4.5) as response variables to treatment type and AMF inoculation combined with either SRL or RD (models 7-10).

$$\log\left(\frac{\text{root mass}}{\text{shoot mass}}\right) \quad \textbf{(4.4)}$$

$$\log\left(\frac{\text{leaf mass}}{\text{stem mass}}\right) \quad \textbf{(4.5)}$$

Model 7: RSR~SRL*AMF*Treatment+(1|cultivar)+(1|block)

Model 8: RSR~RD*AMF*Treatment+(1|cultivar)+(1|block)

Model 9: LSR~SRL*AMF*Treatment+(1|cultivar)+(1|block)

Model 10: LSR~RD*AMF*Treatment+(1|cultivar)+(1|block)

Nitrogen and phosphorus concentration:

To assess the effects of root traits on nutrient concentration in alfalfa leaves we constructed four mixed models the same way as the above biomass models with nitrogen or phosphorus as the response variables (Models 11-14). Phosphorus concentration was log transformed to conform with the assumption of normally distributed residuals. As in the biomass and allocation models, cultivar and replicate were coded as random effects, we also added the run date of the nutrient

measurements, because they were done over three days. The data used in these models excluded the cultivars 2010, Assalt, and Perfection as detailed in the previous section.

Model 11: Nitrogen~SRL*AMF*Treatment+(1|cultivar)+(1|block)+ (1|date)

Model 12: Phosphorus~SRL*AMF*treatment+(1|cultivar)+(1|block)+ (1|date)

Model 13: Nitrogen~RTD*AMF*Treatment+(1|cultivar)+(1|block)+ (1|date)

Model 14: Phosphorus~RD*AMF*treatment+(1|cultivar)+(1|block)+ (1|date)

4.5. Results

Root trait expression was not associated with root colonization. There were no changes in the amount of root length colonized relating to either SRL, RD or RTD under any treatment condition (Table 4.1).

Table 4.1. Summary of ANOVA tables for models of the amount of colonized root length.

Model terms	DF	Chisq	p
SRL	1	0.199	0.656
Treatment	3	4.635	0.201
SRL:Treatment	3	3.341	0.342
RTD	1	0.037	0.847
Treatment	3	2.455	0.483
RD	1	0.175	0.676
RTD:Treatment	3	1.878	0.598
Treatment:RD	3	2.694	0.441

4.5.1. Variance in root trait expression

Fine root trait expression was only altered in drought-stressed plants (Table 4.2). Mean specific root length, and root tissue density depended on AMF inoculation status as well: SRL increased under drought when plants were inoculated ($p=0.003$), meanwhile inoculated plants maintained a low RTD compared to uninoculated ones ($p=0.0001$), and uninoculated plants under drought had a higher RTD than any other plants in any treatment (Fig. 4.1). Root diameter was reduced under drought, but unchanged by AMF.

The CV of SRL was affected by an interaction of stress treatment and AMF inoculation (Table 4.3). Interestingly, the effect of AMF on SRL CV was different in each stress treatment: when unstressed or drought stressed the CV was lower when plants were inoculated, however AMF increase the CV in the low nutrient treatment but did not affect the CV of salt stressed alfalfa at all (p=0.025, 0.024, 0.012 and 0.756 respectively, Figure 4.2). There was a marginal interaction between stress treatment and AMF inoculation affecting root diameter CV, Low nutrient treated roots had higher variation when inoculated with AMF. Root diameter behaves roughly inverse to SRL, but relative to each other, SRL showed higher variation. Inoculation had no effects on RTD plasticity, but drought and low nutrient stress increased the CV compared to unstressed individuals (p=0.014 and p=0.033).

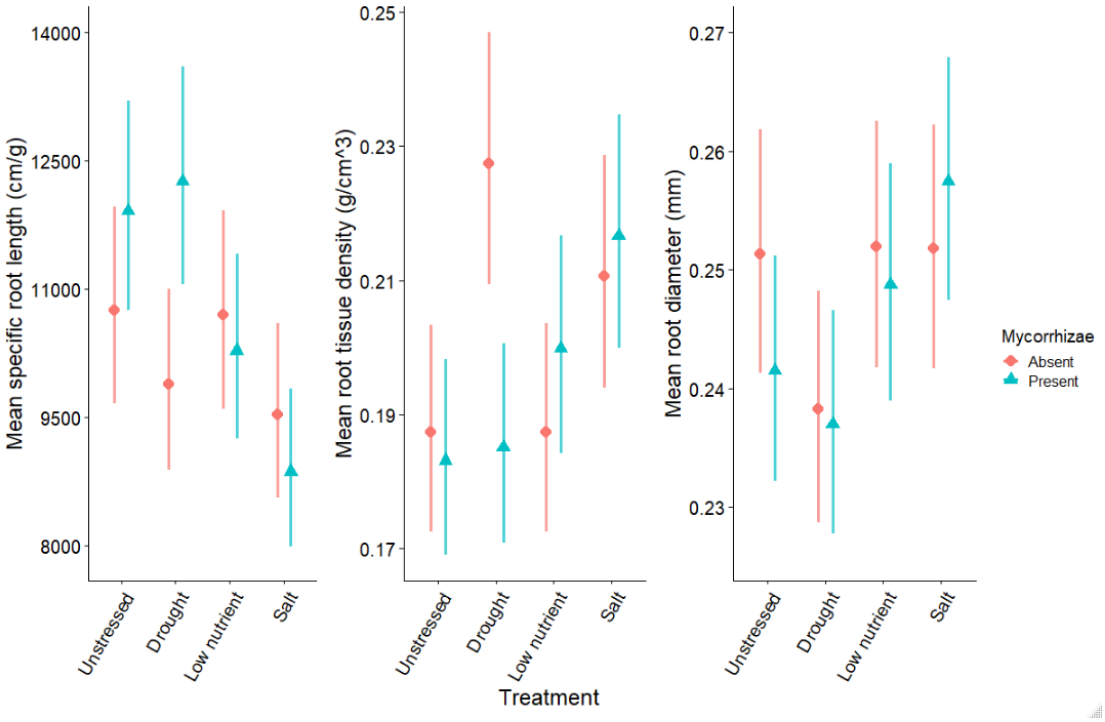

Figure 4.1. The mean trait expression of specific root length (left), root tissue density (center), and root diameter (right) of alfalfa grown with (blue triangles) or without (pink circles) AMF inoculation. The bars represent 95% confidence intervals around the mean.

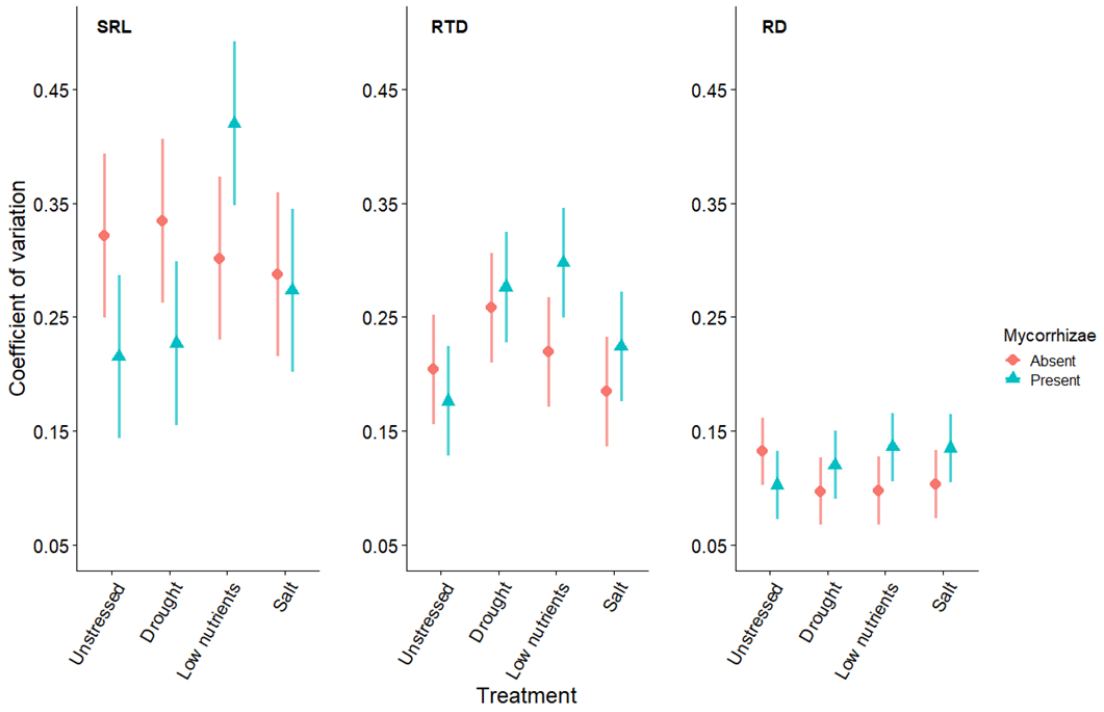

Figure 4.2. The coefficient of variation of specific root length (left), root tissue density (center), and root diameter (right) of alfalfa grown with (blue triangles) or without (pink circles) AMF inoculation. The bars represent 95% confidence intervals around the mean CV.

