SEASONAL INTERACTIONS AND LOCAL ADAPTATION BETWEEN THE PLANT, GERANIUM MACULATUM, AND ARBUSCULAR MYCORRHIZAL FUNGAL SYMBIONTS

By

BRYAN QUIGLEY

(Under the Direction of Shu-mei Chang)

ABSTRACT

We used the gynodioecious study system of *Geranium maculatum* to investigate the intricacies of the complex relationship between plants and arbuscular mycorrhizal fungi (AMF). AMF form associations with over 80% of plant species, and little is known about their effects on the different sexes (female and hermaphrodite) of the gynodioecious systems. We took field colonization measurements during important plant phases to examine yearly fluctuations in association. We also conducted a common garden study to determine if there is adaptation between plants and their local AMF communities. We found evidence of varying colonization levels, coinciding with reproduction and vegetative phases. We also found some evidence of local adaptation and AMF community affects on flowering and leaf production. Better understanding this symbiotic system could lead to applications within the agriculture industry, using AMF mutualisms to enhance gynodioecious crop development, as well as contributing to the knowledge of symbiotic evolutionary relationships.

INDEX WORDS: Gynodioecious, Symbiosis, Local adaptation, Arbuscular mycorrhizal fungi, Sex, Common garden

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CHAPTER 1

INTRODUCTION

Symbiosis with arbuscular mycorrhizal fungi (AMF) is one of the most prevalent associations in the plant kingdom, as they associate with, and typically provide benefit for, over 80% of all plant species (Koide 1991; Allen 1996; Gange and Ayres 1999; Smith et al. 2010; Johnson et al. 2012). These AMF species form mycorrhizal relationships with a plant's root, colonizing it with hyphae, and exchanging nutrients with the host plant through specialized hyphal structures called arbuscules (Mosse and Hepper 1975; Allen 1996; Gange and Ayres 1999). Although AMF can consume up to 20% of the carbohydrates produced via photosynthesis, the plant benefits from the AMF hyphae's greater surface area to soil ratio, acquiring phosphorus and other limiting nutrients from the AMF that, in turn, are better at acquiring these nutrients from the surrounding environment.

This increase in uptake of essential nutrients that occurs with AMF symbiosis has shown to benefit the plant by enhancing growth and reproductive output. Many studies have quantified this increase in plant growth (McGonigle and Fitter 1990; Koide 1991; Johnson et al. 1997; Gange and Ayres 1999; Wilson et al. 2001; Gamper et al. 2005; Mortimer et al. 2005; Castillo et al. 2009; Johnson et al. 2010; Smith et al. 2010; Conversa et al. 2013; Willis et al. 2013), yet the majority of research has been conducted on hermaphroditic plants under artificial conditions. Reproductive benefit can be realized through two avenues: increasing male output or increasing female output. Association with AMF has shown to increase phosphorus content in pollen, enhance pollen size, and increase pollen production (Poulton et al. 2001a; Poulton et al. 2002).

Other effects include increased nitrogen and phosphorus content in seeds coupled with greater seed production (Lu and Koide 1991; Lu and Koide 1994). These responses can be combined in hermaphroditic species to increase reproduction, but in species with dimorphic sexes greater benefit to one sex versus the other may shift the population dynamic, potentially leading plants to develop sex-specific adaptations with AMF.

Gynodioecy, where individual plants may be either female with sterile anthers or hermaphroditic, is one of the most common sexual dimorphisms in plants, representing 7% of all species (Wilcock 1987). Female individuals are constantly at a reproductive disadvantage compared to hermaphrodites, as they can only pass their genetic material through one avenue, ovules, while hermaphrodites have both pollen and ovules to transfer genes. It has been proposed that to persist in a population, females must produce twice as many seeds as hermaphrodites (Lloyd 1976; Charlesworth and Charlesworth 1978; Charlesworth 1981; Delph and Wolf 2005; Chang 2006). There is evidence to support this theory in the gynodioecious species, *Geranium maculatum*, as females typically produce more seeds than hermaphrodites over their lifetime, however the mechanism behind this increased female reproduction is unknown (Chang 2006, 2007). A gynodioecious European congener, *G. sylvaticum*, benefits from AMF association differently between sexes: without AMF, female growth was greater than hermaphrodites, and with AMF inoculation female plants exhibited increased nutrient acquisition compared to hermaphrodites (Varga and Kytoviita 2010b). This information provides indications for a potential mechanism behind female compensation; however it is important to explore this phenomenon further with closely related species.

