THE INFLUENCE OF AN *ASCOPHYLLUM NODOSUM* SEAWEED BIOSTIMULANT ON INTERACTIONS BETWEEN ARBUSCULAR MYCORRHIZAL FUNGI (*RHIZOPHAGUS IRREGULARIS)* AND *MEDICAGO TRUNCATULA*

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Submitted in partial fulfillment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
November 2019

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Abstract

Seaweed biostimulants, such as the *Ascophyllum nodosum* extract (ANE) produced by Acadian Seaplants, are used globally to improve crop quality. The effects ANE have on soil microbial populations, and plant interactions with soil communities, is understudied. The objectives of this study were to (1) evaluate the direct effect of ANE on a globally distributed arbuscular mycorrhizal fungus *Rhizophagus irregularius*; and (2) test whether ANE influences plants ability to form mycorrhizal symbiosis. ANE had a significant effect on growth of *R. irregularius* during *in vitro* trials at concentrations of 0.01% and 0.05%, but not 0.1%. Greenhouse grown *Medicago truncatula* ANE drench treated plants grew larger and were faster to form mature mycorrhizal associations than controls, while foliar results were variable. Genes of *M. truncatula* associated with early signaling for mycorrhizae were expressed at significantly higher levels in plants treated with ANE as a foliar or drench compared to untreated controls.

List of Abbreviations Used

ANE: *Ascophyllum nodosum* extract, all ANE in this research was made by Acadian Seaplants using a proprietary KOH extraction

SSEP: Solid seaweed extract powder, an ANE product made by Acadian Seaplants

AMF: Arbuscular mycorrhizal fungi

Acknowledgements

I would like to thank all the wonderful employees at Acadian Seaplants for their help and support through this process. A special thank you to Kevin Shiell, Katy Shotton, Andy Norman and Dr. Bachar Blal for their support in method development, trouble shooting and data analysis. The molecular part of this project would not have been possible without the patience and guidance from Dr. Timo van der Zwan. Thank you to Dr. Balakrishnan Prithiviriaj for taking me into your lab group and to all members of the Marine Plant and BioProducts Laboratory for being wonderful colleagues. Thank you to students Kevindi Gunasekara and Dani Tonner-Smith for their help and interest in this project. Thank you to committee members Dr. Derek Lynch and Dr. Svetlana Yurgel for input and guidance. I would also like to thank my husband and my Mum for their unwavering support. I would like to acknowledge MITACS Accelerate and Acadian Seaplants for funding this research.

Chapter 1: Introduction

The world's population is estimated to reach 9 billion by the year 2050 (Searchinger *et al*., 2014). To feed a growing population in the face of climate change challenges agriculture needs to be smart and resilient. There is a current trend to move away from synthetic chemicals and towards whole plant management that considers the health of the shoots and roots, but also the organisms below ground (Brown and Saa, 2015). In agriculture the importance of the soil microbial communities is just beginning to be realized. Nurturing the soil microbial communities could help to improve yield in a variety of crops (Van Oosten *et al*., 2017). The use of seaweed based biostimulants is also increasing as it is a natural way to boost the ability of crops to tolerate stress, but mechanisms of action require additional research (Brown and Saa, 2015).

A plant biostimulant is any organic substance that is able to stimulate a natural response in plant regardless of its nutrient content (du Jardin, 2015). The biostimulant industry is growing rapidly, there are estimates that the industry could grow to be worth $2 billion by 2018 (Brown and Saa, 2015). Povero *et al*. (2016) estimate the industry will be worth just over $2.5 billion by 2019. The biostimulant industry includes many products aside from seaweeds, but seaweed based biostimulants are the most common (Brown and Saa, 2015). There are currently several companies that are major players in seaweed based biostimulants worldwide: Valagro, Tradecorp, Stoller, Valent and Acadian Seaplants.

Biostimulants are manufactured from an organic substance, either plant, animal or microbial origin. The term biostimulant has only been officially defined since 2007, but the products have been used for much longer (du Jardin, 2015). The effect of a

biostimulants is caused by more than just the nutrient content of the product (Battacharyya *et al*., 2015). It is thought that biostimulant application improves the plants' ability to respond to their environment, by amplifying or accelerating a response process (Battacharyya et al., 2015). Seaweed products are a large part of the biostimulant market and this study looks at the use of an *Ascophyllum nodosum* extract (ANE) made by Acadian Seaplants LTD, Dartmouth, Nova Scotia. A solid seaweed extract powder (SSEP) made through a proprietary KOH extraction of *A. nodosum* was used in this research by mixing the powder with reverse osmosis water. The SSEP is an ANE product and the terms are both used in this text to refer to the *A. nodosum* extract powder made by Acadian Seaplants.

When studying the effects of a seaweed biostimulant it is intuitive to focus on the plant components of the ecosystem, but there are many other organisms that contribute to plant health. The rhizosphere soil, tightly associated with plant roots, is occupied by millions of microbes that are intimately connected to the health of a plant (Badri *et al.*, 2009). One of the most common groups of microbes found in the rhizosphere and plant root tissues are arbuscular mycorrhizal fungi (AMF), a microscopic obligate symbiont associated with more than 80% of land plants (Smith and Read, 2008). AMF facilitate the uptake of nutrients, while also offering protection from a wide range of abiotic and biotic stresses to the plant (Smith and Read, 2008). AMF have the greatest effect on plant growth in phosphorous poor environments (Smith and Read, 2008). Given that peak phosphorus is likely to happen within the next 30 years these microbes will be of even greater importance into the future (Smith and Read, 2008; Cordell and White, 2015). Plants secrete 20-60% of their total photosynthetic products into the rhizosphere, which

suggest that cultivating microbial associations are import to the plant life cycle (Gillings and Holmes, 2004).

The goal of this research was to study the effects of an *Ascophyllum nodosum* seaweed biostimulant (ANE) on plant-AMF interactions. The main objectives were to explore how the use of ANE effects the early growth of arbuscular mycorrhizal fungi and to examine whether the use of ANE increases the plant molecular response to facilitate a symbiosis with AMF. The model species *Medicago truncatula* and *Rhizophagus irregularis* (syn. *Glomus intraradicies*) were used for this study. To date, few studies have examined how ANE effects root zone microorganisms, but initial findings have shown an increase in total soil respiration, species diversity and in some cases an increase in beneficial microbe populations (Alam *et al*., 2014; Alam *et al*., 2013; Suhail, 2013, Khan *et al*., 2009; Kuwada et al., 2006).

1.1 Effects of Seaweed Biostimulants on Plants

Seaweed has been used as an agricultural amendment for centuries. For example, there are reports of farmers near oceans using seaweed to aid with crop growth long before the biostimulant industry formally developed (du Jardin, 2015). Furthermore, there is also literature showing that ancient Romans used seaweed as an additive in agricultural practices (Battacharyya *et al*., 2015). Agricultural crops have been bred and modified for more than 10 000 years to develop high yielding varieties (du Jardin*, 2015).* High yield is important, but this has come with the sacrifice of lowering plants natural ability to tolerate stresses (Peleg *et al*., 2012). Using an ANE biostimulant has proven to be an

effective way to enhance plants natural ability to tolerate stress like drought, heat, salinity, flood, pest and pathogens (Battacharyya *et al*., 2015).

Seaweed based biostimulants are known to have many effects on plant growth and success under biotic and abiotic stresses (Battacharyya *et al*., 2015). There are over 10 000 known species of seaweeds. Brown seaweed species are most commonly used to make biostimulants (Battacharyya *et al*., 2015). Currently, *Ascophyllum nodosum, Ecklonia maxima, Macrocystis pyrifera* and *Durvillea potatorum* are the most popular species for agricultural biostimulants (Battacharyya *et al*., 2015). The effects of ANE are known to be beyond what would occur if it were simply a plant response to the nutrients in the product (Battacharyya *et al*., 205). That said, not all mechanisms that cause ANE to effect change in a plant are known (Battacharyya *et al*., 2015). As manufacturers begin to mix seaweed extracts with other biostimulants such as humic acid, chitosan, or microbes, the effects of the products on plant success will only continue to diversify. There is evidence to show that biostimulants change gene expression in plants, but what genes are affected are dependent on what crop is being treated and what stress it is being subject to (Battacharyya *et al*., 2015).

From a biochemical perspective, there are many compounds in ANE that have effects on plant growth. Some chemicals in ANE are: complex polysaccharides, fatty acids, vitamins, phytohormones, pholortannins, and mineral nutrients (Battacharyya *et al*., 2015). Inorganic components of extracts made with *A. nodosum* range from nitrogen, phosphorus, potassium, calcium, iron, magnesium, zinc, to sodium and Sulphur (Battacharyya *et al*., 2015). The extracts also contain osmolites like betaines, and other secondary metabolites, like phenolics (Battacharyya *et al*., 2015).

1.2 Seaweed Biostimulants and Soil Microbes

There have been several publications examining the role of ANE on soil microbes. In 2014, Alam *et al*. showed the use of ANE obtained from Acadian Seaplants increased the root zone soil microbial activity in field grown carrots. The ANE was a soluble powder, similar to what was used in this research and was applied as a drench at rates of 0.5, 0.75, and 1g/L with 200mL being given to a row of 45 plants either weekly, biweekly or triweekly. The microbial activity was measured as the amount and diversity of species that could be cultured on plates. There were up to 39% more bacterial populations on plates with soil from the ANE treated plots compared to the control (Alam *et al*., 2014). Fungal population counts also increased up to 16% compared to the non ANE treated plots (Alam *et al*., 2014). The study also found increased soil respiration and microbial metabolic activity when soils were treated with ANE (Alam *et al*., 2014). The application rate of 0.5g/L weekly showed the strongest response in all tested parameters. Similarly, in another study of field and greenhouse grown strawberries it was found that using a drench application of soluble powder ANE increased the number of bacterial colonies, their respiration rate and metabolic rate (Alam *et al*., 2013). These two studies give evidence that using drench applied ANE increases the activity and diversity of the rhizosphere. In 2006 Kuwada *et al*., showed that using an *in vitro* model methanol extracts from red and green seaweeds directly improved the length of AMF spores. As AMF are important fungal symbionts in the rhizosphere it is hypothesized that using ANE will also improve their vigor.

There are very few studies examining the effects of applying both ANE and AMF treatments to plant systems, but initial results are positive. Suhail (2013) found that

inoculating cucumber seedlings with AMF and applying a foliar ANE three times during eight weeks lead to an increase in plant length, fresh weight, dry weight, chlorophyll amount, leaf area, and yield per plant under greenhouse conditions over a control, seaweed alone, or AMF treatment alone. Suhail (2013) found that inoculation of onion seeds with AMF spores, nitrogen fixing bacteria and ANE increased plant height, root length, wet and dry weight and amount of AMF colonization under salt stress in greenhouse conditions. Khan *et al*., (2009) also indicate there is preliminary evidence that ANE application can have a positive effect on AMF growth. The lack of publications examining the effects of ANE on AMF show a clear knowledge gap.

1.3 Growth and Morphology of Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi are a morphologically unique group in the fungal kingdom (Smith and Read, 2008). There are an estimated 230 species in the phyllum Glomeromycota, all form endomycorrhizal associations with land plants (Smith and Read, 2008). This group of fungi is greater than 400 million years old and helped the first plants to colonize on land (Smith and Read, 2008). AMF are microscopic and have both a sporulative and vegetative growth phase, to date it is thought that all reproduction is asexual (Harrison, 2005). Spores can survive dormant in soil for years and while there are differences in spore morphology, the vegetative stages appear similar across all species (Gillings and Holmes, 2004; Smith and Read, 2008). Spores of AMF are quite large compared to many other fungal species, they can be up to 500μm in diameter (Smith and Read, 2008). Each spore contains a significant amount of fat and carbohydrates, as well as thousands of haploid nuclei (Smith and Read, 2008).

There are species in other fungal phyla, mainly the Basidiomycota, that form mycorrhizal associations with plants. However, associations outside the phylum Glomeromycota are termed ectomycorrhizal as the hyphae never penetrate the plant roots (Gillings and Holmes, 2004). AMF species are endomycorrhizal because the fungal hyphae penetrate plant cells and exchange nutrients and carbohydrates intracellularly (Smith and Read, 2008). Unlike parasitic fungi, plants allow, and even facilitate the intrusion of the fungi into their cells through complex signaling that occurs between both the plant and the fungi (Smith and Read, 2008).

To overcome spore dormancy environmental conditions such as temperature, moisture and pH must be ideal (Harrision *et al*., 2005). The first visual sign of germination is the formation of a germ tube (Smith and Read, 2008; Harrison *et al*., 2005). The germ tube then has to quickly branch and form an association with a plant in order to survive (Smith and Read, 2008). The hyphae growing through soil are coenocytic and multinuclear and rely on anastomosis to branch and create a network of mycelium through the soil (Hamel and Plenchette, 2007). Hyphae can grow in varying thicknesses, coarse hyphae usually grow alongside roots sending lateral fine hyphae toward and into the root at irregular intervals (Hamel and Plenchette, 2007). Coarse hyphae often push through soil quickly, while the finer lateral hyphae prioritize nutrient uptake (Hamel and Plenchette, 2007). When penetrating a root cell, the fungi form a penetration structure called an appressorium, similar to the morphology found in pathogenic species (Smith and Read, 2008). Once inside the plant cell, AMF form arbuscules, which are branched hyphal like structures where nutrient exchange takes place with the plant (Smith and Read, 2008) (Figure 1). The AMF also form vesicles

inside plant cells which are bladder like structures, thought to be for storage (Smith and Read, 2008).

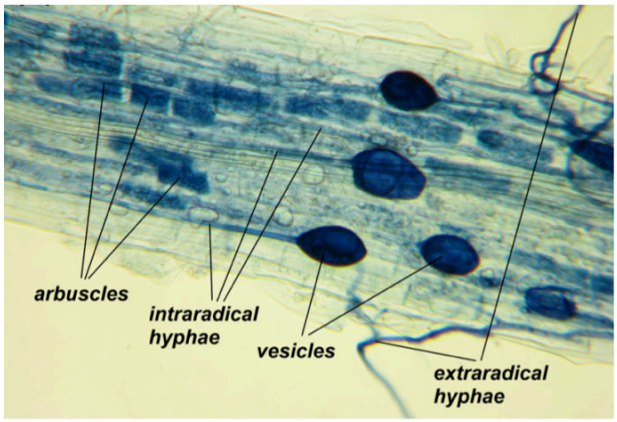


Figure 1: A photo of arbuscular mycorrhizal hyphae growing in association with a root stained with Trypan Blue. Hyphae are extending out of the plant root, while inside the root vesicles are the dark circular structures and arbuscules are the irregular hyphal conglomerates (University Bielefeld, 2013).

1.4 Plant Interactions with Arbuscular Mycorrhizal Fungi

Plants give 20 to 60 percent of their total photosynthates to soil microbes as plant root exudates (Gillings and Holmes, 2004; Badri *et al*., 2009). The majority of plants form associations with AMF, including most agricultural crops (Gillings and Holmes, 2004). Plants receive many benefits from forming associations with AMF, most notably an increase in phosphorous availability (Smith and Read, 2008). Some studies report that AMF are responsible for providing up to 80% of the plants phosphorous (Smith and Read, 2008). Mycorrhizae are also able to provide plants with other nutrients, including water, zinc, copper, potassium, and nitrogen, the AMF work in true symbiosis with their plant host (Hamel and Plenchette, 2007). There are numerous studies that show when a

plant is living with AMF soil borne bacterial and nematode plant pathogens have less significant impacts (Hamel and Plenchette, 2007). Agricultural crops living in association with AMF also typically have higher yields and stronger growth (Hijri, 2016). Literature shows conflicting results when looking at the protective ability of AMF to combat foliar or systemic fungal pathogens and viral diseases, but it is likely that AMF have some positive effects (Hamel and Plenchette, 2007; Rouphael *et al*., 2015). The mechanism behind AMF offering disease protection to the plant host is not well understood.

It is known that plants send signaling molecules, beyond the abundance of carbohydrates, through root exudates to attract fungi toward them in the soil (Hamel and Plenchette, 2007; Badri *et al*., 2009). The plant physiology, genomics and metabolomics behind the signaling is very complicated and not yet well understood (Harrison *et al*., 2005; MacLean *et al*., 2017; Choi *et al*., 2018). Although there are many unknowns, it is confirmed that plants are able to differentiate between AMF and other pathogenic fungi and facilitate the movement of AMF into their cells (Harrison *et al*., 2005, Rouphael *et al*., 2015). Plant cells undergo changes to their cell wall, cellular membrane, and the cytoskeletal structure to allow the fungi space to penetrate (Smith and Read, 2008; Gutjar and Parniske, 2013). Some signaling molecules have also been identified, the most recently discovered fungal branching compound produced by plants are strigolactones, a plant growth regulator (Gomez-Roldan *et al*., 2008; Genre *et al*., 2013).

1.5 The Plant's Molecular Response to Mycorrhizae

Plant cells begin to change in response to AMF before the two organisms make contact in the soil (Bonfante and Genre, 2010; Rouphael *et al*., 2015). Genes examined in

this research and where they are active in the plant cell are shown in Figure 2. In the pre-contact phase, the flavonoid synthesis pathway is triggered, flavonoid production is known to encourage AMF hyphal branching, CHS is expressed as the first part of the synthesis pathway (Bonfante and Genre, 2010; Abdel-Latif *et al*. 2012). Liu *et al*., (2011) has also shown the production of a chloroplastic beta-carotene isomerase, D27 to be increased, which codes for production of a key enzyme in strigolactones synthesis. Strigolactones are signaling molecules that have also been shown to induce AMF hyphal branching, they are thought to be important root exudates for promoting soil microbial symbiosis (Gomez-Roldan *et al*., 2008; Genre *et al*., 2013; Van Zeijl *et al*., 2015).

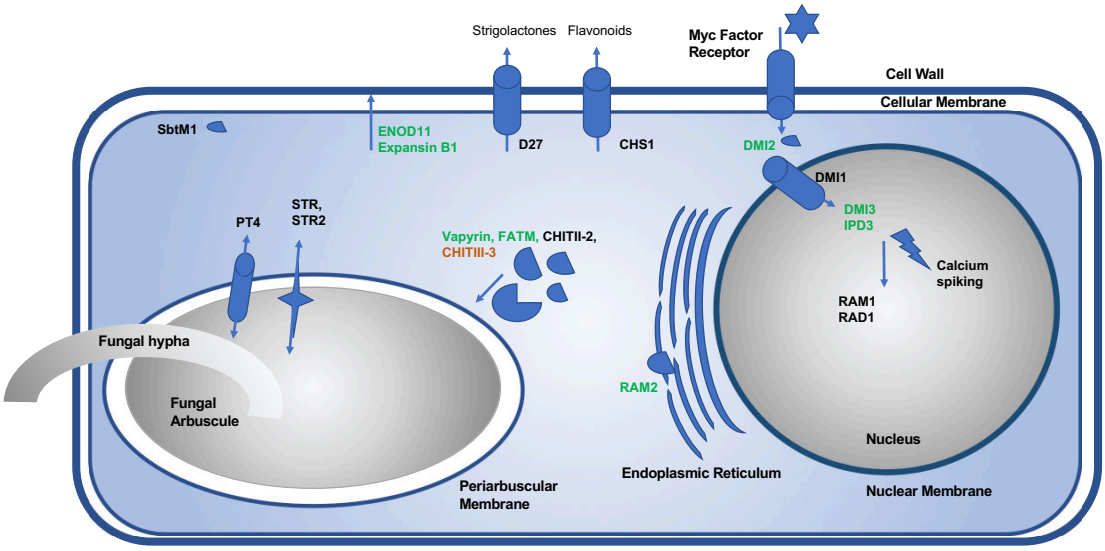


Figure 2: Genes selected for assessment act in many different processes to allow the fungi penetration and sustained growth within the cells. The location or organelle associated with each genes function is indicated above. Genes in green were observed to be upregulated in drench and foliar applications, the orange color indicates the foliar treatment resulted in upregulation, but not the drench. The colonization process begins as the Myc Factor is perceived by the cell. The signal is relayed through a kinase into the nucleus, a signaling cascade and calcium spike then occur in the nucleus, which causes the cell to continue a response. Resulting processes include activity on the cell wall to allow fungal penetration, creation of a specialized periarbulscar membrane to contain the fungi, and eventually nutrient exchange between plant and fungi.

When plants intercept the Myc-LCO's exuded from AMF a signaling cascade begins as a kinase, DMI-2, is activated (Bonfante and Genre, 2010; MacLean, 2017; Olah *et al*., 2005). The signaling cascade leads to a calcium spike in the nuclear membrane of the plant cell as the plant begins to respond to the fungal presence (Bonfante and Genre, 2010; MacLean 2017; Leiato *et al*., 2019). During the calcium spike DMI1 is active helping for a cation channel in the nuclear membrane to function (MacLean *et al*., 2017; Leiato *et al*., 2019). The calcium oscillations are thought to begin the cytoskeleton changes that will occur to accommodate the AMF (Horvath *et al*., 2011).

In the early stages of colonization there are also genes upregulated that are thought to suppress the basal defense response of plants as the fungi begin to form hyphopodium (Siciliano *et al*., 2007; MacLean *et al*., 2017). Siciliano *et al*., (2007) identified expansisn genes to be highly expressed in early AMF contact, ExpanisnB1 is known to help loosen cell walls to allow fungi entry (Siciliano *et al*., 2007). Gene ENOD11 is also important in mediating AMF symbiosis as it creates a cell wall protein that helps the mycorrhizae to penetrate (Journet *et al*., 2001). As the fungi begin to make physical contact the calcium oscillation is continuing to be a major factor in plant response (MacLean *et al*., 2017; Choi, 2018). As well DELLA transcription factors, RAM1 and RAM2 are active and have shown to be essential for AMF colonization (Gobatto *et al*., 2012; Gutjahr and Parniske, 2013; Floss *et al*., 2017; Pimprikar and Gutjahr 2018). In knockout mutants of RAM1 and RAM2 no colonization can occur due to fungal hyphopodium defects (Pimprikar and Gutjahr, 2018). IPD3 is also an essential protein that interacts with DMI3 and RAD1 and helps allow fungal penetration (Horvath *et al*., 2011).

Later phases of colonization are characterized by the presence of AMF arbuscules, which are the site of nutrient exchange between plant and fungi. Plants form a specialized periarbuscular membrane around the AMF arbuscule to facilitate transfer of products (MacLean *et al*., 2017; Choi *et al*., 2018). Recently, as it has been found that AMF receive lipids from plants and the upregulation of lipid transferring molecules STR/STR2 has been confirmed in mycorrhizal plants (MacLean *et al*., 2017; Bravo *et al*, 2017; Choi *et al*., 2018). An enzyme involved in lipid synthesis, FATM is also thought to be a part of the nutrient transfer process and is highly expressed in mycorrhizal cells (MacLean *et al*., 2017; Bravo *et al*, 2017; Choi *et al*., 2018). Breuillin-Sessoms *et al*., (2015) also characterized the presence of a phosphate transporter PT4 in the periarbuscular membrane that is thought to be responsible for moving phosphate from the fungi to the plant. During later phases of colonization there are also many genes expressed that are thought to play a role in supporting the presence of large and well developed fungal arbuscules, such as vapyrin, subtilase (SbtM1) and RAD1 (Pimprikar *et al.,* 2016; Maclean *et al.,* 2017; Choi *et al*., 2018; Salzer *et al*., 2000). Throughout the lifecycle of a colonized cell the defense pathway needs to be suppressed, chitinases CHITIII-3 and CHITII-2 are thought to be involved in this, specifically during late colonization (Chaubaud *et al.,* 2002).

1.6 Arbuscular Mycorrhizal Fungi Interactions with Plants

Current research supports the hypothesis that plant – AMF communication is a reciprocal interaction with AMF also signaling to plants (Smith and Read 2008; Harrison *et al*., 2005; Kousta *et al*., 2003). AMF produce compounds, lipochitoligosachardies,

generally referred to as Myc factors, that induce physiological changes in plant roots to prepare for colonization (Smith and Read, 2008; Malliet *et al*., 2011). An AMF spore does not need a signal from a plant to germinate, but some plant root exudates trigger germination, which suggests the spores can sense more than just the abiotic conditions of the rhizosphere (Harrison *et al*., 2005). When a spore germinates it can grow a few centimeters into the soil looking for a suitable plant host, if it does not find a host some species can revert spore phase to save resources (Harrison *et al*., 2005). Some spores contain enough resources to germinate up to 10 times, increasing the chance a spore can successfully reach a plant host (Harrison *et al*., 2005).

The symbiosis of nutrient transfer between plants and AMF may be more complex than initially thought. Until recently it was believed that AMF could synthesize their own nutrients simply by obtaining carbon from the plant hosts. Bravo *et al*. (2017) have evidence to suggest AMF also receive fats from the plants. Plant root cells that contain AMF arbuscules increase their gene expression for lipid production by 3000 fold in some studies (Gaude *et al*., 2012). Bravo *et al*. (2017) mutated genes in *M. truncatula* to reduce plant lipid production and found the AMF did not form a robust arbuscule population inside the mutated plants. It appears that AMF rely on the host plant to provide simple fats, so they can create their own more complex fats (Bravo *et al*., 2017). It seems logical that the nutrient interactions between AMF and plant hosts are more complex than just providing carbon, or there should be potential to culture the fungi in the absence of a plant host. As genetic techniques advance it is likely that even more complex interactions between AMF and their plant hosts will be realized (Bravo *et al*., 2017).