Table 4.2. Summary of ANOVA results for three models showing the effect of AMF and stress treatment on the mean expression of fine root traits. Results separated by a double line are from different models. Significant terms have been bolded.

	Model terms	**DF**	**F**	**p**
RD	**Treatment**	**3**	**4.863**	**0.003**
	AMF	1	0.409	0.523
	Treatment:AMF	3	1.158	0.326
RTD	**Treatment**	**3**	**6.208**	**$4.13E^{-4}$**
	AMF	1	1.803	0.180
	Treatment:AMF	**3**	**5.581**	**0.001**
SRL	**Treatment**	**3**	**6.936**	**$1.54E^{-4}$**
	AMF	1	2.119	0.146
	Treatment:AMF	**3**	**3.582**	**0.014**

Table 4.3. Summary of ANOVA results for three models showing the effect of AMF and stress treatment on the CV of fine root traits. Results separated by a double line are from different models. Significant terms have been bolded.

	Model terms	**DF**	**F**	**p**
SRL	Treatment	3	3.559	0.027
	AMF	1	1.484	0.233
	Treatment:AMF	**3**	**5.591**	**0.004**
RD	Treatment	3	0.211	0.888
	AMF	1	2.412	0.132
	Treatment:AMF	3	2.299	0.099
RTD	**Treatment**	**3**	**5.355**	**0.005**
	AMF	1	2.606	0.118
	Treatment:AMF	3	1.759	0.178

4.5.2. Alfalfa biomass and allocation

Contrary to our expectations shoot mass was unaffected by root trait expression. There were no interactions of any of the root traits we measured with AMF inoculation in any treatment group (Table 4.4). Treatment and AMF marginally interacted with each other in their effects on shoot mass when modeled with RTD as a predictor (Table 4.4), where shoot mass was reduced by inoculation in drought, salt-stress, and unstressed conditions (p=0.001, 0.003, and 0.034 respectively). There was no treatment:AMF interaction in the SRL model, but this model found that AMF reduced mass across all treatments (Table 4.5, p=0.0001).

Root mass decreased with increasing SRL and increased with RD (Table 4.4) but neither of these root traits interacted with stress treatment or AMF inoculation (Fig. 4.3). Treatment and AMF marginally interacted with each other in their effects on root mass when RD was a predictor, but not in the SRL model. Root mass was reduced by all three stress treatments, but only the root mass of low nutrient treated plants was reduced further by the presence of AMF, despite no change in the shoot mass of those alfalfa (Table 4.5, p=0.003). These changes were not linked to changes in root diameter.

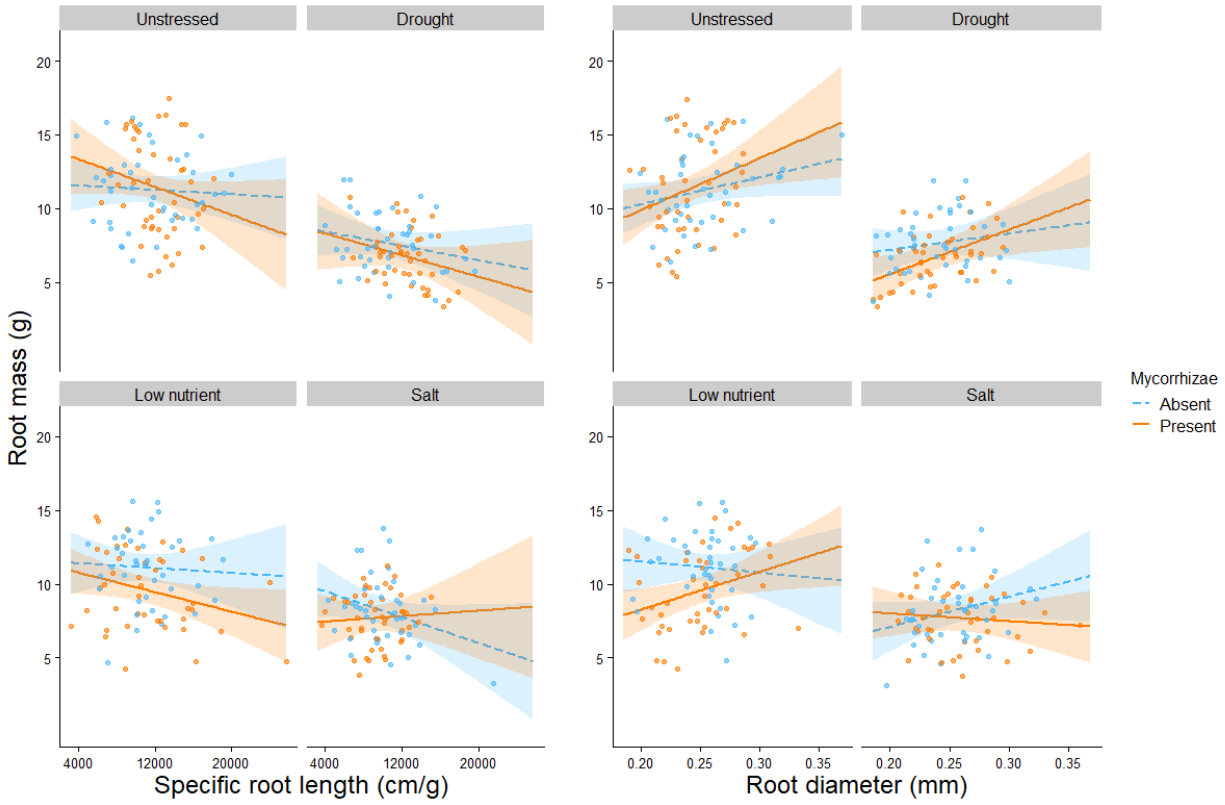

Figure 4.3. The effect of specific root length (left) and root diameter (right) on alfalfa root biomass when grown with AMF (orange solid line) or without AMF (blue dash line). Each panel is subset into four treatment groups. The lines are surrounded by the 95% confidence interval. Points are partial residuals as calculated by interact plot.

Table 4.4. Summary of ANOVA results for the effects of SRL, RD and RTD on alfalfa shoot and root mass. Results separated by a double line are from different models. Model terms with a significant or marginally significant p value referenced in the text are in bold.

	Model terms	**DF**	**F**	**p**
Shoot mass	SRL	1	0.083	0.773
	AMF	**1**	**7.153**	**0.008**
	Treatment	3	1.429	0.234
	SRL:AMF	1	2.385	0.124
	SRL:Treatment	3	1.354	0.257
	AMF:Treatment	3	0.223	0.881
	SRL:AMF:Treatment	3	0.176	0.913
	RTD	1	0.410	0.523
	AMF	1	0.236	0.627
	Treatment	**3**	**6.407**	**$3E10^{-4}$**
	RTD:AMF	1	2.520	0.114
	RTD:Treatment	3	1.564	0.198
	AMF:Treatment	**3**	**2.277**	**0.080**
	RTD:AMF:Treatment	3	1.476	0.221
Root mass	**SRL**	**1**	**7.947**	**0.005**
	AMF	1	0.019	0.891
	Treatment	**3**	**4.176**	**0.006**
	SRL:AMF	1	0.125	0.724
	SRL:Treatment	3	0.123	0.947
	AMF:Treatment	3	1.402	0.242
	SRL:AMF:Treatment	3	1.261	0.288
	RD	**1**	**12.564**	**$5E10^{-5}$**
	AMF	1	2.124	0.150
	Treatment	3	1.192	0.313
	RD:AMF	1	1.477	0.225
	RD:Treatment	3	1.060	0.367
	AMF:Treatment	**3**	**2.209**	**0.087**
	RD:AMF:Treatment	3	2.094	0.101

Table 4.5. Mean shoot and root mass of alfalfa. SE= standard error. Masses followed by different capital letters significantly differ at the α=0.05 level.

