TESTING EFFECTS OF MYCORRHIZAL FUNGI ON GROWTH AND DEVELOPMENT OF *ABRONIA MACROCARPA*

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San Marcos, Texas
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TESTING EFFECTS OF MYCORRHIZAL FUNGI ON GROWTH AND DEVELOPMENT OF *ABRONIA MACROCARPA*

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ABSTRACT

TESTING EFFECTS OF MYCORRHIZAL FUNGI ON GROWTH AND DEVELOPMENT OF ABRONIA MACROCARPA

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SUPERVISING PROFESSOR: PAULA WILLIAMSON

Endangered and threatened species require various management plans for recovery, many of which include reintroductions. *Abronia macrocarpa*, an endangered Texas endemic plant species, has been suggested as a potential candidate for reintroduction. Inoculation with mycorrhizal fungi has been a component of reintroduction plans for some species. Mycorrhizal fungi have a mutualistic, obligatory symbiotic relationship with most higher-order plants. They can increase their host's uptake of nutrients such as P, N, and K, increase plant growth, reduce saline and alkaline toxicity, and increase drought resistance. The effects of mycorrhizae on *A. macrocarpa* had not been studied. I hypothesized that growth and development of *A. macrocarpa* would be increased when plants were inoculated with mycorrhizal fungi. I established 3

transects on private property in Freestone County, TX with 6 plots each. Half of the plots were randomly selected to be planted with inoculated seed while the other half were planted with seed coated with autoclaved inoculant as a control. I collected data for two years and analyzed measurements of growth and development. Results indicated that growth was significantly improved by inoculation in the first year after germination. Mean number of leaves per plant was greater in treatment plots in March 2011 ($P = 0.00544$), and mean aerial diameter of plants in treatment plots was larger in April 2011 ($P = 0.018$). Plants in treatment plots were also larger in aerial diameter and height in the second year of growth. However, these differences were not statistically significant. Germination, survivorship, and development were not affected by treatment, but there was some observable variation in germination due to transect. This suggests that *A. macrocarpa* is extremely sensitive to variations in microhabitat. Positive results in the first year of growth warrant further study of mycorrhizal interaction with this species.

CHAPTER I

INTRODUCTION

Our planet is presently undergoing one of the largest extinction events the world has ever known. Extensive habitat loss and degradation are contributing to species extinctions occurring at an alarming rate, resulting in a rapid loss of overall biodiversity (Novacek and Cleland, 2001). As a result, many extant species of animals and plants are threatened or endangered. Human activities, primarily conversion of natural habitat into agricultural, urban, and recreational areas are the greatest current threats to biodiversity (Seabloom et al., 2002). Ironically, human intervention may be the only means of maintaining, or possibly recovering biota in the face of this environmental catastrophe (Novacek and Cleland, 2001).

Active management strategies such as habitat restorations, artificial outplanting, and reintroductions that might establish populations in historically appropriate habitats are often necessary in these situations (Fisher and Jayachandran, 2002). Reintroduction procedures have become commonplace over the past two decades. As of 1992, nearly one-fourth of the plants listed under the Endangered Species Act have recovery plans that include reintroduction programs (Falk and Olwell, 1992). For example, the recovery plan for *Abronia macrocarpa*, the large-fruited sand-verbena, includes reintroduction as a potential means of downlisting or delisting the taxon. *Abronia macrocarpa* is a Texas

endemic plant species listed as federally endangered on September 28, 1988 (U.S. Fish and Wildlife Service, 1988) and endangered in Texas on December 30, 1988 (U.S. Fish and Wildlife Service, 1992). The U.S. Fish and Wildlife Service designated a recovery priority of 2 for the species, which denotes a high degree of threat, but great potential for recovery (U.S. Fish and Wildlife Service, 1992).

The recovery plan for this species requires at least 20 viable and persistent populations, each of at least 10.11 hectares (25 acres) in size with a population of at least 600 individuals, before it can be delisted (U.S. Fish and Wildlife Service, 1992). There are currently nine known *A. macrocarpa* populations confined to three Texas counties (Leon, Robertson, and Freestone); all populations occur on privately owned property. Eleven new populations of *A. macrocarpa* must be discovered or created in order to meet the recovery goal. If 11 naturally occurring populations are not identified, reintroduction will be critical to recovery. In order to maximize the chances of successful reintroductions, biologists must first become knowledgeable about the optimal conditions required by a given species for it to establish and reproduce.

Much of the information required to develop a reintroduction plan exists for *A. macrocarpa*; therefore, this species should be considered a prime candidate for reintroduction. Studies have been conducted on phenology (Williamson et al., 1994), reproductive biology (Williamson et al., 1994; Williamson and Bazeer, 1997), population genetics (Williamson and Werth, 1999), population structure and habitat characteristics (Meredith, 2006) and seed germination (Goodson and Williamson, 2011). If biologists hope to recover *A. macrocarpa*, it is imperative that researchers continue to add to the growing library of knowledge concerning this endangered species.

In order for a successful reintroduction to occur, a specific set of conditions must be met that mimic the characteristics of a naturally occurring population (Pavlik, 1996). These habitat conditions include the nutrient and biota content of soils in the area. Soils at naturally occurring populations of *A. macrocarpa* are low in nitrates (2-11 ppm), phosphorus (13-29 ppm), and potassium (24-39 ppm) (Meredith, 2006). A pilot reintroduction study by Williamson (2008) found that *A. macrocarpa* had an increased rate of growth and development when planted in soils that were rich in potassium (81 ppm) and contained the upper levels of nitrogen (15 ppm) and phosphorus (29 ppm) known in soils supporting populations of *A. macrocarpa*. In naturally occurring populations, plants remain in the seedling stage the first year after germination and do not reach juvenile and reproductive stages for another one to two years. However, seeds planted in these nutrient-rich soils sometimes reached the juvenile or even reproductive stages within the first year after planting (Williamson, 2008).

One possible method for achieving enriched nutrient conditions is through the presence of mycorrhizal fungi. It is known that there is an existing relationship between *A. macrocarpa* and arbuscular mycorrhizal fungi (AMF) in naturally occurring conditions (Williamson, unpublished data). However, it is not known if there is the potential to create one when reintroducing the species. The effect of AMF on growth and development of the species is also not known.

Arbuscular mycorrhizal fungi have a mutualistic, obligatory symbiotic relationship with most higher-order plants (White et al., 2008). The fungal mycelia extend both into the roots of the host plant and into the surrounding soil. Inorganic compounds found in the substrate can thus flow from fungus to plant, while organic

compounds flow from plant to fungus (Allen, 1991). Plants treated with chemical fungicides often suffer as a result of the death or reduction of their mycorrhizal populations and have to be supplemented with mycorrhizal inoculants in order to recover (Plenchette et al., 1983). Many laboratory and field studies have shown that plants receive a variety of benefits from AMF. These often include improved nutrient absorption and greater tolerance of drought (Smith and Read, 1997). A study on *Araucaria angustifolia* showed that inoculation with AMF greatly improved the growth of this plant, increasing the amount of shoot biomass in comparison to root biomass (Zandavalli et al., 2004). Plants in saline and alkaline soils inoculated with AMF have the potential to increase their survival, growth, and reproduction (Zhang et al., 2011). The same study showed that associations with AMF led to selective nutrient absorption. Uptake of N, P, Ca, and K were increased, and absorption of Na and Cl were reduced. This indicated that a mycorrhizal association had the potential to optimize nutrient content in host plants. These attributes should lead to an increased rate of growth and development for plants with AMF, even in soils with relatively low nutrient concentrations.

Propagules of mycorrhizal fungi that have the potential to initiate colonization include spores, infected root fragments, and mycelia (Harley and Smith, 1983). Inoculations with AMF can be used to reestablish plant communities that have been degraded for reasons such as soil disturbance and erosion (Zhang et al., 2011). Several studies have investigated the usefulness of AMF treatments to enhance the performance of highly threatened or endangered plants and found that the use of AMF has positive effects on nutrient uptake, growth, and survival (Barroetavena et al., 1998; Fisher and

Jayachandran, 2002; Panwar and Vyas, 2002; Zubek et al., 2009). These studies collectively demonstrate that AMF can benefit a wide variety of plant species, and that its use can improve the success of reestablishment efforts. Evidence also suggests that the origin and species of the microbiota used for inoculation are often of little importance. Whether the inoculant is isolated from the plant's natural environment or purchased from a commercial source, the observable benefits are similar (White et al., 2008; Zubek et al., 2009).