To date, most research on AMF at a molecular level is done with Ri-T-DNA plant roots, which changes the quality of the interaction because there is no plant photosynthetic tissue (Koffi *et al*., 2013). It is also challenging to obtain aseptic cultures of AMF spores to use *in vitro* and very laborious to sterilize spores obtained from natural soils. Many studies looking at AMF – plant interactions are done using greenhouse or field trials. These studies are the simplest to establish and negates the problems of culturing AMF under *in vitro* conditions.

1.6 Medicago truncatula as a Model Plant

M. truncatula, or Barrel Medic, is the most common model plant used for genetic studies with AMF (Smith and Read, 2008). *M. truncatula* is a small legume native to the Mediterranean, the plant grows low to the ground like clover and easily reaches 4 meters in length (Barker *et al*., 2006). Leaves are rounded and trifoliate, the plant produces yellow flowers and seeds mature in a spiny pod (Barker *et al*., 2006). The whole genome of *M. truncatula* was sequenced and is 375 million base pairs in size (Young *et al*., 2011). It is difficult to break seed dormancy in this species and mechanical scarification or acid scarification with concentrated sulphuric acid is needed (Barker *et al*., 2006).

1.7 Research Objectives

There are many mechanisms that allow ANE to be an effective agricultural input that supports plant growth. Not all mechanisms are known and there is a knowledge gap surrounding the effects ANE may have on the abundant soil microbe, arbuscular mycorrhizal fungi. It is hypothesized that ANE will have a positive effect on plant–AMF

interactions. Treating with ANE will improve the early growth of AMF (*R. irregularis*) and increase the genes in *M. truncatula* known to be active in creating a symbiosis with AMF, this will lead to a higher percent colonization of AMF in treated plants over time.

The research objectives are:

1. To discover if the use of ANE (produced by Acadian Seaplants, Dartmouth, Nova Scotia) will increase the rate of germination, length of germ tubes, and amount of germ tube branching *in vitro* of *Rhizophagus irregularius* spores
2. To determine whether the use of ANE (applied by substrate drench or foliar) will increase the rate of AMF colonization and increase the total amount of colonization over the course of an eight week trial with greenhouse grown *M. truncatula*
3. To identify if the use of ANE (applied by drench or foliar) will increase relative gene expression in roots of *M. truncatula* of genes known to be associated with signaling for AMF colonization

A separate experiment was conducted to address each of the three research objectives.

Chapter 2: Methods

2.1 *in vitro* Assay (research objective #1)

2.1.1 in vitro Trial Establishment

An aseptic culture of *R. irregularius* spores was obtained from Premier Tech Biotechnologies (Rivere de Loup, Quebec) with a minimum concentration of 400 spores/mL. The early growth of spores was tested on five different media types listed in Table 1. Petri dishes were filled with 20mL of media, six plates of each media were considered one replicate and the whole trial was replicated three times. All media contained 15g/L of agar (BioBasic Canada, Ontario) and was autoclaved at 121^{o}C and 15PSI for 15 minutes. Solid seaweed extract powder (SSEP) was sourced from Acadian Seaplants (Dartmouth, NS) and made by a proprietary KOH extraction method of *Ascophyllum nodosum* (Batch #170-846). The modified Long Ashton Nutrient Solution (LANS) recipe used for the nutrient control can be found in Appendix D. A 3X3 grid was drawn onto each dish and 200µL of well agitated *R. irregularis* spore solution was added dropwise evenly among the quadrants. According to the manufacturer's label 200µL of solution contained a minimum of 80 AMF spores. Plates were wrapped in parafilm and incubated in the dark at 25°C. At day 4 and 14 data were collected for each plate. A preliminary time course trial indicated day 4 was the earliest significant germination was observed and day 14 was the peak of any growth possible in the *in vitro* system. AMF is limited in growth capacity without a plant host and the measured parameters plateaued by day 14.

Table 1: A list of media types tested using the *in vitro* system, % indicates weight/volume. All media was pH adjusted to 6.0 using concentrated KOH.

Controls	ANE Treatments
Water agar (Reverse osmosis H_2O) (15g/L)	0.01% ANE + 15g/L agar
LANS nutrient solution 0.01% + 15g/L agar	0.05% ANE + 15g/L agar
	0.1% ANE + 15g/L agar

2.1.2 in vitro Data Collection and Analysis

On observation dates (day 4 and day 14) the number of spores that had produced a germ tube versus those that did not were tallied with counters by microscopic examination at 40X magnification. Using a Nikon DS-FI1 camera attachment two photos of germinated spores were taken at random from each of the 9 quadrants on the plate. The photos were later analyzed with ImageJ software to record the length of the longest germ tube. Additionally, for photos collected from the day 4 nutrient control 0.01% and SSEP 0.01% plates the number of hyphal branches were also manually counted.

Statistical analysis was done using Excel and the real stats add in (www.real-statistics.com), a one way ANOVA and Tukey's post hoc with $\alpha = 0.05$ was done on the percent germination data for day 4 and day 14 data. The hyphal length differences were assessed with a one way ANOVA and Tukey's post hoc, $\alpha = 0.05$. A t-test, $\alpha = 0.05$, was done on the hyphal branching data comparing the strongest treatment, SSEP 0.01% and the 0.01% nutrient control.

2.2 Greenhouse trial (research objective #2)

2.2.1 Greenhouse Trial Establishment

Seeds of *M. truncatula* Jemalong A-17 were obtained from the U.S. National Plant Germplasm System (Accession number PI6700162011iSD) and mechanically scarified using coarse grit sand paper before seeding in a 200 cell plastic tray filled with moist BX ProMix™. A preliminary trial confirmed that M. truncatula grown in ProMix™ did not form mycorrhizal associations, it is also advertised as not being amended with microbes. Trays were covered and placed in a controlled environment room under 16L:8D, 25C and 60% relative humidity and were watered with reverse osmosis water as needed. At the emergence of the first true leaves lids were removed from seedling trays and two weeks after planting seedlings were transplanted to 6-0-6 cell packs to begin the trial. Cell packs were filled with golf course grade sand obtained from Shaw Resources (Cambridge, Nova Scotia). Again, a preliminary test was done to ensure that over 6 weeks no mycorrhizal colonization occurred from uninoculated sand, ensuring AMF activity came from an equal amount of mycorrhizal inoculum added to each plant. This finding negated the need for sterilizing the sand used in these trials. Agtiv Field Crops Powder (Premier Tech Biotechnologies, Rivere-du-Loup, Quebec) was used as a mycorrhizal inoculant by placing 0.05g in the center of each cell in a shallow pit. The Agtiv Field Crops Powder contains *Glomus intraradices* at a minimum concentration of 6400 viable spores per gram, meaning each plant received approximately 320 spores. For each replicate of this trial 30 boxes, each containing six plants were prepared, this trial was replicated three times. At two weeks after seeding the most even plants with at least one true leaf were selected from the seedling tray and any loose Pro-Mix was gently

removed from the roots manually. The plant was inserted directly on top of the shallow pit containing the mycorrhizal inoculant, ensuring the roots came into direct contact with the powder (Figure 2). Roots were covered in sand and plants were lightly watered. Twenty-four hours later each plant was given 20mL of treatment (either water only control, foliar seaweed or drench seaweed).

2.2.2 Growth Conditions

The 6-0-6 cell packs were labelled by treatment, 9 packs each of control, foliar and drench (Figure 3). They were placed on a greenhouse bench using a randomized complete block design and were rotated daily to account for slight abiotic differences in the greenhouse. Greenhouse conditions were set for 16light:8dark, 60% humidity, and 23.5°C during the trials which ran from September 10 to December 21, 2018. Foliar and drench treated plants received SSEP obtained from Acadian Seaplants (Batch#170-846) the day after transplanting and every 14 days following for a maximum of four treatments. Each drench plant treatment received 20mL of a 1g/L SSEP solution and each foliar plant treatment received 20mL of water and 4mL of 1g/L SSEP via a spray bottle held at 30cm from the substrate, control plants received water only. The foliar solution was applied outside of the greenhouse to avoid spray travelling onto other treatments. All plants received 20mL of a 1g/L 14-0-14 fertilizer weekly for the duration of the trial. A low phosphorous fertilizer was selected to maintain a phosphorous depleted system so as not to interfere with mycorrhizal colonization potential. Treatment rates were based on established methods the Marine Bioproducts Laboratory has used over the past 10 years.

Figure 3: A photo series depicting the growth system in the greenhouse trials. Clockwise from the top left: A) Plants were seeded into sand filled cell packs with 0.05g of mycorrhizal powder at 2 weeks after seeding. B. Example of *M. truncatula* two weeks after establishment in cell packs. 3) Harvesting *M. truncatula,* roots were cut into scintillation vials, shoots were scanned using winFolia for leaf area and sent for nutrient analysis. 4) Roots were stained according to Vierheilg *et al*., 1986) to highlight mycorrhizal structures and examined under a compound microscope at 40x and 100x magnification.

2.2.3 Harvest Methods

A harvest was completed at the beginning of week 3, 4, 6, and 8 after transplanting and inoculation with AMF. For each harvest 10 plants of control, foliar and drench treatments were collected. The plant was gently pulled from the sand substrate and the longest root was measured. The roots were then cut into 2-4cm segments and stored in a glass scintillation vial. For long term storage, roots were submerged in 50% alcohol and kept at 5°C. The leaves were immediately scanned using winFOLIA (Regent Instruments, Quebec) for total leaf area and then dried to send for nutrient analysis at the Nova Scotia Department of Agriculture, Truro.

2.2.4 Root Staining and Microscopic Assessment

Adapted from Vierheilg *et al*., (1998), roots were stained by boiling for five minutes in a 10% KOH solution in a scintillation vial, rinsed with tap water and boiled again in a 5% Shaffer Black Ink, 95% vinegar solution. They were then destained in water acidified with several drops of vinegar for at least 20 minutes. From each plant, 50 root segments, each ranging from 1.5-2cm in length, were placed on microscope slides and examined under a compound microscope at 40X magnification for the presence of mycorrhizal fungi, the colonization intensity, and the level of arbuscule abundance. In total, 500 root segments from each of the three treatments were examined from each weekly harvest.

Roots were assessed for mycorrhizal colonization following the methods outlined in Trouvelot *et al*., (1986). This method looks at the maturity and intensity of the colonization in individual root segments. Intensity is assessed by scoring a root from 1-5,

with 5 given to roots with greater than 90% colonization (Figure 9). The maturity of the colonization is assessed by looking at the abundance of arbuscules which are the specialized structures for nutrient exchange between the host and fungi. Arbuscule scores are on a scale from 0-3, with 0 meaning no arbuscules present and 3 meaning they are abundant throughout the whole root. In early phases of colonization scores of 0 are expected as they fungi first penetrate the root with hyphae before creating arbuscules. This method gives additional information compared to the traditional count cross method which does not measure fungal maturity. A compilation of photos from the microscopic assessments showing different phases of mycorrhizal growth within the roots are shown in Figure 10.

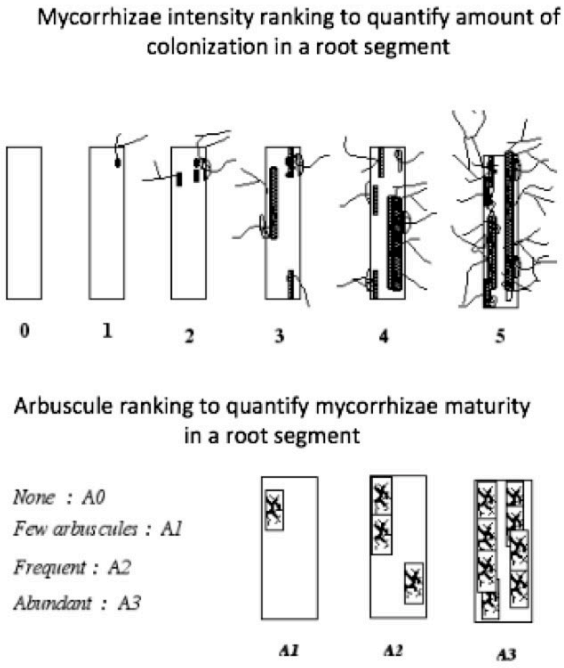


Figure 4: Modified from Trouvelot *et al*., (1986) is a visual guide for how each root segment was assessed for two parameters 1) the intensity of the fungus throughout the root 2) the presence of fungal arbuscules.

Statistical analysis was done in R Studio (version 1.21335). A one way ANOVA was conducted on each tested parameter for each timepoint, with a Kruskall Wallis test and Dunn post-hoc test, significance level was α=0.05.

2.3 Molecular Trial (research objective #3)

2.3.1 Plant Growth and Tissue Collection

Medicago truncatula seeds were germinated and the trial was established as described in section 2.2.1 with the difference being that plants were grown in a controlled environment room. A total of 135 plants were grown, 45 were harvested per sampling date (15 per treatment, either control, drench, or foliar). Conditions were set at 16Light:8Dark, 27^{o}C, 60% humidity and plants were watered as needed. Once weekly each plant received 25mL of a 1g/L 14-0-14 fertilizer. Treatments with SSEP occurred the day after transplanting and AMF inoculation and every two weeks afterwards as described in section 2.2.2. Plant tissue was harvested one week after AMF inoculation, 4 weeks after inoculation and 7 weeks after inoculation. At harvest, plants were gently lifted from the sand substrate, roots were rinsed with tap water and shoots were cut off. Root samples were collected in triplicate, with 5 biological replicates in each tinfoil pouch (15 plants total per treatment, control, foliar, or drench), which were flash frozen in liquid nitrogen within two minutes of being lifted from the substrate. Shoot tissue was scanned using WinFOLIA software (Regent Instruments, Quebec) for differences in leaf surface area.

2.3.2 RNA Extraction and cDNA Synthesis

Following the methods described in Onate-Sanchez and Vincente-Carbajosa (2008) RNA was extracted from 150 mg of root tissue finely ground under liquid nitrogen using 900 µL of lysis buffer (2% SDS, 68 mM sodium citrate) and 300 µL of precipitation buffer (4M NaCl, 16 mM sodium citrate, 32mM citric acid). Tubes were incubated on ice for 10 minutes before centrifugation at 16 000 x g for 5 minutes at 4^0C. Supernatant containing RNA was transferred to new 1.5 mL tubes. RNA was precipitated by adding 600 µL isopropanol, mixing and centrifuging at 16 000 x g for 5 minutes at 4^0C. Supernatant was discarded, being careful not to dislodge the pellet. 500 µL of cold 70% ethanol was added and the tubes were vortexed to dislodge the pellet before centrifuging at 16000 x g for 4 minutes at 4^0C. Supernatant was discarded and RNA was air dried under a fume hood until the pellet turned translucent. RNA was resuspended in 25 µL of H2O before continuing to a DNAse digestion with one unit of DNAse (RQ1 DNase, Promega) on a heat block at 65^0C for 30 minutes. A final precipitation as done by adding 70 µL of H2O, 50 µL 7.5M ammonium acetate and 400 µL ethanol before centrifuging at 16000 x g for 4 minutes. The supernatant was the discarded, 500 µL of cold 70% ethanol was added and the RNA was again centrifuged at 16 000 x g for 4 minutes. The supernatant was discarded and the pellet air dried until turning translucent. The final RNA was put into 30 µL of water. Initially we used the RNAqueous Plant Kit (Invitrogen), but yields were too low to be useable and instead methods from Onate-Sanchez and Vincente-Carbajosa (2008) were adopted.

RNA concentration and purity was assessed through spectrophotometry (NanDrop One, Thermo Scientific). A TAE denaturing gel was run of all 27 RNA samples to check

for integrity before synthesizing cDNA (1% agarose, 0.065% sodium hypochlorite, 0.64 g/L final, 100V, 30 minutes) and stained with SYBR Safe dye (Life Technologies). A DNAse treatment using 1.3 units of DNase (RQ1 DNase, Promega) was done on all RNA samples scaled up to 13µL reactions to get 500ng/µL of RNA into each sample. A No Reverse Transciptase Control qPCR was done for all 27 RNA samples using reference gene PTB and ENOD11 following the qPCR conditions outlined in section 2.3.5, no amplification was found after 40 cycles.

The cDNA synthesis was done by taking 11µL of the DNAse treated RNA and following the manufacturer's instructions for the Invitrogen superscript III kit with oligo$(dT)_{20}$ primers. At the end of cDNA synthesis there were 20 µL of concentrated cDNA (25 ng/µL) per sample which was diluted 8 fold to a total of 160 µL.

2.3.3 qPCR Assay

A qPCR assay was done using 10 µL reactions containing 5 µL PowerUp SYBR Green master mix (Applied Biosystems), 2 µL of 1.5 µm primer mix, 2 µL of 24x diluted cDNA template (1ng/µL), 1 µL H2O. A 2 ng sample was used in qPCR trials. The reactions were carried out in a QuantStudio3 Thermocycler (Applied Biosystems) and the reactions were set up as follows: a UDG activation at 50^{o}C for 2 minutes, DNA polymerase activation at 95^{o}C for 2 minutes, followed by 40 cycles of DNA denaturation at 95^{o}C for 1 second and annealing for 30 seconds. Following the cycles a dissociation step was done at a ramp rate of 1.6^{o}C/second at 95^{o}C for 15 seconds, followed by 1.6^{o}C/second at 60^{o}C for 1 minute and finally increasing to 95^{o}C for 15 seconds at 0.15^{o}C/second. Each gene of interest was run with all 27 samples on one 96 well qPCR

plate using the conditions described above. All reactions were performed in triplicate with no template controls that failed to amplify over 40 cycles. All qPCR experiments were designed for sample maximization (Hellemans *et al*., 2007, Taylor *et al*., 2019), which avoids having to run the reference genes on each plate. Talyor *et al*., (2019) describe that relative expression between samples is the most important consideration, not between genes, this approach negates the need for interplate calibration. The design of these qPCR experiments is consistent with the standards outlined in the Minimum Information for publication of Quantitative real-time PCR experiments (MIQE) (Bustin *et al*., 2009; Taylor *et al*., 2019). The results were analyzed using the ΔΔCt method, calculated in excel and normalized to the geometric mean of the two reference genes, PTB and PP2A (Livak and Schmittgen, 2001; Hellemans and Vandesompele, 2014). A one way ANOVA with a Tukey's post-hoc test (α=0.05) was done on ΔΔCt values for each gene at each week of sample in R Studio.

2.3.4 qPCR Troubleshooting

To obtain amplification without inhibition a 24x dilution had to be done on all cDNA samples. It is likely that the inhibition was caused by the presence of secondary metabolites in the plant roots. The plants that were sampled were growing in a 100% sand substrate and were given a zero phosphorous fertilizer. Creating a nutrient stress was not the intent, but the plants were showing signs of nutrient stress such as dropping leaves, and leaves reddening/purpling (data not shown). A zero phosphorus fertilizer was required as the role of phosphorous abundance limiting plants choice to seek mycorrhizal association was not a variable we wished to consider. Plants under stress are likely to

accumulate various secondary metabolites such as phenolic compounds and polysaccharides. During initial extractions the RNA seemed intact and uncontaminated based on spectrophotometry, but the cDNA was PCR negative. We hypothesized that this was due to polysaccharide contamination. To address this, we added a lithium chloride precipitation step (Colard *et al*., 2011).

Serially diluted cDNA was used as a template for standard curves. With genes ENOD11, DMI1, RAM1, CHITII-2, RAD1 and SbtM1 expression levels were low with the cDNA sample and gDNA was used instead to obtain high quality standard curves. An adequate standard curve for DMI1 was not obtained and it was not included in the final data analysis; it was also expressed at extremely low levels. Gene SbtM1 and CHITII-2 were discounted from results as they were not present at a detectable level in the samples.

2.3.5 Primer Design

Genes of interest were selected based on literature and grouped into three categories based on when in symbiosis they are important: pre-contact signaling, early establishment and arbuscule development. All primers were designed using NCBI Primer Blast with parameters set for a melting temperature >60^{0}C and less than 1^{0}C apart, GC content between 35-65% and targeting across introns if possible with fewer than 3 of the same nucleotides in a row. Primers between 15-25 base pairs long favoring the 3' end of the gene were chosen to avoid 3' bias and products were between 70-150 base pairs long.

Selected primers (Table 3) were screened using the Integrated DNA Technologies OligoAnalyzer tool to check for self dimers, heterodimers and hairpin structures (www.idtdna.com). Primers were commercially synthesized from Integrated DNA

Technologies (Coralville, Iowa, USA) and rehydrated using TE Buffer (10mM Tris, 10mM EDTA, pH8) into a 0.1 µmol/mL concentration. From there, a 1.5 µm primer mix working solution was made using TE Buffer and equal concentrations of forward and reverse primer. Efficiency of all primer pairs was tested through standard curves with composite samples of 24x diluted cDNA. A dilution series was made from the original 24x cDNA composite sample by diluting 3 fold each time to a maximum dilution of 8000 fold. Melt curves were also checked for primer specificity.

2.3.6 Reference Gene Selection

The GeNorm method (Vandesompele *et al*., 2002) was used to select the most stable reference genes from 4 candidates selected from Kakar *et al*., (2008). Each of the candidate reference genes was run against all 27 cDNA samples at 24x dilution in RT-qPCR. Using the QuantStudio3 software (Applied Biosystems) the GeNorm algorithm was applied and PTB and PP2A (also known as PDF2, MTR_6g084690) were chosen as the most stably expressed reference genes across all tissues (Table 2). The M-value, or the variation of a gene compared to all the remaining candidates, was 0.639 for the PP2A and PTB pair, which is acceptable for heterogenous tissues, such as different developmental stages (Hellemans *et al*., 2014). Both primers were redesigned to differ from the Kakar *et al*., (2008) paper to amplify all transcript variants and favor the 3' end of the genes.

Table 2. List of candidate reference genes and information of their primers. PP2A and PTB were chosen as the reference genes for this project.

Gene symbol	Protein	Locus tag	Forward (5' → 3')	Reverse (5' → 3')	Amplicon length (nt)	Location
PP2A [a]	protein phosphatase PP2A regulatory subunit A	MTR_6g084690	GGTTAAGAGGCTGGCTTCTGG	GGCATATCATCTTGGCACAGC	144	Exon 2–3 (Forward primer overlap)
PPRep [b]	pentatricopeptide repeat-containing protein, mitochondrial	MTR_6g079920	AAACTGAAACTATGGACAAACTGC	CATCATCACCACACTCACAGG	82	Exon 1–2 (Forward primer overlap)
PI4KG3 [c]	phosphatidylinositol 4-kinase gamma 3	MTR_3g091400	TGGTTATTGCCTGCCCAAGA	TGGTGTCTAGGGAGTAAGGCT	89	Exon 2–3
PTB [d]	polypyrimidine tract-binding protein homolog 2	MTR_3g090960	GAAGCATTGGAAGGACACTGC	GGCTGAGTGTTCACAACTGG	149	Exon 10–11

[a] Targets both transcript variants; [b] Targets variant X1; [c] In some papers noted as ubiquitin; [d] Targets all three transcript variants

Table 3. List of the genes of interest, the proteins they encode, location in the genome of *M. truncatula* and the primer sets designed for each.