	Treatment	AMF	mean mass	SE	p
Shoot	Unstressed	Absent	11.89	0.38	A
	Unstressed	Present	10.99	0.35	A
	Drought	Absent	9.12	0.34	BC
	Drought	Present	7.36	0.29	CD
	Low nutrients	Absent	11.48	0.38	A
	Low nutrients	Present	11.28	0.36	A
	Salinity	Absent	9.87	0.35	B
	Salinity	Present	8.46	0.31	C
Root	Unstressed	Absent	11.27	0.46	A
	Unstressed	Present	11.4	0.44	A
	Drought	Absent	7.62	0.46	C
	Drought	Present	6.72	0.45	C
	Low nutrients	Absent	11.11	0.46	AB
	Low nutrients	Present	9.58	0.45	B
	Salinity	Absent	8.19	0.46	C
	Salinity	Present	7.69	0.45	C

Root diameter and specific root length influenced mass allocation in alfalfa (Table 4.6). The leaf to stem ratio of inoculated alfalfa increased with increasing RD (p=0.004) across all treatments (Fig. 4.4 right), but not in uninoculated plants. Leaf to stem ratio marginally decreased with SRL independent of AMF inoculation(p=0.092; Fig. 4.4 left). The root to shoot ratio of alfalfa also increased with root diameter, and decreased with SRL (Table 4.6), though in this case the relationship was consistent between AMF treatment groups (Fig. 4.5). Root diameter and root mass are highly correlated with each other across all treatment groups, so root mass is most likely driving this relationship.

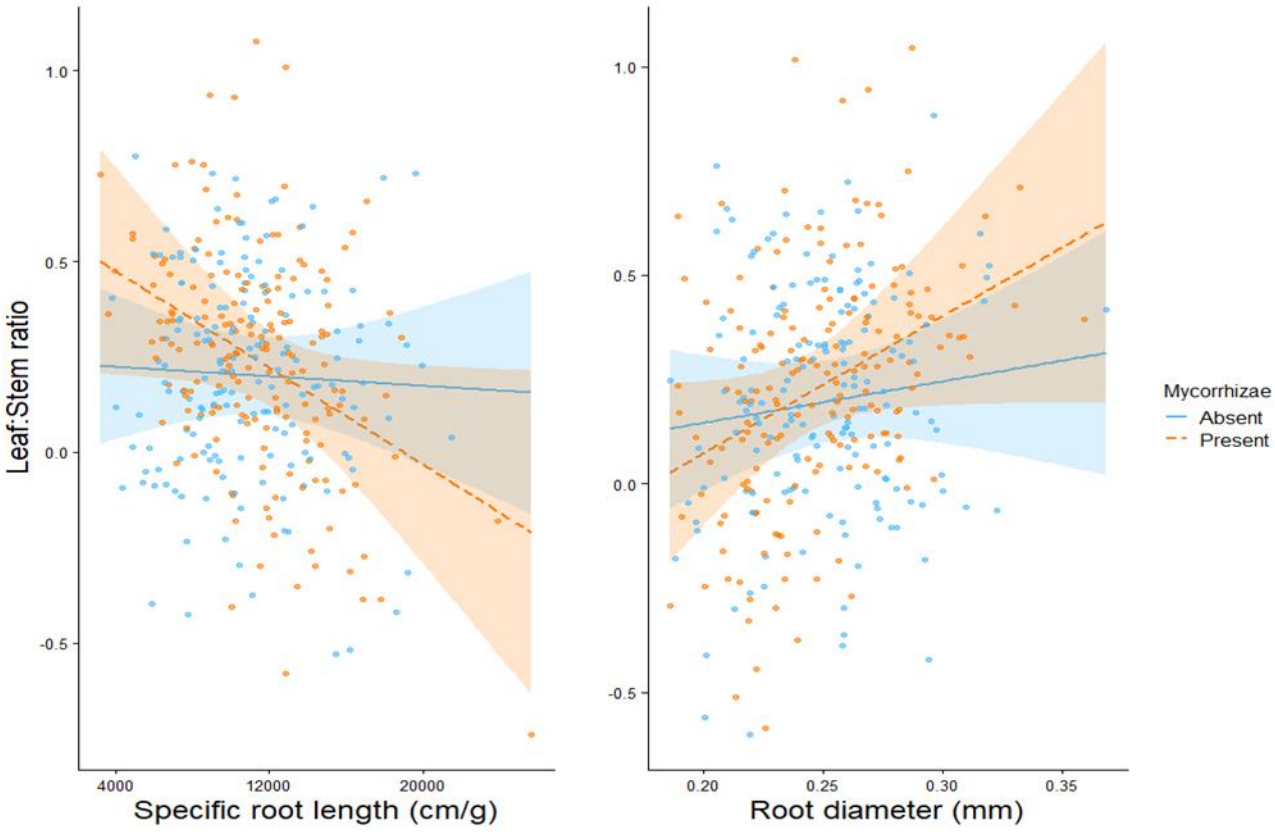

Figure 4.4. The relationship between SRL (left) and root diameter (right) on the leaf to stem ratio across all treatments groups of alfalfa when grown with (orange solid line) or without (blue dash line) AMF. Bars around lines are the 95% confidence interval. Points are partial residuals as calculated by interact plot. The slopes of inoculated and uninoculated plants do not differ from each other in the SRL plot but do in the RD plot.