The primary objective of this study was to determine if *A. macrocarpa* would benefit from AMF inoculation under field conditions. I hypothesized that inoculation with mycorrhizal fungi should increase nutrient uptake in *A. macrocarpa*, resulting in increased rates of growth and development, even in low-nutrient soils. If *A. macrocarpa* showed increased growth and rate of development when inoculated with AMF, inoculation could be used as a reintroduction tool in recovery of this species.

CHAPTER II

MATERIALS AND METHODS

Study Species

Abronia macrocarpa, commonly known as the large-fruited sand-verbena, was first described by Galloway in 1972. It is not a true verbena, but a member of the Nyctaginaceae family which is also referred to as the four o'clock family because the flowers of its plants open at around 3:00 p.m. to 4:00 p.m (Galloway, 1972). *Abronia macrocarpa* blooms from February to June, with peak flowering in March and April, and produces inflorescences composed of 27 to 40 flowers each (Williamson et al., 1994). They range from light pink to fuchsia in color and are tubular in shape, growing up to 3.2 centimeters in length. This species is an herbaceous perennial that grows up to 20 centimeters in height and has a large taproot. The leaves are oval-shaped, covered with glandular hairs, and are oppositely arranged along the stem.

The fruit produced by *A. macrocarpa* is an anthocarp. This consists of a dry, papery outer portion formed by the lower calyx which encases an achene. Achenes are dry, indehiscent, single-seeded fruits that are unattached to the pericarp. Interestingly, the papery anthocarp develops whether the flower has been pollinated or not. However, in the absence of pollination, the achene within fails to develop (Williamson et al., 1994). This species relies solely on pollinators for fruit set because it is incapable of successfully

self-fertilizing (Williamson and Bazeer, 1997). Common pollinators of this species include hawk moths (Sphingidae) and noctuid moths (Noctuidae) (Williamson et al., 1994). Anthocarps of *A. macrocarpa* are wind-dispersed, but the majority fall within 30 centimeters of the parent plant (Williamson and Werth, 1999). Spacial distribution in naturally occurring populations is therefore described as "clumped-contagious", meaning that if there is an individual of the species present, there is a high probability that others are nearby (Williamson and Werth, 1999). This distribution pattern may also be a contributing factor to the taxon's rarity and small range size.

Mycorrhizal Inoculation Experiment

The mycorrhizal inoculation experiment was conducted on private property in Freestone County, Texas and was chosen based on its potential to support populations of *A. macrocarpa* based on edaphic features and community composition. Landowner permission to conduct this experiment was obtained. *Abronia macrocarpa* seeds were collected from an existing population at Hilltop Lakes (Leon Co., Texas). At the study site, I established three transects, each measuring 30 m in length and containing six plots each. I placed each transect at least 12 m away from the others. The plots measured 1 m^2 and were separated by a buffer zone of 4 meters in order to avoid inadvertent colonization of AMF in the control plots due to the spreading of mycorrhizae from the inoculant. I then randomly assigned each plot to one of two treatments. Experimentally treated plots were planted with *A. macrocarpa* seed that had been coated with an AMF inoculant in powder form. The other half (control plots) were planted with seeds that I treated with an inoculant which had been autoclaved to kill the mycorrhizae. The AMF

inoculant was supplied by Mycorrhizal Applications, Inc. and contained four species of mycorrhizal fungi: *Glomus intraradices, Glomus mosseae, Glomus aggregatum*, and *Glomus etunicatum*. The inoculant contained 220 propagules/g. I planted seventy-five *A. macrocarpa* seeds in each plot in the spring of 2010. I also applied a supplemental dose of inoculant to the treatment plots in the spring of 2011 to ensure that the mycorrhizae made contact with roots. Each plant in the experimental plots received 2 ml of a suspension containing 10% mycorrhizal inoculant and 90% DI water. I administered 2 ml of plain deionized water to plants in control plots.

In March of 2011, I identified individuals of *A. macrocarpa* that had successfully germinated and calculated percent germination for treatment and control plots. I also marked plants numerically to keep track of individuals in each plot. However, I was unable to consistently track individuals because of multiple soil disturbances caused by animals at the field site, most likely feral hogs. *Abronia macrocarpa* only grows in height from late February to late April or early May. After this time, the above-ground part of the plant dies back, leaving only the taproot and shoot apex buried in the soil (Williamson and Bazeer, 1997). For this reason, I only recorded measurements of growth in the spring months. Since *A. macrocarpa* is endangered, it was important not to destroy the resulting plants. Therefore, I used several proxies to measure growth in lieu of measuring dry biomass of the plants.

In March of 2011, I recorded number of leaves, developmental stage, and number of inflorescences present. Developmental stage was classified as seedling, juvenile, or at anthesis. I classified plants as seedlings if they had only emerged cotyledons, as juveniles if they had three or more leaves but no inflorescences, and at anthesis if

inflorescences were present. In April 2011, March 2012, and April 2012, I recorded number of leaves, aerial diameter, height, developmental stage (seedling, juvenile, at anthesis), and number of inflorescences present. I also calculated percent survivorship from spring of 2011 to spring of 2012 for treatment and control plots.

I analyzed percent germination using a two-factor ANOVA with the experimental treatment as one factor and individual transects as the other in order to determine if there was a statistically significant influence due to differences in location in addition to the presence or absence of AMF inoculation. I used a separate two-factor ANOVA to analyze percent survivorship from March 2011 to March 2012.

I conducted separate Chi-Squared tests of independence on developmental stage data collected in March and April 2011. Frequencies of plants at each developmental stage were often fewer than 5 per treatment regime, so Fisher's Exact tests were used for data collected in March and April 2012.

For leaf count data collected in March 2011, I used a three-factor ANOVA with treatment and transect crossed and plot nested within transect. For all other data sets, I used separate multifactorial MANOVAs for analysis of growth. Once again, treatment and transect were crossed while plot was nested within transect. Univariate equivalent analyses were conducted when significant results were found in MANOVAs. All statistical analyses were performed using R software version 2.9.2 (R Development Core Team, 2009).

Examination of Naturally Occurring Mycorrhizae

Root samples from associated plants in the area were screened during spring 2011 to determine if a mycorrhizal association already existed in the area. The staining technique I used was implemented following procedures from Vierheilig et al. (1998). The procedure employs a non-toxic stain composed of a mixture of regular fountain pen ink and white household vinegar. However not all brands of ink are functional in this task (Vierheilig et al., 1998). In addition, most ink brands used in the study performed by Vierheilig et al. (1998) are not readily available in the United States. For these reasons, it is recommended that a preassay be conducted with several different inks.

I conducted the preassay using roots of green lentil plants (*Lens culinaris*). The inks used were Speedball Super Black India Ink (waterproof), Bombay Black Ink (waterproof), Shaeffer Black ink (non-waterproof), Higgins Black Ink (waterproof), and Higgins Black Ink (non-waterproof). All were chosen based on their availability in the United States.

I planted 50 green lentil seeds in soil inoculated with the same mycorrhizal formula used to treat seeds of *A. macrocarpa* in the field study. To accomplish this, I placed regular potting soil in planting trays and made indentations for each seed before sprinkling 5 g of inoculant in each cavity. I then placed one green lentil seed in each cavity and covered it with approximately 1 cm of soil. I kept the resulting plants on a day/night cycle of 16h/8h with a constant temperature of 25^0 C and harvested them 8 weeks after sprouting. I rinsed the roots in tap water to remove soil and debris and separated them from the rest of the plant. I cut the samples into segments measuring 2 cm in length and soaked them in water overnight.

I submerged the root segments in boiling aqueous 10% KOH solution until cleared before rinsing them repeatedly with tap water. I equally divided the cleared segments among the five ink treatments and boiled each segment group for 5 min in a 5% ink-vinegar solution comprised of one of five brands of black writing ink (Speedball Super Black India Ink, Bombay Black Ink, Shaeffer Black ink, Higgins Black Ink waterproof, and Higgins Black Ink non-waterproof) and plain white vinegar containing 5% acetic acid. I then rinsed the samples several times with tap water slightly acidified with vinegar. I observed each segment under a compound microscope and scored it for presence/absence of mycorrhizal infection in order to calculate percent infection for each sample group. If there was any visible trace of mycorrhizal infection in a given root segment, I scored it as "present," and if there was no visible infection in a given root segment, I scored it as "absent." I compared percent infection for each of the sample groups to determine if there was a statistically significant difference in the amount of observed root colonization due to ink brand and used a 95% Confidence Interval to calculate error for each sample group. I assessed the usefulness of each ink brand based on the ability to stain AMF and the ability to differentiate between stained fungal cells and the surrounding plant tissue. I used the ink brand determined to be most suitable based on this preassay to test native plants collected from the field site for presence and extent of AMF infection.

