Effect of Mycorrhizae on *Artemisia annua*

A Major Qualifying Project Report

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By

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Abstract

Malaria is a life-threatening disease that is prevalent in tropical areas, leaving more than half of the world's population at-risk. Although Artemisinin Combination Therapy (ACT) is recognized by the WHO as an effective malaria treatment, the low yields and difficulty associated with purifying artemisinin, a sesquiterpene lactone with antimalarial properties found only in the plant *Artemisia annua* L., make the cost for malaria therapy too burdensome for people in the affected developing nations. This project expanded upon prior research by exploring the symbiotic effects of mycorrhizae, a root fungus, on *A. annua* in hopes of increasing the artemisinin and flavonoid content in the plant to lower the costs of anti-malarial treatments. To accomplish this, a high producing *A. annua* cultivar, SAM from the Weathers lab, was grown in two types of soil, one seeded with spores of the mycorrhizal fungus, *Glomus intraradices*, to determine whether a further increase in biomass, flavonoids, and artemisinin could occur. Plants grown in Pro-Mix HP Mycorrhizae soil only experienced an increase in root biomass when grown from cuttings and stem biomass when grown from tissue culture. Interestingly, compared to plants grown from rooted cuttings, plants derived from tissue culture and grown in the different test soils produced substantially less artemisinin. Overall, the SAM cultivar did not show a significant increase in flavonoid or artemisinin content, nor did it appear to be colonized by mycorrhizae, while the Glandless cultivar in the Weathers lab was colonized. Ultimately, this research is beneficial in providing insight into the ability of the arbuscular mycorrhizae *G. intraradices* to colonize cultivars at WPI. The differences in biomass, flavonoid, and artemisinin between plants grown from cuttings and tissue culture suggest that there is perhaps a signal or specific mechanism that results in mycorrhizal colonization in the SAM cultivar of *A. annua* that should be further studied.

1. Introduction

Malaria is a life-threatening disease that primarily affects sub-Saharan Africa, Asia, and Latin America, leaving half of the world's population at-risk. The disease is caused by several different species of *Plasmodium* parasites that are passed to humans bitten by infected *Anopheles* mosquitoes. Transmission occurs most frequently at nighttime, when the mosquito lifespan is longer, and often under specific climate conditions. Other factors that favor transmission are a mosquito preference for biting humans instead of animals and low immunity rates among the human population (WHO, 2014).

Malaria is an acute febrile illness, meaning that it is categorized by sudden onset and fever. Symptoms, including chills, vomiting, and headache, typically appear about 10 days after the infectious bite. The most deadly forms of malaria, *P. falciparum* and *P. knowlesi*, can become severe if not treated within the first 24 hours. This may lead to anemia, respiratory issues, and malarial infection of multiple organs in adults and children. The worst type of malaria is cerebral malaria. However, in areas where malaria is endemic, asymptomatic infections also often occur due to partial immunity in the population. For other types of malaria, such as *P. vivax* and *P. ovale*, dormant liver forms of the parasite, known as hypnozoites, may cause reinfection (WHO, 2014). It is not known how activation of the hypnozoites occurs. In order to prevent this relapse, patients infected with these two forms of infection should be treated with a 14-day course of primaquine phosphate (CDC, 2013).

Although spreading awareness, adoption of prevention methods, and medical advancements have reduced global mortality rates by 42% since 2000, malaria remains a major health concern in many parts of the world. In 2012, there were approximately 207 million cases of malaria and 627,000 deaths. Figure 1 shows the global malaria epidemic for 2012. Some areas in South America, Africa, and Southeast Asia reported over 10 million cases of malaria in 2012. Currently in Africa, death rates are the highest among children with one death every minute. In areas where malaria is a concern, other susceptible individuals include low-immunity international travelers from non-endemic areas, pregnant women, and people with the autoimmune condition HIV/AIDS (WHO, 2014).

Despite the fact that malaria is now a preventable disease, 97 countries reported ongoing malaria transmission in 2013 (WHO, 2014). Although a malaria vaccine is not yet available, clinical trials have been run, and several other prophylactic and therapeutic methods exist.

Insecticide-treated bed nets are a moderately successful prevention method due to their low cost; however, only 54% of African households reported owning a net in 2013 (The Henry J. Kaiser Family Foundation, 2014). Of the people who do own bed nets, many use them for other purposes, such as fishing (Minakawa et al., 2008).

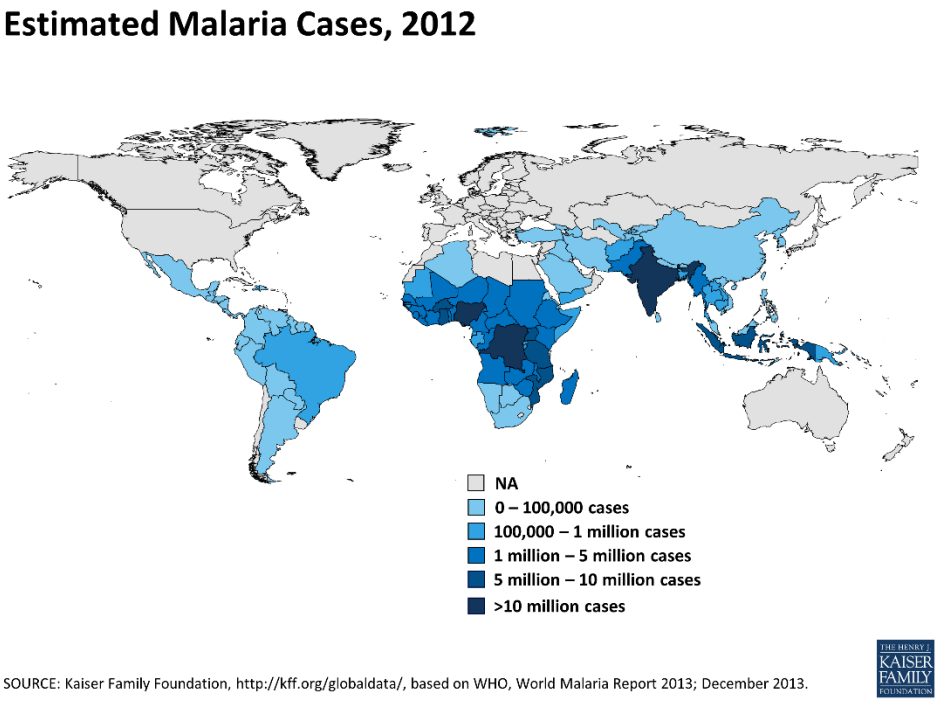

Figure 1: Estimated cases of malaria on a global scale for 2012

Throughout many parts of the world indoor residual spraying is used to prevent malaria, but resistance to insecticides has become an emerging problem. For pregnant women who are especially at-risk for malaria, Intermittent Preventive Treatment in Pregnancy is used as a prevention method. The treatment is a complete course of antimalarial medicine given to pregnant women during routine visits before the birth of their child. Intermittent Preventive Treatment in Pregnancy is used to reduce the risk of malaria in mothers, anemia in mothers and fetuses, parasitaemia of the placenta, low birth weight, and death of the fetus. Although only 38% of African women who attended clinics post-birth received the second dose of the treatment in 2012, it provided this group with increased protection (WHO, 2013).

Although malaria is now a curable disease, over the years drug resistance has built up against older antimalarial medications such as quinine, chloroquine and sulfadoxine-

pyrimethamine. Because nearly all malaria cases occur in developing nations, economic feasibility must be considered when developing new malaria treatments (WHO, 2014).

Artemisia annua (Figure 2), produces a potent antimalarial drug, artemisinin (Figure 3); however, resistance is also emerging when the drug is used as a monotherapy or even now as a two-drug combination therapy. The efficacy of artemisinin-based therapies in treating falciparum malaria specifically has declined on the Thai-Cambodian border (Dondorp et al., 2012). The World Health Organization has developed the Global Plan for Artemisinin Resistance Containment to stop the spread of resistant parasites and continue to eliminate the disease (WHO, 2011). Still, artemisinin-based combination therapy (ACT) is the best available treatment today according to many researchers, especially for severe cases of malaria (WHO, 2014). However, ACT and the cost of artemisinin is often burdensome for people living in developing nations as a consequence of low yields and the cost of other anti-malarial drugs used in combination with artemisinin (Barbacka et al., 2011). The current prevalence of malaria indicates that awareness efforts, financial support, research, education, prevention methods, disease control, and treatment options are not adequate for the most at-risk or affected populations (WHO, 2014).

To develop more effective and economically feasible alternative malaria treatments, the Weathers lab has been studying the plant *Artemisia annua* and its derivatives, such as artemisinin. In 2014, Weathers and colleagues used a mouse malaria model to demonstrate that dried whole plant material was a more effective and less expensive antimalarial therapy (Weathers et al., 2014). Even more recently, the Weathers lab demonstrated that the whole plant material can even be used to treat mice with pure artemisinin-resistant forms of malaria, and that whole plant material is more resilient against the evolution of artemisinin drug resistance (Elfawal et al., 2015). Our project will enhance this research for a more efficient anti-malarial treatment by investigating the potential of symbiotic effects of the mycorrhizal fungus, *Glomus intraradices*, in hopes of further increasing the production of artemisinin and flavonoids by the plant *Artemisia annua.*

2. Background

2.1 Artemisia annua

Artemisia annua L. *(A. annua),* from the family Asteraceae, is a fragrant annual wormwood that originates from China (Figure 2; Abdin et al., 2003). This shrub can achieve heights ≥ 2 m (Dhinga et al., 2004).

Figure 2: *Artemisia annua* (CDC, 2012)

Many of this plant's secondary metabolites are known to be medicinally active and are playing important roles in treatment of diseases, such as malaria and cancer (de Ridder et al., 2008; Firestone and Sundar, 2009). These secondary metabolites are synthesized in and isolated from different parts of the plant. The isolated secondary metabolites from *A. annua* consist of monoterpenoids, sesquiterpenoids, triterpenoids, flavonoids, coumarins, steroids, phenolics, purines, lipids and aliphatic compounds (Bhakuni et al., 2001). Of interest to this project are flavonoids and the sesquiterpene lactone, artemisinin, a potent anti-malarial drug (Figure 3; Collaboration research group for Qinghaosu, 1979).