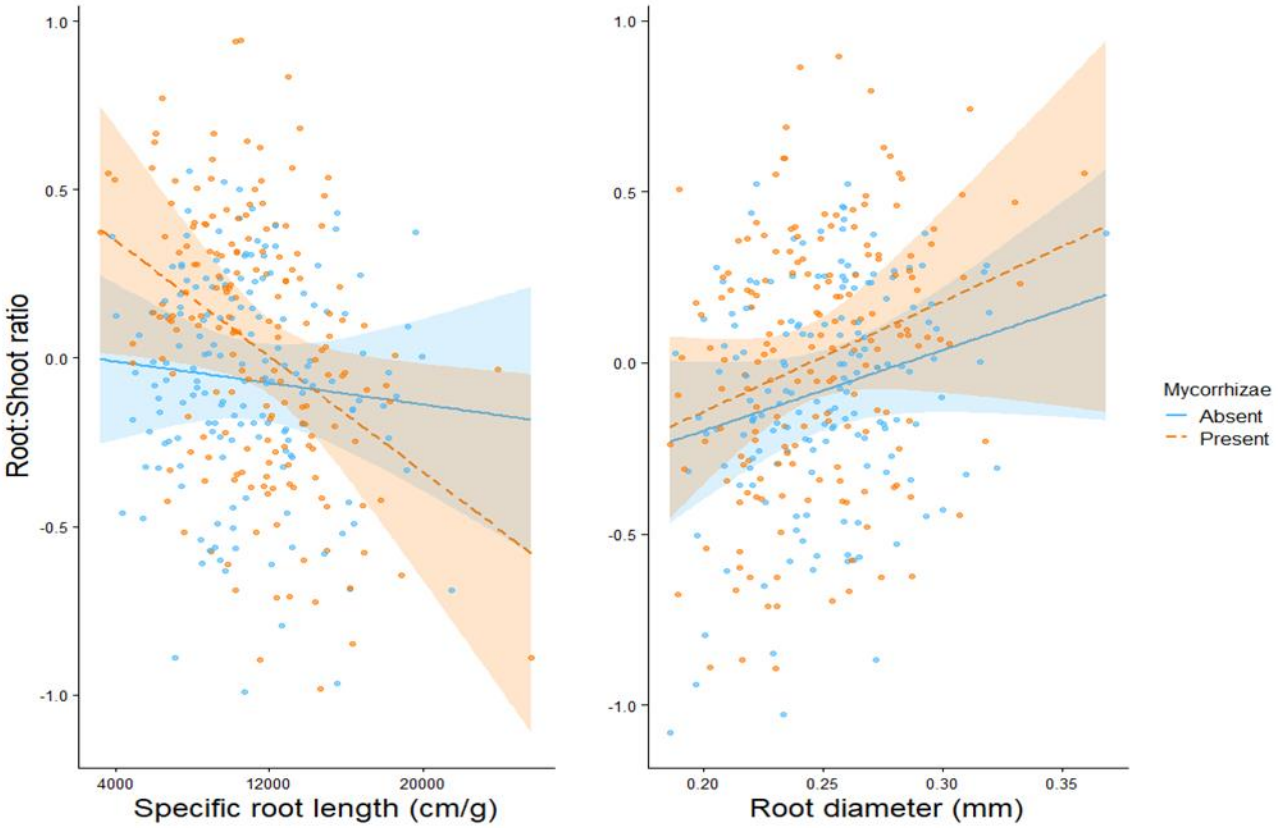

Figure 4.5. The relationship between SRL (left) and root diameter (right) on the root to shoot ratio across all treatments groups of alfalfa when grown with (orange solid line) or without (blue dash line) AMF. Bars around lines are the 95% confidence interval. Points are partial residuals as calculated by interact plot. The slopes of inoculated and uninoculated plants do not differ from each other in either plot.

Table 4.6. Summary of ANOVA table for models describing the effects of SRL, RTD, and RD on the leaf to stem ratio and root to shoot ratio of alfalfa. Results separated by a double line are from different models. Model terms that reached significance or marginal significant as referenced in the text are in bold.

	Model terms	DF	F	p
Leaf:stem ratio	**SRL**	**1**	**2.902**	**0.089**
	Treatment	3	0.249	0.862
	AMF	1	2.166	0.142
	SRL:Treatment	3	0.425	0.735
	SRL:AMF	1	0.638	0.425
	Treatment:AMF	3	1.034	0.378
	SRL:Treatment:AMF	3	1.521	0.209
	RD	**1**	**4.796**	**0.029**
	Treatment	3	0.668	0.573
	AMF	1	2.180	0.141
	RD:Treatment	3	0.567	0.637
	RD:AMF	**1**	**3.123**	**0.078**
	Treatment:AMF	3	1.939	0.123
	RD:Treatment:AMF	3	1.783	0.150
Root:Shoot ratio	**SRL**	**1**	**7.834**	**0.005**
	Treatment	3	0.845	0.471
	AMF	1	2.233	0.136
	SRL:Treatment	3	0.237	0.871
	SRL:AMF	1	1.349	0.246
	Treatment:AMF	3	0.619	0.603
	SRL:Treatment:AMF	3	0.777	0.508
	RD	**1**	**9.482**	**0.002**
	Treatment	3	0.519	0.670
	AMF	1	1.207	0.273
	RD:Treatment	3	0.675	0.568
	RD:AMF	1	1.546	0.215
	Treatment:AMF	3	0.848	0.468
	RD:Treatment:AMF	3	0.715	0.544

4.5.3. Nitrogen and Phosphorus Content

Nitrogen concentration in alfalfa leaves increased with SRL (Fig. 4.6). This relationship did not interact with AMF or treatment (Table 4.7). There were marginal interactions between RTD and treatment, and RTD and AMF (Table 4.7): AMF inoculation flattened the negative relationship between RTD and N concentration found in uninoculated plants across all treatments, though the negative relationship is most evident in low nutrient and salt stressed plants (Fig. 4.7).

Phosphorus concentration increased with SRL across all individuals (Fig. 4.8), this relationship did not interact with AMF or treatment (Table 4.7). There was a three-way interaction between root diameter, treatment, and AMF (Table 4.7). Nutrient limited inoculated plants had a (marginally) negative relationship between RD and P concentration ($p=0.063$), whereas uninoculated salt stressed plants had a positive relationship between RD and P content ($P=0.015$, Fig. 4.9).

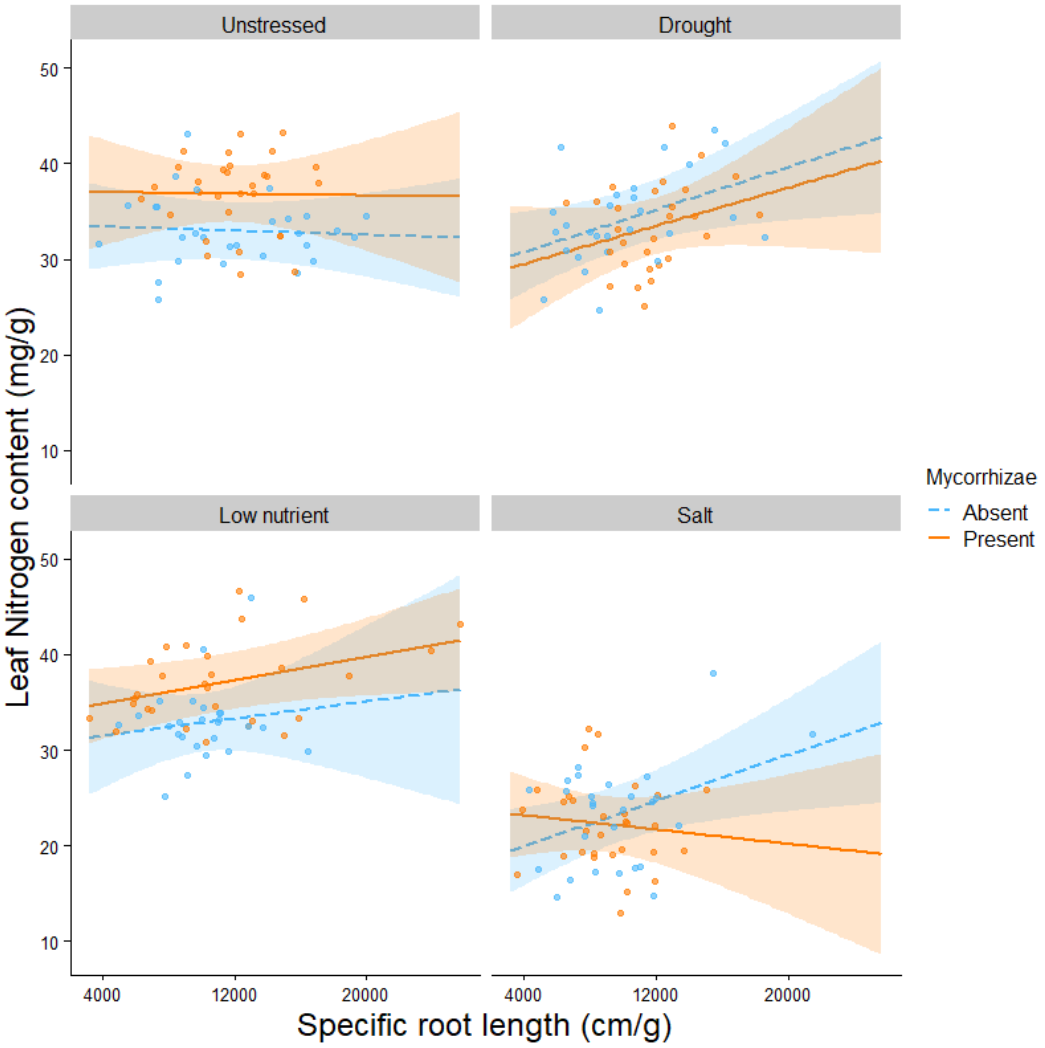

Figure 4.6. The relationship between specific root length and leaf nitrogen content when grown with AMF (orange solid line) or without AMF (blue dash line). The lines are surrounded by the 95% confidence interval. Points are partial residuals as calculated by interact plot.