CATEGORY	GENE SYMBOL	PROTEIN	LOCUS TAG	FORWARD (5' → 3')	REVERSE (5' → 3')	AMPLICON LENGTH (NT)	LOCATION
PRE-CONTACT SIGNALING	*ENOD11*	cell wall repetitive proline-rich protein	MTR_3g415670	TATGGTAACCAGCCTCCACC	CAAGCATTGGTAAACCTTGTTGC	71	Exon 1 (no introns)
	CHS1 [a]	chalcone synthase 2	MTR_7g016780	GCAAATTGTGTTGAACAAAGCAC	TTGATCATGGATTTATCACACATGC	116	Exon 1–2 (Reverse primer overlap)
	DMI2 [b]	receptor kinase	LOC1140732 6	GCTTGGTTGAATGGGCTAAACC	ACTCTCCACAATGCCTCTGC	103	Exon 13–14 (Forward primer overlap)
	DMI1 [b]	nuclear ion channel for signal transduction	LOC1143385 1	GCAGATCAGAGTGATGCACG	GGGTTCATTGTCTAGGTCGC	111	Exon 5–6 (Forward primer overlap)
	D27	beta-carotene isomerase, chloroplastic	MTR_1g471050	ATCCAGCACTCAAGCAACCC	CTGACCTGAAAGCCTGCTGC	95	Exon 6–8 (Reverse primer overlap 7, 8)
EARLY ESTABLISHMENT	*IPD3* [b]	transcription factor, "CYCLOPS"	MTR_5g026850	CATGGAAACAAGTGGGAGAAAGC	CCATCTTGCCATCTGAAACAGC	144	Exon 6–9 (Reverse primer overlap 8–9)
	DMI3	calcium and calcium/calmodulin-dependent serine/threonine-protein kinase	LOC1140524 0	TGATGGAACAGTTGACATGCG	TCATACATCTGGAAGCACAAACG	99	Exon 5–6 (Reverse primer overlap)
	RAM1	DELLA transcription factor	MTR_7g027190	GCTAGTGCTTCTCTTTCACAAGC	GTCCACTGTCTTGCTCCTGC	130	Exon 1–2 (Reverse primer overlap)
	RAM2	glycerol-3-phosphate 2-O-acyltransferase 6	MTR_1g040500	CATGGCTTTGTGCAAGGAGG	TTTGTCACTTGTCACTGTTGTTACC	70	Exon 1–2 (Forward primer overlap)
	ExpB1	expansin-like B1	MTR_4g099400	CGTGATCCTCTACGTTGGTGG	CCATTTGTGCTGTTCTTTCTGCC	73	Exon 3–4 (Reverse primer overlap)
ARBUSCULE DEVELOPMENT	*ChitIII-3*	acidic endochitinase	MTR_0027s0260	CAGGCAGTGGCTTCATTCCT	ACCACAGCATAACACCGCC	93	Exon 1 (no introns)
	ChitIII-2	acidic endochitinase	MTR_2g102020	TGGAGAATTAGGCGTCGCAC	CCATCGCTTCCACGAATCCA	127	Exon 1
	STR	ABC transporter G family member 20	LOC1141290 1	GACATAGCTCAGGGTGGTAGC	GAGTGTGAAGTGCATCTGGC	139	Exon 3–4
	STR2	ABC transporter G family member 17	LOC1140783 8	AGCTCACAAGAACATCTAGGTCC	ATGTTTGTGAAGTTGCGGCG	86	Exon 3
	FATM	palmitoyl-acyl carrier protein thioesterase, chloroplastic	MTR 1g109110	CGACGAGACTGGCTCATAAGG	CATCACCCATGTGCTTGTTGC	72	Exon 3–4 (Reverse primer overlap)
	RAD1	DELLA transcription factor	MTR_4g104020	ACATGGACACCAATGGCGAG	CCTAAGCCTCCGAACACGG	73	Exon 1 (no introns)
	PT4	inorganic phosphate transporter 1-11	MTR_1g028600	ACATGCCATGCTTTCAGTGC	TCTTCCTTGGCGTACCATCC	93	Exon 2 (but not spanning)
	Vapyrin	putative ankyrin repeat protein	MTR_6g027840	ACAATTTATGTGCGGCTGCG	TGATCCCTTCCATTGATCACCC	88	Exon 1 (no introns)
	SbtM1 [c]	subtilisin-like protease	MTR_5g011310	GTTTGTACTGATAACATTCCATGCG	GCAATTGGGTCTTCAAAGAATGG	137	Exon 2 (but not spanning)

[a]Targets several other chalcone synthases (homologs with very high sequence similarity); [b] Targets both transcript variants; [c] Targeted to isoform X1, which is most homologous to the well-characterized *SbtM1* in *Lotus japonicus* of the two transcript variants

Chapter 3: Results

3.1 in vitro Assay Results

The direct effect of SSEP on the growth potential of *R. irregularius* (syn. *G. intraradicies*) was evaluated by pipetting spores onto a Petri dish containing water agar and various rates of SSEP. Spores were assessed at day 4 and day 14 for germination, hyphal length and amount of branching.

In the *in vitro* assay the rate of spore germination was improved beyond the water and nutrient controls when using rates of 0.05% and 0.01% SSEP at day 4 in all three replicates. In replicate 3 the day 4 treatments showed the germination rate of 0.01% SSEP was 84.47%, which is statistically significant compared to all other treatments, the equivalent LANS nutrient control only reached 60.9% germination (Figure 4). The SSEP 0.05% treatment was also statistically significant compared to the controls in day 4. Trends in the other two replicates are similar, even showing the same statistical differences between 0.01% SSEP and the controls at day 4, they are pictured in Appendix A. By day 14 there were minimal differences between treatments in all replicates of this experiment, this is likely because the maximum germination potential was reached in most plates. Spores have a finite lifespan after germination if a host is not found (Smith and Read 2008; Kuwada *et al*., 2006). In replicate 3 the 0.01% SSEP and 0.05% SSEP rates were higher than the controls at day 14, but the differences were much less pronounced and were not statistically significant. The three replicates of this experiment were not combined because spore germination is asynchronous, and it was not possible to determine if the spores at day 4 and day 14 were at the same developmental phase in all

three replicates. The goal was to examine the response of the spores at day 4 and day 14 during three independent replicates of the experiment to validate results.

The 0.1% SSEP treatment responded differently than all other treatments by appearing to inhibit the germination potential of *R. irregularius* spores in all three replicates. Germination percentage of the 0.1% SSEP treatment did not exceed 70% in any trial and was below 60% at both day 4 and day 14 in replicate 1 and 2 (Appendix A). In all three replicates the germination percentage of 0.1% SSEP was statistically different than the water control at α=0.05% showing decreased spore performance (Figure 5).

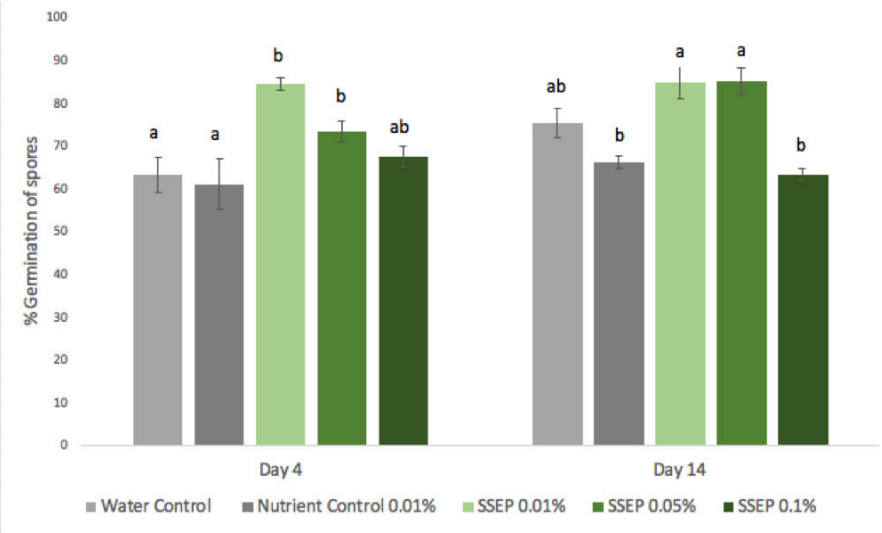
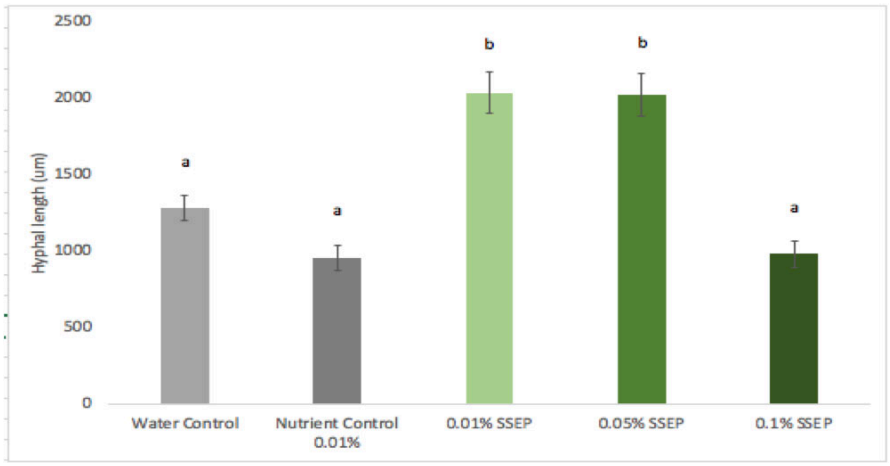


Figure 5: Replicate 3 of *Rhizophagus irregularis* spore percent germination results taken at day 4 and day 14. 0.01% and 0.05% SSEP consistently resulted in the highest germination percentage that was statistically different from other treatments (one way ANOVA, Tukey's post-hoc, α=0.05). Each bar represents 6 biological replicates, each Petri Dish contained at least 80 AMF spores.

When assessing the longest hyphae, all treatments showed increased hyphal lengths between the first and second sampling date which indicate the spores were growing as expected in the *in vitro* system. Spores growing on 0.01% SSEP media had longer hyphae than other treatments in all three replicates. In replicate 3 at day 14 the

average length of the longest hyphae was 52.4% longer in the SSEP 0.01% treatment than the LANS 0.01% nutrient control, which was statistically significant (Figure 6). The 0.05% SSEP treatment also showed improved length at day 4, but there was no difference between 0.05% SSEP and the nutrient control at day 14 (Figure 6). Consistent with percent germination data, the 0.1% SSEP treatment does not show a statistically significant difference in hyphal length compared to the controls. Overall, trends are similar between replicates with 0.01% SSEP treatment resulting in the longest hyphae, 0.05% SSEP showing longer hyphae than controls and 0.1% SSEP with no significant growth difference compared to controls, additional graphs are in Appendix A.

A



B

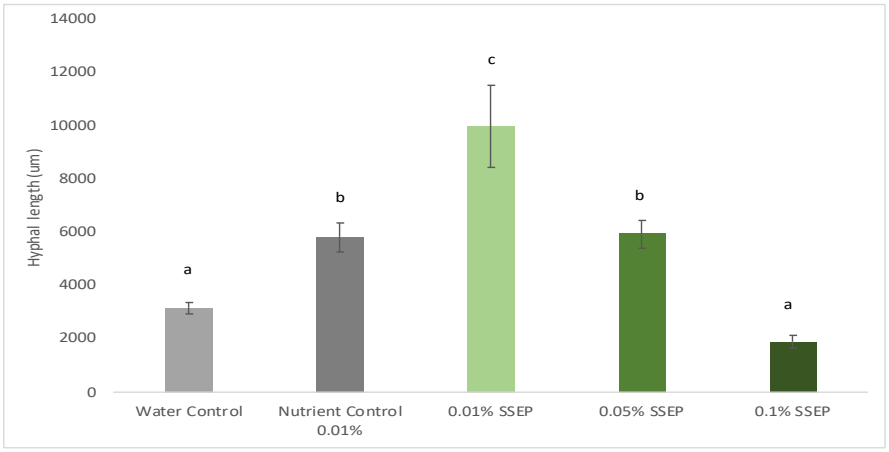


Figure 6: Replicate 3 results for *in vitro* hyphal length of germinated *Rhizophagus irregularis* spores (α=0.05, one way ANOVA, Tukey's post-hoc). A) hyphal lengths at day 4 show the 0.01% and 0.05% SSEP have a significant effect on hyphal length B) hyphal lengths at day 14 show that 0.01% SSEP promotes rapid growth. The nutrient control and 0.05% are also significantly larger than the control. At least 100 germinated spores were measured per treatment.

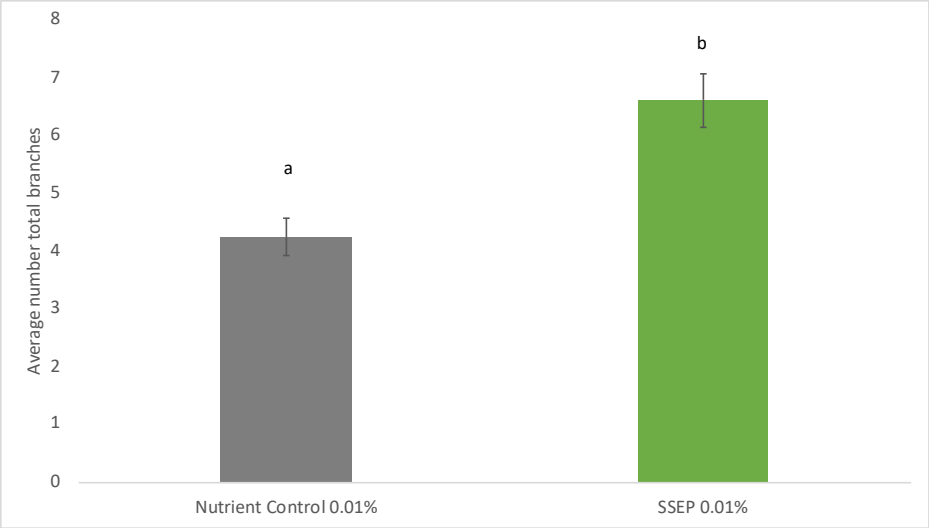


Figure 7: A comparison of the average total number of *Rhizophagus irregularis* hyphal branches at day 4 between the nutrient control and SSEP at 0.01%. The SSEP treatment showed significantly more branching compared to the nutrient control (Student's T-test, α=0.05, n= 100 spores per treatment).

As an additional assessment measure the number of hyphal branhes were determined between 0.01% SSEP treatment and the equivalent nutrient control at day 4 (Figure 7). The 0.01% SSEP treatment was chosen for additional assessment because it's use resulted in a higher rate of germination and longer hyphal length than the other seaweed treatments. Data from all three replicates were pooled and assessed for this measure, there were 100 spores assessed from each treatment. The SSEP treated spores had more branching compared to the control and the difference was statistically significant (Figure 7). Branching is a sign of vigorous presymbiotic growth, and the more hyphae grow the more likely they are to find a host to form a symbiosis.

In all three lines of evidence, percent germination, hyphal length and hyphal branching the SSEP treatments at 0.01% and 0.05% outperformed the water control and the 0.01% nutrient control. These experiments consistently show that at low application

rates ANE has a positive effect on the early growth of *R. irregularis* spores using an *in vitro* model.

3.2 Greenhouse Trial Results

Using *Medicago truncatula* and a commercial powder of *R. irregularis* spores, greenhouse trials were established to assess differences in mycorrhizal colonization in the presence of ANE applied by drench or foliar treatment. Plants were treated with ANE bi-weekly and the trials were eight weeks in length with harvests occurring at 3, 4, 6 and 8 weeks after inoculation. Trends were consistent when this experiment was replicated two additional times, results for experiment 2 and experiment 3 are available in Appendix B.

3.2.1 Greenhouse Leaf Tissue Results

At weeks 3, 4 and 8 of experiment 1 there was statistical significance among treatments showing drench treated plants grew larger than control and foliar treatments (Figure 8). Replicate 1 and 2 results are similar and available in Appendix B. Foliar treated plants did not differ statistically from the controls during any week of harvest.

Although there is significance in this data there is also high variability. Due to having a limited number of *M. truncatula* seeds it was not possible to grow extra plants to select the most even individuals for assessment. The sand substrate combined with the fertilizer (14-0-14) caused plants to show signs of nutrient stress and Phosphorous deficiency (purpling leaves). These factors contributed to the large variation in plant growth. However, this phenotypic assessment shows that drench treatment of ANE positively affected plant growth.

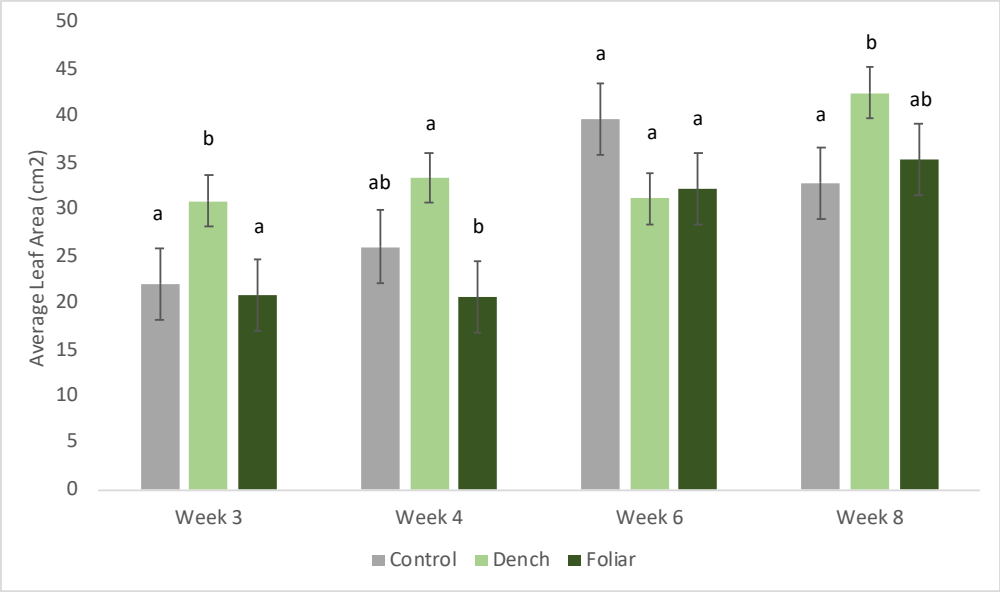
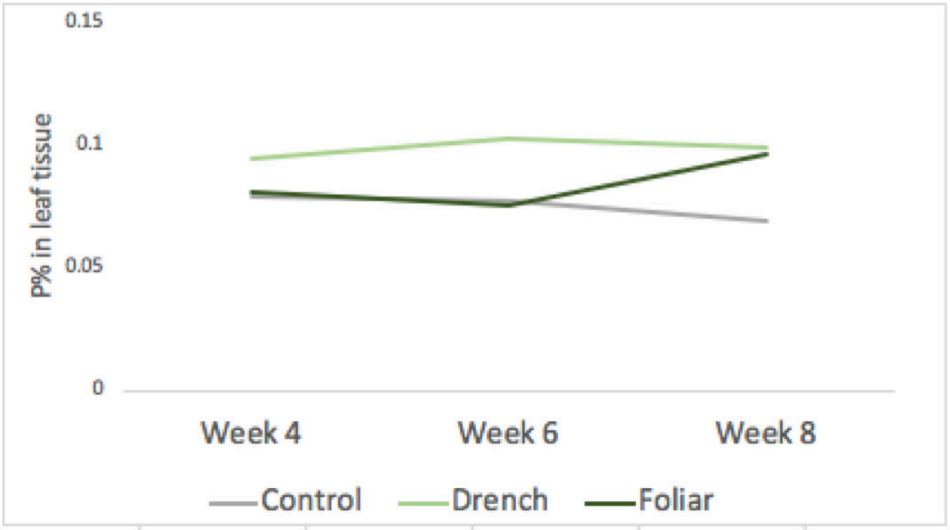


Figure 8: In experiment 1 drench plants were often statistically different in leaf area compared to the control and foliar treatments (α=0.05, one way ANOVA, Tukey's post-hoc). Foliar treatments were not statically different from the control. A large data spread shows the biological variability within treatments.

Results of the leaf nutrient analysis done on experiment 1 tissues confirmed there was nutrient stress occurring. The amount of phosphorous decreased over the course of the trial and was low from the beginning (Figure 9). There were no significant differences between treatments in the phosphorus content of leaves. This is not surprising because there was very little phosphorous available in the system and negligible amounts were added over the course of the trial. If there were differences in phosphorous content, we would have to question where the phosphorous originated from in the system. Leaf tissue results confirm that plants were under nutrient stress (Figure 9).

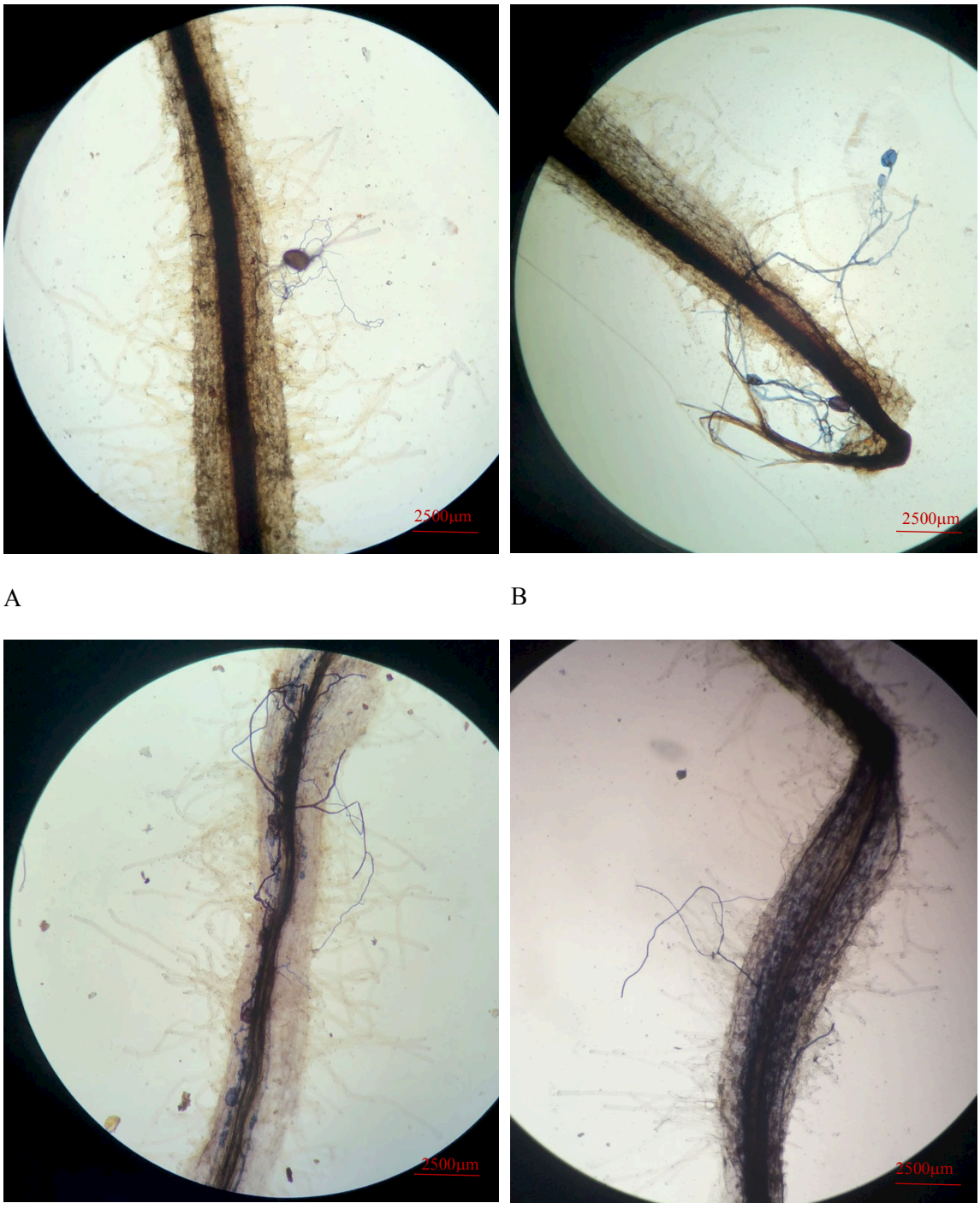


	Week 4	Week 6	Week 8
Control	0.079	0.077	0.0688
Drench	0.094	0.102	0.099
Foliar	0.081	0.075	0.096

Figure 9: Phosphorus content in leaf tissue from replicate 1 of the greenhouse trial as reported by the Nova Scotia Department of Agriculture. Each value is an average from a pooled sample of 8 biological replicates.

3.2.2 Microscopic Assessment of Fungal Colonization Intensity

The process of fungal colonization begins as spores produce hyphae and penetrate the root cells. The hyphae then branch intercellularly develop arbuscule and vesicle structures to exchange and store nutrients (Figure 10). Across the 8 weeks of harvest in all three replicates of this experiment fungal intensity root scores (based on Trouvelot *et al*., 1986) changed to reflect greater mycorrhizal colonization, approaching a point where each root segment contained mycorrhizal fungi (Figure 11). The intensity that each root was colonized by mycorrhizae also increased over time, an average of 30/50 root segments per plant were classified as having more than 90% coverage by week 8 of the trial (Figure 11).



A B

C D

Figure 10: A compilation of photos showing how *Rhizophagus irregularis* colonizes *Medicago truncatula.* Starting from mycorrhizal branching external to the root (A), to early hyphal penetration (B) through to heavy presence of vesicles and arbuscules within the root (C, D). All photos were taken at 100X magnification and blue structures are mycorrhizae.