Flavonoids, which are classified as polyphenols, are located in many tissues of plants, including the glandular trichomes of *A. annua* (Ferreira, 2010; Kuman and Pandey, 2013). These phytochemicals are made especially under stressful conditions when they are produced in a defense response (Ferreira, 2010). As many as nine flavonoids that are produced by *A. annua* have exhibited therapeutic applications for malaria (Weathers and Towler, 2014). As an example, the flavonoid quercetin, produced by *A. annua*, is shown in Figure 3. Part of the role of flavonoids as antioxidants involves their ability to chelate metals such as iron (Mira et al., 2002). One of the proposed mechanisms of artemisinin requires the oxidation of iron; therefore, it is

believed that by chelating iron, flavonoids could increase availability of iron and thus the efficacy of artemisinin as a treatment for malaria (Ferreira, 2010).

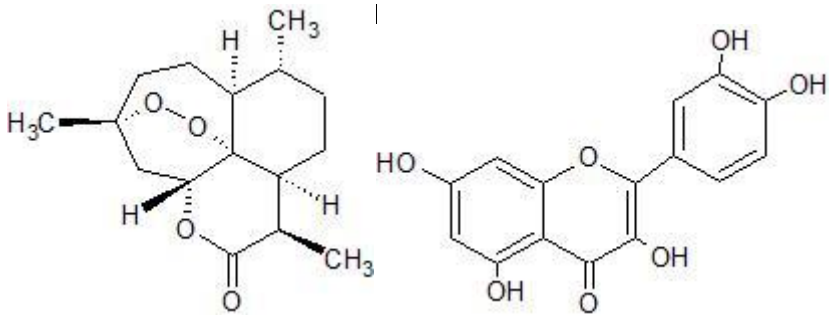

Figure 3: Chemical structure of sesquiterpene artemisinin (left) and quercetin flavonoid (right)

Trichomes are appendages located on the aerial surface of plants including the leaves, floral buds, and flowers (Oloffson et al., 2011). They vary in shape, size, and other characteristics, including whether they are glandular or nonglandular (Nguyen et al., 2011). Glandular trichomes (Figure 4) play a role in chemical defense by producing phenolics, alkaloids, terpenes, and other noxious compounds that are triggered for release when herbivore or insect damage occurs (Cheng et al., 2007). Dicotyledonous angiosperms commonly possess glandular trichomes. For example, the genera *Nicotiana*, *Cleome*, *Primula, Petunia,* and *Artemisia* all possess glandular trichomes that secrete defensive compounds (Oloffson et al., 2011).

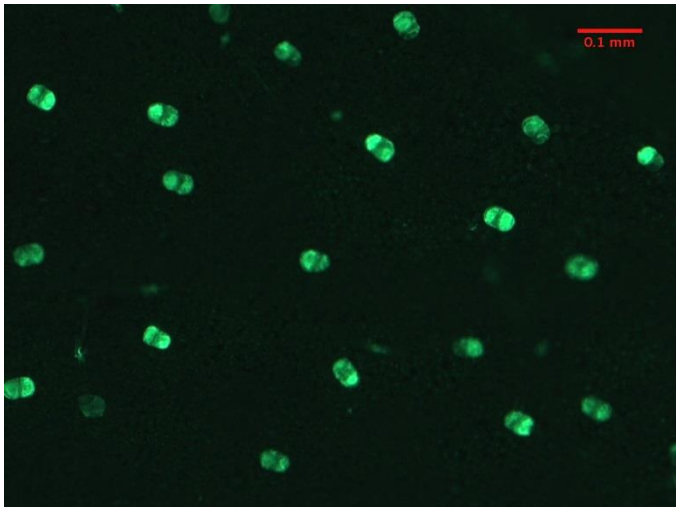

Figure 4: Glandular trichomes in cultivar #15 (obtained from: Sibo Wang)

Specifically, the glandular secretory trichomes are located mainly on the leaves and flowers, of *A. annua* (Ferreira and Janick, 1996). Artemisinin is made and stored in the glandular trichomes of *A. annua*, so these structures are important for artemisinin biosynthesis (Duke et al., 1994; Ferreira and Janick, 1996). The pathway showing the biosynthesis of artemisinin and its main derivatives is shown in Figure 5.

IPP/DMAPP isoprene units are produced in the cytosol and plastid and condensed in the cytosol forming farnesyl diphosphate (FPP). FPP is cyclized via amorphadiene synthases (ADS) to amorphadiene, and this is the first committed step in artemisinin biosynthesis. A cytochrome P 450/reductase (CYP) complex then converts amorphadiene to artemisinic alcohol that is then converted to artemisinic aldehyde again by CYP. From there the pathway branches, and one route forms artemisinic acid through the enzymatic activity of CYP and aldehyde dehydrogenase 1 (ALDH1), which then results in arteannuin B through a nonenzymatic photo-oxidative reaction. The other route involves conversion of artemisinic aldehyde to dihydroartemisinic aldehyde by the enzyme double bond reductase 2 (DBR2). At this step the pathway branches again, with one route leading to the synthesis of dihydroartemisinic alcohol by the enzyme dihydroartemisinic aldehyde reductase 1 (RED1). In the second route, aldehyde dehydrogenase 1 (ALDH1) converts dihydroartemisinic aldehyde to dihydroartemisinic acid. From dihydroartemisinic acid, either artemisinin or deoxyartemisinin is synthesized (Nguyen et al., 2011). Artemisinin is also the result of a nonenzymatic photo-oxidative reaction.

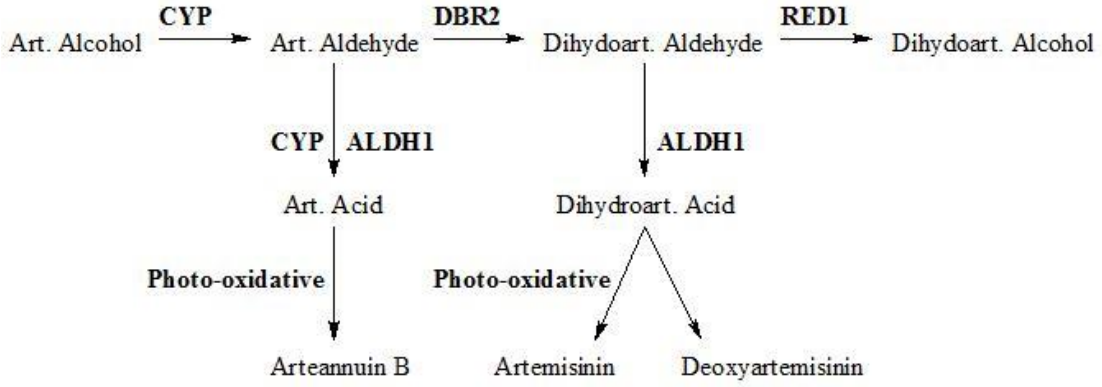

Figure 5: Biosynthetic pathway of artemisinin (adapted from Nguyen et al., 2011)

Several studies showed that artemisinin concentration increases with an increasing number of trichomes, with younger leaves developing more trichomes, and thus more artemisinin (Ferreira and Janick, 1996; Kapoor et al., 2007; Arsenault et al., 2010; Graham et al., 2010).

In many plant species, it is common for sesquiterpene content to be higher in younger plants than older plants (Rapparini et al., 2007). Artemisinin levels seem to be highest right before full flowering when the floral bud is formed, but the best cultivars only have a yield of ~1.4% (Ferreira and Janick, 1996; Elfawal et al., 2015). Currently, the large production costs and low artemisinin yields have hindered the process of plant Artemisinin Combination Therapy (pACT) becoming a commercialized treatment for malaria (Weathers et al., 2014). Over the past several years, the Weathers team demonstrated that whole plant material is less expensive and more effective at treating malaria in mice, especially those with artemisinin-resistant forms of the disease (Elfawal et al., 2015). It is also of interest to this project to explore a feasible, effective, and inexpensive way in which production of artemisinin and other secondary metabolites can be increased in *A. annua*.

2.2 Mycorrhizae

Plant roots interact with various rhizospheric microorganisms in soil, including mycorrhizal fungi, which are able to inhabit the root cortical cells and induce a symbiotic relationship with the plant (Bagyaraj, 1991; Harrison, 2005; Rapparini et al., 2007). This symbiotic relationship constitutes a progression of complex feedbacks that are controlled by the physiology and nutrition of the plant and the fungus (Rapparini et al., 2007). When a plant becomes infected with mycorrhizae, it is able to acquire phosphorus, nitrogen, and other nutrients more efficiently from the soil (Read, 1999). The fungus also benefits from the interaction, as it obtains photosynthetically-derived carbon compounds from the infected plant (Harley and Smith, 1983). Without the carbon supply from the host plant, most mycorrhizae would not be able to form or function (Jakobsen, 1999). Often the symbiotic relationship is so well balanced that no tissue damage occurs, and plant growth, along with other characteristics, is enhanced. In recent years, interest in this symbiosis has increased dramatically due to the benefits that mycorrhizae have on the growth and resilience of the host plant. Several different types of the fungus exist: ecto, arbutoid, monotropoid, ericoid, orchidaceous, and vesicular-arbuscular (Table 1). This project focuses on vesicular-arbuscular mycorrhizae, also known as arbuscular mycorrhizae, which colonize the majority of plants that are important in agriculture, horticulture, and tropical forests (Bagyaraj, 1991).