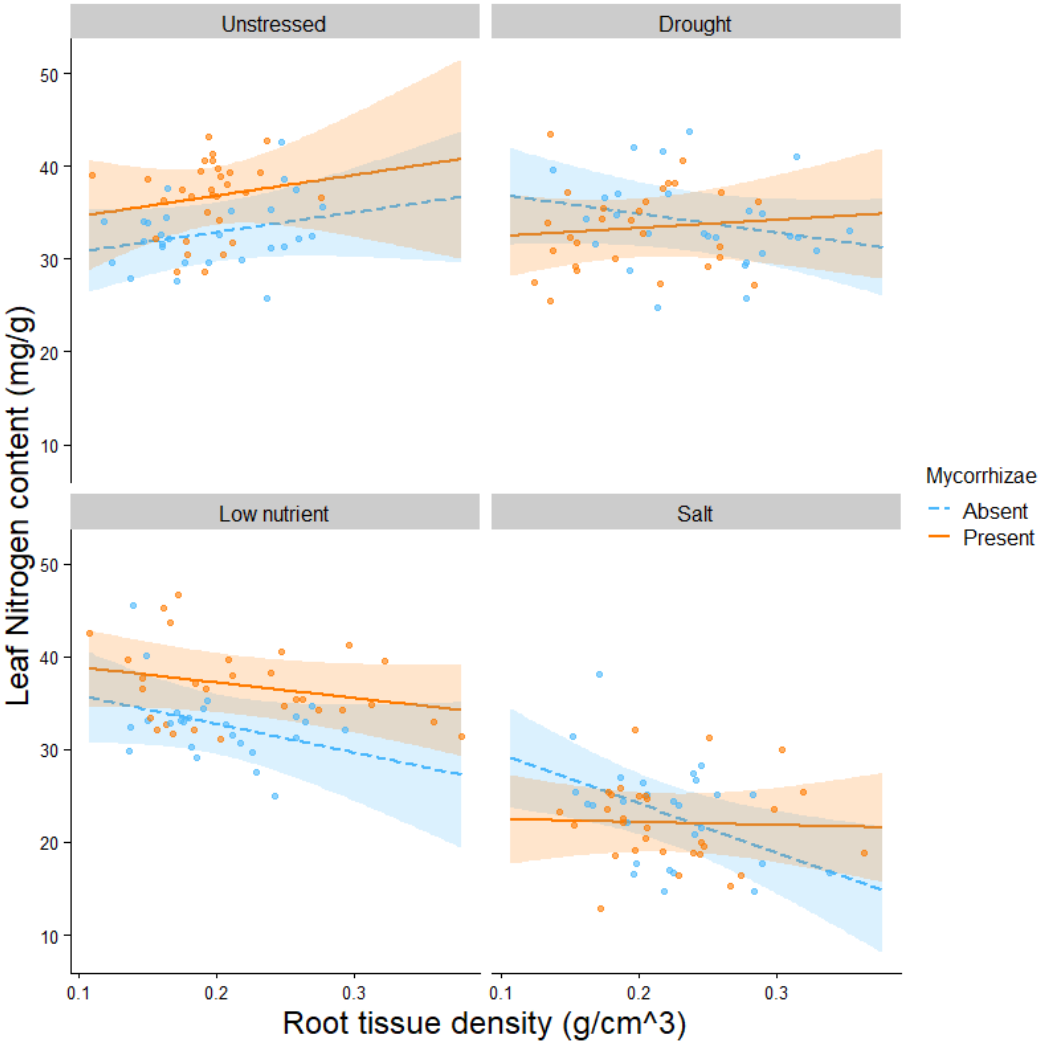

Figure 4.7. The relationship between root tissue density and leaf nitrogen content when grown with AMF (orange solid line) or without AMF (blue dash line). The lines are surrounded by the 95% confidence interval. Points are partial residuals as calculated by interact plot.

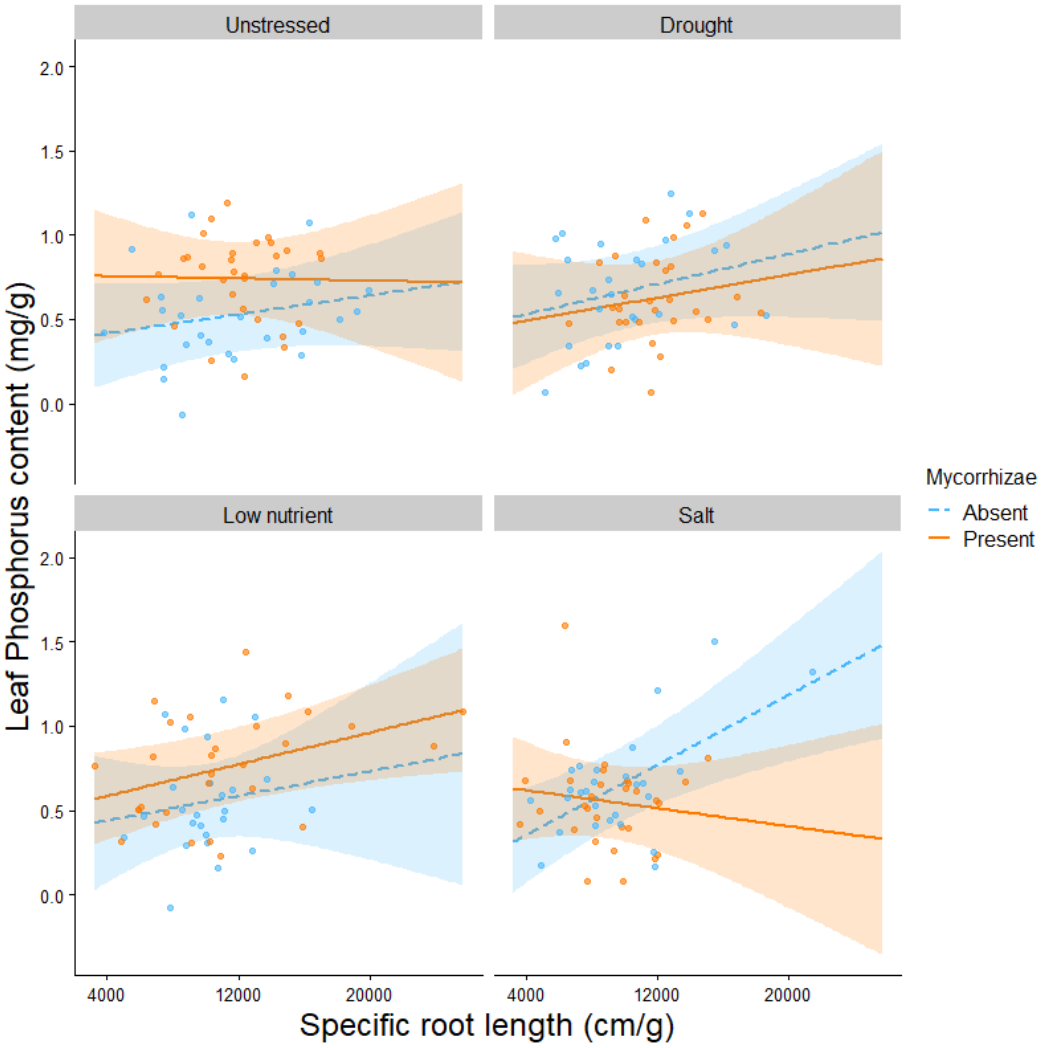

Figure 4.8. The relationship between specific root length and leaf phosphorus content when grown with AMF (orange solid line) or without AMF (blue dash line). The lines are surrounded by the 95% confidence interval. Points are partial residuals as calculated by interact plot.