Texas sandmint (*Rhododon ciliatus*), silver croton (*Croton argyranthemus*), and the common plantain (*Plantego major*) are plants commonly associated with suitable *A. macrocarpa* habitat (Meredith, 2006). I collected at least 10 samples from each of these three species, and screened their roots for AMF using the aforementioned procedure with

the most suitable ink. I then calculated percent infection and error using a 95% Confidence Interval for each associated plant species and used these results to help determine the degree of AMF colonization already present at the field site excluding experimental treatments.

CHAPTER III

RESULTS

Mycorrhizal Inoculation Experiment

Total germination for control plants was 16.19% while total germination for plants treated with AMF inoculant was 17.63% (Fig. 1). A two-factor ANOVA indicated that there was no significant difference in percent germination due to treatment ($F = 0.2685$, $df = 1$, $P = 0.6137$), but there was a difference due to transects ($F = 7.9785$, $df = 2$, $P = 0.0063$). Transect 1 had mean germination of 13.33%, transect 2 had mean germination of 12.67%, and transect 3 had mean germination of 24.73% (Fig. 2). There was also no significant difference in survivorship between treatments according to a two-factor ANOVA ($F = 0.0863$, $df = 1$, $P = 0.7739$). From March 2011 to March 2012, total control plant survival was 14.67%, and total inoculated plant survival was 17.0% (Fig 3).

Developmental stage was classified as seedling, juvenile, or at anthesis. A Chi-Squared test of independence for developmental stage of plants in March of 2011 revealed that developmental stage of plants was not dependent on treatment ($X^2 = 0.2938$, $df = 1$, $P = 0.5878$) (Fig. 4). Developmental stages in April of 2011 were also independent of treatment ($X^2 = 0.0001$, $df = 1$, $P = 0.9927$) (Fig. 5). Fisher's Exact tests on developmental stage data collected in March ($P = 1.0$) (Fig. 6) and April of 2012 ($P =$

0.7036) (Fig. 7) also revealed no dependence on treatment. Throughout the experiment, only one plant reached anthesis.

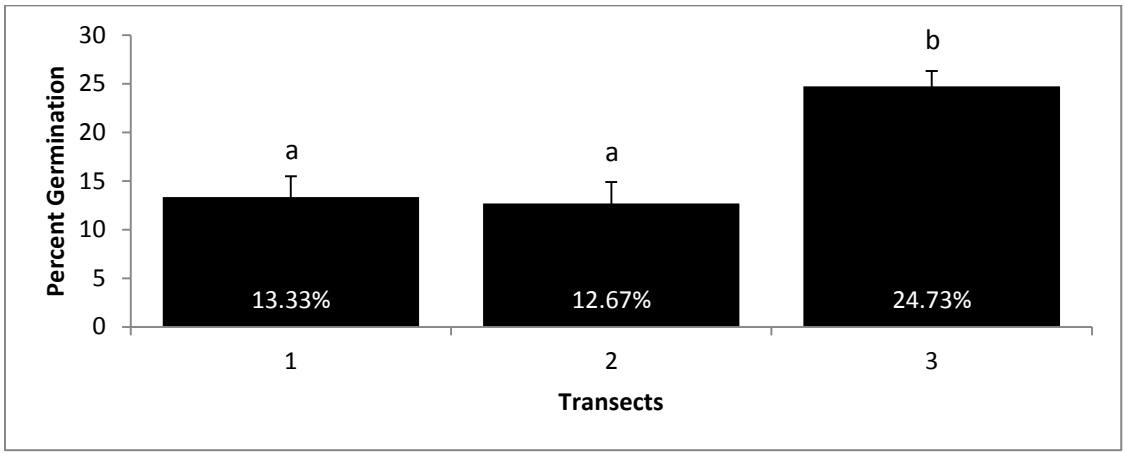

Figure 1. Germination percentages as of March 2011 due to transect. ($F = 7.9785$, $df = 2$, $P = 0.0063$). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

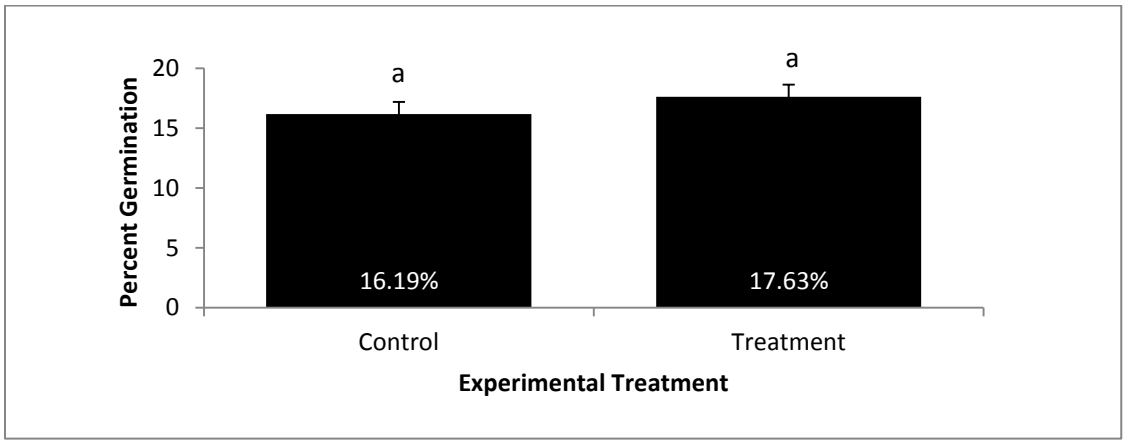

Figure 2. Germination percentages as of March 2011 due to treatment. ($F = 0.2685$, $df = 1$, $P = 0.6137$). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

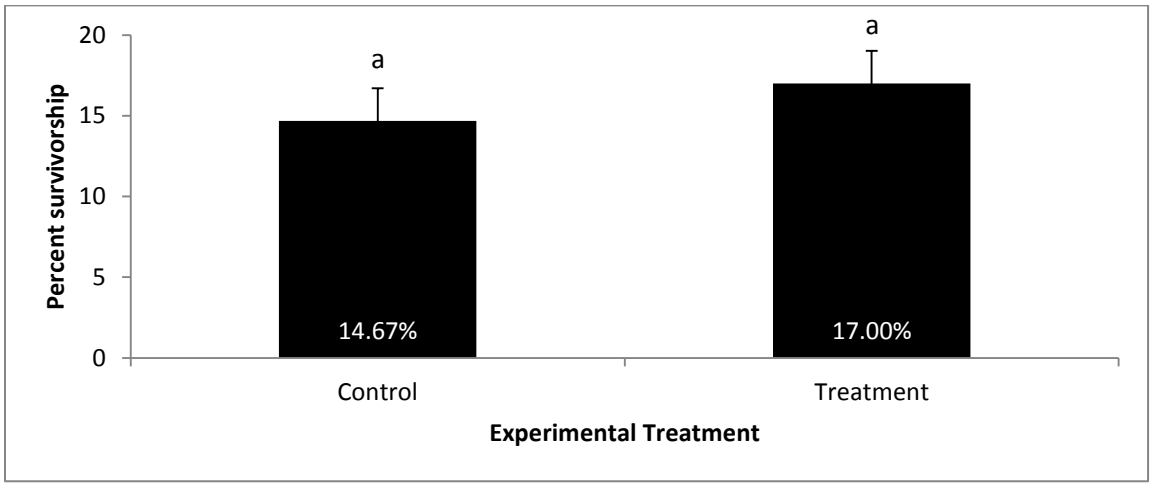

Figure 3. Mean percent survivorship from March of 2011 to March of 2012 for control and treatment categories. Effect due to treatment (F = 0.0863, df = 1, P = 0.7739). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