Table 1: Different types of mycorrhizae, category, and defining structures (adapted from Read, 1999)

Type	Category	Defining Structures
Ectomycorrhiza	Sheathing	External mycelial network
Arbutoid	Sheathing	Hartig net, intracellular penetration
Monotropoid	Sheathing	Fungal pegs
Ericoid	Endo	Hyphal complexes in hair roots
Orchidaceous	Endo	Pelotons
Vesicular-Arbuscular	Endo	Arbuscules, vesicles, hyphal coils

Arbuscular mycorrhizae (AM) contain arbuscules within cortical cells, vesicles within or between cortical cells, and hyphal coils (Read, 1999; Harley and Smith, 1983). Although it is not usually possible to detect AM infection without microscopic observation, some plant roots turn yellow upon infection (Harley and Smith, 1983). Over 120 species of AM exist and are known to establish symbiotic relationships with over 1,000 genera of plants including >80% of angiosperms (Harley and Smith, 1983; Harley and Harley, 1987; Newman and Reddell, 1987; Smith and Read, 1997). This broad host range is not yet fully understood, but it suggests that AM do not have very specific nutritional requirements. However, it is known that AM fungi are completely dependent on the host plant for a source of carbon so growth is significantly diminished in the absence of a host plant. When not in contact with a plant, AM fungi are present in soil as dormant spores (Harrison, 2005). Once the spore germinates, the hyphal germ tube grows through the soil searching for a host root, contacts a plant root, and establishes an appressorium, or hyphal organ with an infection appendage, on the surface of the root through which the fungi is able to enter the plant. Despite the fact that AM can colonize 90% of vascular plants, the degree to which they affect plant species within a single family or even different cultivars of the same species may vary greatly (Harley and Smith, 1983). AM most commonly infect the root cortical cells, and a typical root system is pictured in Figure 6.

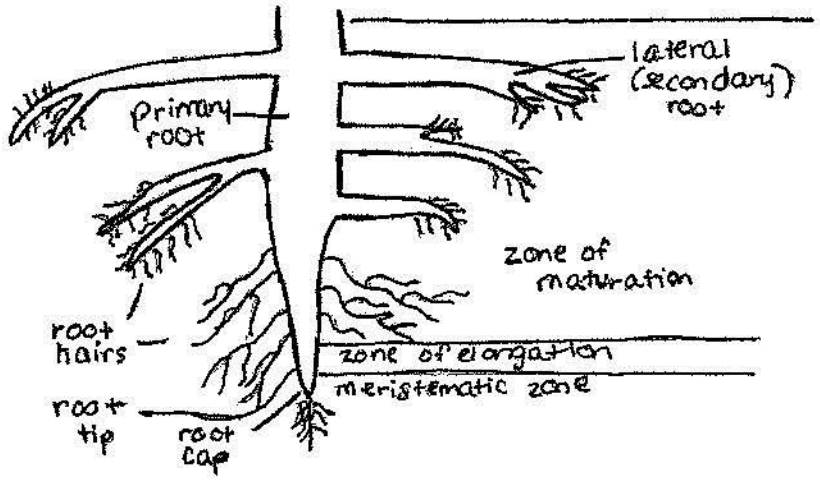

Figure 6: Typical root system in plants (adapted from http://diggingdazza.files.wordpress.com/2011/04/rootstructure.gif)

By 1987, all species of AM described were classified into the Endogonaceae family, and classified into six different genera that form a mycorrhizal association: *Glomus, Sclerocystis, Entrophospora, Acaulospora, Scutellospora,* and *Gigaspora*. The most common genus of AM fungi, *Glomus,* has over 50 species, which form irregularly-shaped spores that vary greatly in size. The spores are produced near the plant roots in the soil, at the surface of the soil, or even in the roots themselves (Bagyaraj, 1991).

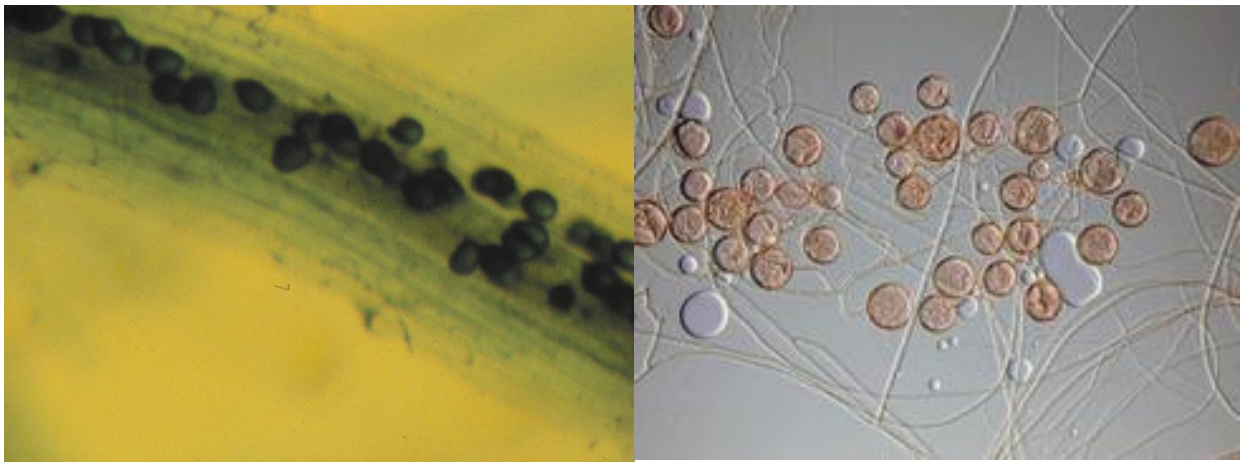

Figure 7: Arbuscular mycorrhizae infection of clover plant vesicles, stained with Trypan blue - left (Ingham, n.d.), *Glomus intraradices* - right (https://biomesfirst09.wikispaces.com/file/view/glomus_intraradices.jpg/104191029/240x180/glomus_intraradices.jpg).

2.3 Mycorrhizal effects on secondary metabolites in plants

Araim et al. (2009) studied the effect of AM colonization by *Glomus intraradices* on *Echinacea purpurea* L. After 13 weeks of growth, the plants that had been infected with AM reached almost 100% root colonization determined by the presence of one fungal structure in each root stained with aniline blue dye. The colonized plants had greater root and shoot mass as well as length. In addition, some minerals and proteins increased in AM-colonized roots. Secondary metabolites produced by *E. purpurea*, specifically phenolics, also increased as a result of the AM treatment (Araim et al., 2009).

The effect of *G. intraradices* on the primary and secondary metabolites produced by *Medicago truncatula* was examined throughout 8 weeks of mycorrhizal association (Schliemann et al., 2007). The roots with AM infection had higher levels of primary metabolites, amino acids and fatty acids after about 4 weeks of AM colonization. Meanwhile for secondary metabolites, the mycorrhizal-infected roots only exhibited increased levels of isoflavonoids at the late stages (8 weeks) of root mycorrhizal infection.

The effect of AM infection by *G. intraradices* on the flavonoid production of *Trifolum repens* (white clover) was identified using methods such as nuclear magnetic resonance (NMR) spectroscopy (Ponce et al., 2004). Several previously known flavones, two newly reported flavones in the roots, and two newly reported flavones in the shoots were isolated from AM-inoculated and non-inoculated plants. The flavonoids quercetin, acacetin, and rhamnetin were detected only in the roots of clover plants infected with AM. That study indicated that AM colonization of roots played a role in the production of secondary metabolites, specifically flavonoids, in the shoots and roots of white clover plants (Ponce et al., 2004). Their research supported the hypothesis of this project: AM colonization of plant roots, and in our case roots of *A. annua,* would increase production of the plant's secondary metabolites.

2.4 Mycorrhizal effects on artemisinin in A. annua

Several studies have been performed to observe the effects of AM mainly on the production of artemisinin in *A. annua*. Those studies showed that *A. annua* was infected by *Glomus* species of AM and that the infection increased the concentration of artemisinin along with other terpenes (Rapparini et al., 2007). However, to our knowledge there are no published findings about the effect of AM on flavonoid concentration in *A. annua.*

In a study by Rapparini et al. (2007), *A. annua* plant roots were colonized by AM fungi and associated bacteria in order to examine the effects on volatile organic compound emissions and content of the plant. Plant roots were infected with two different inocula: one containing a single *Glomus* AM species isolated from a peach orchard and the second containing a mixture of *Glomus* species (*G. mosseae*, *G. intraradices*, and *G. viscosum*) plus associated soil bacteria. These AM-infected plants were also compared to non-mycorrhizal plants ± additional phosphorus, which stimulates plant growth. Compared to the single species infection, plants infected with several different species of AM and bacteria showed significant increases in shoot length, leaf number, leaf biomass, stem dry weight, and root dry weight. The emission of two monoterpenes, limonene and artemisia ketone, also generally increased after mycorrhizal infection.

Kapoor et al. (2007) investigated the effects of AM fungal inoculation ± phosphorus fertilization on artemisinin concentration and glandular trichome density in *A. annua* L. plants. The AM fungal inocula consisted of either *Glomus macrocarpum* (GM) or *Glomus fasiculatum* (GF). There were six treatments: control (no mycorrhizae), GM ± P-fertilizer, GF ± P, and soil + P. Overall, plants inoculated with *G. fasiculatum* performed better than those inoculated with *G. macrocarpum* or plants provided only with phosphorus fertilization. Phosphorus addition increased the fresh and dry weight of the shoots, but decreased the percent AM colonization in the roots and did not affect the glandular trichome density. Plants colonized by *G. fasiculatum* had the highest artemisinin concentration and were unaffected by phosphorus fertilization.

Chaudhary et al. (2008) studied the effect of two different species of arbuscular mycorrhizal fungi (AMF), *G. macrocarpum* (GM) and *G. fasiculatum* (GF), on *A. annua*. Seedlings that were 35 days old were transplanted into soil with indigenous AMF spores and chopped, AM-colonized sorghum roots of each respective fungus. The plants were harvested after being grown for 12 weeks under natural field conditions. In addition to an increase in dry weight and nutrient content in the shoots, the concentration of artemisinin in the leaves increased as a result of infection by both fungal species. However, *A. annua* colonized with GM produced more artemisinin than plants infected with GF when compared to the controls. The experiment also used three different accessions or cultivars of *A. annua* and found that they varied in response to the two different *Glomus* species. This supported other studies (Graham and

Eissenstat, 1994; Sylvia et al., 2003), which observed that the effect of mycorrhizal colonization varied significantly between plant species and varieties.