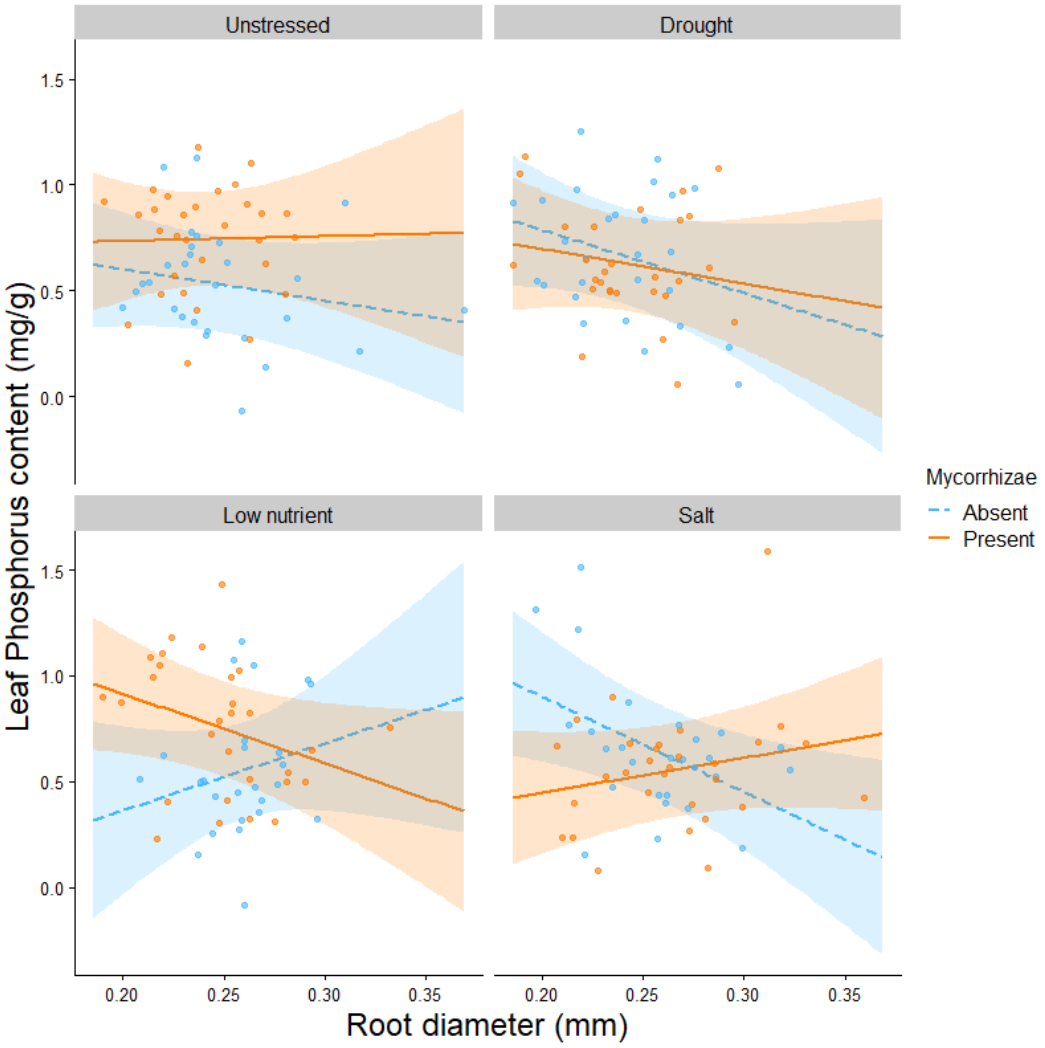

Figure 4.9. The relationship between root diameter and leaf phosphorus content when grown with AMF (orange solid line) or without AMF (blue dash line). The lines are surrounded by the 95% confidence interval. Points are partial residuals as calculated by interact plot

Table 4.7. Summary of ANOVA table for models describing the effects of SRL, RTD, and RD on leaf nitrogen and phosphorus concentration. Results separated by a double line are from different models. Model terms where the p value was less than 0.05 have been bolded.

	Model terms	**DF**	**F value**	**p**
Nitrogen	**SRL**	**1**	**5.875**	**0.016**
	Treatment	**3**	**9.49**	**$6.81e^{-6}$**
	AMF	1	1.754	0.187
	SRL:Treatment	3	1.431	0.235
	SRL:AMF	1	0.818	0.367
	Treatment:AMF	3	0.481	0.696
	SRL:Treatment:AMF	3	1.065	0.365
	RTD	1	1.737	0.118
	Treatment	**3**	**3.342**	**0.021**
	AMF	1	1.498	0.223
	RTD:Treatment	3	2.29	0.08
	RTD:AMF	1	3.023	0.083
	Treatment:AMF	3	1.663	0.177
	RTD:Treatment:AMF	3	0.558	0.643
Phosphorus	**SRL**	**1**	**6.681**	**0.011**
	Treatment	3	0.244	0.866
	AMF	1	3.22	0.074
	SRL:Treatment	3	0.327	0.806
	SRL:AMF	1	2.463	0.118
	Treatment:AMF	3	0.788	0.502
	SRL:Treatment:AMF	3	1.474	0.223
	RD	1	2.538	0.114
	Treatment	3	0.688	0.726
	AMF	1	0.047	0.768
	RD:Treatment	3	0.681	0.712
	RD:AMF	1	0.157	0.621
	Treatment:AMF	**3**	**3.272**	**0.01**
	RD:Treatment:AMF	**3**	**2.713**	**0.021**

4.6. Discussion

We found little evidence in this study to support our hypothesis that SRL, RTD, or RD are mediating factors of an alfalfa-AMF relationship. Our hypothesis in this chapter assumed that the root traits we measured would indicate alfalfa growth strategies under different stresses and would therefore be linked to their final biomass. On the contrary, alfalfa biomass was reduced by both stress treatment and the presence of AMF (in some cases) seemingly uncoupled from root trait expression, while root traits were linked to nutrient uptake, and investment into leaf tissue. Alfalfa biomass accrues over time, but the fine roots turn over. This could explain the lack of relationship between trait expression and biomass if trait expression changed significantly over time. Alfalfa fine root production plateaus after 9 weeks of growth, with a life span of approximately 2.5 to 4.4 months (Goins & Russelle, 1996). It possible that early root growth differed from later growth in the 4-month growing period of this study, but we were able to detect a consistent relationship between SRL and nutrient concentration, so if root trait expression changed enough over time to disrupt a trait-biomass relationship that relationship may have been negligible. It is unlikely that harvesting root samples at an earlier time point would have yielded very different results.

Root trait expression was affected by some stresses and AMF inoculation. Under drought stress inoculated plants increased mean SRL while maintaining mean RTD expression similar to that when unstressed. Uninoculated plants under drought increased their RTD. These divergent responses represent two different drought tolerance strategies: increasing SRL increases soot surface area and hydraulic conductance (Fitter, 2002; Franco et al., 2011), where increased RTD is linked to drought resistance through withstanding dehydration, and maintaining rather than increasing growth (Bristiel et al., 2019). Inoculation also reduced the CV of SRL in the drought treatment group, indicating that trait expression was in response to AMF. This AMF induced shift may be part of the overall reduced growth of inoculated drought stressed alfalfa. Low nutrient stressed plants had no changes in their mean trait expressions from AMF inoculation, but AMF did increase the variation of trait expression. Low nutrient treated plants inoculated with AMF improved their nutrient uptake, so they may not have been as pressured to change root expression in a single direction.

Leaf nutrient concentration consistently increased with SRL, regardless of treatment, showing that higher SRL is an effective strategy in increasing nutrient uptake. Even though we saw AMF interacting with SRL expression in all treatments except salt stress, this never changed the basic SRL-nutrient relationship. Root tissue density and RD should also be important parameters to nutrient uptake because they control surface area. The relationships between these traits and nutrients were dependant on treatment conditions unlike SRL. AMF did not alter RTD expression in salt treated plants, but inoculation removed a negative relationship between RTD and N concentration. Saline soil is commonly reported to reduce N in alfalfa (Ashrafi et al., 2018; Khan et al., 1994), and it was the only stress treatment that significantly reduced nitrogen concentration in our plants. However, changing the RTD-N relationship did not equate to an increased N concentration for inoculated plants overall , but higher RTD could be associated with a marginal increased N input from AMF here.

The relationship between AMF and P concentrations depended somewhat on root traits. Average P concentration was higher in inoculated unstressed and low nutrient treated alfalfa, inoculated low-nutrient treated alfalfa had a negative RD-P relationship not found in other treatments. This result is opposite to our expectation that plants with thicker roots would rely more on AMF to forage, it could be a result of "additive" P uptake where inoculated plants were better able to forage themselves when root diameters were small, as well as receive an AMF bump in nutrients. AMF also affected the RD-P relationship in the salt treatment, despite no net effect of AMF inoculation on P nutrition under these conditions. Salt treated plants showed a negative RD-P relationship only when AMF were absent. This could mean that these plants had a reduced capacity to forage when root diameter was high that was relieved by AMF. As we saw in the first chapter, salt stress (and drought) was far more stressful to the plants than the low nutrient treatment that produced no stress response, so, even if the low nutrient plants technically had less nutrients available to them, they were probably not hindered in their ability to forage themselves. Even though neither drought nor salt stress reduced P concentrations in alfalfa, they reduced the effectiveness of AMF to supply P in these conditions.