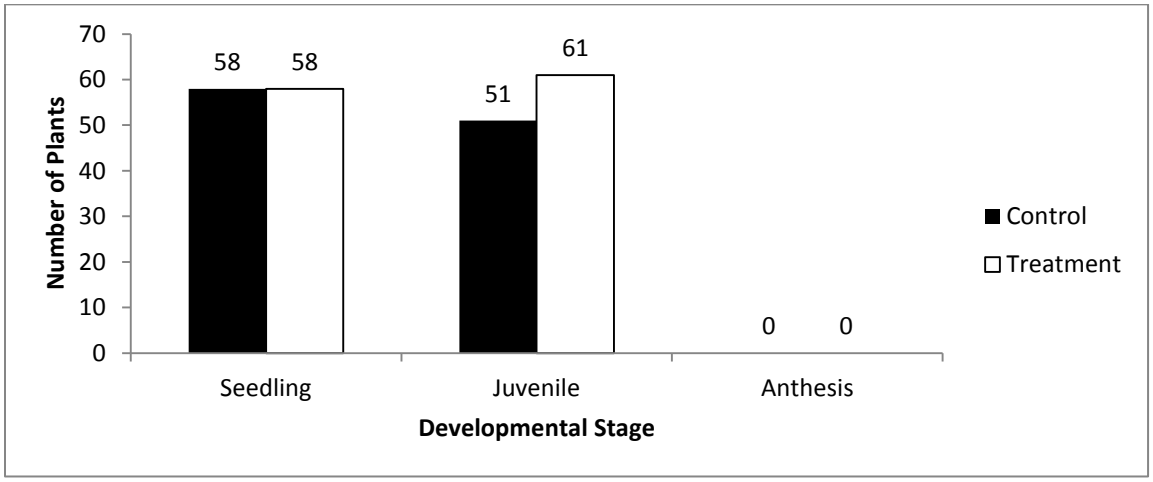

Figure 4. Total number of plants in each developmental stage for each treatment category in March of 2011. (X^2 = 0.2938, df = 1, P = 0.5878).

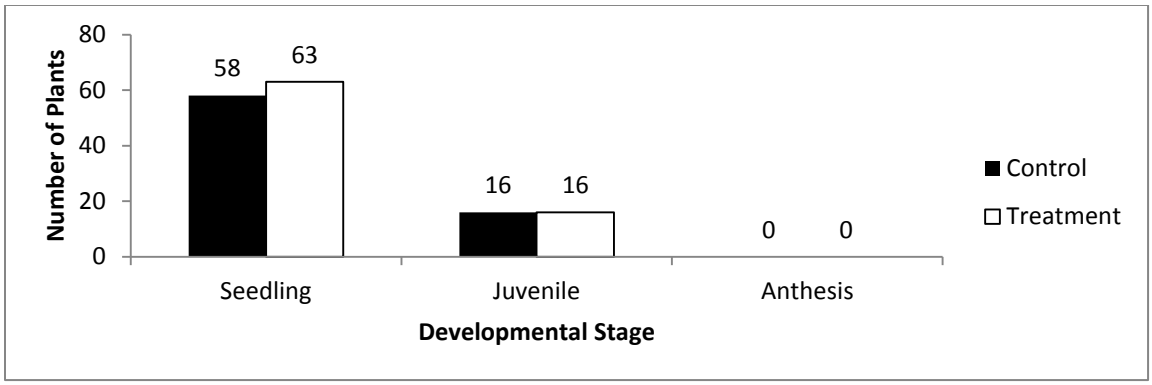

Figure 5. Total number of plants in each developmental stage for each treatment category in April of 2011. ($X^2 = 0.0001$, df = 1, P = 0.9927).

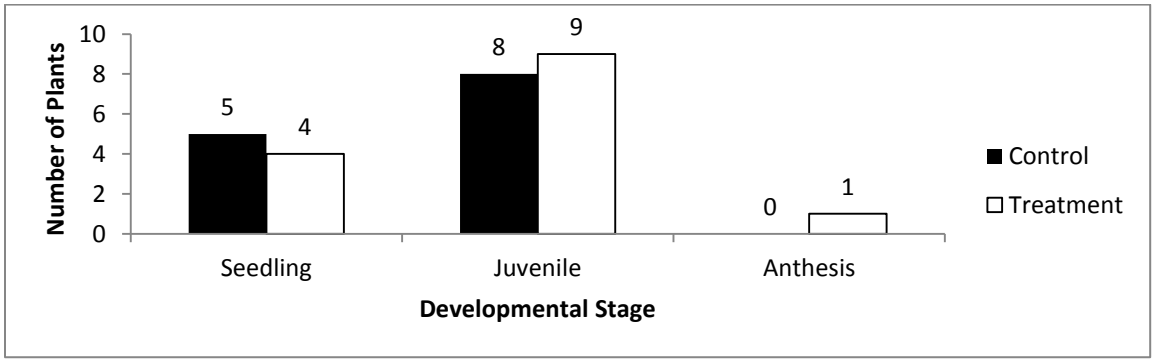

Figure 6. Total number of plants in each developmental stage for each treatment category in March of 2012. (P = 1.0).

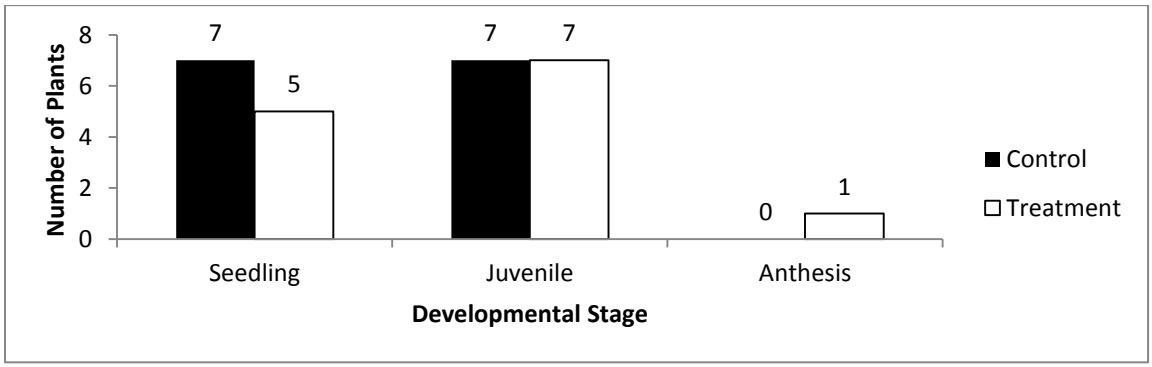

Figure 7. Total number of plants in each developmental stage for each treatment category in April of 2012. (P = 0.7036).

Analysis of leaf count data collected in March 2011 showed that there were significant differences in leaf numbers of plants due to treatment ($F = 7.8896$, $df = 1$, $P = 0.00544$) with inoculated plants having higher leaf counts (Figure 8). Leaf counts also differed significantly among transects ($F = 14.1677$, $df = 2$, $P = 2.8 \times 10^{-13}$) and plots ($F = 2.348$, $df = 14$, $P = 0.00488$). Individuals in transect 1 averaged 3.072 leaves per plant, those in transect 2 averaged 1.765 leaves per plant, and those in transect 3 averaged 2.82 leaves per plant (Figure 9).

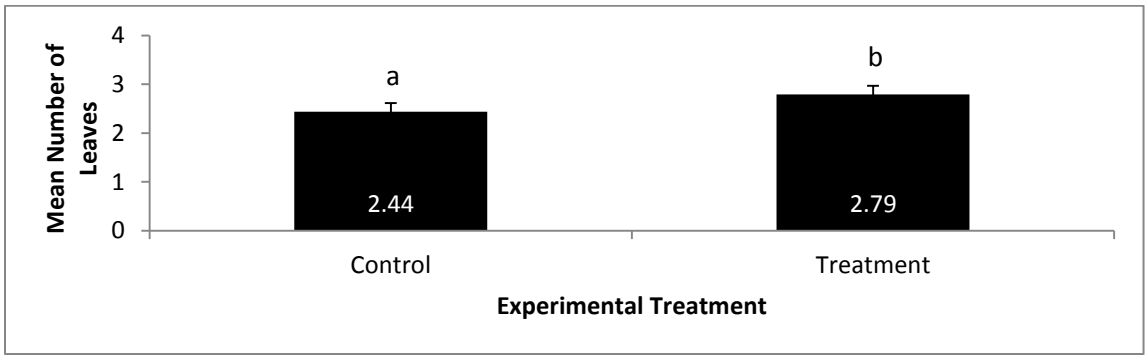

Figure 8. Mean number of leaves per plant in control and treatment plots in March of 2011. ($F = 7.8896$, $df = 1$, $P = 0.00544$). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