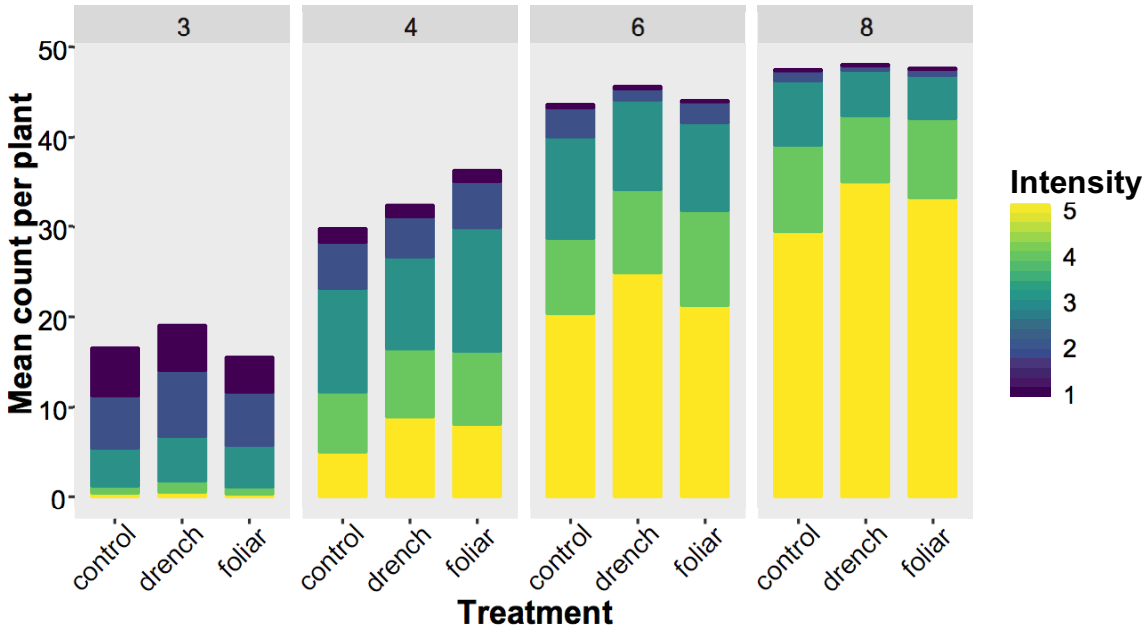


Figure 11: A graph showing trends across all three experiments of the distribution of fungal intensity scores assigned according to Trouvelot *et al*., (1986). Each bar represents the mean count of 24 biological replicates. Across time more AMF was observed in roots, but also root segments became more intensely colonized. There is a trend showing drench plants typically received a rank of 5 more often, meaning more than 90% of the root was colonized.

The data can be examined in greater detail by looking at how the intensity ranks (from 1-5) are distributed per week (Figure 12). This shows ANE has a positive effect on how intensely roots become colonized by AMF. Replicate 1 is discussed here, but trends between experiments are consistent and results of experiment 2 and 3 are in Appendix B. During week 3 of harvest there is a trend showing drench roots are more likely to receive a rank of 5, indicating the root is more than 90% colonized by mycorrhizae (Figure 12a). This is interesting as week 3 is very early to be observing such advanced fungal development. It is clear that week 3 is capturing the early phases of colonization because most roots receive a rank of 1, regardless of treatment, which indicates less than 1% colonization. Phenotypically a rank of 1 usually appears as hyphae just beginning to penetrate the roots (Figure 10).

Week 4 results showed the foliar treatment had the most fungi present in root segments (Figure 12b). This is not consistent with other reps (Appendix B), it is likely that there is a lot of biological variability at play in these experiments. There is little statistical significance at this timepoint, but there is a trend showing control roots receive ranks less frequently, indicating less mycorrhizal colonization overall.

By week 6 the colonization is increasing in roots, across treatments it is more likely a rank of 4 or 5 is given as compared to the previous weeks where ranks were clustered around scores of 1-3 (Figure 12c). Again, there is little statistical significance in this week of harvest, but there is a trend showing drench roots are being assigned a rank of 5 more often than the other two treatments. Control roots receive a rank of 3 more often than in ANE treatments, showing that they are more likely to be in this category as opposed to the 4 or 5 categories for higher colonization.

In the final week of harvest all treatments showed heavy colonization with nearly 70% of observed root segments showing a rank of 5, which indicates greater than 90% mycorrhizal colonization. There is still a trend showing more mature colonization in the treatments compared to the control, however, there is no statistical significance (Figure 12d). Plants were able to reach maximum mycorrhizal colonization because of the nutrient depleted conditions that they were kept in. Had there been excess phosphorus in the system the need for mycorrhizae would not have been as great and it would have introduced another variable into the experiment. Maintaining phosphorous depletion allowed for the trackability of mycorrhizae from the early stages where most fungi had barely begun to penetrate the host's roots through to the point where nearly 70% of roots in all treatments were more than 90% colonized.

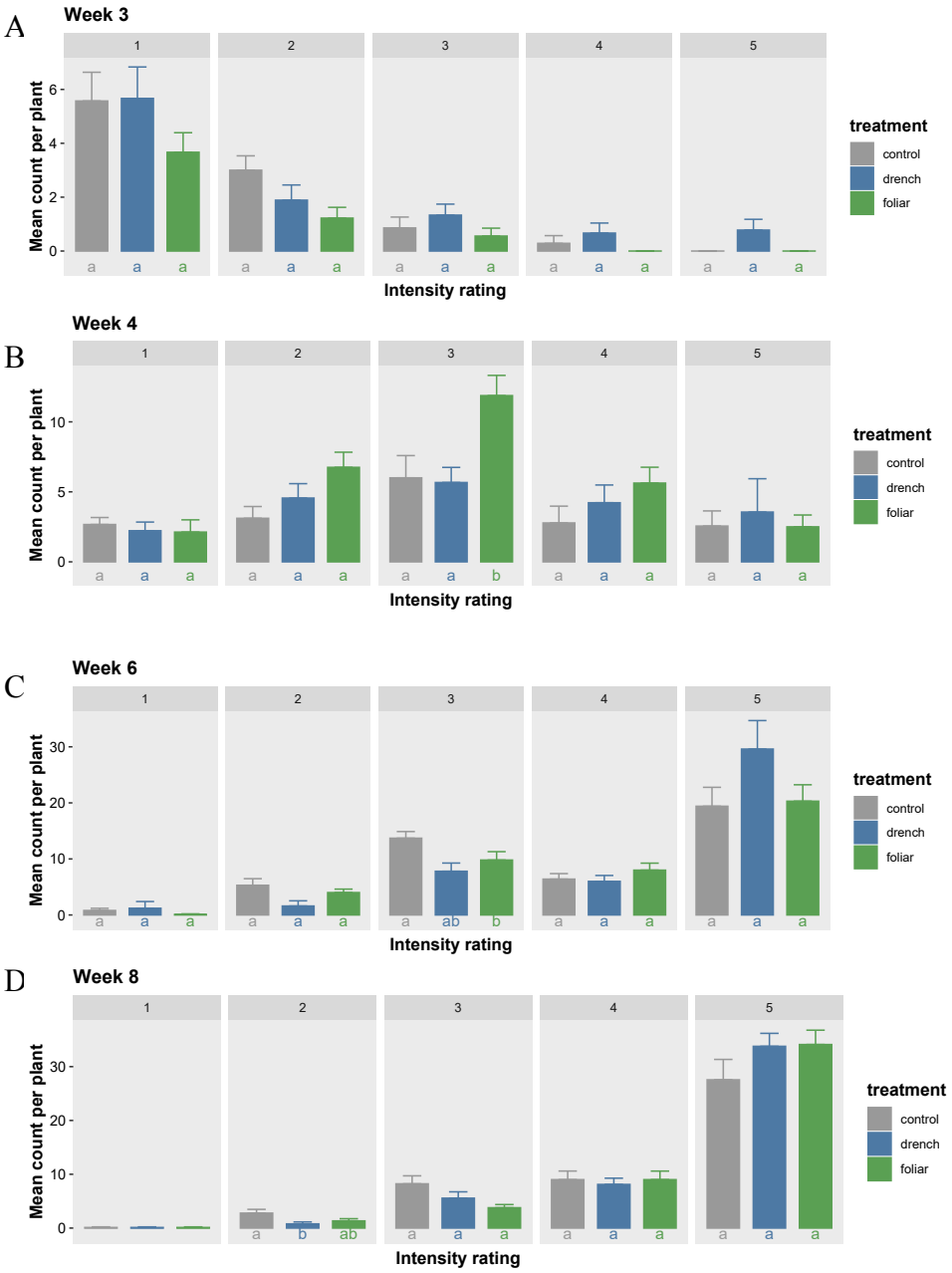


Figure 12: The mean count per plant for experiment 1 that a rank from 1-5 was given in each treatment during the 4 harvest timepoints according to the methods outlined in Trouvelot *et al*., (1986). Grey plants are control, blue are drench and green are foliar ANE application. Each bar represents 8 biological replicates and 50 roots were examined per biological replicate. A) In week 3 the drench plants receive a rank of 5 more often compared to other treatments, showing an early boost to roots reaching heavy colonization. B) Foliar plants have the most fungal colonization in week 4, but differences do not show a lot of significance. C) Week 6 plants have become more heavily colonized and regardless of treatment are more likely to receive a rank of 4 or 5. D) All plants are heavily colonized, ANE treated plants show a trend towards more colonization, but differences are not significant. Statistical significance is calculated using a Kruskal-Wallis and Dunn Post-Hoc, α=0.05.

3.2.3 Microscopic Assessment of Fungal Arbuscules

Quantifying arbuscules is an excellent measure of AMF maturity as arbuscules are complex structures created for the transfer of nutrients between the AMF and plant. Arbuscules are a sign of a vigorous symbiosis where the benefits are being realized by both partners. The assessment of arbuscule abundance followed similar trends to the overall intensity data, where very few were present in in week 3 during all three experiments, all the way to most plants receiving a maximum rank of 3 by week 8 of harvest, regardless of treatment (Figure 13). Again, this trend shows the success of the system, regardless of treatment, nearly all examined root segments are colonized by AMF with many arbuscules present within the time frame of the experiment.

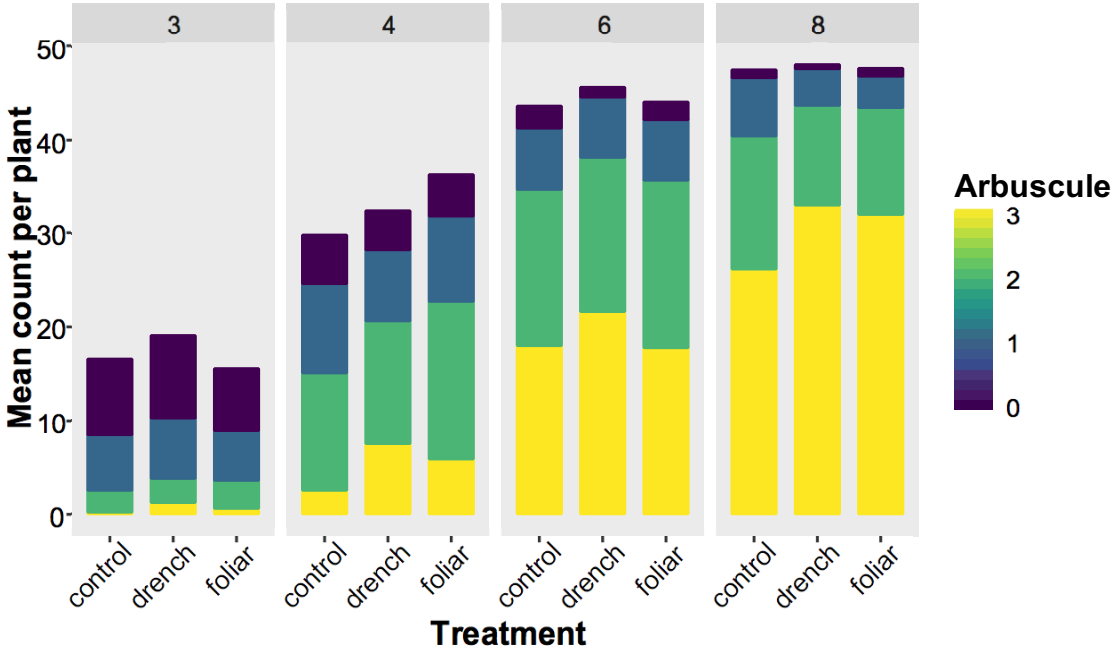


Figure 13: The mean count per plant that the fungal arbuscule development scores from Trouvelot *et al*., (1986) were given to each treatment during each week of sampling. This graph combines all three replicates of this experiment, for a N of 24 plants per column. A score of 0 indicates no arbuscules present and a score of 3 is for a significant presence. Over time the colonization matures, and scores of 3 are more common. By week 8 nearly 80% of roots have arbuscules. During every week of harvest there is a trend showing drench plants have more mature arbuscules (ranking of 3) than the other two treatments.

Examining the breakdown of how treatments respond during each week of colonization shows that ANE has some effect on the arbuscule development in root segments (Figure 14). Experiment 1 is discussed here, but trends between experiments are consistent and results of experiment 2 and 3 are in Appendix B. In week 3, control plants are more likely to receive a rank of 0, though this difference is not statistically significant (Figure 14a). A score of 0 is given if a root has some AMF hyphae and is ranked on the intensity score but does not yet show arbuscules. Similar to the intensity data there is a trend showing drench roots are more likely to receive a rank of 3, for very mature arbuscules at this early timepoint. Week 4 does not have any statistical significance between treatments, but there is a trend showing foliar roots have more ranked segments, showing greater mycorrhizal colonization overall (Figure 14b). Week 6 also does not show significance. However, drench roots are less likely to receive a rank of 1, and instead more drench treated roots receive a rank of 3 (Figure 14c). In week 8 control roots are more likely to receive a rank of 2 than 3, showing less fungal maturity at the end of the trial (Figure 14d). Although the drench and foliar treatments are not significantly from the control there are trends suggesting that ANE treatment has some effect on the maturity of colonization.

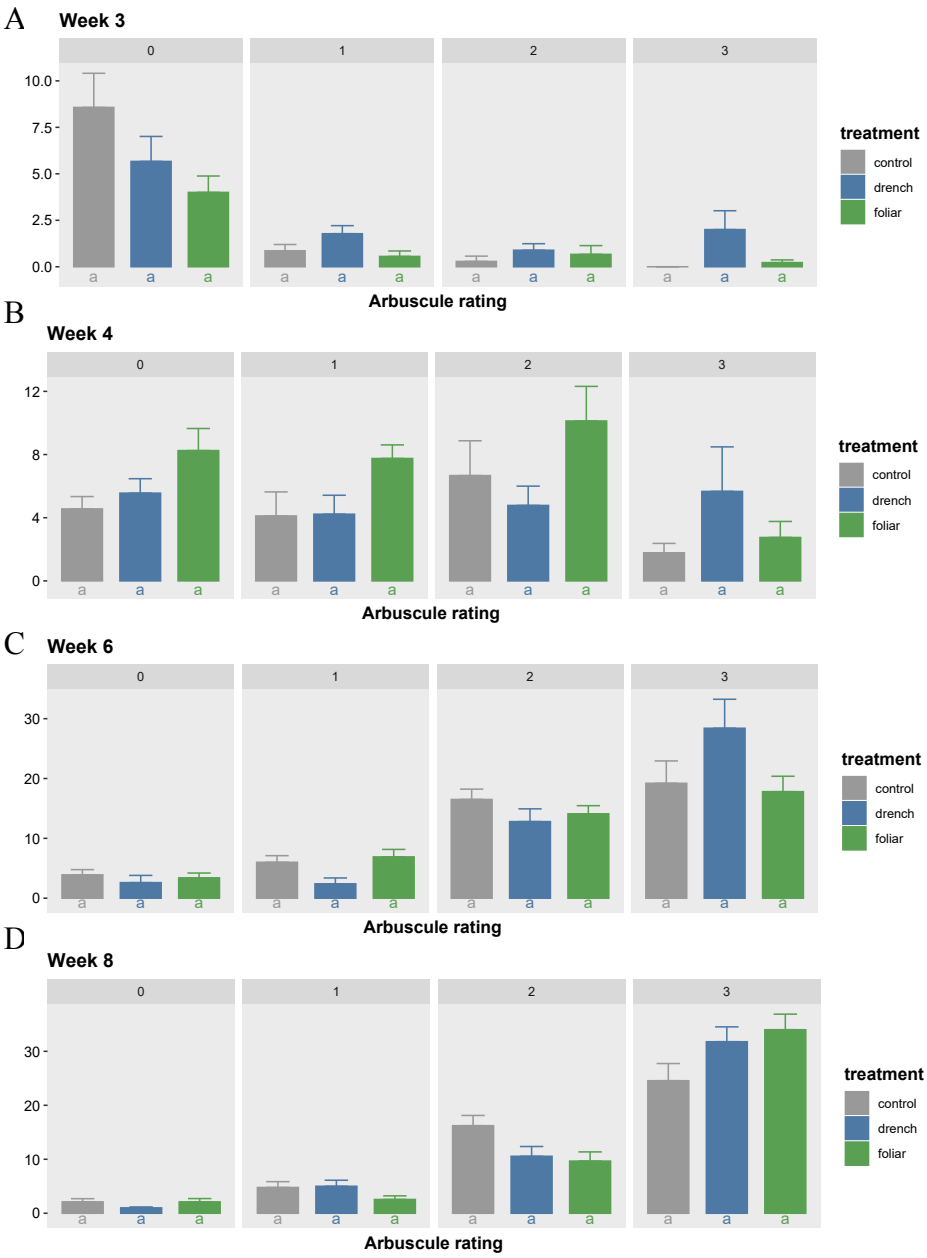


Figure 14: The mean count per plant for experiment 1 that a ranking from 0-3 was given in each treatment during the 4 harvest timepoints according to the methods outlined in Trouvelot *et al*., (1986). Grey bars are control, blue are drench and green are foliar treated plants. Each bar represents 8 biological replicates and 50 roots were examined per biological replicate. A) In week 3 more control plants receive a rank of 0, showing they are not as mature as ANE treated plants. B) Little significance is observed in week 4, but there is a trend showing more foliar plants have arbuscules in general. C) Week 6 drench plants are less likely to receive a rank of 1 and more likely to receive ranks showing greater arbuscule maturity. D) In week 8 foliar plants have greater arbuscule maturity and there is a trend showing differences in the amount ANE treatments receive a rank of 3 over the control. Statistical significance is calculated using a Kruskal-Wallis and Dunn Post-Hoc, α=0.05.

3.2.4 Microscopic Assessment Summary

Overall there is a phenotypic response showing more fungi in the roots of plants treated with ANE throughout the greenhouse time course harvest. It appears that ANE stimulates initial colonization, though at the end of trials there was still greater arbuscule presence in treated plants compared to the control. To gather a sense for how ANE affected roots reaching maximum colonization the number of times a root received a score of 3 for arbuscules and 5 for intensity during all three replicates of this experiment was tallied. This data is interesting because it shows that drench plants are significantly more likely to reach maximum colonization during weeks 4 and 8 (Figure 15). Across all weeks the ANE treatments tend to show more maximally colonized roots as compared to the control.

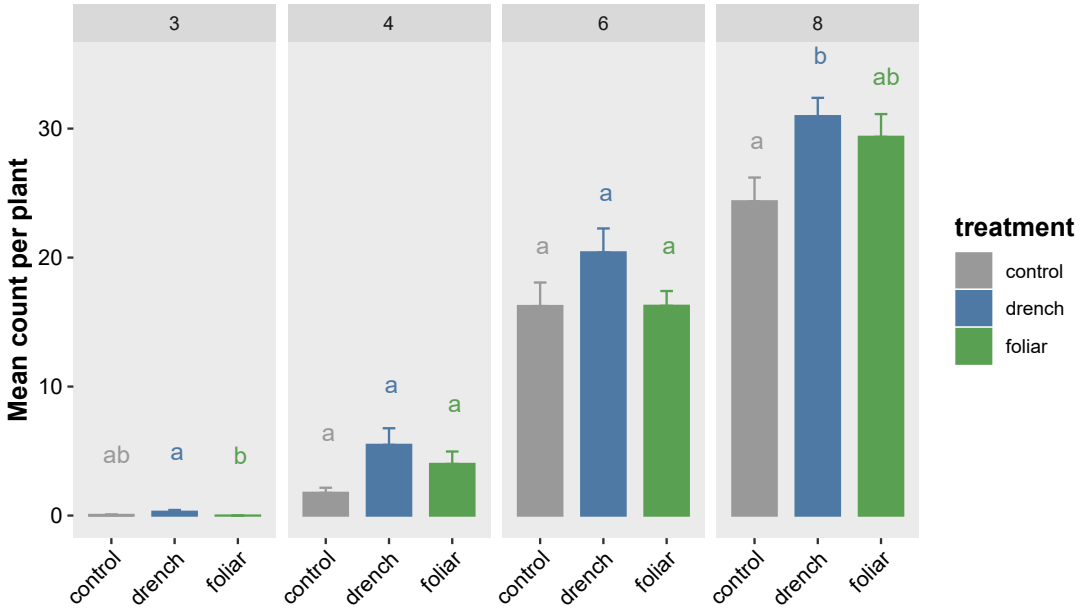


Figure 15: Each root segment examined during the 3 experiments was given an arbuscule score and an intensity score. The most heavily colonized plants received a score of 3 for the arbuscules and a 5 for intensity, meaning there were many arbuscules for nutrient exchange and greater than 90% of the root was colonized. The mean count per plant for each treatment and week is shown here. There are significant differences in week 3, 4 and 8 showing that ANE treatment has an effect on the number of plants reaching maximum colonization (Kruskal-Wallis with Dunn post-hoc α=0.05).

3.3 Molecular Mechanisms Behind Colonization

Following phenotypic results that suggested ANE affected the colonization process the next step was to examine how plant signaling at a molecular level was affected by the application of ANE through foliar and drench applications. *M. truncatula* plants were grown as described in section 2.3.1, following the same methods as in the first greenhouse experiments. Harvests were completed at 1 week following inoculation, 4 weeks and 7 weeks after inoculation with the goal being to capture the mycorrhizal symbiosis at the pre contact, early establishment and later, full arbuscule development phases. As in the greenhouse trial, plants were treated bi-weekly with ANE. During each harvest there were three technical replicates, all containing 5 biological replicates. RNA was extracted, cDNA synthesized, and qPCR was conducted on all 27 samples. During this trial 19 genes of interest were examined representing pre-contact signaling for AMF, early establishment of the symbiosis and AMF arbuscule creation (Figure 2, Table 3).

3.3.1 Molecular Results of Colonization

A heatmap of the ΔΔCt results shows the expression of all tested genes known to be associated with mycorrhizal colonization increased over time in all treatments (Figure 16). This is evidence that the molecular response was similar to what was observed phenotypically in the greenhouse trial with an increase in mycorrhizal colonization across time. The expression levels of gene PT4 were very high in all samples regardless of treatment. At weeks 4 and 7, the expression was greater than 32 0000 fold higher than in week 1 (Figure 17). As PT4 is involved in the exchange of phosphorous between the

fungi and its plant host this is another line of evidence that the symbiosis was well developed during the timeframe of the trial.

When looking at clustering of the heatmap the week 4 and week 7 plants are all very similar with no clear differences between ANE treatments, with the exception of week 4 drench which stood out as being unique to other treatments (Figure 14). Seeing the week 4 and week 7 plants cluster is an indication that the sampling times could have been improved. The sample times were chosen based on the phenotypic results from the greenhouse trial, but the window for maximum differences at a molecular level may be different. Grouping by category did not show clear trends, the categories were created based on when literature suggested the genes had the greatest activity, or whether a knockout of the gene completely prevented colonization, minimized colonization or prevented arbuscule development. Although the functions were loosely linked to time the process of colonization is asynchronous, so genes were active at various time points depending on when the fungi made contact with the continually growing plant roots. The genes linked to arbuscule development did cluster some, especially when looking at how few of these genes were present above the threshold of detection in the week 1 samples. Gene SbTm1 was not included in the results as it was not present at detectable levels in any sample. Results from DMI1 must also be discounted as a standard curve was not obtained.