In a study by Mandal et al. (2014) the mechanism behind the increase in secondary metabolites in AM colonized *A. annua* plants was explored. Using seedlings that were 30 days old of a high artemisinin yielding (1.3%) variety of *A. annua*, plants were grown in autoclaved soil with a soil-based culture containing roots of trap plants colonized by *Rhizophagus intraradices* (newer name for *G. intraradices*) as well as spores for 33 days (Yadav et al., 2013). When compared to the control, Mandal found that dry weight artemisinin concentrations were greater in the plants colonized by the fungus. The study suggested that this increase in artemisinin was due to an increase in glandular trichome density and increased jasmonic acid levels that controlled transcription patterns (Mandal et al., 2014). Jasmonic acid along with its methyl ester, methyl jasmonate, are naturally occurring phytohormones and growth regulators that are particularly responsive during stress from biotic and abiotic factors (Creelman and Mullet, 1995). Other studies show that jasmonic acid, besides increasing the production of secondary metabolites, is able to help establish the symbiotic relationship between an AM fungus and a plant (Hause et al., 2002; Vierheilig & Piche, 2002; Meixner et al., 2005; Stumpe et al., 2005; Landgraf et al., 2012). Mandal and colleagues showed that leaves of mycorrhizal plants, which had elevated jasmonic acid levels, and leaves of non-mycorrhizal plants, that were supplied with various methyl jasmonic acid concentrations, had similar concentrations of artemisinin, trichome densities, and transcriptional activation of genes for artemisinin synthesis (Mandal et al., 2014). This study also determined that arbuscular mycorrhizae increased isoprenoids by stimulation of the methyl erythritol phosphate (MEP) pathway.

In addition to those published studies, a preliminary experiment in the Weathers lab used cuttings of a high-producing cultivar (SAM) and a lower-producing cultivar (#15) of *A. annua* that were planted in two different soil types - one that contained spores of *G. intraradices* and one that, to our knowledge, did not. Upon inspection of the roots, plants grown in soil allegedly containing spores of *G. intraradices* did not appear to be colonized by the fungus, while plants grown in soil supposedly without *G. intraradices* present did show characteristics of AM colonization. Therefore, it appeared that the plants grown in soil without fungal spores might already be infected with AM. It was unclear why these results were observed, and the research carried out through this project duplicated this experiment and studied other differences in plant

characteristics besides root colonization by AM. Overall, this project aimed to further the research that had already been carried out involving the colonization of *A. annua* by AM, specifically *G. intraradices*, in hopes of increasing the concentration of artemisinin and other secondary metabolites, flavonoids in particular.

3. Hypotheses and Objectives

The goal of this project was to determine whether high producing *Artemisia annua* cultivars, such as SAM in the Weathers lab, could experience a further increase in artemisinin and flavonoid production when inoculated with AM. It was hypothesized that:

- Plants grown in Pro-Mix HP Mycorrhizae soil would have greater biomass, flavonoid, and artemisinin content than plants grown in MetroMix 360 standard potting soil.
- Plants grown in autoclaved soil would not experience mycorrhizal infection.

These hypotheses were tested through the completion of the following objectives on all experimental groups:

- To measure the differences in biomass between plants grown in Pro-Mix HP Mycorrhizae soil and plants grown in MetroMix 360 standard potting soil and their autoclaved controls.
- To extract and measure total flavonoids from plant material.
- To extract and measure artemisinin content from plant material.
- To determine extent of root colonization by mycorrhizae.

4. Materials and Methods

4.1 Propagation of Artemisia annua by cuttings

The *Artemisia annua* L. cultivar used was SAM, which produces about 1.4% AN (Weathers & Towler, 2012; Weathers & Towler, 2014). The cultivar was isolated at Worcester Polytechnic Institute in the Weathers laboratory. Initially, propagation was performed by planting nodal cuttings of SAM in four soil types: MetroMix 360 (Sungro Horticulture Canada Ltd.) potting mix (see Appendix A for composition); autoclaved MetroMix 360; Pro-Mix HP Mycorrhizae (Premier Tech Horticulture) growing medium, which contains spores of *Glomus intraradices* (see Appendix B for composition); and autoclaved Pro-Mix HP Mycorrhizae. Table 2 summarizes the different experimental groups of plants grown from cuttings or tissue culture, along with their abbreviations. There were 4-8 (n) replicates in each experimental group.

Table 2: Experimental groups

SAM Cultivar Abbreviation	MetroMix 360 Soil	Pro-Mix HP Mycorrhizae Soil	Auto-claved	Cutting	Tissue Culture
S (n=8)	+	-	-	+	-
SA (n=7)	+	-	+	+	-
SM (n=8)	-	+	-	+	-
SMA (n=5)	-	+	+	+	-
ST (n=8)	+	-	-	-	+
SAT (n=4)	+	-	+	-	+
SMT (n=7)	-	+	-	-	+
SMAT (n=5)	-	+	+	-	+

Before planting, cuttings were treated with Hormodin 2 root-inducing hormone containing 0.3% indole-3-butyric acid from OHP, Inc. (Mainland, PA). Cuttings were grown in glass-filtered natural sunlight under a vegetative photoperiod (>13 hour light) at a temperature maximum of 25.1 °C and minimum of 15.6 °C and a humidity maximum of 76% and minimum of 26% until they achieved ~20 cm height with at least 16 fully expanded leaves. Plants were watered at least every other day and were fertilized once per week. The harvested plants were then used for data analysis of biomass, secondary metabolite concentrations, and root colonization.

4.2 Artemisia annua tissue culture

Nodal cuttings from *in vitro* cultivated SAM *A. annua* plants were sub-cultured into 50 mL of medium containing 20 g/L sucrose (PhytoTechnology Laboratories Lot #11B0391133A), 2.215 g/L Murashige and Skoog medium salts at full strength (PhytoTechnology Laboratories Lot #12E0519061B), and 5 g/L Agargellan (PhytoTechnology Laboratories Lot #11C0133030A) in magenta boxes (see Appendix C). After 6 weeks of growth, rooted plantlets were transplanted into pots containing all four types of soil and were grown under the same conditions as the plants grown from cuttings. See Table 2 for a summary of the four experimental groups of plants grown from tissue culture, along with their abbreviations.

When transplanting, the plants were grouped into three different developmental stages and divided evenly amongst soil types. During initial growth in soil, plants were placed in a clear plastic bag to minimize evaporation. Plants were harvested when they achieved the height and leaf number previously described. After harvesting, the amounts of artemisinin, flavonoids, and biomass were extracted and measured, and the root colonization was examined.

4.3 Leaf harvest, and extraction and analysis of metabolites

To compare data across plants, leaves were numbered as depicted in Figure 8. The numbering began at the top of the plant after the shoot apical meristem (ShAM) and the first fully expanded leaf counted as leaf 1. Leaves were grouped in order to ensure enough plant material, and different developmental stages were measured. Each plant had four groups with two leaves in each group, except for the ShAM (i.e., ShAM, leaves 5 and 6, leaves 11 and 12, and leaves 15 and 16). The four groups were used for analysis of artemisinin and total flavonoids.

To quantitatively determine the differences in the concentrations of various metabolites among plants of both cultivars grown in each of the four soil conditions, gas chromatography-mass spectrometry was used with a method adapted from Weathers & Towler (2012). Metabolites were extracted from selected plant material by adding 4 mL $MeCl_2$ to weighed, dried leaf material in glass test tubes and sonicated in a water bath for 30 minutes. The leaf material was removed and the resulting solvent was dried under nitrogen gas. If preparation of the samples for analysis was not performed on the same day as the extraction, the samples were stored in the freezer at -20 °F. To prepare the dried extracts for analysis of metabolites, the samples were resuspended in 200 µL $MeCl_2$. A 20 µL aliquot of each sample was transferred

to GC/MS sample vials and dried using compressed air. The samples were injected into the GC/MS, with He carrier gas at 1 mL/min. The temperatures of the ion source, inlet, transfer line, and oven were dependent upon the various compounds that were being measured, and are noted in Weathers and Towler (2014). Artemisinin was identified by comparing retention times and signature ion peaks in the plant extracts with purchased standards.

Figure 8*: Artemisia annua* with numbered leaves – left, ShAM - right

Flavonoid analysis was performed using a spectrophotometer using quercetin as a standard and following the method of Arvouet-Grand et al. (1994). First, 200 µg of quercetin was solubilized in 400 µL of MeOH. Aliquots of 40, 30, 30, 10, and 4 µL were transferred into separate test tubes. The plant samples were prepared by transferring a 20 µL aliquot of the sample resuspended in 200 µL $MeCl_2$ into a new test tube. Aliquots were dried under nitrogen gas and resuspended in 1 mL of 1% $AlCl_3$ in MeOH. Samples were immediately vortexed and a marble was placed over the opening of the tube to prevent evaporation. After 30-40 minutes at

room temperature, a sample was transferred into a glass cuvette and assayed at 415 nm in a spectrophotometer. A blank of 1 mL 1% $AlCl_3$ in MeOH was run first to zero the spectrophotometer. The remaining plant extracts were dried under nitrogen gas and stored in the freezer at -20 °F.

4.4 Transplantation of GLS Artemisia annua plant

To investigate whether an artemisinin-glandular trichome null mutant cultivar in the Weathers lab, glandless (GLS) (Duke et al., 1994), was able to be colonized by *G. intraradices*, a plant was transplanted into Pro-Mix HP Mycorrhizae soil after growing in the standard soil in a climate-controlled growth chamber. When transplanted, the GLS plant was of approximately the same developmental age as the harvested SAM plants. The GLS plant was grown on the windowsill under the same conditions as plants of the SAM cultivar for two weeks. The GLS plant was harvested, and its roots were examined for mycorrhizal colonization.

4.5 Determination of root colonization

To visualize root colonization by mycorrhizae, the method of Phillips and Hayman (1970) modified by Dr. Melissa Towler was used. Fresh root samples were taken from all four soil conditions, cleaned in tap water to remove soil particles, and soaked in a 50% EtOH solution (prepared with d_iH2O) for at least one week after soil was removed. For some plants, including all SAM plants grown from cuttings in Pro-Mix HP Mycorrhizae soil, the roots were cleared with 10% KOH by autoclaving them in this solution for 60 minutes. After cooling and rinsing with distilled water, the roots were stained with 0.05% Trypan blue (Lot #MKBP3291V) in a 1:1:1 mixture of water, glycerol, and 85% lactic acid (w/w) (Lot #109H0006) (Phillips and Hayman, 1970) and autoclaved for an additional 60 minutes. In the remainder of the plant samples, an altered method was used. The roots were cleared with 10% KOH by autoclaving for 20 minutes, cooled and rinsed with distilled water, and stained with 0.05% Trypan blue (Lot #MKBP3291V) (Phillips and Hayman, 1970) by autoclaving for an additional 10 minutes. For both methods, the roots were de-stained with a 1:1:1 mixture of water, glycerol, and 85% lactic acid (w/w) (Lot #109H0006). The stained root samples were observed using a compound light microscope. A typical root structure can be seen in Figure 6. Mycorrhizal colonization was measured by observing root segments for the presence or absence of fungal structures, such as

hyphae, vesicles, arbuscules, or spores that would stain blue. The number of roots with those structures was recorded.