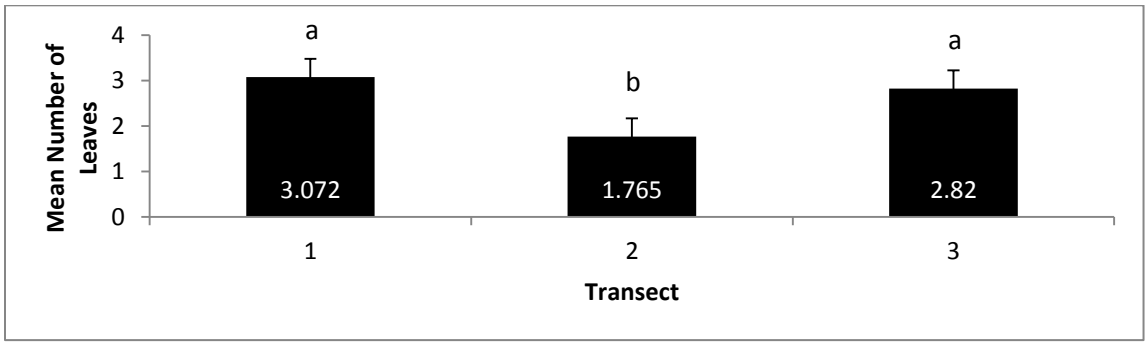

Figure 9. Mean number of leaves per plant in each of the experimental transects in March of 2011. ($F = 14.1677$, $df = 2$, $P = 2.8 \times 10^{-13}$). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

MANOVA analysis of growth data collected in April 2011 showed significant effects due to treatment (Pillai Trace = 0.0766, P = 0.0048), transect (Pillai Trace = 0.1877, P = 2.287×10^{-5}), and plot (Pillai Trace = 0.2953, P = 0.021). I therefore conducted equivalent ANOVAs for both leaf count and aerial diameter of plants. These revealed that leaf count was not significantly influenced by treatment (F = 0.0006, df = 1, P = 0.9811) but was significantly influenced by transect (F = 4.224, df = 2, P = 0.014), while aerial diameters of plants were significantly influenced by treatment (F = 5.707, df = 1, P = 0.018) and plot (F = 2.0915, df = 14, P = 0.0158), with inoculated plants having larger aerial diameters (Figs. 10, 11, 12).

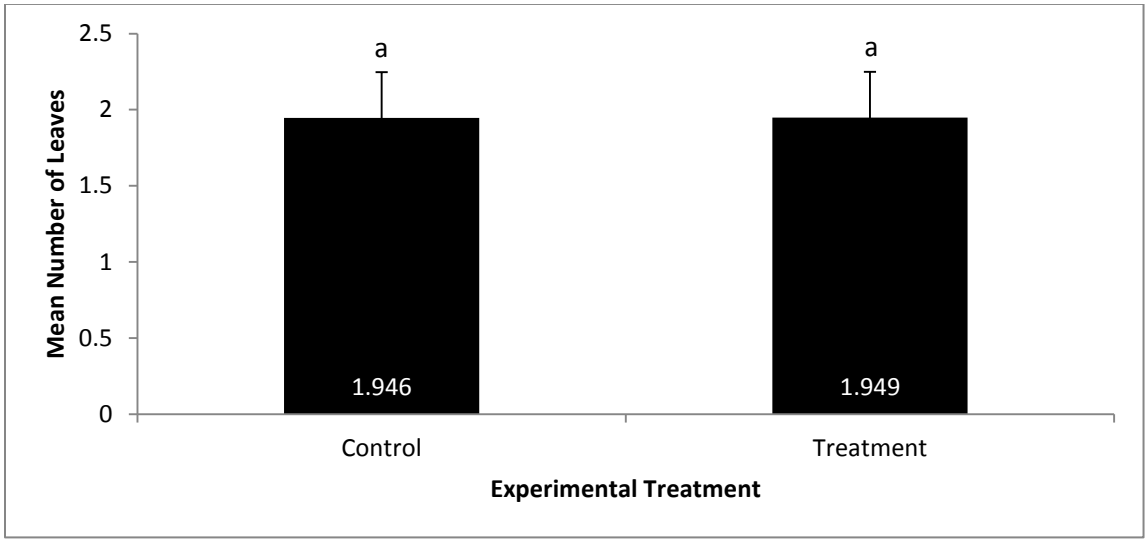

Figure 10. Mean number of leaves per plant in control and treatment plots in April of 2011. (F = 0.0006, df = 1, P = 0.9811). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

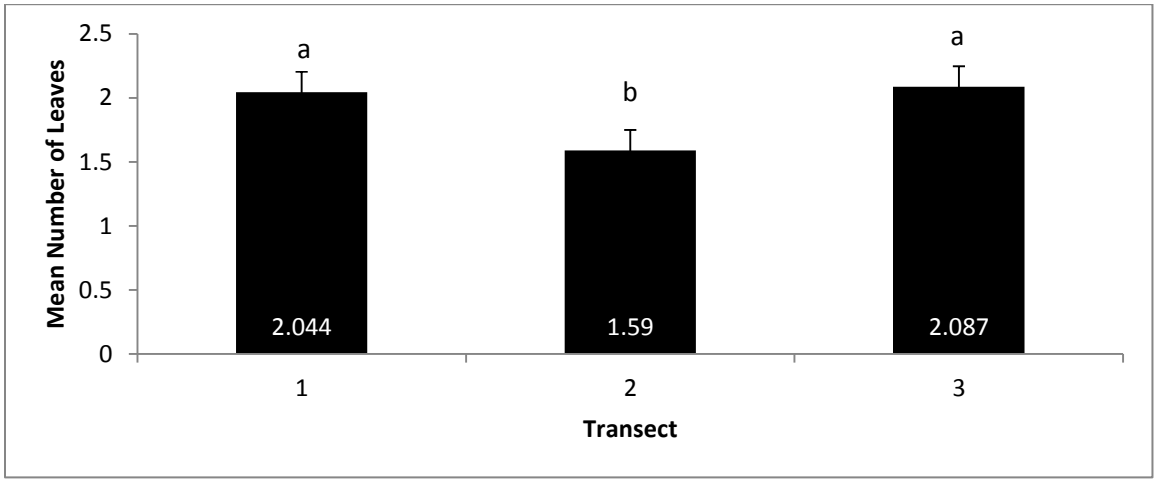

Figure 11. Mean number of leaves per plant in each of the experimental transects in April of 2011. ($F = 4.224$, $df = 2$, $P = 0.014$). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

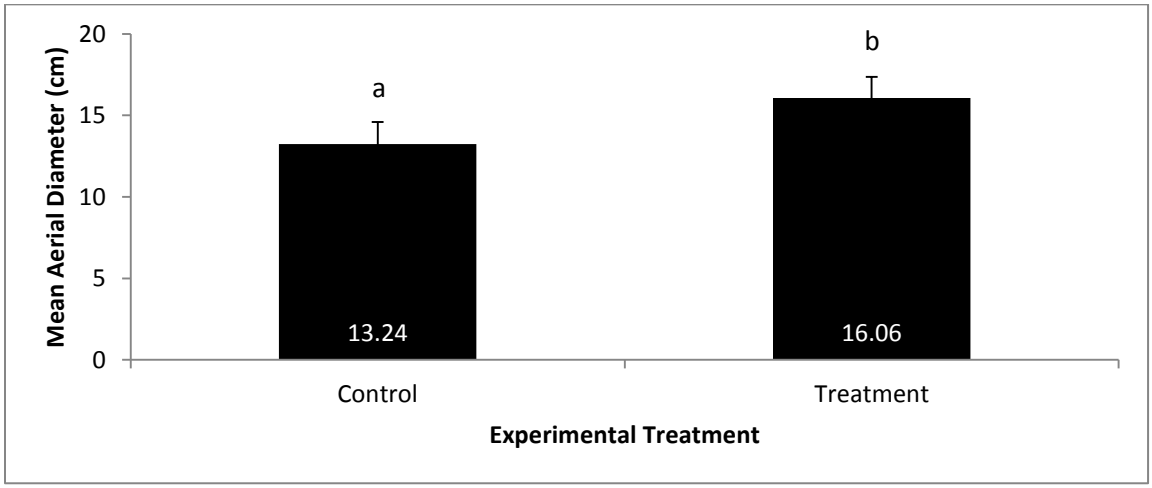

Figure 12. Mean aerial diameter of plants in control and treatment plots in April of 2011. ($F = 5.707$, $df = 1$, $P = 0.018$). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

Analysis of growth data collected in March 2012 showed that leaf count (Fig. 13), aerial diameter of plants (Fig. 14), plant height (Fig. 15), and number of inflorescences were all not significantly different between plants in treatment and control plots (Pillai Trace = 0.32804, P = 0.36), among transects (Pillai Trace = 0.77032, P = 0.149), or among plot (Pillai Trace = 1.47386, P = 0.817). Since there was only one plant to reach anthesis, the experiment-wide total number of inflorescences was 2. Growth data collected in April 2012 also yielded insignificant differences due to treatment (Pillai Trace = 0.24193, P = 0.51), transect (Pillai Trace = 0.78733, P = 0.099), and plot (Pillai Trace = 1.41595, P = 0.69) (Figs. 16, 17, 18).