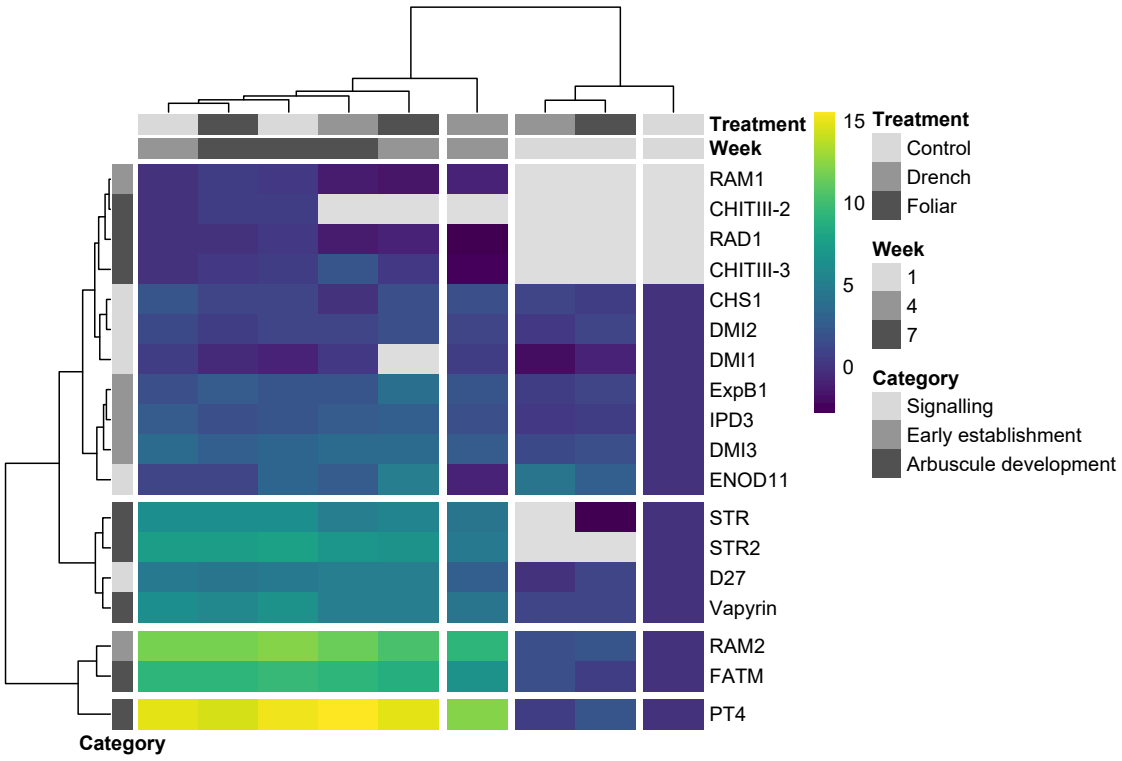


Figure 16: A heatmap showing the expression of all genes (ΔΔCT) that were detectable during weeks 1, 4 and 7 of sampling in the control, drench and foliar treatments. Grey squares indicate genes were present at levels below the limit of detection in the sample. Clustering is calculated using the complete linkage clustering method, genes RAM1, RAM2, STR, STR2, CHITIII-3, RAD1 and PT4 were not present at detectable levels in week 1, so were normalized to control week 4 values. Week 1 results show the control is significantly different from the ANE treatments. Week 4 drench is unique and clusters alone, but is quite similar to other week 4 and week 7 gene expression profiles. Clustering by category did not show any results of interest.

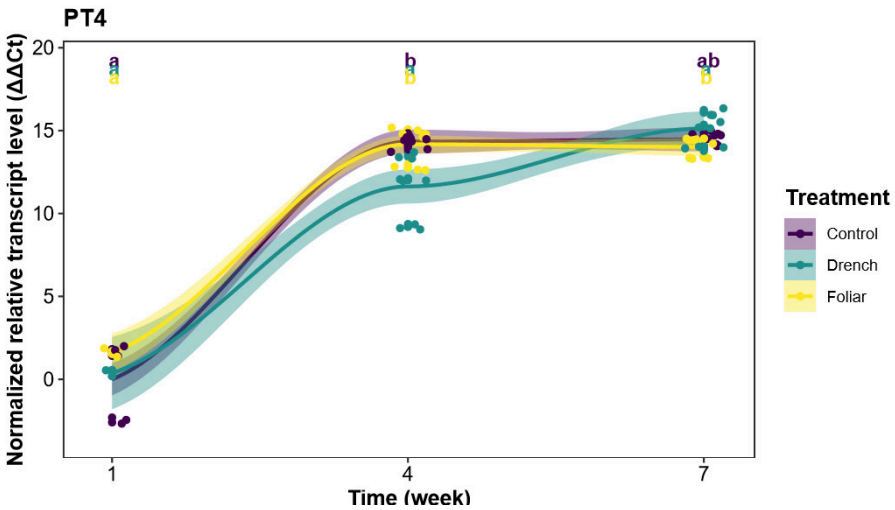


Figure 17: Expression levels of gene PT4 were extremely high in this trial for all three treatments. In week 4 and 7 there is more than a 32 000 fold increase in expression levels over week 1.

Week 1 results were the most interesting as it showed the control was different from the foliar and drench treated plants in the clustering of the heatmap and in the expression of key genes. In this week some genes attributed to early establishment and pre-contact phases are upregulated by the use of ANE as both a foliar and drench treatment. Many of genes associated with arbuscule development and later stages of colonization were not present at detectable levels in the week 1 tissue samples, which is to be expected as based on the phenotypic data few arbuscules were forming at three weeks after inoculation.

ENOD11 expression was significantly affected by the application of ANE, in week 1 the foliar and drench treatments both showed expression levels significantly higher than the control (Figure 18). In week 4 all treatments were significantly different from one another, the foliar was higher than the control and the drench was lower. At week 7 there are significant differences between the foliar and control treatments, but visually the means are similar, and the differences are beginning to plateau which is

expected as the function of ENOD11 is to modify cell wall changes needed for early fungal penetration (Journet *et al*., 2001).

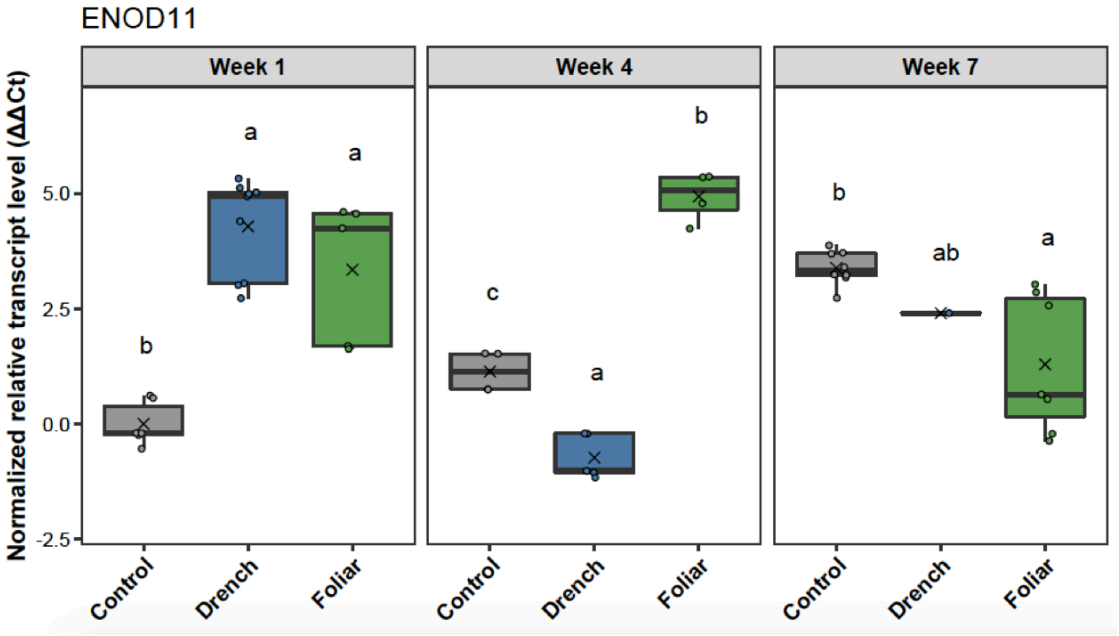


Figure 18: Normalized relative transcript level (ΔΔCt) of ENOD11 across weeks of tissue harvest and treatments. Week 1 shows ANE having a significant effect on the expression patterns.

DMI2 is a signaling kinase involved in the initial interpretation of the mycorrhizae near the plant cell, it is part of a signaling cascade that is involved in the changes that occur inside the nucleus for fungal accommodation to occur (MacLean *et al*., 2017). In week 1 there are significant differences between the foliar and control treatments (Figure 19). The drench treatment is not significantly different from the control, but there is a trend showing higher data spread and the mean is greater than control. In week 4 and week 7 there are no significant differences between any of the treatments.

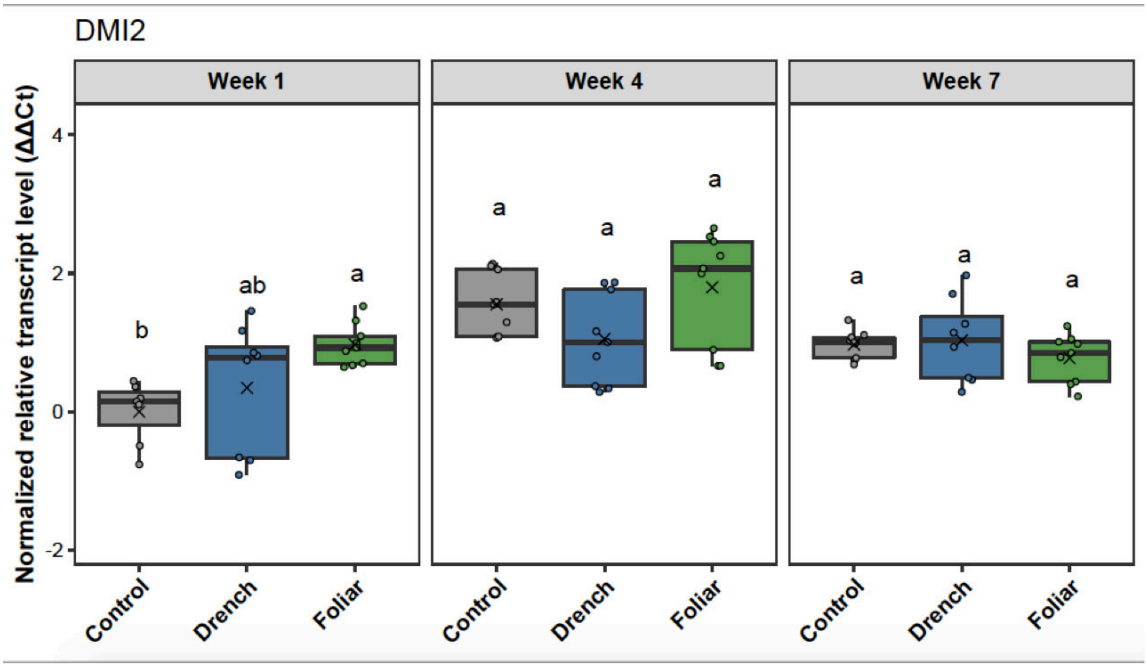


Figure 19: Normalized relative transcript level (ΔΔCt) of DMI2 across weeks of tissue harvest and treatments. Week 1 shows the foliar expression levels are significantly higher than the control and the drench shows a larger data spread compared to the control.

DMI3 is another kinase involved in the signaling cascade that triggers the cellular accommodation process for mycorrhizal fungi (Pimprikar, 2016). The expression is similar to ENOD11, in week 1 there are significant differences between the control and the two ANE treatments (Figure 20). At week 4 the drench expression levels have again dropped to levels below the control, but the foliar remains high. In week 7 the expression of DMI3 is consistent between treatments.

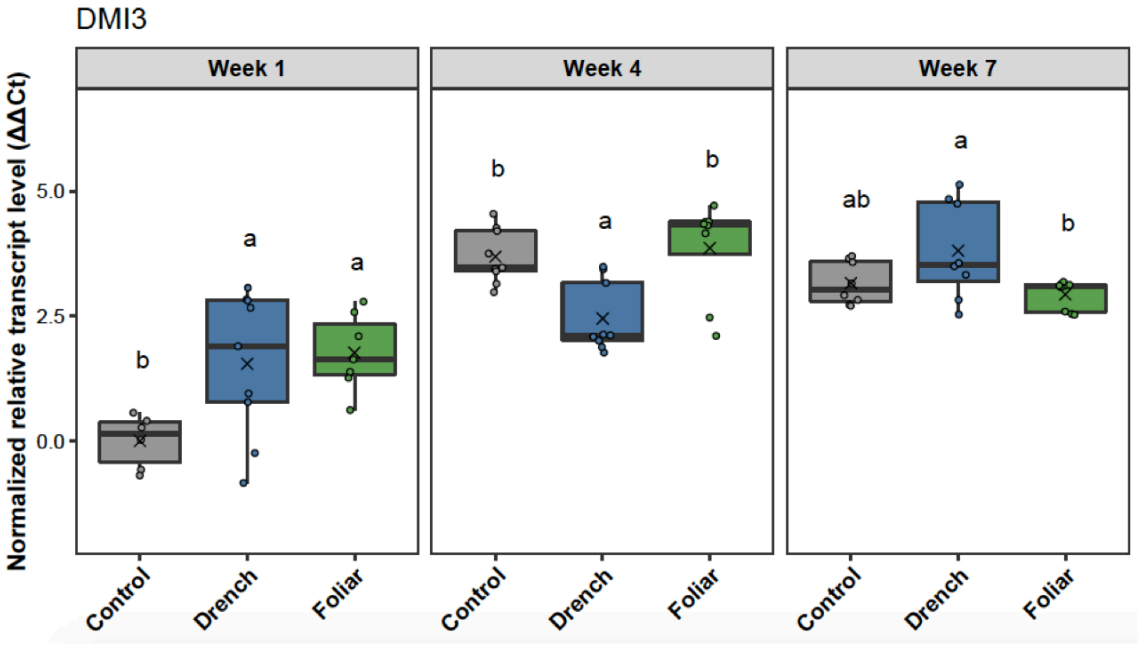


Figure 20: Normalized relative transcript level (ΔΔCt) of DMI3 across weeks of tissue harvest and treatments. Week 1 results show the ANE treatment has a significant effect on expression.

Expanisn B1 is a gene involved in the creation of proteins that loosen cell walls to allow for fungal entry (Choi *et al.*, 2018). In week 1 the gene is expressed at significantly higher levels in the drench and foliar treatments as compared to the control (Figure 21). At week 4 there are no significant difference between the treatments, but the extremely large spread in the foliar and drench treatments shows there are some detectable differences between plants receiving ANE as compared to the controls. As with many of the other genes that are involved in early accommodation processes there are no differences between treatments at week 7.

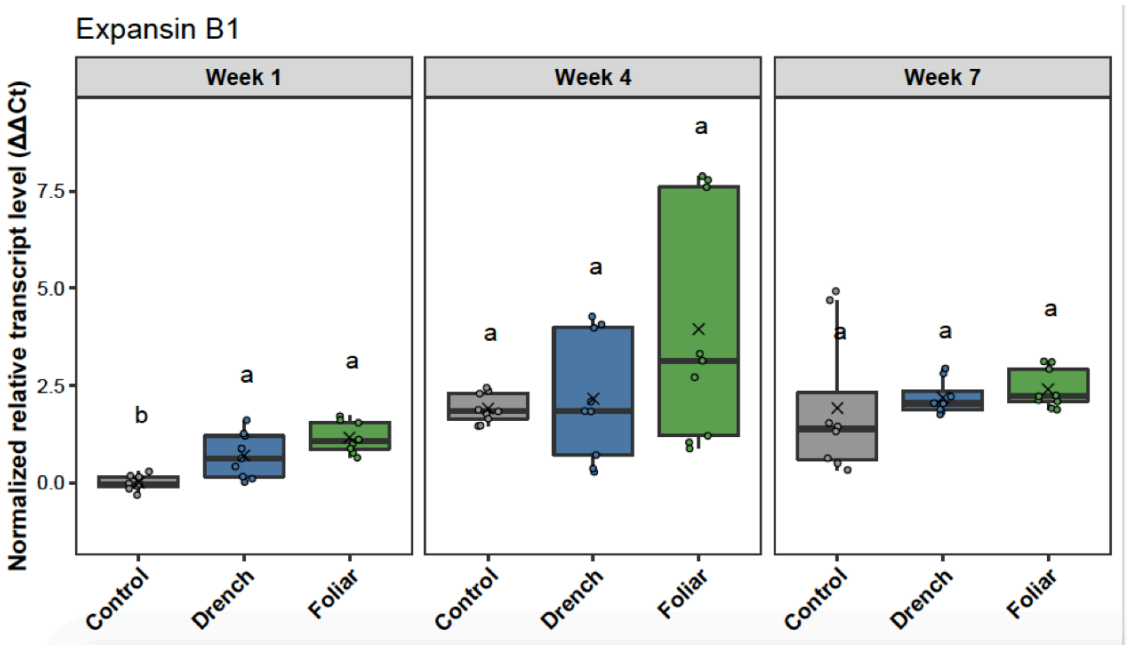


Figure 21: Normalized relative transcript level (ΔΔCt) of Expansin B1 across weeks of tissue harvest and treatments. Week 1 shows statistical significance between the control and ANE treatments, week 4 is not statically significant, but the large spread suggests some effects are still being experienced.

IPD3 is a protein that interacts with DMI3 and is involved in allowing the fungi to successfully form the prepenetration apparatus (Horvath *et al*., 2011). The foliar and control treatments are significantly different from one another at week 1 (Figure 22). At week 1 the drench is not significantly different from the control, but the data spread is larger suggesting high biological variability within the treatment. In week 4 expression is lower in the drench treatment, but in week 7 the drench treatment shows the highest expression.

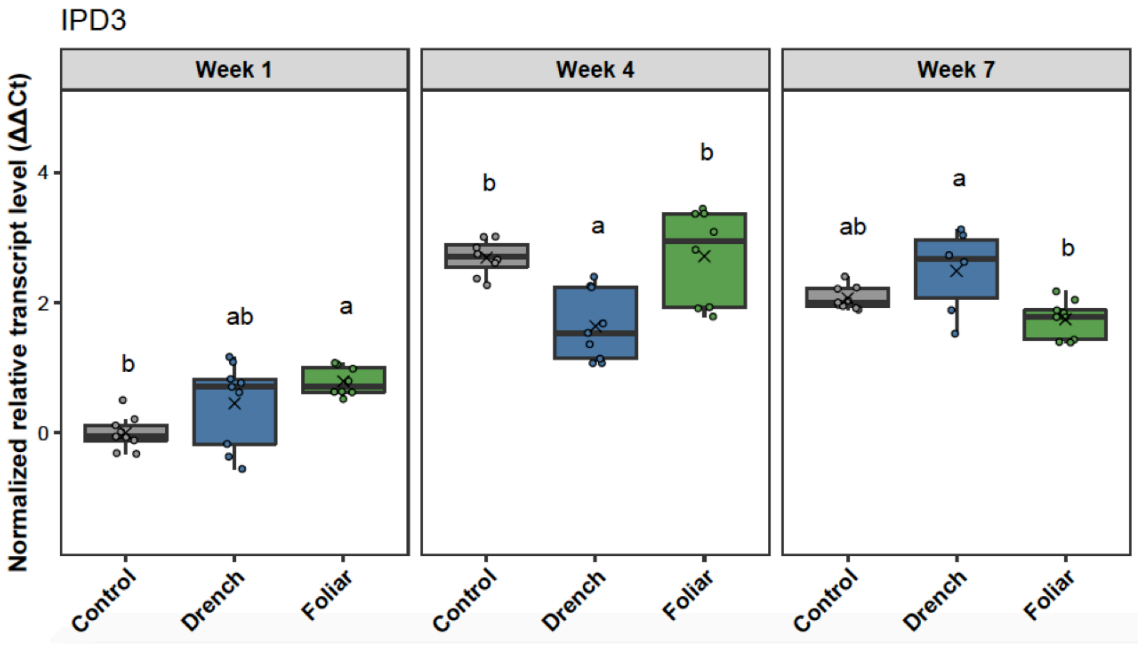


Figure 22: Normalized relative transcript level (ΔΔCt) of IPD3 across weeks of tissue harvest and treatments. Week 1 shows the ANE having an effect compared to the control. Results in week 4 and week 7 are variable, which is consistent with results of other genes.

Vapyrin was not expressed at high levels in week 1, but it is interesting because the ANE treatments did show significant differences over the control (Figure 23). In weeks 4 and 7 the differences are not as pronounced, and expression of the gene increases in all treatments. Vapyrin is a gene involved in maintaining arbuscules, but also could be involved in defense pathway suppression which would be useful during early colonization (Pumplin *et al*., 2010).

Vapyrin

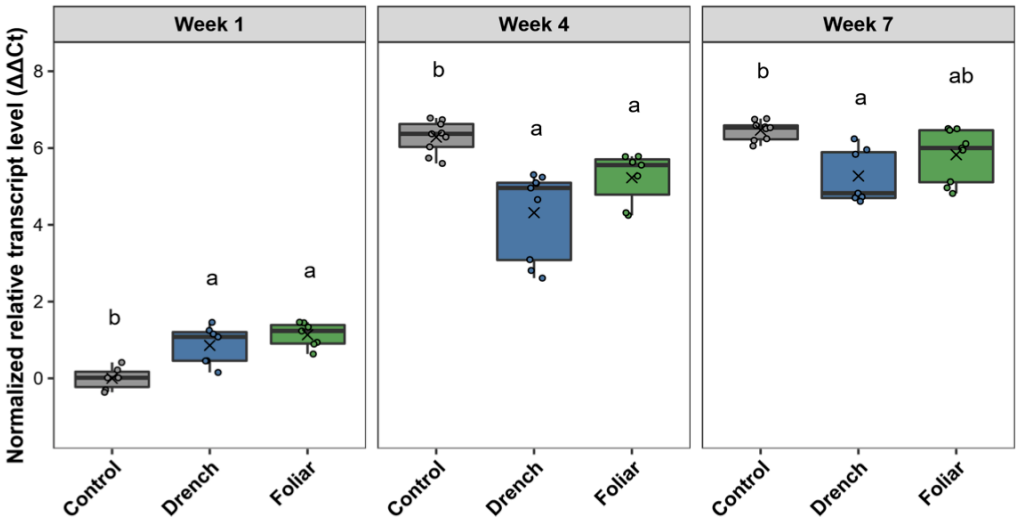


Figure 23: Normalized relative transcript level (ΔΔCt) of Vapyrin across weeks of tissue harvest and treatments. Expression is statistically significant between the control and ANE treatments in week 1. Expression levels also increase significantly in week 4 and week 7, and is most highly expressed in the control.

In weeks 4 and 7 there are no clear trends among multiple genes suggesting an effect from ANE treatment. Many of the genes involved in later stages of colonization were not expressed at detectable levels in week 1. This is not surprising as the phenotypic evidence shows very few arbuscules in week 1, they are markers of late, well developed colonization. Some individual genes show changes that suggest an effect, but the evidence is not as robust as for the relevant early establishment genes. The most consistent trend is CHITIII-3 expression is one of the more interesting genes involved in later colonization events because of the significant differences that occur in the week 7 tissues showing drench plants to have a much higher expression (Figure 24). CHITIII-3 is a gene involved in defense pathway suppression (Salzer *et al*., 2000). Results for the genes not highlighted here are in Appendix C.

CHITIII-3

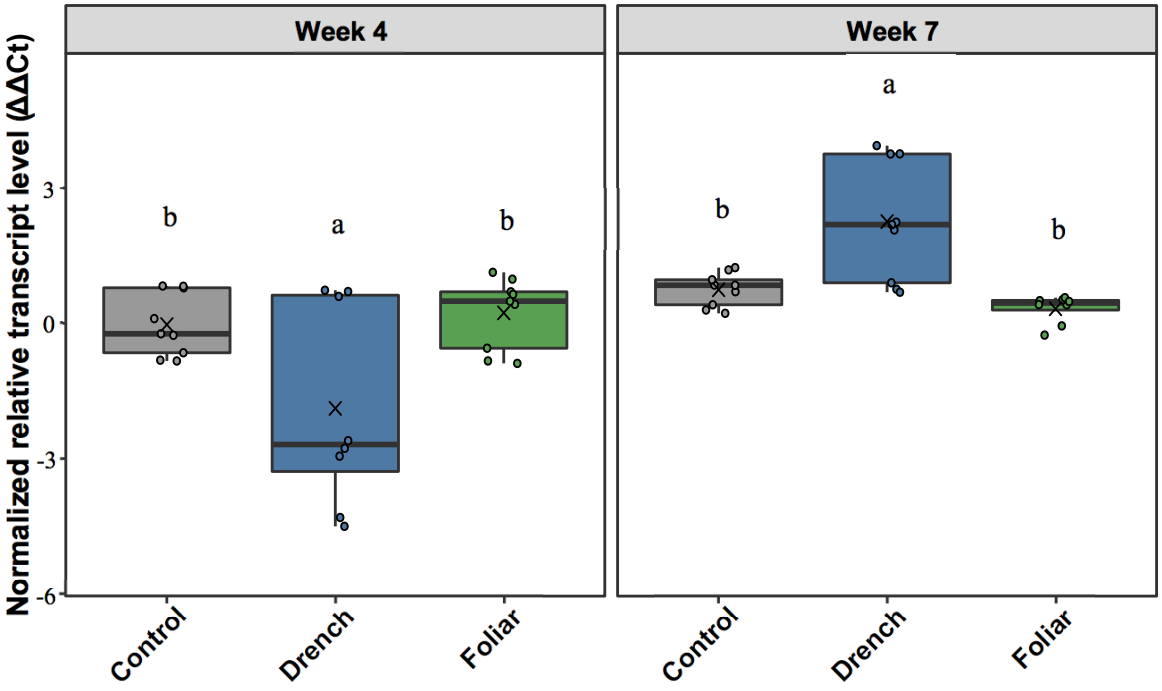


Figure 24: CHITIII-3 expression was affected by ANE treatment in weeks 4 and 7. Foliar results showed increased expression and drench results showed significantly lower expression.