4.6 Statistical analysis

All experimental conditions had at least 4 replicates, and all values were represented as the mean ± standard deviation. Statistical significance was determined using a two sample *t* test assuming unequal variances with a *p* value of ≤ 0.05.

5. Results

5.1 Effect of mycorrhizae on biomass – total leaf, stem, root, and overall mass

The SAM cultivar grown in Pro-Mix HP Mycorrhizae soil from cuttings had a significant increase in average biomass for root mass when compared to SAM grown in standard soil (Figure 9a). When grown in autoclaved soil, there was a significant decrease in stem, root, and total biomass compared to plants grown from cuttings in the respective unautoclaved soils. There was no significant difference between any of the experimental groups when comparing total leaf mass. Interestingly, these trends were not observed when the SAM cultivar was grown from tissue culture (Figure 9b). The stem biomass for SAM grown from tissue culture in Pro-Mix HP Mycorrhizae soil was significantly greater than SAM in standard soil. No other significant differences were observed between the experimental groups for plants originating from tissue culture. The tissue culture results suggested that autoclaving the two soil types did not affect the biomass of those plants.

5.2 Effect of mycorrhizae on growth – height, leaf number, inter-nodal length

The SAM cultivar grown in autoclaved soils from cuttings achieved significantly less height compared to plants grown in unautoclaved soils (Table 3). No significant differences were observed between the experimental groups for plants grown from cuttings in terms of number of leaves. All plants were harvested at approximately the same developmental stage with 19-27 mature leaves. The SAM cultivar grown in Pro-Mix HP Mycorrhizae soil from cuttings had significantly greater inter-nodal length when compared to SAM grown in standard soil and autoclaved soil (Table 3 top). Plants grown in unautoclaved soils had a significantly greater inter-nodal length than those grown in autoclaved soils. This trend was not observed in the SAM cultivar grown from tissue culture, as differences between all experimental groups were not significant (Table 3 bottom). Although plants grown from cuttings and tissue culture were grown for 4-6 weeks under the same conditions and then harvested with approximately the same number of leaves, the heights and inter-nodal lengths of plants grown from cuttings were about 50% greater than plants grown from tissue culture.

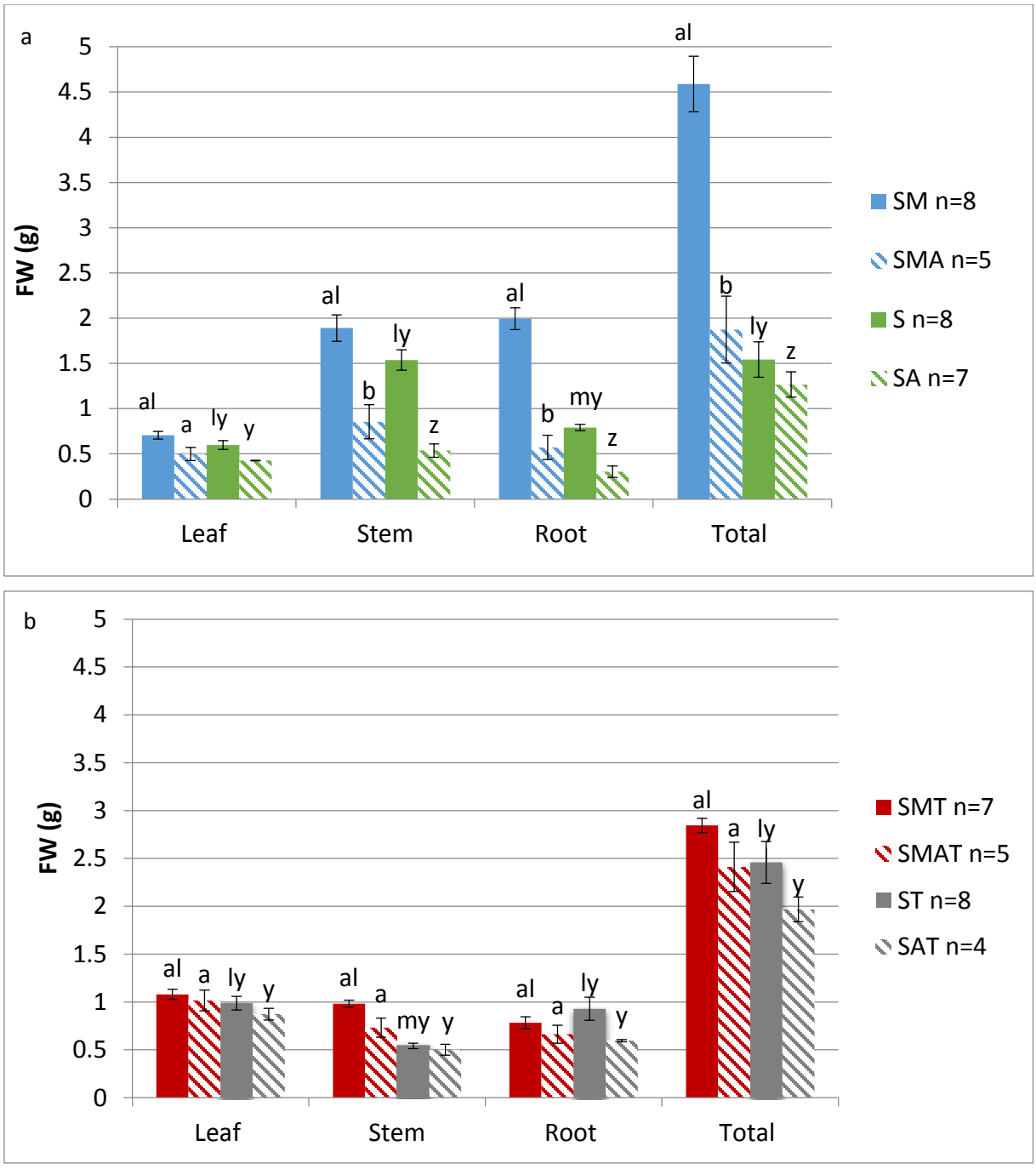

Figure 9: Change in biomass of *A. annua* SAM cultivar after growth in two soils ± autoclaving: (a) Plants grown from cuttings and (b) plants grown from tissue culture. At harvest, plants had 19-27 leaves. Leaf mass represents the sum of the masses of all leaves on the plant. Total mass represents the sum of total leaf, stem, and root mass. Three pair-wise comparisons were made. a,b compares Pro-Mix HP Mycorrhizae ±autoclaving (SM v. SMA, SMT v. SMAT); l,m compares non-autoclaved Pro-Mix HP Mycorrhizae with MetroMix 360 (SM v. S, SMT v. ST); y,z compares MetroMix 360 ±autoclaving (S v. SA, ST v. SAT). Bars showing different letters indicate significant differences between treatments according to the Student's t test assuming unequal variances ($p \leq 0.05$).

Table 3: Average growth of *A. annua* SAM cultivar after growth in two soils ± autoclaving.

Experimental Group	Height (cm)	Number of Leaves	Inter-nodal Length (cm)
SM	28.94 ± 0.43 al	22.88 ± 0.15 al	1.26 al
SMA	20.88 ± 1.91 b	22.20 ± 0.97 a	0.93 b
S	25.79 ± 0.19 ly	24.38 ± 0.32 ly	1.06 my
SA	18.56 ± 0.64 z	21.43 ± 0.07 y	0.87 z
Experimental Group	**Height (cm)**	**Number of Leaves**	**Inter-nodal Length (cm)**
SMT	11.71 ± 0.41 al	24.14 ± 0.60 al	0.48 al
SMAT	11.04 ± 0.42 a	24.60 ± 0.68 a	0.45 a
ST	10.34 ± 0.48 ly	23.00 ± 0.27 ly	0.45 ly
SAT	9.60 ± 0.17 y	22.5 ± 0.43 y	0.42 y

Top: plants grown from cuttings; Bottom: plants grown from tissue culture. At harvest, plants had 19-27 leaves. Inter-nodal length was calculated by dividing the stem height by the leaf number. Three pair-wise comparisons were made. a,b compares Pro-Mix HP Mycorrhizae ±autoclaving (SM v. SMA, SMT v. SMAT); l,m compares non-autoclaved Pro-Mix HP Mycorrhizae with MetroMix 360 (SM v. S, SMT v. ST); y,z compares MetroMix 360 ±autoclaving (S v. SA, ST v. SAT). Bars showing different letters indicate significant differences between treatments according to the Student's t test assuming unequal variances ($p \leq 0.05$).

5.3 Effect of mycorrhizae on flavonoids

SAM grown in autoclaved standard soil from cuttings had significantly less total flavonoids than SAM grown in unautoclaved standard soil, but only in leaves 15 and 16 (Figure 10a). No other statistically significant differences were observed among any experimental groups for plants grown from cuttings. Total flavonoid content was greatest in the ShAM for all four experimental groups from cuttings. Similarly, there were no statistically significant differences in total flavonoid content among all four experimental groups grown from tissue culture (Figure 10b). The ShAM produced the greatest total flavonoid content for all four experimental groups grown from tissue culture.