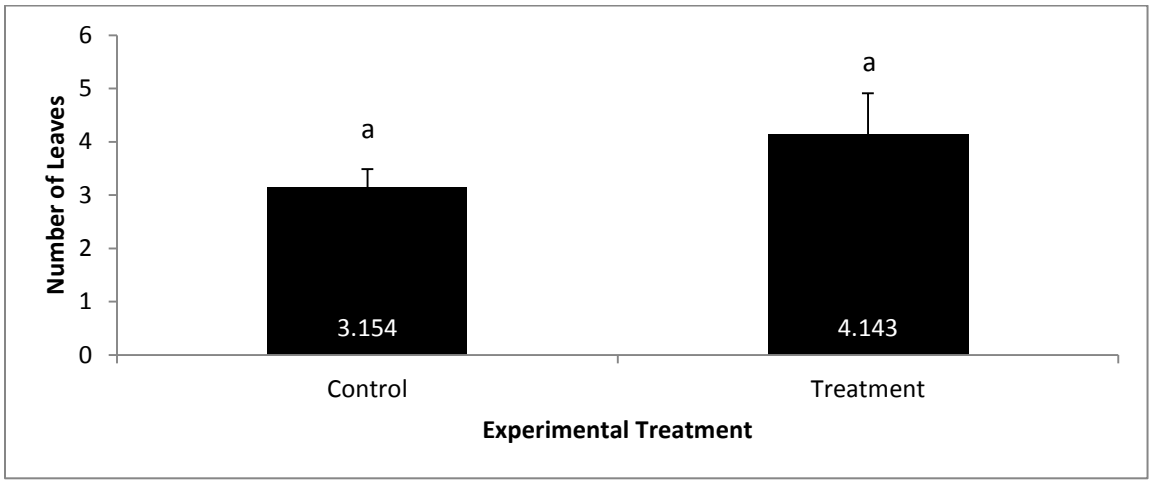

Figure 13. Mean number of leaves per plant in control and treatment plots in March of 2012. (Pillai Trace = 0.32804, P = 0.36). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

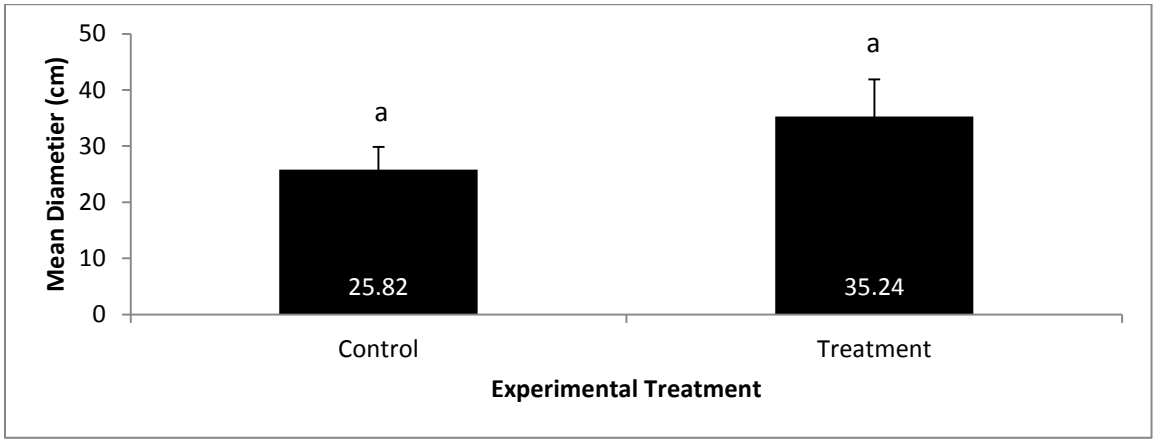

Figure 14. Mean aerial diameter of plants in control and treatment plots in March of 2012. (Pillai Trace = 0.32804, P = 0.36). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

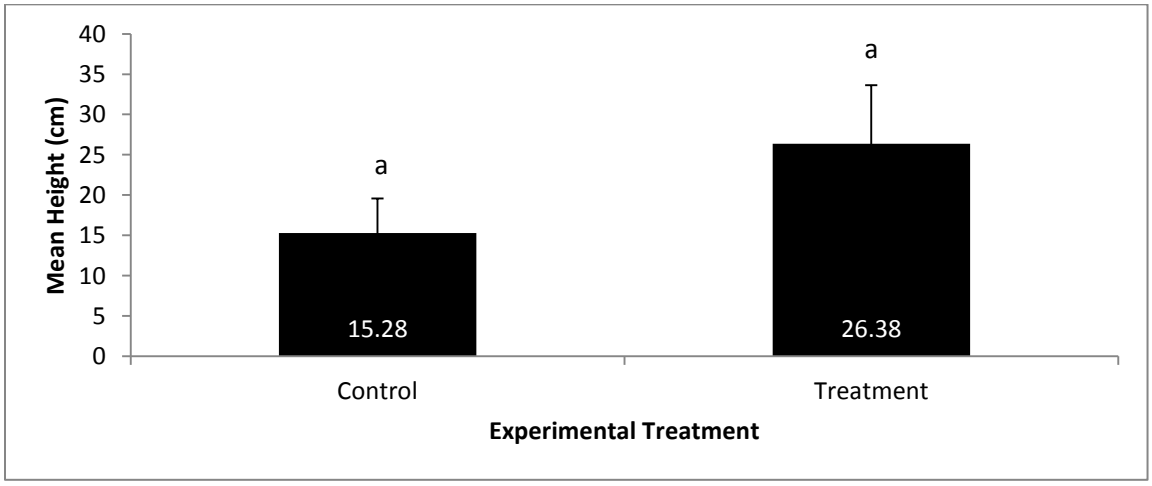

Figure 15. Mean height of plants in control and treatment plots in March of 2012. (Pillai Trace = 0.32804, P = 0.36). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

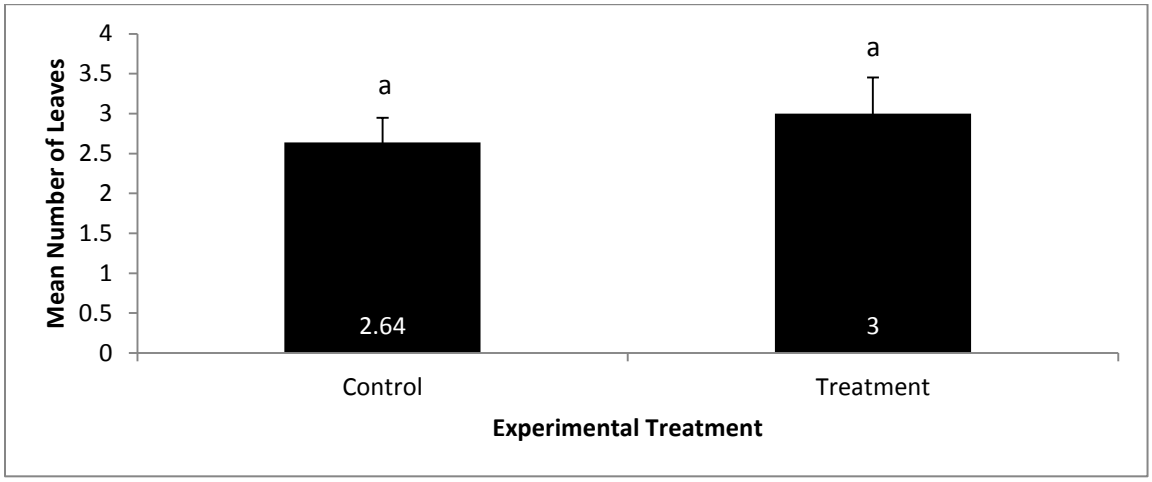

Figure 16. Mean number of leaves per plant in control and treatment plots in April of 2012. (Pillai Trace = 0.24193, P = 0.51). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