3.4 Results Summary

The results indicate that ANE has a direct effect on the performance of early spore growth in *R. irregularis*. The 0.01 and 0.05% SSEP treatments had a significant response on tested parameters: percent germination, hyphal length and hyphal branching. In the greenhouse a phenotypic response was seen indicating drench application of ANE affected plant growth and the amount and quality of mycorrhizal colonization. Foliar application results were more variable, but there was evidence of some effect. A molecular trial examination of genes involved in mycorrhizal colonization indicated there

was a response from the plant during early stages of the colonization process when treated with a foliar or drench application of ANE.

Chapter 4: Discussion

4.1 ANE at Lower Concentrations Directly Stimulates Early Mycorrhizal Growth

When spores sense the environmental conditions are suitable for growth and signaling molecules are interpreted AMF can begin using carbon reserves for pre-symbiotic growth (Smith and Read, 2008). The *in vitro* spore assay showed consistently improvements in early AMF growth when ANE was present at low concentrations. A nutrient control was run at the best performing rate (0.01%) and the growth response was consistently significantly different between the nutrient control and ANE. This shows that there was a biostimlulatory effect at play, as the growth of the spores was beyond what would occur from nutrients (Shukla *et al*., 2019). ANE is a complex mixture with several components that could be the active ingredients stimulating the AMF to keep growing and use their carbon reserves to a greater degree. It is known that strigolcatones stimulate AMF growth (Gomez-Roldan *et al*., 2008; Liu *et al*., 2011, Besserer *et al*., 2006). Genre *et al*., (2013) showed that strigolactones induced AMF branching which increases the likelihood that AMF will make contact with a plant host. Results obtained in this project show that ANE presence increased branching and hyphal length, perhaps due to strigolactone-like effects. More research is needed to confirm if strigolactone-like molecules are in the ANE. ANE also contains flavonoids which are also known to trigger AMF pre symbiotic growth (Badri *et al*., 2009; Khan *et al*., 2009; Abdel-Lateif *et al*., 2012; Siqueria *et al*., 1991). The introduction of ANE flavonoids, strigolactone-like molecules or other signaling molecules into the *in vitro* assay may be having an effect on the positive early AMF germination and hyphal length results. Other compounds in ANE like ethylene or polyamines have also been reported to have AMF growth promoting

effects (Kuwada et al., 2006). It is likely that a combination of several of these compounds are together creating the observed growth effects on AMF spores.

4.2 Plant Response Mode of Action

This research has shown through phenotypic and molecular lines of evidence that ANE application has some influence on plant signaling to encourage AMF symbiosis. ANE contains many compounds that could influence how plants respond at a molecular level. Khan *et al*., (2013) hypothesized that ANE was able to elicit a response from plants that made them more likely to form associations with rhizobium. Rhizobium are known to use similar pathways to AMF in plant communication (Malliet *et al*., 2011; Olah *et al*., 2005). In Jayaraj *et al*., (2007) ANE was shown to enhance fungal disease resistance when applied at a rate of 0.2%. Treated plants showed increased defense gene expression and accumulation of phenolics without affecting the phenotypic growth of the plants. Phenolic accumulation can help plants resist stress and many consider it to be a priming effect of ANE application (Jayaraj *et al*., 2007; Shukla *et al*., 2019). Colonization with AMF is also reported to increase phenolic content in plants, suggesting that treatment with AMF and ANE together has potential to improve the resilience of plants (Lone *et al*., 2015). ANE is known to contain many oligosaccharides and polysaccharides which serve many functions including affecting signal transduction (Jayaraj *et al*., 2007). If these compounds are present in higher quantities, it could help explain how molecular signaling in the plant was affected with the application of ANE.

Understanding what components of ANE affect plant communication with mycorrhizae requires much more research. ANE is complex and it is likely that multiple

compounds exert effects on multiple genes and plant processes (de Sager *et al*., 2019). Two recent review papers on ANE show that the mode of action understanding is still very preliminary, we are a long way from attributing certain effects to the various compounds in ANE (Shukla *et al*., 2019; de Sager *et al*., 2019). There is still debate on which compounds are found consistently in ANE and how much variety there is between time of seaweed harvest, species used, and extraction method (de Sager *et al*., 2019; Van Oosten *et al*., 2017). ANE is a complex substance and in other research papers it is common to attempt to use organic fractions of ANE as a way to narrow down which classes of molecules could be providing the biostimulant response. However, the whole product often performs better than the individual fractions, suggesting that the interaction of multiple types of compounds is central to the effectiveness of ANE (Khan *et al*., 2013; Shukla *et al*., 2018).

To our knowledge this is one of the first studies looking at the three-way interaction between plants, mycorrhizae and ANE. Our pioneering study suggests the focus of future research examining AMF interactions with ANE could focus on early stage processes of mycorrhizal colonization. However, even the process of mycorrhizal colonization is complex, and a lot is left to be understood before we can confidently understand how AMF and ANE interact (Choi *et al*., 2018; MacLean *et al*., 2017). An extensive literature review was done to select genes that are involved in key cellular accommodation activities, but it is entirely possible that unsearched genes and processes are more important. As well, in the future it would be valuable to have a no AMF control in molecular studies. As the molecular understanding of ANE effects improves we will be

better equipped to answer what is happening within the plant to induce changes with mycorrhizal interactions.

4.3 Understanding the Molecular Response of Mycorrhizal Colonization

During this research 19 genes of interest were identified in the literature as important during the process of mycorrhizal colonization. When selecting genes function was considered, there is representation from all phases of colonization, from pre contact signaling to early establishment inside root cells to the development and maintenance of arbuscules, the fungal structures used for nutrient exchange (Figure 2). A large list of genes was initially compiled and narrowed down to choose those that were differentially regulated, referenced by multiple papers and representative of all phases of mycorrhizal establishment (Table 3).

In our study we looked at two signaling molecule related genes, CHS and D27, these genes were not expressed at different levels in treated versus non treated plants, which does not support the hypothesis that ANE application increases the way plants send exudates to look for fungal partners (Figure A11, A12). CHS is related to flavonoid production and D27 to strigolactone synthesis (Bonfante and Genre, 2010; Abdel-Latif *et al*., 2012; Liu *et al*., 2011 MacLean *et al*., 2017). However, to attribute all exudate signaling to two genes is not valid, and we cannot say for sure if ANE is affecting early signaling via exudates at this time. ANE did seem to positively effect genes involved in the cellular accommodation processes, like ENOD11, Expansin B1 and IPD3 (Journet *et al*., 2001; Siciliano *et al*., 2007; Horvath *et al*., 2011. All three of these genes affect cell wall changes and showed significantly increased expression during week 1 in the drench

and foliar treated plants. Vapyrin is often classified as being involved in arbuscule development, but Pumplin *et al*., (2010) report Vapyrin knockouts as having poor epidermal penetration. If Vapyrin is also involved in cell wall changes the observed response of ANE fits well with the other genes we had classified as being involved in early establishment. The genes DMI2 and DMI3 are also worth noting for their roles in early establishment and the observed effects of ANE on their activity. The genes are two signaling kinases that are key in plant cells interpreting AMF in the environment (Olah *et al.*, 2005; Bonfante and Genre 2010). DMI2 helps plants to perceive the Myc factors from AMF and then helps to signal changes to begin in the nucleus of the cell (MacLean *et al*., 2017). DMI3 interacts with transcription factors like DELLA proteins (RAM1, RAM2, RAD1) to create further transcriptional changes after it becomes active through phosphorylation (MacLean *et al*., 2017). Seeing the DMI2 and DMI3 expression significantly increased by ANE application suggests that plants are able to process the Myc factors more efficiently. The greenhouse phenotypic evidence does support this as in drench plants there are more roots receiving a maximum ranking for colonization earlier than in the other treatments (Figures 11-15). It is interesting to note that the foliar expression levels were consistently as high as drench levels and often had less spread. It is clear that there is a foliar response on plant signaling, despite the phenotypic data from the greenhouse not showing as consistent a response. Perhaps two different periods in development were captured comparing the foliar treatment and the drench. Drench treated plants phenotypically achieved higher AMF colonization during early timepoints compared to foliar treatment (Figures 11-15). During week 1 it is possible that the foliar

molecular response is often more pronounced because the plants are developmentally at an earlier phase of mycorrhizal colonization requiring higher expression of key genes.

In genes known to be important to later stages of colonization ANE treatment did not appear to have a strong effect at the timepoints we tested. The expression levels of most of these genes become very high during week 4 and week 7 sampling which shows the genes are very important to the well-developed symbiosis that was observed phenotypically in the greenhouse study. For example, PT4 is expressed 32 000 fold higher than in week 1 (Figure 17). Gene FATM is also expressed more than 1000 fold higher in late weeks compared to week 1 (Figure A18). Other lipid transfer genes STR and STR2 are also expressed more than 100 fold higher in later weeks despite not showing differences between treatments (Figure A16, A17). This response is evidence that mycorrhizal colonization is a very important process that our system has captured well because plant cells are investing a lot of resources into the upregulation of these genes.

4.4 Stress and the ANE Mediated Response

As research continues to discover the mode(s) of action of ANE the focus on how abiotic and biotic stresses may be involved is growing. This is fitting as ANE is often used to protect crops from stresses in a changing climate (Battacharyya *et al*., 2015). Nutrient stress was induced during the plant growth experiments for several reasons. The first, is that plants have some ability to regulate how much they want to encourage mycorrhizal associations (Smith and Read, 2008). In a symbiosis there is a benefit to each partner, but also a cost. If nutrients (especially phosphorus) are available in excess the plant has no need to give resources to the mycorrhizae in exchange for nutrients (de

Miranda and Harris, 1994). The goal was to measure mycorrhizal colonization up to a maximum, so negligible amounts of phosphorus were added to the system. Fertilizer containing no phosphorous (14-0-14 1g/L) was applied once per week because the sand substrate provided very few nutrients and plants could not have survived for 8 weeks without fertilizer. The second reason for applying a nutrient stress was to maximize the chance of seeing an effect from the ANE application. There is growing evidence to suggest that ANE has a maximum effect when a stress is present. Zamani-Babgohari *et al*., (2019) found that there was no positive effect from ANE application on biomass in the absence of a freezing stress treatment.

The idea that ANE application "primes" plants to prepare for stress events is gaining traction in the literature (Nair *et al*., 2012; Van Oosten *et al*., 2017; de Saeger *et al*., 2019). For example, Santaniello *et al*., (2017) found that ANE application pre-activated key pathways in plants leading to earlier stomatal closure during drought stress. In our research early phases of mycorrhizal colonization were affected by ANE application and the sand substrate and fertilizer regime did not provide ample nutrition. It is possible that the ANE application had a priming effect that lead to plants choosing to prioritize mycorrhizal symbiosis in an attempt to aid with nutrient acquisition.

4.5 Effect of ANE Application Rate on Fungal Response

The highest rate of ANE tested in the *in vitro* system consistently underperformed compared to the control. This could be a salinity affect as a rate of 0.1% (1g/L) is high to use in a Petri dish system with no buffering capacity. Lower rates in the range of 0.01% are typically used in *in vitro* systems to avoid the ionic imbalance that can occur. An *in*

vitro assessment of *Sinorhizaobum meliloti* growth on media containing 0.1% ANE also found no positive effect on the microbe growth (Khan *et al*., 2013). The high rate of ANE likely made the Petri dishes into an inhospitable environment as it inhibited % germination and growth. In all media the pH was adjusted to 6, which is within a range that *R. irregularis* spores can grow (Kuwada *et al*., 2013). A pH effect likely does not account for the inhibition observed in the 0.1% SSEP treatment.

In Bajpai *et al*., (2019) growth of strawberry pathogen *Podosphaera aphanis* was assessed on individual leaves treated with the same rate of 0.1% ANE. The study found that 0.1% ANE. inhibited fungal spore germination of this common pathogen. In the assays it was unclear if the ANE had a direct effect on fungal growth, or if results were due to plant defense response stimulation. There are many studies indicating ANE application is able to combat a variety of fungal diseases (Battacharyya *et al*., 2015; Shukla *et al*., 2019). It is interesting to consider how varying rates of ANE could affect beneficial and pathogenic species growth.

Plants are able to differentiate between beneficial and pathogenic fungal species, but the direct effects of ANE on the fungus would be far less selective. However, microbes are diverse and are often fill small niches. Beneficial species can likely use more complex carbohydrate sources than pathogens due to their roles in the environment (Badri *et al*., 2009). This can be likened to human health, where beneficial gut microbes are better able to metabolize complex fibers. It is possible that the complex carbohydrates that are abundant in ANE are experienced differently by microbes with different functions in the environment.

4.6 Microbial Interactions as a Mode of Action of ANE to Help Plants Resist Stress

Seaweed extracts are known to be very complex and exert many effects onto plants (Battacharyya, 2015; Shukla *et al*., 2019). Phenotypically, these modes of action are diverse and well characterized; from drought stress, salt stress, temperature stress to biotic disease resistance (Van Oosten *et al*., 2017; Shukla *et al*., 2019). Plant communication with soil microbes is also known to be influenced by all stress situations (Vandenkoornhuyse *et al*., 2015). The mode of action ANE has on microbial interactions can be thought of in isolation, but it can also be considered as one of the response pathways that help plants to become resilient in the face of abiotic and biotic stress conditions. If plants change their root exudate profile because of ANE application that would have many indirect effects, including helping plants resist stress (Vandenkoornhuyse *et al*., 2015; Lucini *et al*., 2019). This paradigm shift is being considered in agriculture as growers look at creating optimal environments to for plants, instead of focusing solely on the plant (Van Oosten *et al*., 2017). This study builds on previous work done by Alam *et al*., (2013), showing that ANE can affect soil microbial activity. Most biostimulants that make soil health claims contain microbes or microbial byproducts (Brown and Sea, 2015). This research helps to suggest that ANE not only affects plants, but also microbial health. As growers continue to focus on holistic methods and policies change to limit traditional chemistries this work will hold increasing value (de Saeger *et al*., 2019).

4.7 Directions for Future Research

The *in vitro* spore plate assay resulted in positive data showing that ANE has an effect on pre-symbiotic growth of AMF. To build on the research to determine active ingredients in the ANE it would be interesting to replicate the trials using organic fractions as outlined in Khan *et al*., (2013). However, to examine all components of the ANE it would be interesting to test not only what initially goes into solution with the initial methanol extraction, but also what is insoluble (Khan *et al*., 2013). It is likely that molecules like alginates could be left in the insoluble part and they could have AMF growth promoting activates (Khan *et al*., 2009).

The next step in examining the molecular mechanisms of colonization in the presence of ANE would be to focus on gene expression in the fungi. The genome of *R. irregularius* was sequenced in 2013 (Young *et al*., 2011). Fungal genetics are just beginning to be understood, the Myc factor, which is the key signal plants interpret to begin colonization was only fully described in 2011 (Maillet *et al*., 2011). Due to the complex nature of fungal genetics this part of the signaling relationship was beyond the scope of this project.

The molecular data show ANE has an effect on foliar and drench treated plants with the upregulation of key early establishment genes in week 1. In the future, time points beyond week 1 could be refined to hopefully yield more telling results for genes involved in arbuscule creation and other mature symbiosis functions. The week 1, week 4 and week 7 harvests were chosen based on the phenotypic data from the greenhouse study and several papers where the first sampling occurred no earlier than 5 days after inoculation and several weeks afterwards to a maximum of 9 weeks (Gutjahr *et al*., 2012;

Colard *et al*., 2011). Review papers have shown that key genes are expressed at different phases of the colonization process, it was thought that targeting these phases when we saw the phenotypic response would yield the best results (Choi *et al*., 2018; MacLean *et al*., 2017). However, no strong correlation between the week 4 and week 7 timepoints and which gene function groups were most active. This is likely in part because the process of AMF colonization is asynchronous. The week 1 tissues showed the strongest difference in gene expression due to ANE treatment regardless of the genes function in the cell. For example, Vapyrin differences were significant in week 1 and it is commonly reported to be involved in arbuscule development (Choi *et al*., 2018). During week 3 of the greenhouse study some arbuscules were observed, it is possible that as early as week 1 these processes were being stimulated at a molecular level. This also leads to question if timepoints earlier than one week may have shown different responses. In the future it would be interesting to consider timepoints mere hours after inoculation to see how quickly there is a response.

Another question is to consider the extent the ANE is affecting the plant–AMF communication versus simply improving the AMF growth. In the greenhouse assay a drench application showed a more consistent improvement to the colonization process than the foliar application. One key difference between the two application methods was the amount of extract that made direct contact with the soil. Perhaps the drench application was more effective because the soil microbes were more directly stimulated. Drench plants also grew larger but attributing all the results to increased growth is an oversimplification. Molecular results showed a change in the plant communication profile, where key genes for early mycorrhizal establishment were upregulated. The foliar

plants also showed changed molecular profiles, and some improvements to the phenotypic colonization, but the rate of growth did not improve the same way compared to drench plants. If improved plant growth was the primary mode of action leading to improved mycorrhization the foliar molecular response should have been less pronounced to correlate with less plant growth. In future studies it would be interesting to see how dependent the expression profiles of these AMF related genes are on there being AMF present in the system. This also would help to tease out the effects of ANE on the microbe versus the plant.

Chapter 5: Conclusions

This study has confirmed that ANE has a direct positive effect on the beneficial mycorrhizal species *R. irregularis*. To our knowledge this is one of the first studies to examine effects of ANE on mycorrhiza. There was a phenotypic response in the greenhouse experiments that suggested ANE treatment improved the quality and quantity of mycorrhizal colonization in host plant *M. truncatula*, especially during the early stages of symbiosis formation. Looking at over 11 500 root segments to quantify mycorrhizae also confirmed that drench plants were significantly more likely to have reached maximum colonization than the untreated controls at 8 weeks. Molecular evidence strongly supports a positive change in expression levels of key genes associated with forming AMF symbiosis when ANE treatment is applied as a foliar or drench. The genes showing the greatest effects were those involved in early cellular accommodation processes to facilitate the AMF entering plant cells, ENOD11, IPD3, Expansin B1, DMI2, DMI3 and Vapyrin. At the conclusion of this project it has been found that ANE has an effect directly on mycorrhizal spore growth, on the phenotypic response of mycorrhizal colonization in host plant roots, and on the molecular processes of host plant roots. This research adds to the developing body of literature indicating ANE improves soil health.

Soil microbial interactions are an increasing area of interest in agriculture and there is much to learn about how plants curate their rhizosphere symbionts. As agriculture moves to embrace green chemistries the use of ANE biostimulants is projected to increase. Similarly, the focus on soil health as a way to promote plant health will only continue to grow in its relevancy. This study has shown that ANE application can

improve interactions with mycorrhizae, a globally important microbe. ANE application has been shown to improve plants abilities to tolerate abiotic and biotic stresses. The mode of action is complex but affecting soil community function as a direct or indirect effect is a plausible new line of thought. As climate change continues to alter growing conditions worldwide the use of ANE to protect against stresses will increase. There is now additional evidence that ANE application will improve the health of plants, but also the mycorrhizae that support their growth.

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Appendix A: Results From Experiment 1 and 2 of the *in vitro* Spore Plate Assay

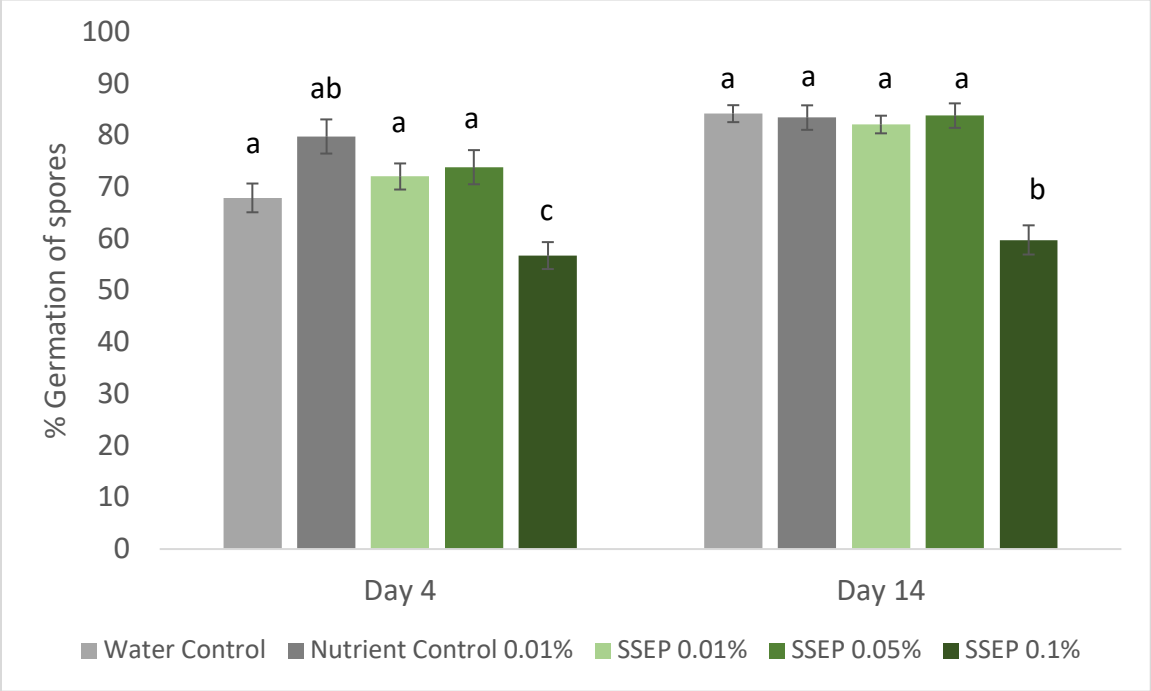

Figure A1: Percent germination results from experiment 1, at day 4 the nutrient control was performing the best, though differences between treatments are not as pronounced as in other replicates. At day 14 % germination was very high and consistent between treatments, with the exception of the high rate of 0.1% SSEP. A one way ANOVA and Tukey's post-hoc was done on each sample date, $\alpha = 0.05$. Each bar represents 6 biological replicates, each Petri Dish contained at least 80 spores.

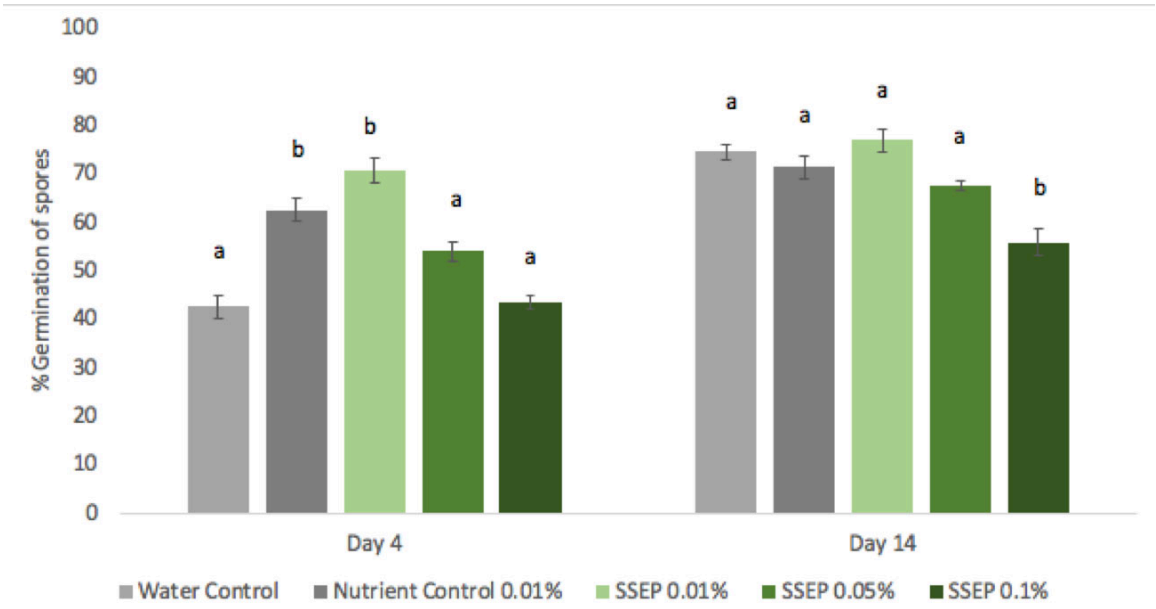

Figure A2: Percent germination results from experiment 2, at day 4 there were significant differences between treatments with the nutrient control and 0.01% SSEP performing the best. At day 14 the 0.01% SSEP treatment reaches slightly higher % germination, but differences are not significant between treatments, with the exception of the poor performance of 0.1% SSEP. A one way ANOVA and Tukey's post-hoc was done on each sample date, $\alpha = 0.05$. Each bar represents 6 biological replicates, each Petri Dish contained at least 80 spores.