Root diameter and root tissue density are important to the plant-AMF relationship not only because coarser roots are less adept at absorbing nutrients, but because roots with a higher proportion of cortex tissue are better suited to mycorrhizal colonization (Brundrett, 2002; Kong

et al., 2017). In a global analysis Bergmann et al. (2020) found root diameter was negatively correlated with root tissue density because the ratio of less dense cortex increases compared to more dense root components. Root diameter and RTD were not correlated in our measurements. We did not measure the amount of root cortex, so we can only speculate about our root composition, but the lack of correlation could mean either that root cortex was not scaling with root diameter and therefore having no effect on AMF colonization, or that the range of root diameter size we utilized was too narrow to produce such a correlation. The fine roots in the global analysis were of root orders 1-3 or had a diameter less than 2mm, a much larger range than we measured.

In conclusion, alfalfa fine root traits interacted with AMF treatments to affect nutrient acquisition and biomass partitioning, but not plant biomass. We expected to see a positive relationship between RTD, RD, and nutrient concentration when plants were inoculated, but we instead found that AMF either had no effect, or in the case of salt stressed plants, inoculation flattened negative relationships between RTD, RD and plant nutrient concentrations. Therefore, the hypothesis that root traits determined mycorrhizal benefits was not supported.

5. CONCLUSIONS

The goal of our work presented here was to investigate the context dependency of plant-AMF relationships. Our study showed growth conditions mattered more for determining AMF affects on growth and stress response than cultivar identity did. AMF amplified the effects of drought and salt stress on biomass, a result that is rarely reported. In chapter 3 we found some interaction between AMF, stress, and reproduction, but they were inconsistent with the biomass stress responses we measured. We had expected to identify differences between cultivar stress responses in most of the parameters we measured, but in fact biomass and nutrient concentrations were fairly consistent across cultivars in each treatment group. Flowering and seed production were more cultivar dependant; AMF interactions with flowering differed between most cultivars, and non were consistently negative or positive, these changes did not seem to affect pollinator visitation or seed production in the end, so they may be largely inconsequential other than demonstrating that AMF were affecting whole plant systems.

Because the cultivars we used in the first analysis lacked distinctive growth responses, we examined the relationship between trait expression and AMF. Root trait expression has been used with some success to indicate plant's reliance on mycorrhizal symbiosis (Yang et al., 2015). The objective here was to discover if fine root trait expression predicted mycorrhizal response, and if AMF affected root trait expression in ways that altered the host plant's growth. We found that alfalfa trait expression was affected by AMF and stress, but this trait expression was not indicative of plant growth in any of the treatment groups. Specific root length correlated with the amount of N and P plants took up, but AMF did not interact with this relationship. This indicates that the plants were taking up a significant amount of nutrients on their own, not solely relying on AMF, this corresponds with the lack of a major growth or nutrient advantage in unstressed inoculated plants.

In conclusion neither cultivar identity, nor fine root trait expression were useful indicators for understanding alfalfa-AMF interactions in our study system. There were clear differences in

alfalfa stress response, biomass, and nutrient acquisition induced by AMF inoculation but we could not connect these to either inherent alfalfa traits, or trait changes caused by AMF or stress conditions. Qu et al. (2021) found similarly contradictory negative mycorrhizal responses under a range of light and phosphorus treatments where positive results are typical. They suspect that a combination of high colonization and low P transfer through the mycorrhizal pathway was responsible. We found low colonization in the more stressful treatments, but it is still likely that the negative affects seen in inoculated plants were a result of stressed AMF acting as a drain on resources. Negative mycorrhizal effects are not often reported, whether this is due to their true rarity or a publication / dissemination bias (Møller & Jennions, 2001) is up for speculation. Even unstressed conditions only facilitated marginal improvements in nutrient acquisition for alfalfa that did not translate to biomass or seed mass benefits. Using alternative AMF species or inoculant mixtures, especially if the AMF were adapted to stressful conditions could provide an interesting addition when addressing similar questions as our work here.

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Appendix A

Table A.1 The mean shoot mass of each alfalfa cultivar in each treatment group.

Treatment	AMF	Cultivar	Mean shoot mass (g)	Standard deviation
Unstressed	Without	2010	11.04	1.16
Unstressed	Without	3010	10.50	0.84
Unstressed	Without	ACB	11.90	1.97
Unstressed	Without	Asalt	12.14	2.23
Unstressed	Without	Foothold	11.20	1.21
Unstressed	Without	Perfection	13.14	2.39
Unstressed	Without	Rugged	11.06	2.61
Unstressed	Without	TH2	14.15	1.39
Unstressed	Without	Vision	12.70	3.09
Unstressed	With	2010	9.58	3.17
Unstressed	With	3010	10.48	1.64
Unstressed	With	ACB	10.78	1.10
Unstressed	With	Asalt	12.14	2.21
Unstressed	With	Foothold	11.62	0.73
Unstressed	With	Perfection	11.40	1.72
Unstressed	With	Rugged	11.48	3.33
Unstressed	With	TH2	10.96	1.69
Unstressed	With	Vision	11.40	1.92
Drought	Without	2010	9.05	2.78
Drought	Without	3010	9.98	0.60
Drought	Without	ACB	9.42	3.37
Drought	Without	Asalt	7.90	2.00
Drought	Without	Foothold	9.62	1.77
Drought	Without	Perfection	8.84	1.58
Drought	Without	Rugged	9.86	2.53
Drought	Without	TH2	10.42	3.23
Drought	Without	Vision	8.96	3.77
Drought	With	2010	6.92	1.15
Drought	With	3010	6.98	1.72
Drought	With	ACB	6.64	1.32
Drought	With	Asalt	7.24	0.47
Drought	With	Foothold	6.60	1.90

Drought	With	Perfection	7.34	1.06
Drought	With	Rugged	8.30	1.42
Drought	With	TH2	8.16	3.16
Drought	With	Vision	8.90	2.74
Low nutrient	Without	2010	10.70	1.81
Low nutrient	Without	3010	11.68	2.10
Low nutrient	Without	ACB	12.92	1.76
Low nutrient	Without	Asalt	11.40	0.89
Low nutrient	Without	Foothold	12.00	1.82
Low nutrient	Without	Perfection	10.78	2.77
Low nutrient	Without	Rugged	9.98	2.65
Low nutrient	Without	TH2	13.00	1.52
Low nutrient	Without	Vision	12.28	3.66
Low nutrient	With	2010	10.46	2.67
Low nutrient	With	3010	11.25	1.21
Low nutrient	With	ACB	10.84	3.05
Low nutrient	With	Asalt	12.94	3.47
Low nutrient	With	Foothold	12.20	1.51
Low nutrient	With	Perfection	11.02	0.98
Low nutrient	With	Rugged	11.48	2.75
Low nutrient	With	TH2	11.20	1.03
Low nutrient	With	Vision	11.68	4.04
Salt	Without	2010	9.48	2.67
Salt	Without	3010	10.90	1.10
Salt	Without	ACB	7.76	1.38
Salt	Without	Asalt	12.08	2.09
Salt	Without	Foothold	12.04	3.73
Salt	Without	Perfection	9.84	2.27
Salt	Without	Rugged	9.06	1.03
Salt	Without	TH2	8.48	0.79
Salt	Without	Vision	9.82	2.28
Salt	With	2010	8.00	2.02
Salt	With	3010	9.60	3.79
Salt	With	ACB	7.92	2.28
Salt	With	Asalt	6.86	0.54
Salt	With	Foothold	8.18	1.37

Salt	With	Perfection	8.72	0.84
Salt	With	Rugged	10.84	1.52
Salt	With	TH2	8.70	2.83
Salt	With	Vision	7.98	1.68

Table A.2. The mean root mass of all alfalfa cultivars in each treatment group.