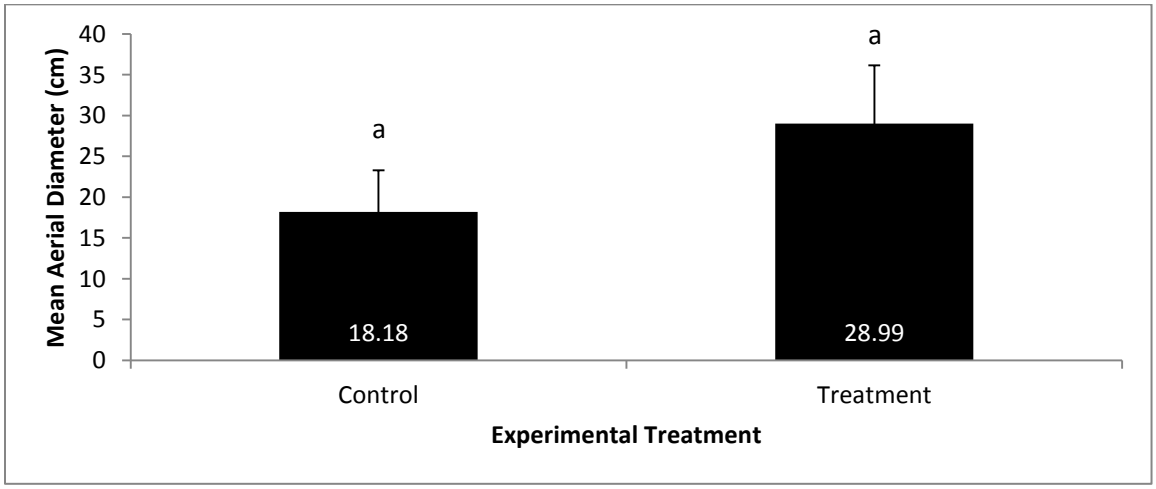

Figure 17. Mean aerial diameter of plants in control and treatment plots in April of 2012. (Pillai Trace = 0.24193, P = 0.51). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

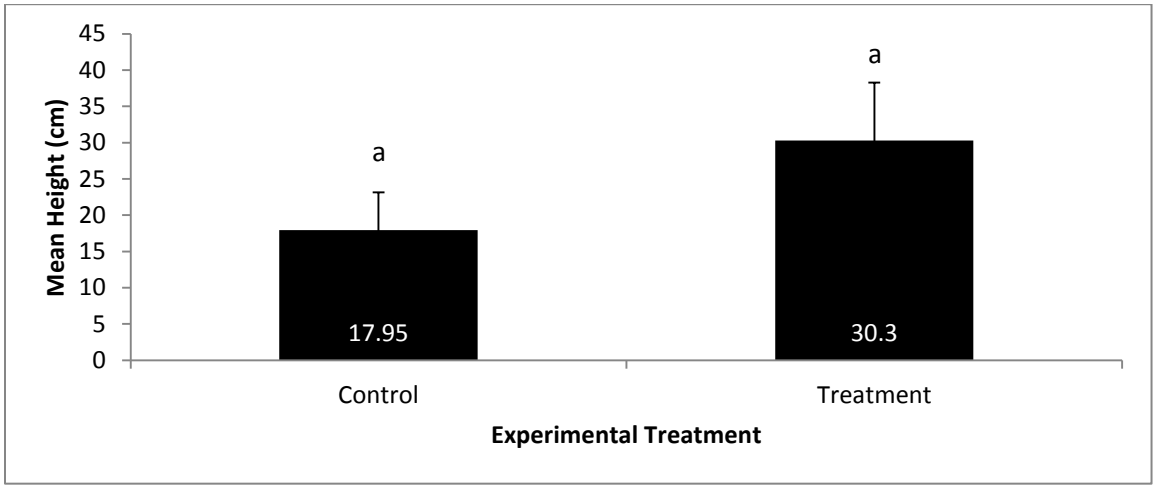

Figure 18. Mean height of plants in control and treatment plots in April of 2012. (Pillai Trace = 0.24193, P = 0.51). Error bars represent percent ± 1 SE. Letters above bars are an indicator of significance. Bars with the same letters are not significantly different, and bars with different letters are significantly different.

Although there were no statistically significant differences in growth between control and treatment plots in the second year of growth, plants in treatment plots were still larger than those in control plots. Mean aerial diameter and height of plants were generally greater for plants in treatment plots, but these differences were not captured by statistical analysis. This was most likely due to low numbers of surviving plants in the second year. There were only 14 surviving treatment plants and 13 surviving control plants in 2012. The low sample sizes for this year increased the possibility of Type I error beyond a threshold where differences could be found significant.

To summarize, results showed that treatment with AMF inoculation has a positive effect on growth of *Aboronia macrocarpa* following germination. Since there were also

significant effects on plant growth due to transect and plot, results indicate that *A. macrocarpa* is extremely sensitive to variations in microhabitat. Inoculation had no significant effect on germination or survivorship and no effect on developmental stage in either year. Although there was greater growth shown from plants in treatment plots in the second year, these differences were not significant.

Examination of Naturally Occurring Mycorrhizae

Speedball Super Black India Ink (waterproof), Bombay Black Ink (waterproof), and Higgins Black Ink (waterproof) each immediately precipitated out of solution once vinegar was added. I therefore determined that none of these inks were suitable for use in this staining procedure. Higgins Black Ink (non-waterproof) and Shaeffer Black ink (non-waterproof) both stayed soluble in the vinegar solution and were suitable for staining.

Root segments stained in the Higgins (non-waterproof) solution had moderate contrast and appeared dark and slightly blurry under magnification (Fig. 19a). Fungal structures were stained dark black, while the surrounding root tissue was comparatively more translucent, but difficult to see through. This stain combination appeared to be highly soluble and kept bleeding out of root samples, darkening the water even after being stored for weeks and rinsed again. This made working with the root samples slightly more difficult, but had no effect on my ability to detect fungal structures under microscopic observation.

The degree of contrast with Shaeffer ink (non-waterproof) was excellent (Fig. 19b). Roots stained in this solution remained clear while fungal structures were stained a

reddish brown or occasionally a bright blue. This result differs from those obtained by Vierheilig et al. (1998) in which AMF structures appeared black after staining with Shaeffer black ink (non-waterproof).

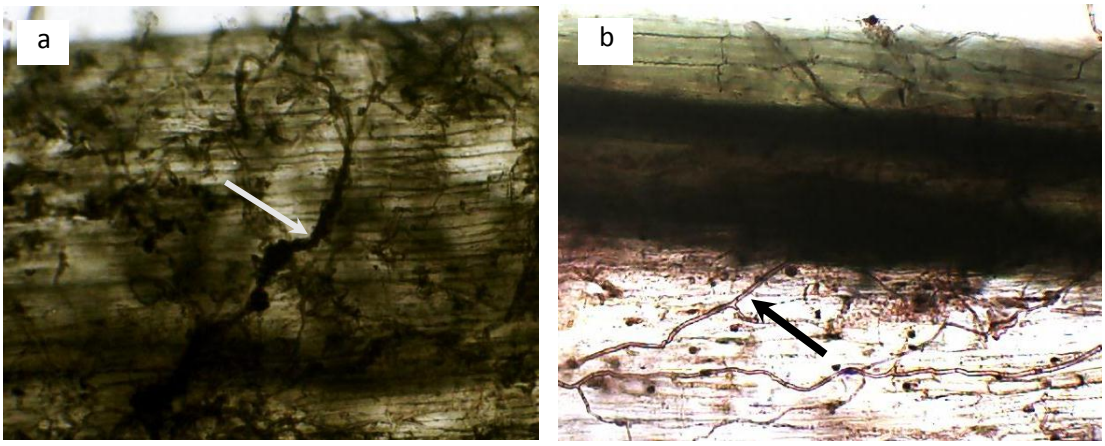

Figure 19. Lentil root samples with observable AMF colonization . Micrographs taken at 10x magnification after staining with either (a) Higgins black ink (non-waterproof) or (b) Shaeffer black ink (non-waterproof). Arrows indicate fungal structures within the root tissue.

Infection in samples stained with Higgins black ink (non-waterproof) was 90% (± 3.84%) and infection in samples stained with Shaeffer black ink (non-waterproof) was 96% (± 5.88%). This indicates no difference in the utility of these ink brands for the purposes of detecting AMF infections. However, the clarity and degree of contrast were better with Shaeffer brand ink (non-waterproof). Since Shaeffer ink (non-waterproof) provides better staining, it was used for staining the root samples collected from the experimental site.