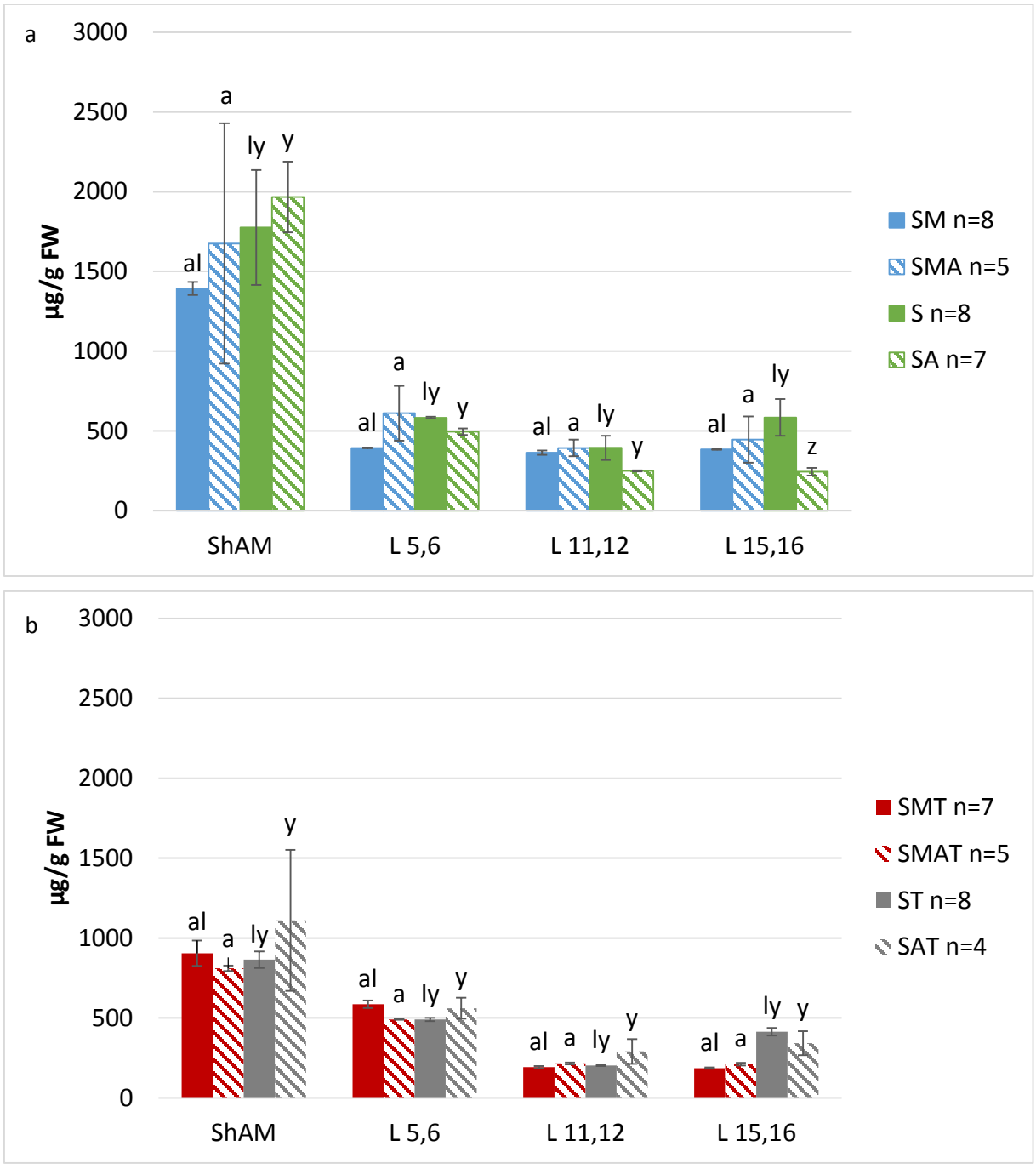

Figure 10: Change in total flavonoid concentration in *A. annua* SAM cultivar after growth in two soils ± autoclaving. (a) Plants grown from cuttings (b) Plants grown from tissue culture. At harvest, plants had 19-27 leaves. ShAM, shoot apical meristem; L, leaf number. Three pair-wise comparisons were made. a,b compares Pro-Mix HP Mycorrhizae ±autoclaving (SM v. SMA, SMT v. SMAT); l,m compares non-autoclaved Pro-Mix HP Mycorrhizae with MetroMix 360 (SM v. S, SMT v. ST); y,z compares MetroMix 360 ±autoclaving (S v. SA, ST v. SAT). Bars showing different letters indicate significant differences between treatments according to the Student's t test assuming unequal variances ($p \leq 0.05$).

5.4 Effect of mycorrhizae on artemisinin

No statistically significant differences were observed in the amount of artemisinin present in plants grown from cuttings (Figure 11a). No trends were observed when comparing experimental groups across different leaf sample points. Interestingly, artemisinin content in the ShAM and leaves 5 and 6 of all plants grown from tissue culture was much less than that observed for the developmentally identically tissues in plants grown from cuttings. In leaves 11 and 12, SAM grown in autoclaved Pro-Mix HP Mycorrhizae soil from tissue culture had a statistically significant increase in artemisinin content compared to SAM grown in unautoclaved Pro-Mix HP Mycorrhizae soil (Figure 11b). In contrast, leaves 15 and 16 of SAM grown in Pro-Mix HP Mycorrhizae soil from tissue culture experienced a statistically significant decrease in artemisinin content compared to SAM grown in standard soil. No other statistically significant differences were observed between experimental groups of plants grown from tissue culture.

5.5 Root Colonization

No mycorrhizal fungal structures were observed in the roots of plants from any of the experimental SAM groups; there was no Trypan blue staining. A representative root sample for each experimental group is shown in Figure 12. The artemisinin-glandular trichome null mutant cultivar, glandless (GLS), was transplanted as a plant that was similar in developmental age to the SAM plants that were harvested and grown in Pro-Mix HP Mycorrhizae soil for 2 weeks. Roots of GLS had blue-stained mycorrhizal fungal structures (Figure 13).

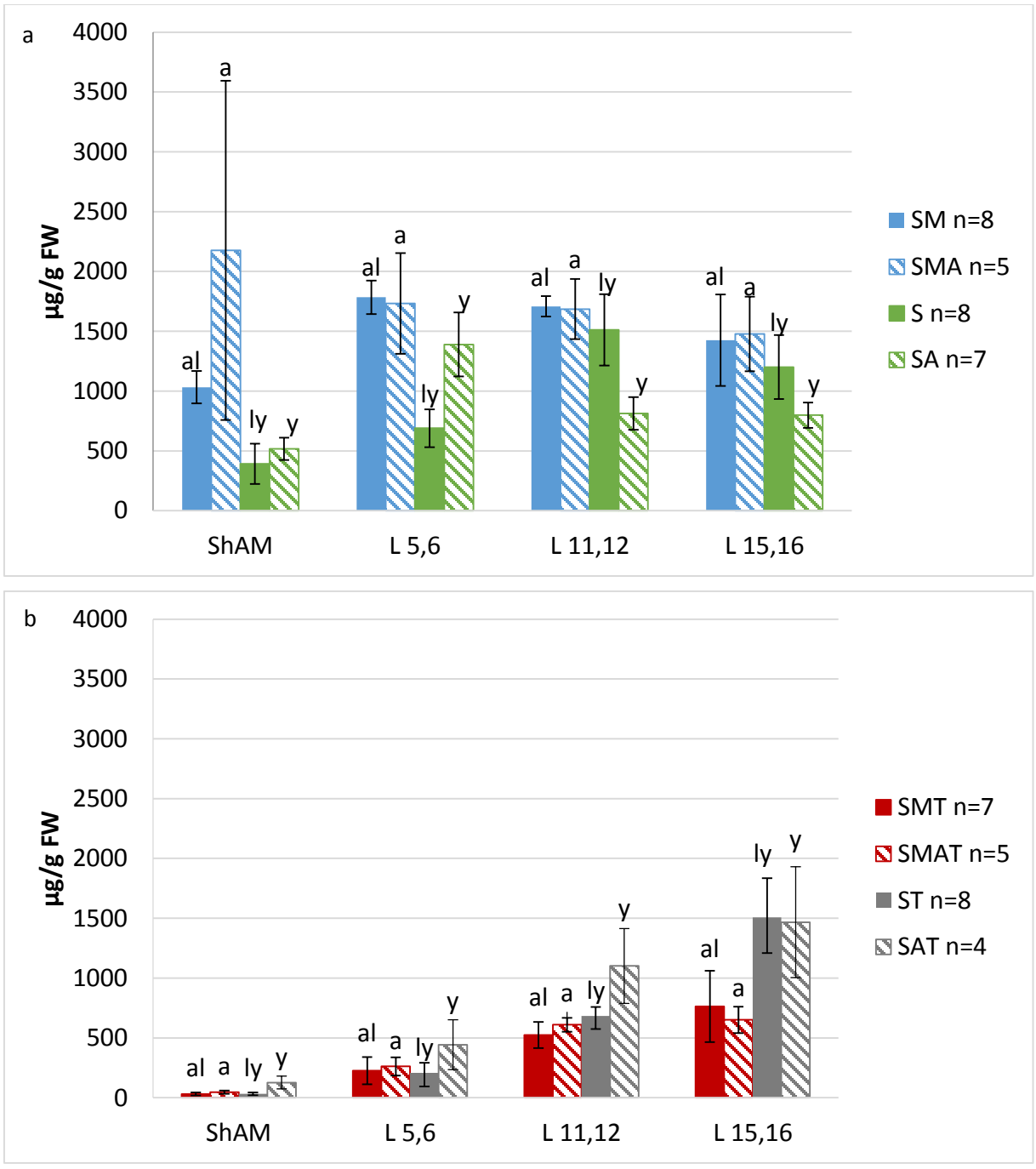

Figure 11: Change in artemisinin concentration in *A. annua* SAM cultivar after growth in two soils ± autoclaving. (a) Plants grown from cuttings (b) Plants grown from tissue culture. At harvest, plants had 19-27 leaves. ShAM, shoot apical meristem; L, leaf number. Three pair-wise comparisons were made. a,b compares Pro-Mix HP Mycorrhizae ±autoclaving (SM v. SMA, SMT v. SMAT); l,m compares non-autoclaved Pro-Mix HP Mycorrhizae with MetroMix 360 (SM v. S, SMT v. ST); y,z compares MetroMix 360 ±autoclaving (S v. SA, ST v. SAT). Bars showing different letters indicate significant differences between treatments according to the Student's t test assuming unequal variances ($p \leq 0.05$).