Treatment	**AMF**	**Cultivar**	**Mean root mass (g)**	**Standard deviation**
Unstressed	Without	2010	13.28	2.48
Unstressed	Without	3010	14.43	1.04
Unstressed	Without	ACB	12.34	2.55
Unstressed	Without	Asalt	11.28	1.76
Unstressed	Without	Foothold	11.28	2.09
Unstressed	Without	Perfection	11.06	1.34
Unstressed	Without	Rugged	10.26	1.67
Unstressed	Without	TH2	8.83	1.47
Unstressed	Without	Vision	9.08	3.10
Unstressed	With	2010	13.68	3.65
Unstressed	With	3010	10.52	4.04
Unstressed	With	ACB	10.14	4.85
Unstressed	With	Asalt	11.44	3.98
Unstressed	With	Foothold	12.18	2.48
Unstressed	With	Perfection	11.72	3.82
Unstressed	With	Rugged	10.32	3.23
Unstressed	With	TH2	11.78	3.11
Unstressed	With	Vision	10.86	3.88
Drought	Without	2010	8.00	1.07
Drought	Without	3010	7.28	2.42
Drought	Without	ACB	8.08	2.81
Drought	Without	Asalt	7.12	2.37
Drought	Without	Foothold	6.96	2.37
Drought	Without	Perfection	7.18	1.74
Drought	Without	Rugged	7.50	1.24
Drought	Without	TH2	9.48	2.68
Drought	Without	Vision	6.10	2.25
Drought	With	2010	5.54	2.08
Drought	With	3010	7.45	3.08

Drought	With	ACB	6.20	1.08
Drought	With	Asalt	7.22	0.98
Drought	With	Foothold	7.22	2.68
Drought	With	Perfection	6.24	1.92
Drought	With	Rugged	7.76	1.49
Drought	With	TH2	5.80	0.71
Drought	With	Vision	7.04	2.25
Low nutrient	Without	2010	10.60	2.57
Low nutrient	Without	3010	12.16	2.98
Low nutrient	Without	ACB	11.04	3.14
Low nutrient	Without	Asalt	11.66	3.18
Low nutrient	Without	Foothold	11.15	1.86
Low nutrient	Without	Perfection	9.70	3.64
Low nutrient	Without	Rugged	11.16	1.90
Low nutrient	Without	TH2	11.12	2.67
Low nutrient	Without	Vision	11.86	1.65
Low nutrient	With	2010	8.74	3.42
Low nutrient	With	3010	9.25	2.24
Low nutrient	With	ACB	8.70	2.29
Low nutrient	With	Asalt	10.22	1.90
Low nutrient	With	Foothold	9.40	2.88
Low nutrient	With	Perfection	10.32	2.70
Low nutrient	With	Rugged	12.04	2.70
Low nutrient	With	TH2	9.82	4.01
Low nutrient	With	Vision	7.54	1.84
Salt	Without	2010	8.15	3.29
Salt	Without	3010	9.70	1.94
Salt	Without	ACB	5.82	1.95
Salt	Without	Asalt	8.16	2.87
Salt	Without	Foothold	9.54	2.63
Salt	Without	Perfection	7.64	2.45
Salt	Without	Rugged	7.62	1.01
Salt	Without	TH2	8.40	0.29
Salt	Without	Vision	8.72	2.59
Salt	With	2010	8.46	1.49
Salt	With	3010	7.46	1.75

Salt	With	ACB	5.70	1.29
Salt	With	Asalt	8.12	2.44
Salt	With	Foothold	8.68	1.07
Salt	With	Perfection	8.36	1.76
Salt	With	Rugged	7.54	1.69
Salt	With	TH2	7.24	1.55
Salt	With	Vision	6.76	2.06

Appendix B

Table B.1. The mean nitrogen content of alfalfa leaves in each treatment group.

Treatment	**AMF**	**Cultivar**	**Mean nitrogen**	**Standard deviation**
Unstressed	Without	3010	28.07	2.51
Unstressed	Without	ACB	34.81	2.66
Unstressed	Without	Foothold	34.00	2.42
Unstressed	Without	Rugged	33.14	3.38
Unstressed	Without	TH2	36.33	7.11
Unstressed	Without	Vision	32.79	3.87
Unstressed	With	3010	35.34	1.77
Unstressed	With	ACB	37.53	2.71
Unstressed	With	Foothold	40.35	4.74
Unstressed	With	Rugged	37.60	6.17
Unstressed	With	TH2	36.69	7.16
Unstressed	With	Vision	35.12	5.28
Drought	Without	3010	NA	NA
Drought	Without	ACB	37.91	3.61
Drought	Without	Foothold	37.13	5.00
Drought	Without	Rugged	34.46	5.46
Drought	Without	TH2	33.38	2.93
Drought	Without	Vision	31.92	6.27
Drought	With	3010	33.02	5.64
Drought	With	ACB	33.30	7.05
Drought	With	Foothold	37.63	5.12
Drought	With	Rugged	35.25	4.39
Drought	With	TH2	NA	NA
Drought	With	Vision	29.41	6.09

Low nutrient	Without	3010	NA	NA
Low nutrient	Without	ACB	34.36	6.35
Low nutrient	Without	Foothold	37.24	7.21
Low nutrient	Without	Rugged	31.68	3.27
Low nutrient	Without	TH2	NA	NA
Low nutrient	Without	Vision	30.22	2.10
Low nutrient	With	3010	36.10	7.55
Low nutrient	With	ACB	38.96	3.07
Low nutrient	With	Foothold	40.55	3.73
Low nutrient	With	Rugged	36.07	6.19
Low nutrient	With	TH2	37.48	5.60
Low nutrient	With	Vision	34.48	4.06
Salt	Without	3010	19.08	5.90
Salt	Without	ACB	24.60	5.56
Salt	Without	Foothold	25.47	2.49
Salt	Without	Rugged	24.01	10.16
Salt	Without	TH2	23.59	5.18
Salt	Without	Vision	22.57	3.52
Salt	With	3010	20.46	4.02
Salt	With	ACB	20.98	5.47
Salt	With	Foothold	24.20	3.50
Salt	With	Rugged	23.62	5.67
Salt	With	TH2	26.59	5.94
Salt	With	Vision	19.72	2.12

Table B.2. The mean phosphorus content of alfalfa leaves in each treatment group.

Treatment	**AMF**	**Cultivar**	**Mean phosphorus**	**Standard deviation**
Unstressed	Without	3010	1.64	0.35
Unstressed	Without	ACB	1.81	0.32
Unstressed	Without	Foothold	1.78	0.76
Unstressed	Without	Rugged	1.74	0.77
Unstressed	Without	TH2	1.82	0.79
Unstressed	Without	Vision	1.77	0.45
Unstressed	With	3010	2.14	0.33
Unstressed	With	ACB	2.13	0.33
Unstressed	With	Foothold	1.92	0.49

Unstressed	With	Rugged	2.09	0.70
Unstressed	With	TH2	1.78	0.44
Unstressed	With	Vision	2.71	0.71
Drought	Without	3010	NA	NA
Drought	Without	ACB	2.35	0.59
Drought	Without	Foothold	1.79	0.62
Drought	Without	Rugged	1.96	1.03
Drought	Without	TH2	1.63	0.45
Drought	Without	Vision	2.16	0.82
Drought	With	3010	1.89	0.72
Drought	With	ACB	1.99	0.95
Drought	With	Foothold	1.78	0.79
Drought	With	Rugged	1.96	0.63
Drought	With	TH2	NA	NA
Drought	With	Vision	2.37	0.60
Low nutrient	Without	3010	NA	NA
Low nutrient	Without	ACB	2.05	0.70
Low nutrient	Without	Foothold	1.84	1.16
Low nutrient	Without	Rugged	1.30	0.46
Low nutrient	Without	TH2	NA	NA
Low nutrient	Without	Vision	2.43	0.91
Low nutrient	With	3010	2.87	0.42
Low nutrient	With	ACB	2.35	0.11
Low nutrient	With	Foothold	1.97	0.41
Low nutrient	With	Rugged	1.90	1.24
Low nutrient	With	TH2	1.88	0.52
Low nutrient	With	Vision	2.26	0.69
Salt	Without	3010	2.13	0.33
Salt	Without	ACB	2.34	1.07
Salt	Without	Foothold	1.53	0.26
Salt	Without	Rugged	2.11	1.49
Salt	Without	TH2	2.02	0.87
Salt	Without	Vision	2.01	0.29
Salt	With	3010	1.67	0.35
Salt	With	ACB	1.71	0.23
Salt	With	Foothold	1.71	0.26

Salt	With	Rugged	1.70	0.40
Salt	With	TH2	1.39	0.34
Salt	With	Vision	2.51	1.65