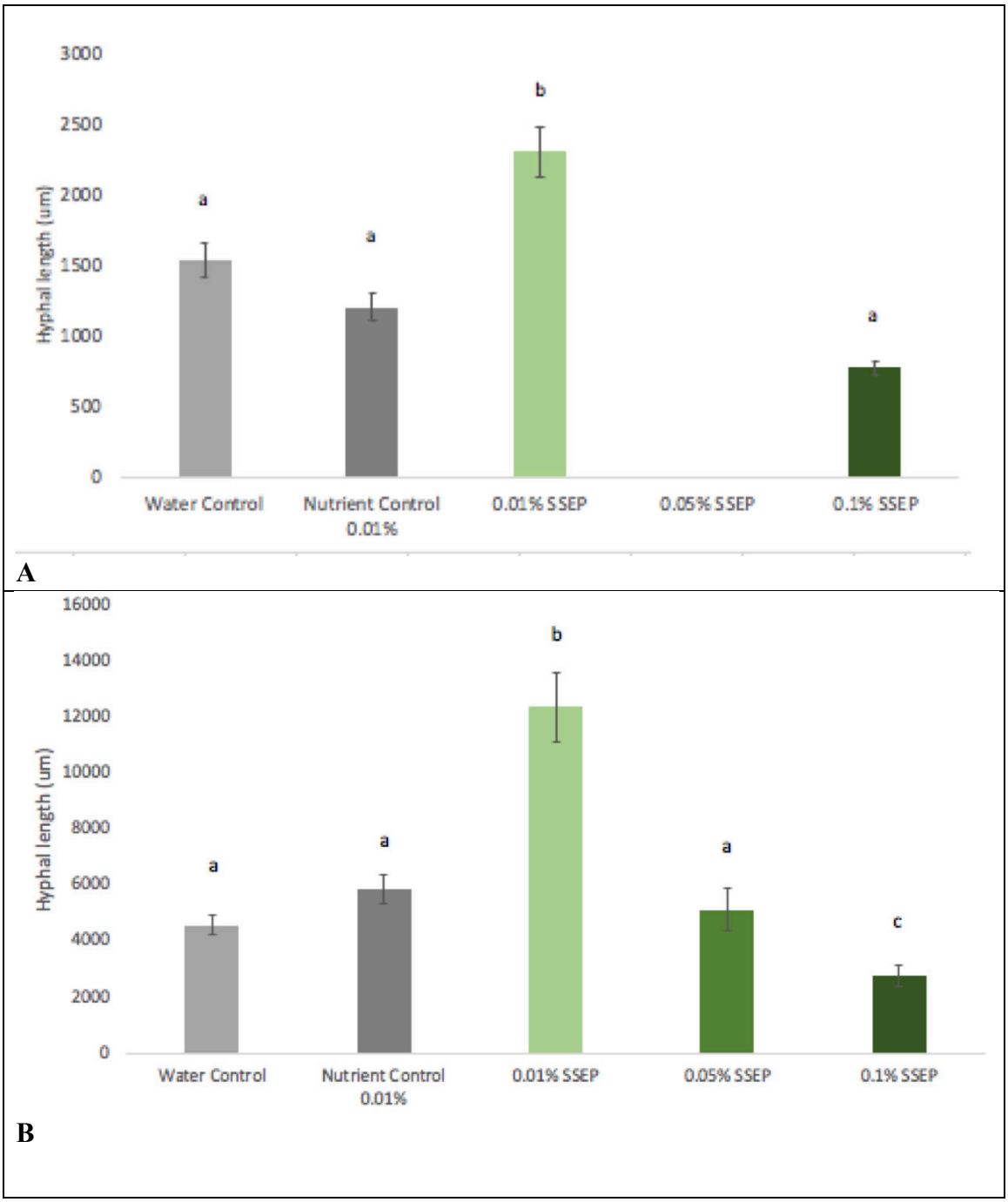

Figure A3: In experiment 1 of the *in vitro* trial the longest hyphae from germinated spores were measured at day 4 (A) and day 14 (B). At both timepoints 0.01% SSEP had significantly longer hyphae and differences among other treatments were not significant. Across time the spores from all treatments grew significantly, showing the success of the *in vitro* system. A one way ANOVA and Tukey's post-hoc was done on each sample date, $\alpha = 0.05$. Each bar represents 6 biological replicates, each Petri dish contained about 80 spores. At least 100 germinated spores per treatment were measured. Data was not available for 0.05% SSEP at day 4.

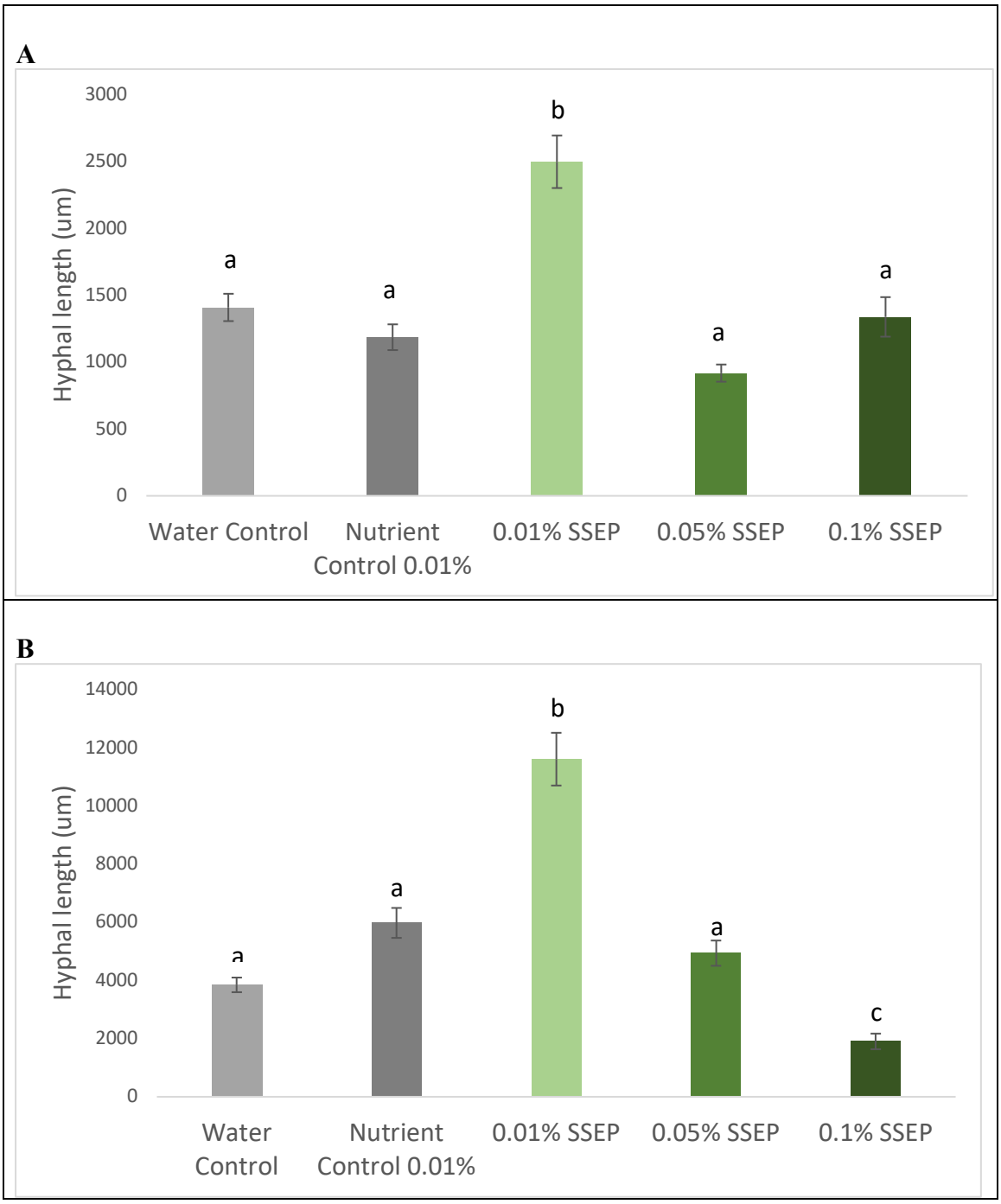

Figure A4: In experiment 2 of the *in vitro* trial hyphal lengths of the longest germinated spores were measured at day 4 (A) and day 14 (B). At both timepoints 0.01% SSEP had significantly longer hyphae. Across time the spores from all treatments grew significantly, showing the success of the *in vitro* system. A one way ANOVA and Tukey's post-hoc was done on each sample date, $\alpha = 0.05$. Each bar represents 6 biological replicates, each Petri Dish contained at least 80 spores. At least 100 germinated spores per treatment were measured.

Appendix B: Results From Replicate 1 and 2 of the Greenhouse Trial

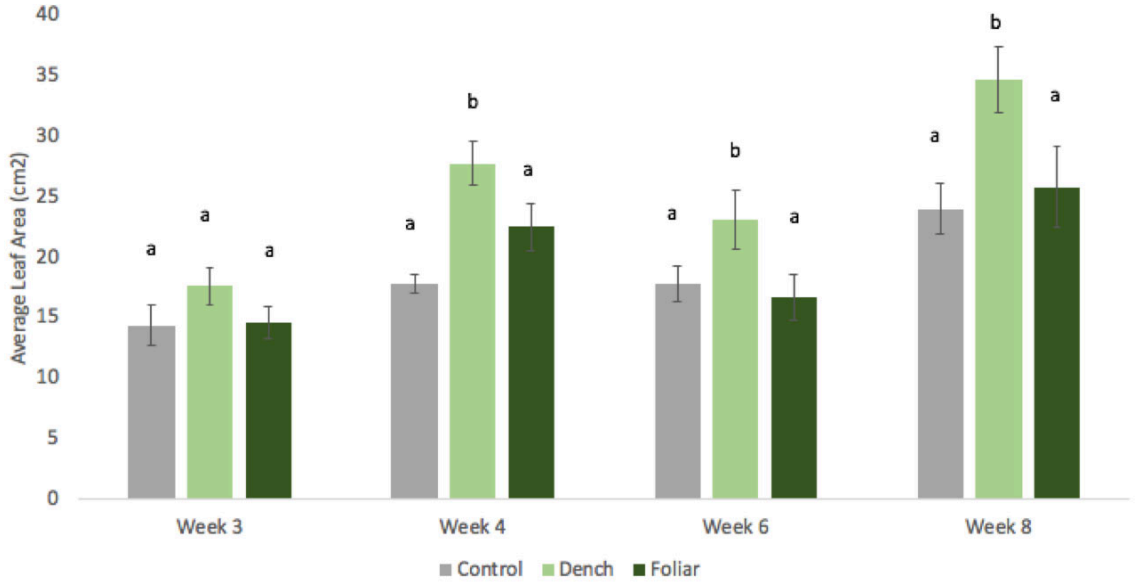

Figure A5: Experiment 2 of the greenhouse trial showed significance between treatments, the drench plants had significantly more leaf area than the controls during weeks 4, 6 and 8. The biological variability between plants is also evident from the large error bars. A one way ANOVA and Tukey's post-hoc was done on each sample date, $\alpha = 0.05$, each bar represents 8 plants.

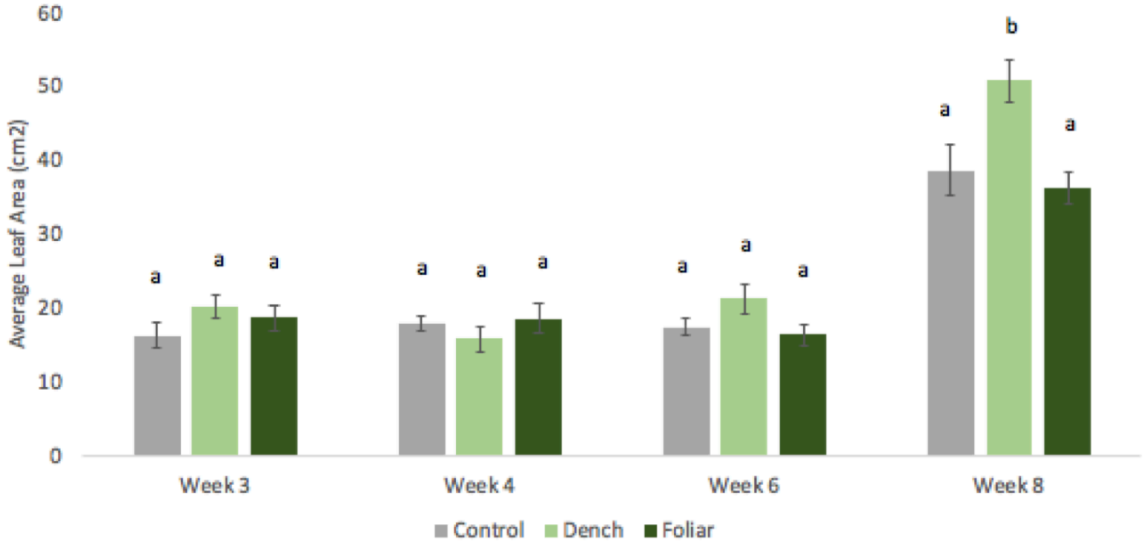

Figure A6: In experiment 3 of the greenhouse trial leaves were slower to grow compared to the two other experiments. Differences among treatments are also not significant until week 8, when the drench plants are larger than the other treatments. A one way ANOVA and Tukey's post-hoc was done on each sample date, $\alpha = 0.05$, each bar represents 8 plants.

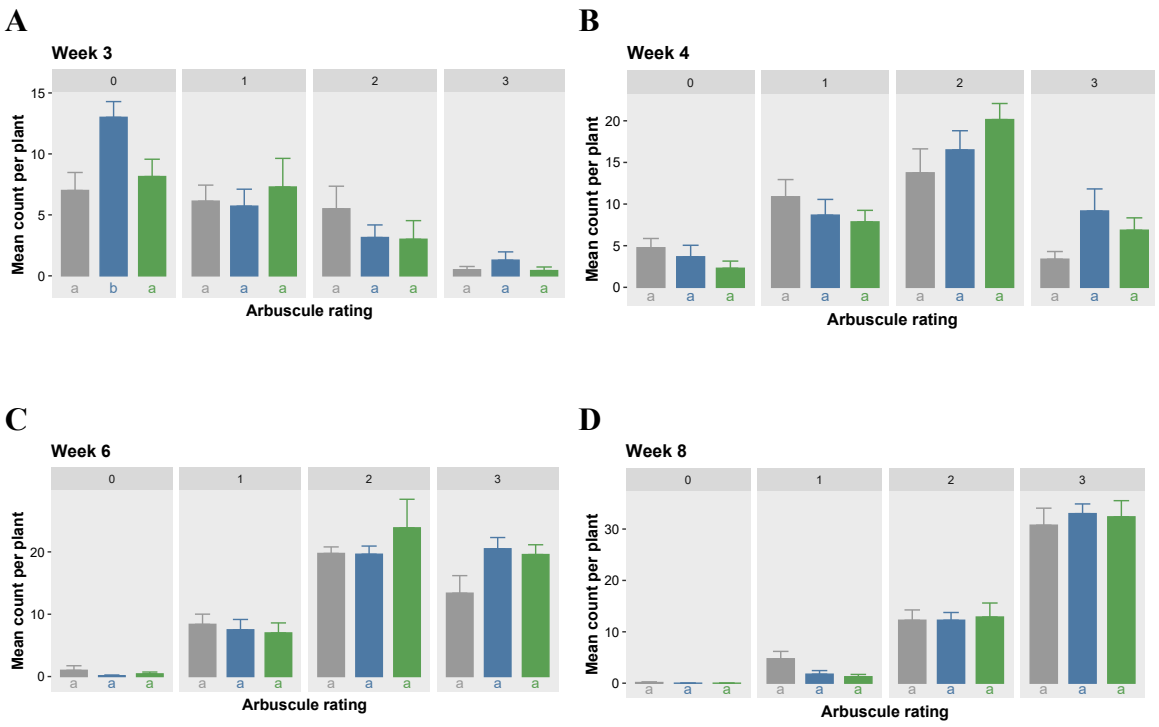

Figure A7: Mean count of the arbuscule ranking data across time and treatment in experiment 2, each bar represents 8 biological replicates. Grey is control, blue is drench, green is foliar ANE treatment. A) In week 3 there are very few incidences of plants ranked as having many arbuscules, but the drench is the most likely to have reached this advanced stage of colonization early on, this trend stays consistent during weeks 4 and 6. B) Although there is no significance there is a trend showing ANE treatments have more plants ranked in the 2 and 3 categories. C) Across time all treatments progress towards having more arbuscules showing the progression of the mycorrhizal colonization process. D) A trend showing high presence of arbuscules continues regardless of treatment. However, the control roots are more likely to receive a rank of 1 than either ANE treatment, indicating less mature arbuscules. A one way ANOVA was conducted on each tested parameter for each timepoint, a Kruskal Wallis post-hoc test was done, significance level was $\alpha=0.05$

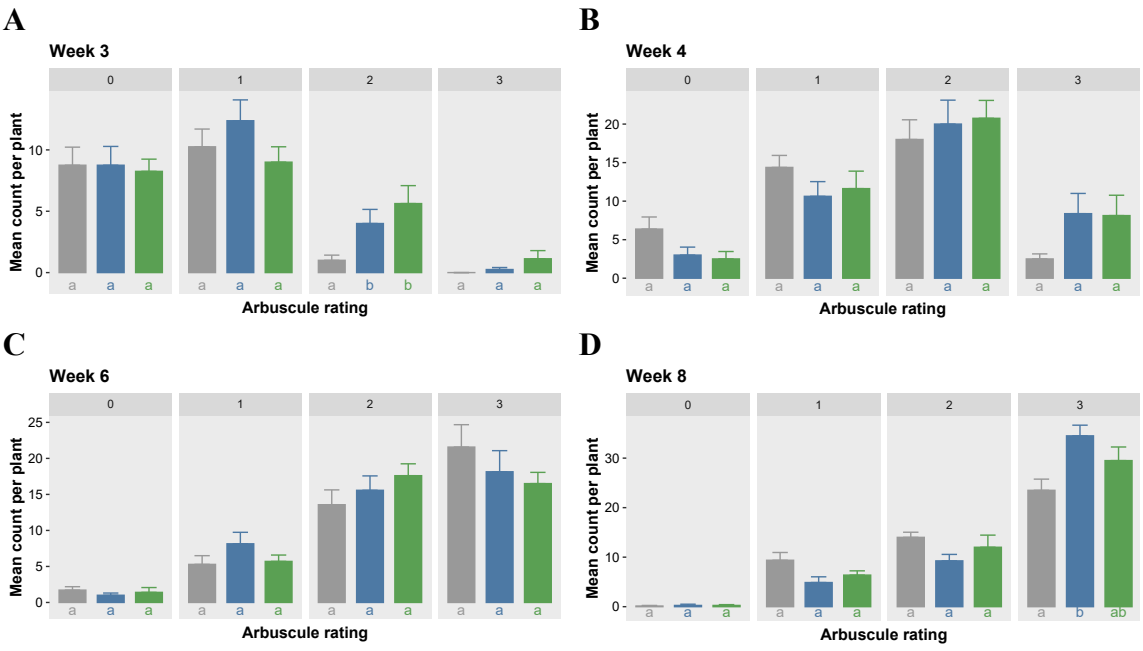

Figure A8: Mean count of the arbuscule ranking across time and treatment in experiment 3, each bar represents 8 biological replicates. Grey is control, blue is drench, green is foliar ANE treatment. A) In week 3 control roots receive a rank of 2 and 3 less often than ANE treatments. B) Most roots are starting to develop more arbuscules, there are no significant differences among treatments. C) There is a trend showing control roots have more well developed arbuscules at week 6 in this experiment, but the differences are not significant. D) There is significance showing drench treated roots have more well developed arbuscules than the control. There is also some level of significance between the control and drench, if the α value was increased to account for the high biological variability there would likely be greater significance in all of these results. A one way ANOVA was conducted on each tested parameter for each timepoint, a Kruskal Wallis post-hoc test was done, significance level was $\alpha=0.05$

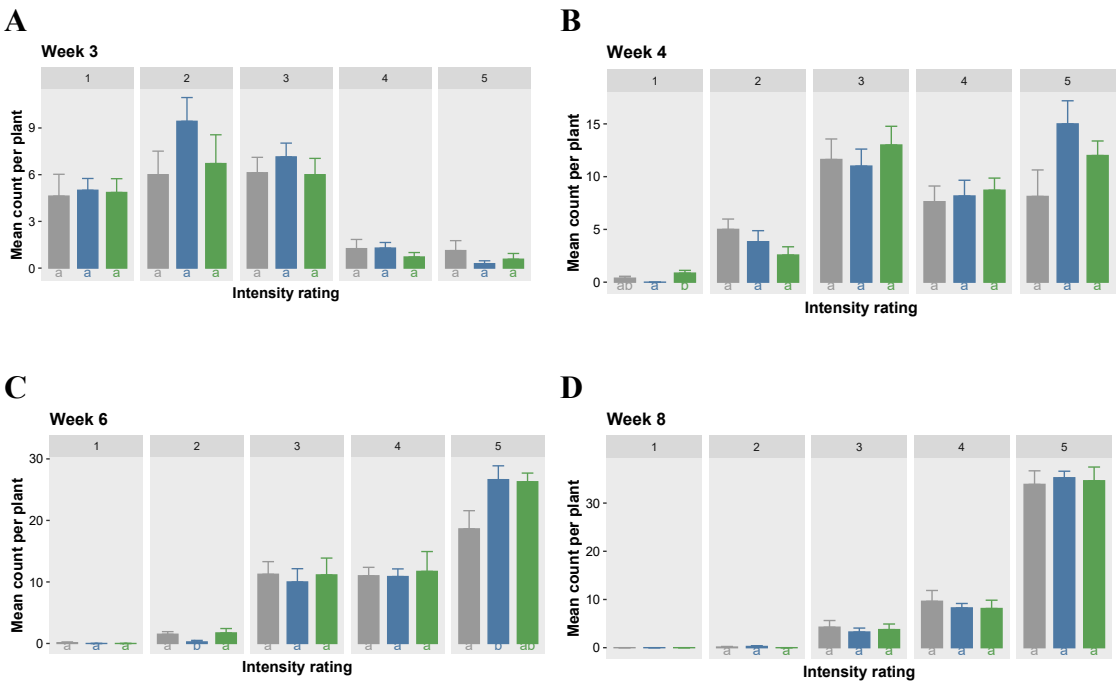

Figure A9: Mean count per plant of the fungal intensity ranking across time and treatment in experiment 2, each bar represents 8 biological replicates. Grey is control, blue is drench, green is foliar ANE treatment. A one way ANOVA was conducted on each tested parameter for each timepoint, a Kruskal Wallis post-hoc test was done, significance level was α=0.05. A) In week 3 there is no statistical significance among treatments and all are performing similarly. B) There are significantly more foliar roots receiving a rank of 1 in week 4, indicating less than 1% colonized by mycorrhizae. Drench roots are not as likely to receive this ranking. C) Most roots are moving towards heavy colonization, there is statistical significance between the drench and control roots showing drench roots are more heavily colonized. D) It appears that by week 8 the differences between treatments have leveled out as more than 30/50 of the observed roots per biological replicate have reached greater than 90% colonization regardless of treatment. No roots received a rank of 1 during week 8 of this experiment.

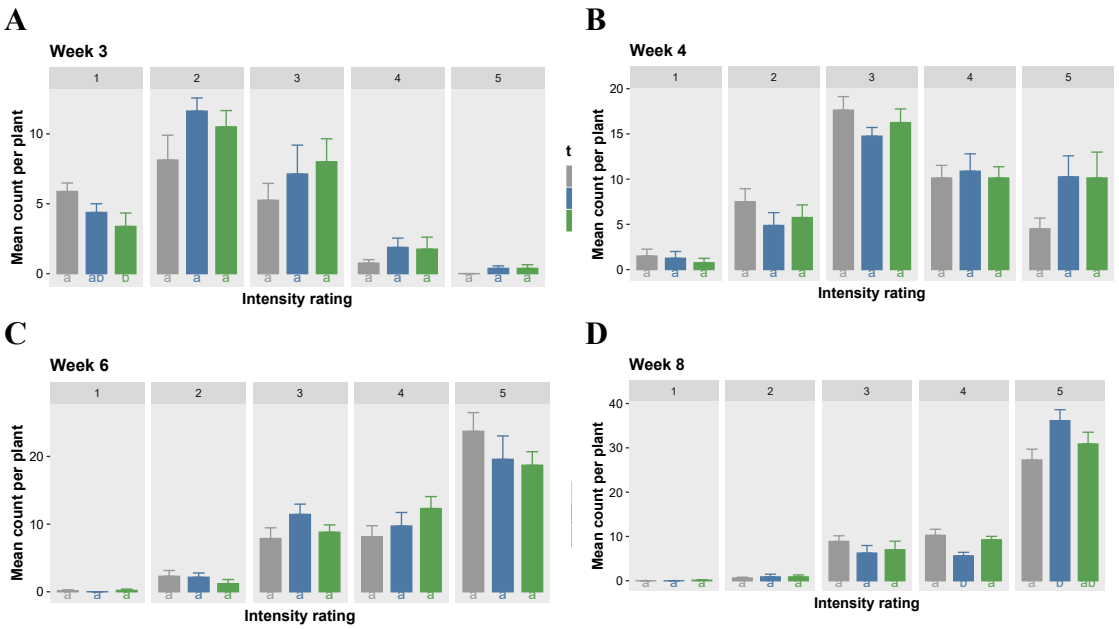

Figure A10: Mean count per plant of the fungal intensity ranking across time and treatment in experiment 3, each bar represents 8 biological replicates. Grey is control, blue is drench, green is foliar ANE treatment. A one way ANOVA was conducted on each tested parameter for each timepoint, a Kruskal Wallis post-hoc test was done, significance level was α=0.05. A) In week 3 control roots receive significantly more rankings of 1 compared to the foliar. The trend holds that ANE treatment result in more rankings of 4 and 5 at early timepoints. B) There is no significance among results, but there is a trend showing ANE increases the roots reaching 90% colonization at week 4. C) In week 6 there are no significant differences, all treatments are improving their levels of colonization compared to previous weeks. D) There are significantly more drench plants reaching 90% colonization as compared to the control during week 8 of this experiment.