AMF infection rates were 66.3% (± 6.94%) in *Plantego* sp., 60.1% (± 7.19%) in *Rhododon ciliatus,* and 46.5% (± 11.6%) in *Croton argyranthemus* (Figure 20). This indicates that there is a relatively strong preexisting mycorrhizal component to the community structure at the experimental field site.

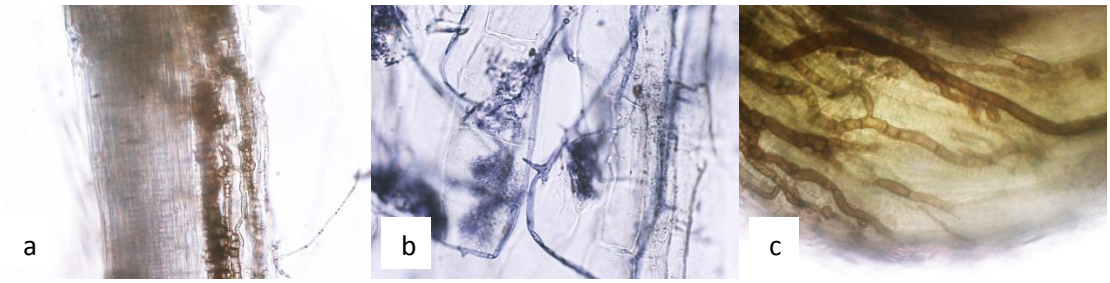

Figure 20. Naturally occurring mycorrhizae after staining with Shaeffer black ink (non-waterproof). (a) *Croton argyranthemus* roots at 40x magnification. Dark brown areas indicate fungal structures. (b) *Plantego* sp. roots at 40x magnification. Bright blue areas indicate fungal structures. (c) *Rhododon ciliatus* roots at 40x magnification. Dark brown areas indicate fungal structures.

CHAPTER IV

DISCUSSION

The importance of arbuscular mycorrhizal fungi for improving the fitness of vascular plants has been well-known for decades (Allen, 1991; Smith and Read, 1997). Endangered and threatened plant species may be able to benefit from this association if AMF inoculations are utilized in their recovery plans. A study of multiple endangered Hawaiian plants revealed that individuals inoculated with AMF were generally larger and had higher survivorship than control plants (Gemma and Koske, 1995). A separate study of four more endemic Hawaiian plants, two of which are endangered, yielded similar results, showing that inoculated plants had higher P levels in their tissues, had higher root biomass, and had shoots that were up to seven times larger than plants that were not inoculated (Gemma et al., 2001). In another study, two endangered plant species located in south Florida, *Jacquemontia reclinata* and *Amorpha crenulata*, were both reported to have crucial relationships with AMF (Fisher and Jayachandran, 2002). These species displayed fitness benefits such as increased seedling growth and greater P uptake when inoculated with mycorrhizal fungi (Fisher and Jayachandran, 2002). The endangered plants *Pulsatilla slavica* and *Plantago atrata*, as well as the ecologically extinct *Senecio umbrosus* all had positive responses to AMF inoculation (Zubek et al., 2009). In this study the inoculated specimens displayed "increased efficiency for energy conservation

and increased stability" as well as enhanced growth, photosynthetic activity, and nutrient content (Zubek et al., 2009, p. 121).

In more extreme cases, a species can be completely dependent on mycorrhizal associations and cannot survive without them. *Astragalis applegatei*, another endangered species, is able to survive in artificial or sterilized substrates only when inoculated with soil containing AMF (Barroetavena et al., 1998). Cases such as this illustrate how critical it can be to have knowledge on the mycorrhizal status of a plant species of concern when developing conservation strategies.

In addition to increasing nutrient uptake in deficient soils, mycorrhizae have the ability to reduce the threat of toxic ions in saline and alkaline soils (Zhang et al., 2011). Specifically, the increased P uptake provided by AMF colonization can mitigate the negative effects of salinity by equalizing concentrations of Na and Cl ions (Zhang et al., 2011).

There was an observable effect on growth and germination of *A. macrocarpa* in 2011. However, significant differences in percent germination were due to transect, not inoculation. This could have been due to subtle differences in moisture levels, soil nutrient availability, preexisting microbiota assemblages, or other uncontrolled environmental factors. Although I attempted to establish three transects that were as uniform as possible by placing them within several meters of one another and by placing them parallel to one another, the subtle differences that existed between them were enough to produce differences in germination success. There were no observable differences in survivorship from March 2011 to March 2012 due to treatment or transect. Growth was improved due to inoculation. Leaf counts on inoculated plants were higher

than control plants in March and April of 2011, suggesting an advantage in photosynthetic potential for these individuals. This result is in agreement with other studies such as Zubeck et al. (2009) in which inoculated plants showed increased efficiency for photosynthesis. Aerial diameters of inoculated plants in April of 2011 were also greater than control plants, once again suggesting increased photosynthetic capability due to higher surface area. These advantages in size and photosynthetic capabilities in the first growth season could provide more energy for plants to establish robust taproot systems early in life.

The three counties where *A. macrocarpa* is known to occur have an average annual precipitation between 96.5 and 99.8 centimeters (Natural Fibers Information Center 1987), but the past few years have been unusually dry. Throughout 2011, Freestone County received only 80.264 centimeters of precipitation (National Climatic Data Center, 2012a). During March of 2011, various parts of Freestone County were simultaneously at drought intensities of D1 (moderate), D2 (severe), and D3 (extreme), progressing to D3 for the entire county by the end of April (National Climatic Data Center, 2012b). Leon and Robertson counties were already in D3 at the beginning of March 2011 and had progressed to D4 (exceptional) by the end of April 2011 (National Climatic Data Center, 2012b). Since both plants and fungi are highly moisture-dependent, the lack of rainfall may have impacted growth and survivorship as well as prevented thorough AMF colonization from the inoculant. In fact, there is evidence that mycorrhizal fungi have the potential to become parasitic to their host plants when environmental conditions are stressful and the cost of symbiosis becomes too great (Johnson et al., 1997; Karst et al., 2008). Had the amount of precipitation at the

experimental site more closely reflected normal patterns throughout the course of this experiment, there may have been more of an effect due to AMF inoculation, and the observed effects may have been significant in the second year of growth. Furthermore, there were already mycorrhizal fungi present in the habitat preceding the experiment, so the addition of more may have had little to no effect.

In the second year of growth (2012), there were no longer any statistically significant benefits in growth due to inoculation. If there had been higher survivorship in this study, differences between growth of plants in treatment and control plots might have been detected by the statistical analyses. Since sample sizes were so low in the second year, it would have been very difficult to find significant results.

No difference in survivorship was observed between control and treatment plots either. Once again, this may have been due to unfavorable environmental conditions. However, this phenomenon is evidently not unheard of in areas where mycorrhizae are already present. A study evaluating the effectiveness of AMF inoculations for the restoration of roadside prairies found that although there were colonization benefits in the first growth season, there was no difference between inoculated and control plots after 27 months (White et al., 2008). Inoculant from the treatment plots, or the preexisting mycorrhizae in the area, could have spread to control plots by that time. In other words, the inoculant may have served as a jump-start for colonization, but the naturally occurring mycorrhizae in the area would have colonized control plants eventually. Inoculation may have only sped up the process, which may have been the case in this study as well.

Observable benefits provided by mycorrhizal symbiosis vary greatly based on fungal species, plant species, and habitat types (Smith and Read, 1997). They can even vary among members of the same genus (Sigueira and Saggin-Junior, 2001). Generalizations concerning AMF dependency should therefore be avoided, and each species of concern should be evaluated individually. Large-scale inoculations can be time consuming and costly, so some authors advise that mycorrhizal inoculations be utilized only when existing AMF colonization is lacking or when soil nutrient levels are low (White et al., 2008).

This study has shown that AMF inoculation has the potential to improve growth in *A. macrocarpa*, especially in the first year, suggesting that mycorrhizal inoculation could be a desirable part of a reintroduction plant for this species in the future. Further evaluation is necessary to determine the utility of inoculations in the context of reintroduction procedures. In the coming years, I would recommend research that investigates the effectiveness of different inoculation methods with *A. macrocarpa*, such as trenching, drilling, broadcasting, or even the use of different mycorrhizal species. I would also recommend research to explore how long there is an observable benefit to mycorrhizal inoculation for various plant species in field conditions. Finally, I would like to see studies that determine if *A. macrocarpa* is ecologically dependent upon mycorrhizae, or if they are negatively affected by being planted in soils that have been treated with fungicide or sterilized.

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VITA

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