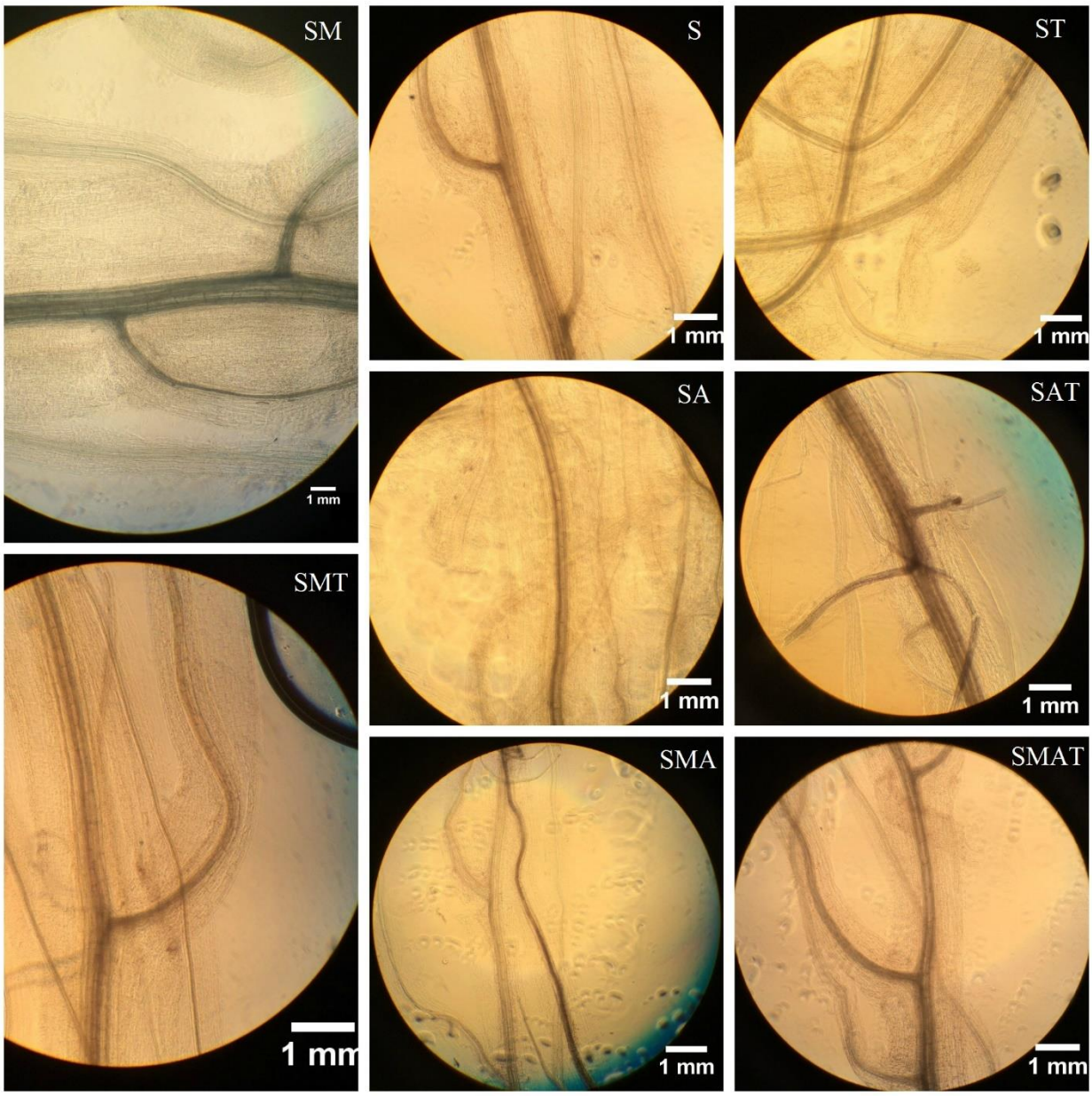

Figure 12: Segments of SAM roots stained with Trypan blue at 100x magnification under a compound light microscope. Roots from SAM cultivar from cuttings or tissue culture grown in the two soil types ± autoclaving. SM, cuttings in Pro-Mix HP Mycorrhizae; SMT, rooted tissue culture in Pro-Mix HP Mycorrhizae; S, cuttings in standard soil (Metro Mix 360); ST, rooted tissue culture in standard soil; SA, cuttings in autoclaved standard soil; SAT, rooted tissue culture in autoclaved standard soil; SMA, cuttings in autoclaved Pro-Mix HP Mycorrhizae; SMAT, rooted tissue culture in autoclaved Pro-Mix HP Mycorrhizae.

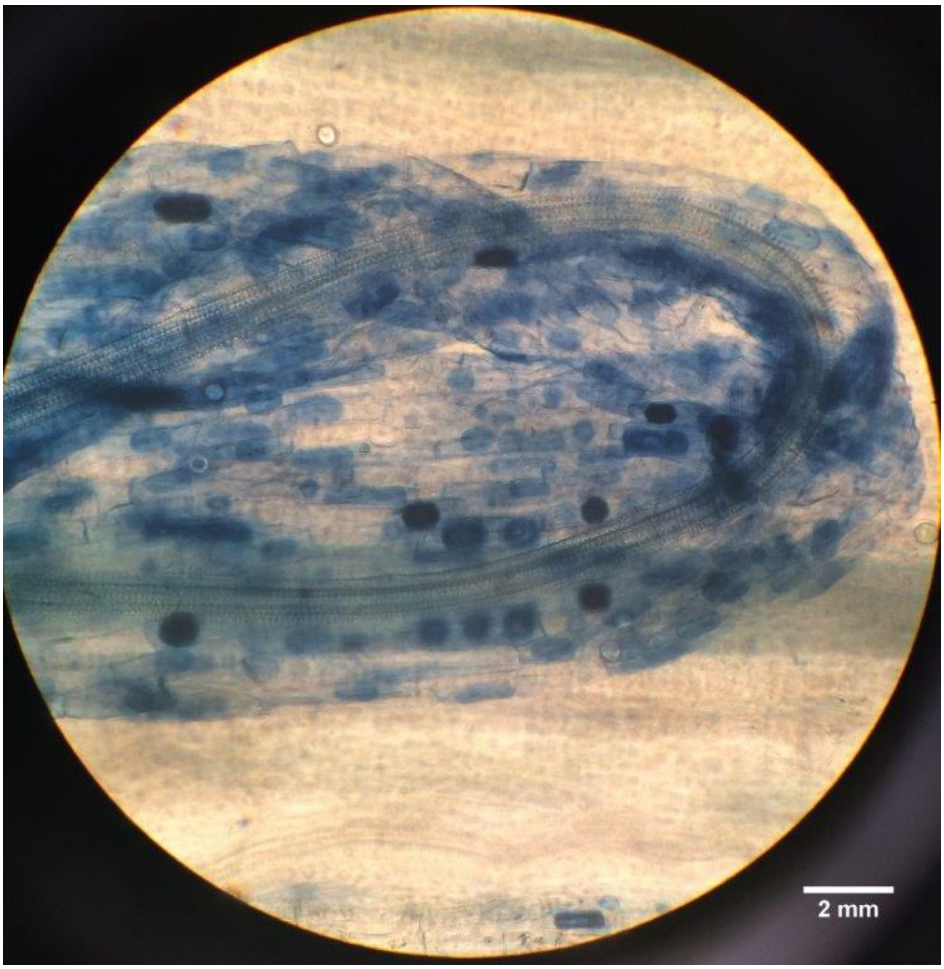

Figure 13: Segments of GLS roots stained with Trypan blue at 400x magnification under a compound light microscope. Roots from GLS cultivar from plant transplanted and grown in Pro-Mix HP Mycorrhizae soil for 2 weeks. Fungal structures including vesicles and arbuscules are observed in the root cortical tissue.

6. Discussion

Our project analyzed artemisinin concentration and biomass for the SAM cultivar of *A. annua* when grown in Pro-Mix HP Mycorrhizae soil and in the standard soil used by the Weathers lab. Plant propagation by cuttings resulted in a significantly greater root biomass when grown in Pro-Mix HP Mycorrhizae soil than in the standard soil (Figure 9a). Mycorrhizal colonization increases lateral branching of roots which may explain the increased root biomass (Hanlon and Coenen, 2011). However, this was not observed in plants propagated by tissue culture as only stem biomass was significantly greater (Figure 9b). This observation supported prior research that showed an increase in *A. annua* stem biomass of plants colonized by two species of mycorrhizal fungus but no increase in root or leaf biomass (Rapparini et al., 2007).

In general, the presence of mycorrhizae in the soil did not affect the amount of artemisinin produced by the plants (Figure 11). Although studies showed that *A. annua* increased in artemisinin after mycorrhizal colonization, different cultivars varied in their response (Chaudhary et al., 2008). These findings suggested that the SAM cultivar in the Weathers lab may not experience a significant response to exposure to mycorrhizae. Since artemisinin is produced in the glandular trichomes of the leaves, glandular trichomes should be observed in future experiments and compared amongst the experimental groups described here since these findings suggest that the Pro-Mix HP Mycorrhizae soil would not affect the density of glandular trichomes. This may explain why no significant change in leaf biomass or artemisinin content occurred.

There may be other explanations for the contrast in our results with others who have studied mycorrhizal infection of *A. annua*. For example, all of the previous studies used seeds rather than cuttings or tissue cultured plantlets for plant propagation (Chaudhary et al., 2008; Mandal et al., 2014; Kapoor et al., 2007; Rapparini et al., 2007). The SAM cultivar is clonally maintained so seeds were not available. Furthermore, the mycorrhizal inoculum used in this experiment varied from those previously used. As mentioned in the Methodology section of this report, our project used a commercially available soil with mycorrhizal spores. The four prior studies added isolated spores to soil in addition to using chopped AM-colonized sorghum roots (Chaudhary et al., 2008; Mandal et al., 2014; Kapoor et al., 2007; Rapparini et al., 2007). Upon replication of this study, it is recommended that this method of adding isolated spores or chopped AM-colonized roots to the soil be used. An alternative method would be to use two different

types of the same brand of soil, one with an inoculum of fungal spores added and one without, to ensure that the only variable between the experimental groups is the presence of the commercially added mycorrhizal spores, not nutrient composition. To better follow the prior studies, chopped, colonized GLS roots could also be added to the soil to promote mycorrhizal colonization of plants of the SAM cultivar.