Appendix C: Gene Expression Box Plots for Genes Not Included in the Body of the Thesis

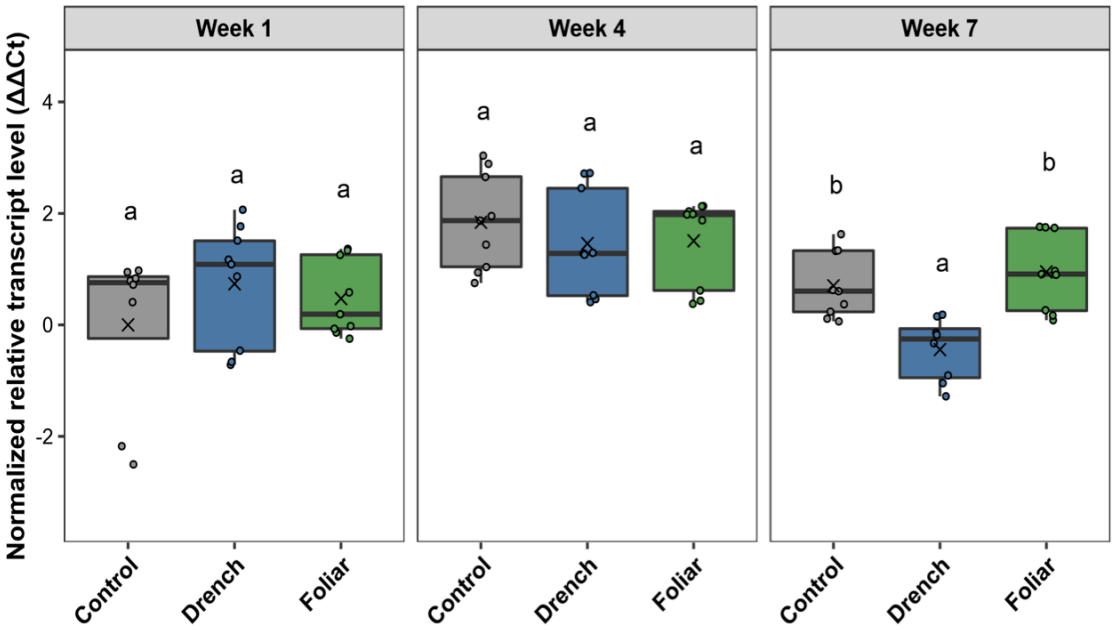

Figure A11: Normalized relative transcript level (ΔΔCt) of CHS1 across weeks of tissue harvest. This gene is involved in flavonoid synthesis and there are no differences between treatments at the early sampling timepoints when it would be expected to be most active.

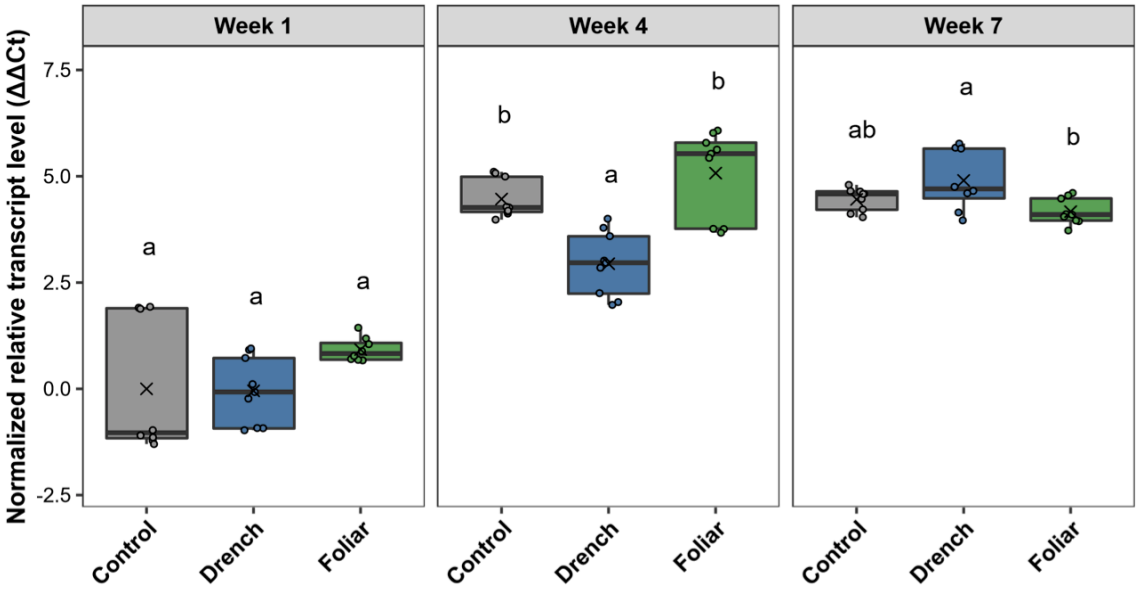

Figure A12: Normalized relative transcript level (ΔΔCt) of D27 across weeks of tissue harvest. D27 is thought to be involved in strigolcatone synthesis. There is no clear pattern of difference between control and treated plants. Based on function differences in activity would be expected during week 1. There is no statistical difference, but it is apparent that the foliar plants have much less variability and are clustered towards higher expression suggesting a possible effect of ANE on D27 production.

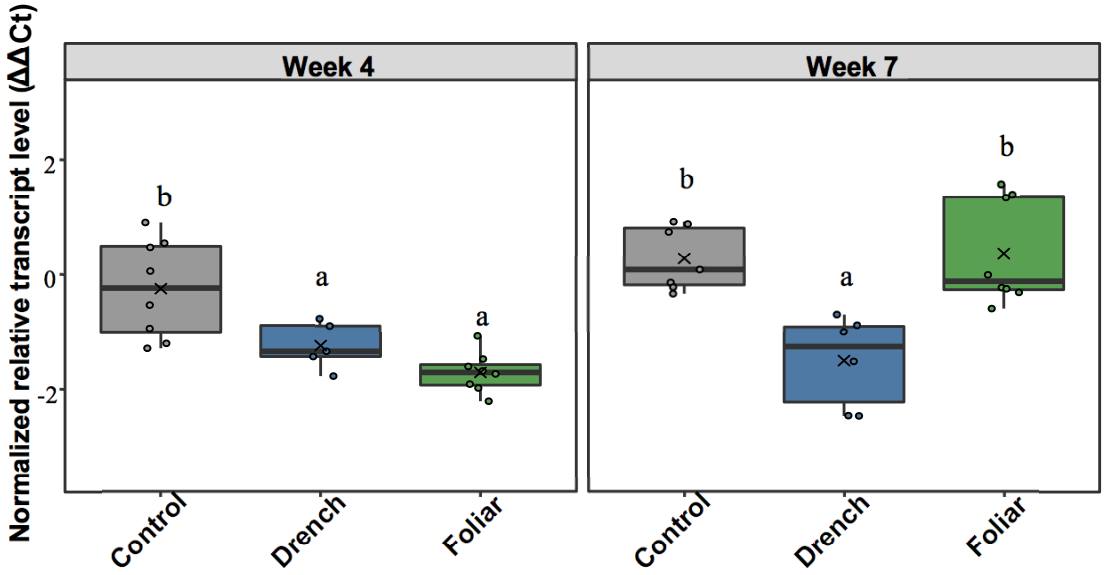

Figure A13: Normalized relative transcript level (ΔΔCt) of RAM1 across weeks of tissue harvest. This gene was not present at detectable levels in week 1. Expression levels of this gene remain low at week 4 and week 7 with only slight differences between treatments.

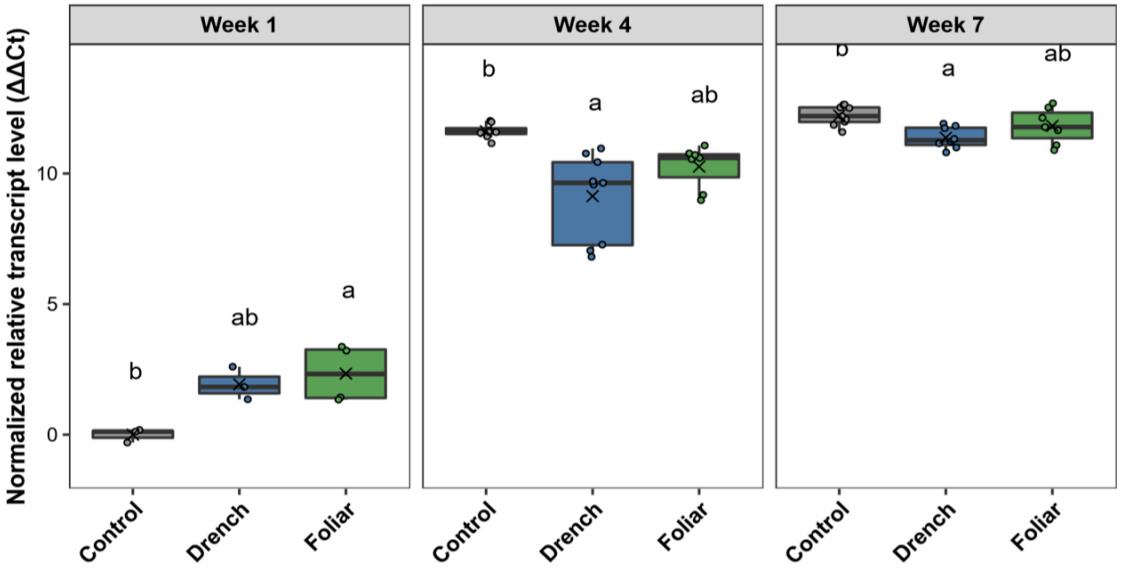

Figure A14: Normalized relative transcript level (ΔΔCt) of RAM2 across weeks of tissue harvest. At week 1 expression is higher in ANE treated plants, which could indicate there is more activity to encourage AMF colonization early on, this would correlate well with phenotypic evidence. By week 4 expression is significantly higher in all three treatments and it remains high through to week 7.

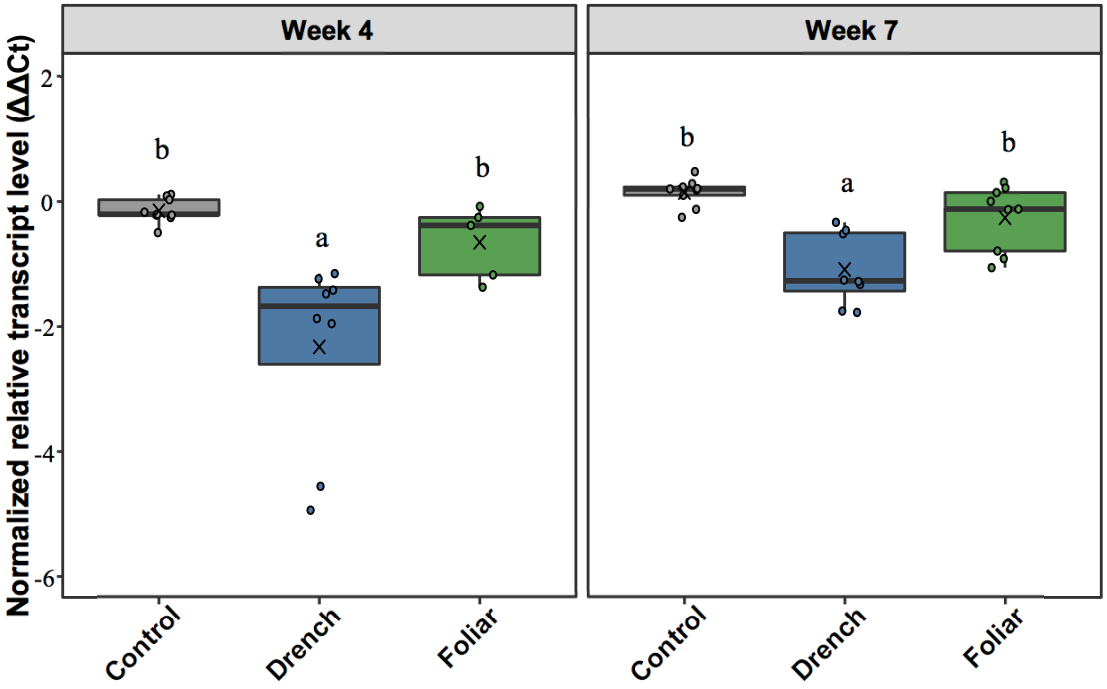

Figure A15: Normalized relative transcript level (ΔΔCt) of RAD1 across weeks of tissue harvest and treatments. RAD1 was not expressed above detectable levels in week 1. RAD1 is a DELLA transcription factor like RAM1 and RAM2, overall these genes do not show clear response patterns to ANE treatment.

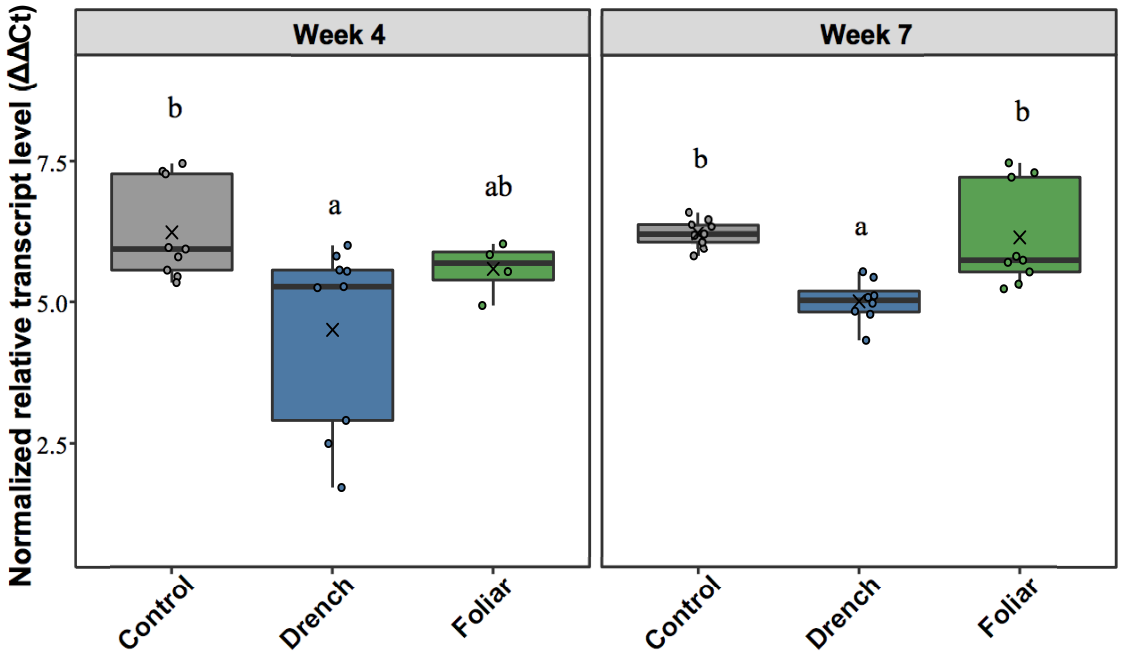

Figure A16: Normalized relative transcript level (ΔΔCt) of STR across weeks of tissue harvest when expression was detectable. STR is involved in lipid transfer across the periarbuscular membrane between the plant and fungi. Expression levels are high and fairly consistent between treatments during week 4 and week 7 giving evidence that lipid transfer was occurring, this correlates well with the increased arbuscule presence seen in the greenhouse trials.

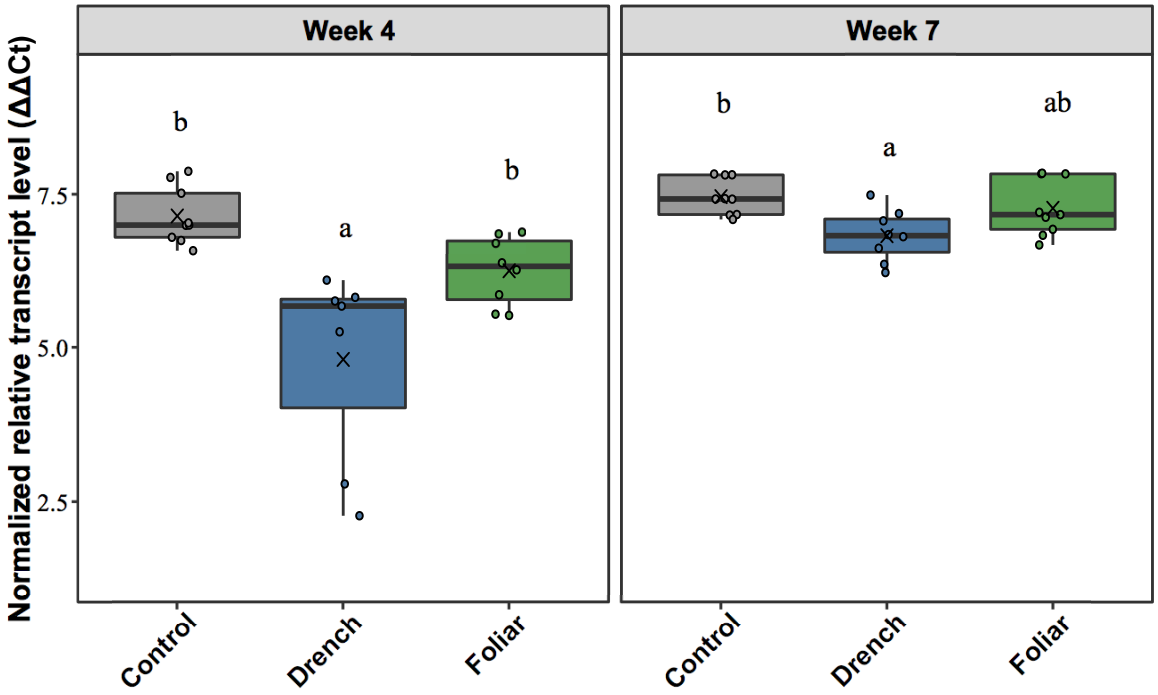

Figure A17: Normalized relative transcript level (ΔΔCt) of STR2 across weeks of tissue harvest.STR2 is a transport protein that helps with nutrient exchange between the plant and fungi. Like STR1, expression is high and fairly consistent between treatments in week 4 and week 7. Expression was not at detectable levels in week 1.

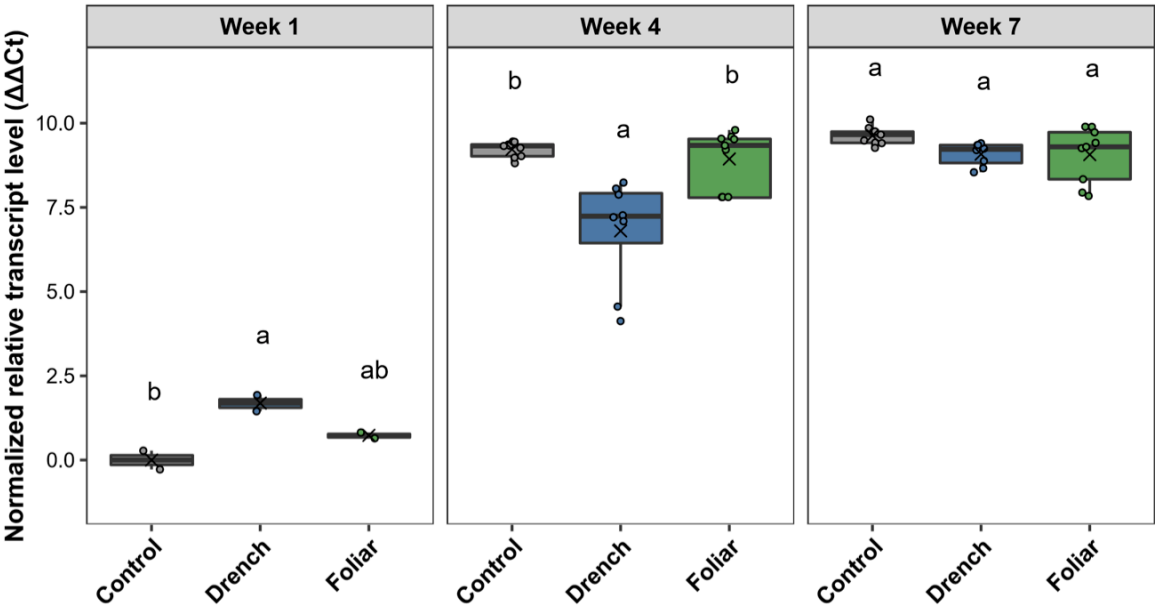

Figure A18: Normalized relative transcript level (ΔΔCt) of FATM across weeks of tissue harvest. In week 3 drench plants have slightly higher expression, in week 4 expression is statistically lower and in week 7 there are no differences. FATM is involved in nutrient transfer, it is interesting to note how expression is up to 1000 fold higher in weeks 4 and 7 compared to week 1.

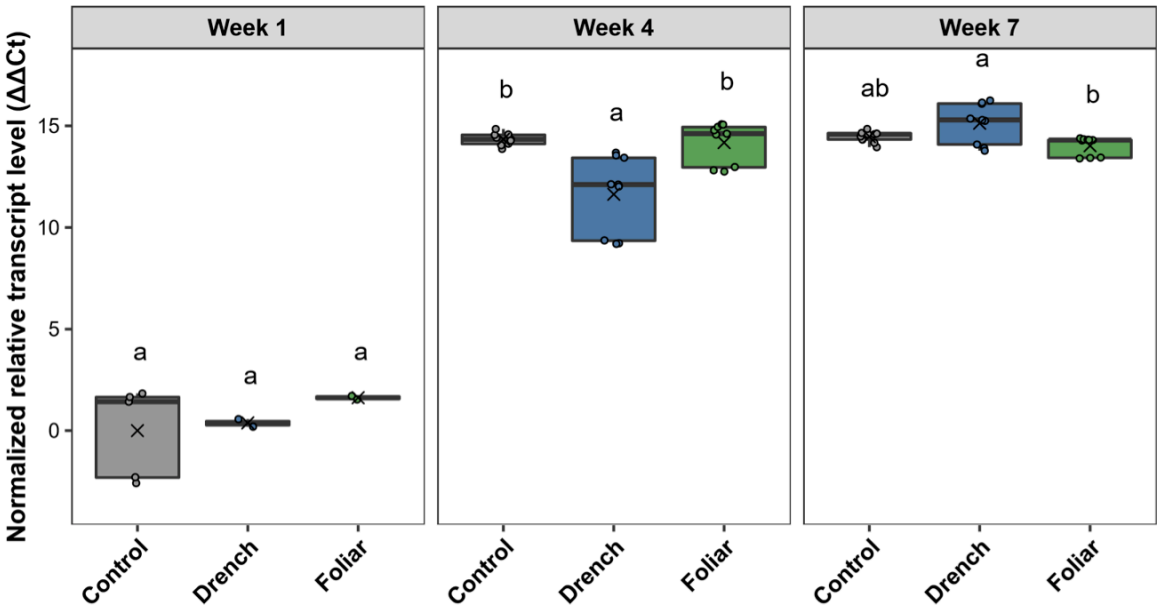

Figure A19: Normalized relative transcript level (ΔΔCt) of PT4 across weeks of tissue harvest. There are no significant differences between treatments, but expression over time increases by significant amounts, up to 32 000 fold higher in weeks 4 and 7 compared to week 1.

Appendix D: Modified LANS recipe used for *in vitro* spore assay controls

Compound	**LANS-M SSEP 0.1x, pH 8 Final conc'n (ppm=mg/L)**
KNO_3	K = 15.6 N = 5.6
$Ca(NO_3)_2$ **$4H_2O$**	**Ca = 3.3** **N = 2.2**
$MgSO_4$ $7H_2O$	Mg = 3.6 S = 4.8
NaH_2PO_4 **H_2O**	P = 4.1 Na = 3.1
Fe-citrate H_2O	Fe = 0.28
$MnSO_4$ **H_2O**	Mn = 0.05
$CuSO_4$ $5H_2O$	Cu = 0.003
$ZnSO_4$ $7H_2O$	Zn = 0.013
H_3BO_3	B = 0.032
Na_2MoO_4 $2H_2O$	Mo = 0.005
NaCl	Cl = 0.36 Na = 0.23
$CoSO_4$ $7H_2O$	Co = 0.0012
KCl	**K = 65** **Cl = 60**
KOH	**K = 65**
MES Bufffer	5mM