To determine root colonization, this project used the procedure of Phillip and Hayman (1970). The previous research obtained a range of 50-80% root colonization as a percentage of examined root length (Chaudhary et al., 2008; Mandal et al., 2014; Kapoor et al., 2007). In this project, root colonization did not appear to be observed in any experimental group. However, the literature did not provide the duration that the samples should be autoclaved, which resulted in an investigation of an appropriate length of time to obtain clear images of the roots. To validate the efficacy of the adapted staining procedure and the Pro-Mix HP Mycorrhizae soil, the roots of another cultivar in the Weathers Lab, Glandless, were stained and observed (Figure 13). This process demonstrated that the Pro-Mix HP Mycorrhizae soil, with *G. intraradices* spores, can indeed infect the GLS cultivar and that the resulting mycorrhizal structures can be viewed through the use of the staining procedure. Therefore, the adapted staining procedure described here should be used in future studies. Additionally, mycorrhizal colonization of the GLS cultivar should be explored in future work in order to observe the effects of AM fungi on biomass, flavonoids, and artemisinin like this project accomplished for the SAM cultivar.

To our knowledge, no published studies about the effects of arbuscular mycorrhizal colonization on flavonoid production in *A. annua* exist, and limited studies discussing the effects in other species of plants were found. Ponce et al. (2004) studied the effect of AM colonization by *G. intraradices* on *Echinacea purpurea* L. and observed an increase in flavonoid production. Therefore, this project aimed to explore these effects on total flavonoid concentration in the Weathers lab cultivar, SAM. The results, seen in Figure 10, show no significant difference in flavonoid content with plants grown in Pro-Mix HP Mycorrhizae soil. However for both plants propagated by cuttings and by tissue culture, the shoot apical meristem contained the greatest amount of flavonoids, which decreased with leaf age. This result was also observed for the SAM cultivar in a recent developmental study by Towler and Weathers (2015) that tracked the production of about 20 phytochemicals including artemisinin in *A. annua.*

Overall, our study suggested that the mycorrhizal fungus, *G. intraradices*, may not be competent at infecting the SAM cultivar of *A. annua.* It is interesting to note, however, that the method of propagation yielded quite different results. As previously mentioned, there was a statistically significant increase in root biomass for plants grown in Pro-Mix HP Mycorrhizae from cuttings but a statistically significant increase in stem biomass for plants grown in Pro-Mix HP Mycorrhizae derived from tissue culture (Figure 9), and there was also a difference in flavonoid concentration between the different propagation methods used, with plants derived from tissue culture producing less total flavonoids than plants from cuttings (Figure 10). Plants derived from tissue culture experienced markedly reduced heights and inter-nodal lengths by approximately 50% (Table 3), along with a decrease in artemisinin (Figure 11).

Other studies showed that phytohormones, such as auxins, cytokinins, and gibberellins, may play an important role in initiating mycorrhizal colonization, and those could certainly be different in plants propagated by cuttings versus by tissue culture (Hanlon and Coenen, 2011). Auxins are primarily responsible for cell elongation during phototropism and gravitropism, and many commercially available root hormones contain the naturally-occurring auxin called indole acetic acid (IAA). In propagating the plants derived from cuttings, Hormodin 2 root-inducing hormone, which contains 0.03% IAA, was added; this is important to note, as it provided a possible explanation for the increase in biomass, flavonoids, and artemisinin in plants from cuttings compared to plants derived from tissue culture. Cytokinins are another type of growth regulator that are important in promoting cell division, particularly in plant roots; the synergistic relationship between cytokinins and other phytohormones like auxins are known to effect plant development. Gibberellins are plant hormones that have a number of effects on plants, including increasing shoot length and delaying aging of leaves (Boundless, 2014).

Additional research has also indicated that signaling occurs to and from the plant root throughout different stages of the symbiosis (Harrison, 2005; Elias and Safir, 1987). Typically, before direct physical contact between the mycorrhiza and the plant roots, molecular signaling occurs, although the specific signaling events vary depending on the organisms involved. For example, during the interaction between rhizobium and legumes, the plant releases flavonoid molecules which results in the synthesis and release of Nod factor from the bacterium. The Nod factor signal is processed by legume root receptors, which initiates development of nodules and physical contact (Harrison, 2005). The plant signal is richest in the root exudates of plants

derived of phosphate; therefore, examining root exudate from this cultivar, with and without phosphorous, may provide insight into this relationship (Harrison, 2005). Even more recently, strigolactones, which are thought to result from the carotenoid biosynthetic pathway and phosphate deprivation, have been identified as a possible plant signal that is released during the establishment of the initial mycorrhizal symbiosis (Mohanta and Bae, 2015). Strigolactones are hormones responsible for stimulating AM spore germination, and they have been identified as a component of plant root exudates. In turn, AM fungi produce Myc factors, which are perceived by plants and are analogous to the Nod factors from bacteria. Myc factors result in an increase in intracellular calcium, cell structural alterations, and changes in root transcriptional programming. In addition to Myc factor, AM release lipochitooligosaccharides to initiate symbiosis. With this knowledge, it would be advantageous to pursue a study that explores these signals in cultivars in the Weathers lab along with the spores of *G. intraradices.* Although the findings from this project suggested that the SAM cultivar is at its highest artemisinin production, the investigation of the symbiotic relationship with this plant and arbuscular mycorrhizal fungi should be continued by implementing the aforementioned suggestions.

7. Conclusions

Overall, the high-producing cultivar in the Weathers lab (SAM), propagated through the use of nodal cuttings or rooted tissue culture, did not show a significant increase in flavonoid or artemisinin content nor did it appear to be colonized by mycorrhizae. On the other hand, a null mutant cultivar in the Weathers lab (GLS), which does not produce any artemisinin, was colonized. The differences in biomass, flavonoid, and artemisinin between plants grown from cuttings and tissue culture, along with the variations in mycorrhizal colonization between the two *A. annua* cultivars in the Weathers lab, suggest that there is perhaps a signal or specific mechanism that should be further studied. In conclusion, this research is beneficial in providing insight into the ability of the arbuscular mycorrhizae *G. intraradices* to colonize *A. annua* cultivars at WPI which could ultimately lead to more affordable and efficacious antimalarial treatment.

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Appendix A – Ingredients in Metro Mix 360

Ingredients:

Formulated Canadian Sphagnum peat moss, coarse perlite, bark ash, starter nutrient charge (with Gypsum) and slow release nitrogen and dolomitic limestone.

Retrieved from: http://sungro.com/products_displayProduct.php?product_id=106&brand_id=17

Appendix B - Pro-Mix HP Mycorrhizae Specification Sheet

PRO-MIX HP MYCORRHIZAE™

PRO-MIX HP MYCORRHIZAE™ is a high porosity peat-based growing medium ideal for water sensitive crops, rooting cutting and/or low-light growing conditions that contains a beneficial mycorrhizal inoculum (*Glomus intraradices*). These microscopic fungi attach to and colonize root systems to benefit plants by increasing water and nutrients acquisition (especially Phosphorus, Copper and Zinc). This symbiotic relationship between fungi and plant results in overall improved plant growth. **PRO-MIX HP MYCORRHIZAE™** is suitable for a wide variety of horticultural plants, especially when high air capacity and extra drainage are required.

INGREDIENTS

- Canadian Sphagnum Peat Moss (65-75%)
- Perlite - horticultural grade
- Dolomitic and Calcitic limestone (pH adjuster)
- Wetting Agent
- **MYCORRHIZAE™** mycorrhizal inoculum (*Glomus intraradices*)

FEATURES

- High perlite content, peat-perlite based growing medium
- Low water retention
- Designed for water sensitive crops
- Ideal for cultural conditions with low-light and excess moisture/humidity
- Includes **MYCORRHIZAE™** mycorrhizal inoculum (*Glomus intraradices*) fungi

BENEFITS

- Dries out more frequently than general purpose, peat-based mixes
- Well suited for low-light growing conditions and high humidity
- Less difficult to overwater crops
- Reduces incidence of water related problems (root diseases, algae and fungus gnats)
- Holds less water for improved leaching of fertilizer salts during crop cycle
- **MYCORRHIZAE™** improves overall growth of plants and increases yields of flowers/ fruits.

CHEMICAL CHARACTERISTICS:

- pH Range: ..... 5.2 - 6.2 (S.M.E.)
- pH Incubated: ..... < 6.2 after 7 days saturation (S.M.E.)
- Electrical Conductivity: ... 1.0 - 1.8 mmhos/cm (S.M.E.)
- S.M.E.[1] Nutrient Analysis:

ppm (mg/l) *										
NO3-N nitrate	P04-P phosphate	K potassium	Ca calcium	Mg magnesium	S-SO4 sulfate	Fe iron	Zn zinc	Cu copper	Mn manganese	B boron
70-130	5-40	50-130	100-180	20-45	30-100	0.8-2.2	0.1-1.2	< 0.3	0.3-1.0	< 0.6

1 - Saturated Medium Extract

PHYSICAL CHARACTERISTICS:

- Air Porosity: ..... 14 - 20% by volume (6 inch pot)
- Bulk Density: ..... 8 - 10 lb./cu.ft. (0.13-0.16 g/cm3)
- Moisture Content: ..... 30 - 50 % by weight
- Saturated Weight: ..... 55 lb./cu.ft. (880 g/l) avg.
- Water-Holding Capacity: .. 50 - 70 % by volume

PACKAGE SPECIFICATIONS:

Product Code	Package Size	Units / pallet	Weight / unit	Minimum Yield
PT20281	2.8	57	25-35 lb	2.8 cu ft
PT20381	3.8	30	60-75 lb	7 cu ft

*West only.

PREMIER TECH HORTICULTURE

Appendix C – Plant Tissue Culture Rooting Media

To make 1 L (adapted from Dr. Melissa Towler):

20 g/L Sucrose (Prod. #S391, Lot #11B0391133A) (PhytoTechnology Laboratories)

2.215g/L Murashige and Skoog medium (Prod. #M519, Lot #12E0519061B) (PhytoTechnology Laboratories)

5 g/L Agargellan (Prod. #A133, Lot #11C0133030A)

d_iH2O

pH = 5.8