There has been a recent call for more field AMF observations and studies done within natural populations (Smith et al. 2010), and in that vein we proposed to examine the natural association

of *Geranium maculatum* with AMF. We predicted that our studies would provide evidence that AMF association naturally varies depending on season and sex, and that this varied association has lead to plant adaptation to their local arbuscular mycorrhizal fungi communities. To explore these theories we conducted two field experiments, one looking at natural populations and the other creating artificial gardens in the field. For our observational field study we measured plant morphological traits and quantified AMF colonization rates on plant roots during plant growth phases. Roots were sampled during vegetative, reproductive and dormant seasons of the perennial's life from 2011 to 2013. Noticing differences in association between populations, we became interested in testing for local adaptation between plants and their home fungal suites. To examine this, we collected plants from three geographically diverse populations of *G. maculatum* in the Southeastern United States, and planted them in common gardens with one of four fungal treatments; home AMF, one of two away AMF, and no-AMF. We hope that information from these studies will help better our understanding of how AMF association affects natural populations of *G. maculatum*, and yield broad scale implications for the adaptation of gynodioecious species in the wild.

CHAPTER 2

ASSOCIATIONS BETWEEN A GYNODIOECIOUS PLANT, *GERANIUM MACULATUM*, AND ARBUSCULAR MYCORRHIZAL FUNGI DURING IMPORTANT PLANT GROWTH PHASES IN NATURAL POPULATIONS

Introduction

Over 80% of all plant species form a symbiotic relationship with arbuscular mycorrhizal fungi (AMF) (Koide 1991; Allen 1996; Gange and Ayres 1999; Smith et al. 2010). These fungi infect a host plant's roots with hyphae that facilitate the plant's uptake of phosphorus and nitrogen through structures called arbuscules, in exchange for plant photosynthates (Koide 1991; Allen 1996; Smith et al. 2010; Willis et al. 2013). This association has been shown to enhance plant growth (Gange and Ayres 1999; Wilson et al. 2001; Castillo et al. 2009; Sherrard and Maherali 2012), increase reproductive output (Stanley et al. 1993; Pendleton 2000; Wilson et al. 2001; but see Varga and Kytoviita 2010b; Conversa et al. 2013) and increase plant resistance to herbivory and pathogens (Al-Whaibi 2009; Garrido et al. 2010). The general effects of mycorrhizal symbiosis on plants has been widely examined in the laboratory setting, showing increased concentrations of N and P in seeds (Lu and Koide 1994), greater seed production and size (Lu and Koide 1991), as well as greater pollen size, production, and P content (Poulton et al. 2001aa; Poulton et al. 2002). However, this relationship has been shown to be highly dynamic, and not always wholly beneficial.

Though arbuscular mycorrhizal fungi are often seen as beneficial for the plant, recently there has been evidence showing the contrary: AMF treatments can yield smaller plants than control treatments (Johnson et al. 1997; reviewed in Smith et al. 2009; Varga 2010). An explanation for this detrimental effect may be that the fungus is limiting the plants growth due to its high carbon needs. Percent root colonization by fungi typically exhibits a curvilinear relationship with its host's fitness, improving fitness up to a certain colonization rate, but often decreasing the productivity of its host beyond this threshold (Gange and Ayres 1999). AMF have been shown to require up to 20% of the photosynthates produced by the plant (Pfeffer et al. 1999), providing a potential explanation for this observed decrease in growth. However, Smith et al. (2010) propose that this widely accepted view may be incorrect, and that the reduction in growth may not be the overabundance and carbon demands of AMF on the root structures but the lack of AMF colonization and consequently ineffectiveness of nutrient transfer in certain individuals. Another potential explanation is that plant genotypes within a species may have profound, and yet incompletely recognized in the literature, effects on the symbiosis.

Extensive work has been published on the general effects of AMF on plant growth (Koide 1991; Sudova and Vosatka 2008; Siddiqui and Akhtar 2009), yet there is little evidence of how dynamic the levels of association are throughout natural growing seasons (noteworthy exceptions Kennedy et al. 2002; Titus et al. 2002; and Apple et al. 2005). These variations can have significant effects on the plant depending on what life phase it is in, as certain macronutrients, such as phosphorus, are in greater demand during reproduction (Poulton et al. 2001a, b; Poulton et al. 2002). A plant's nutritional needs vary throughout the growing season (Korkmaz et al. 2012) as do many of the ecological factors, for example precipitation (Al-Whaibi 2009; Hawkes et al. 2011), that are known to affect association .

Interactions between organisms in the wild shape the evolution of species, and much can be inferred about the future of a symbiosis through *in situ* observations. Because of the ubiquitous nature of AMF symbioses, it is important to quantify this relationship at not only a plant species level, but also on the population and genotype levels. Communities of fungi can vary drastically depending on ecological and geographic location (Morton et al. 1995; Smith and Smith 2011; Brundrett and Ashwath 2013; Velazquez et al. 2013), but alongside these larger variances, it is important to examine the small scale variation occurring within plant populations. Examining plant sex in *gynodioecious* systems will provide a subtle, but potentially compelling heterogeneity in a population. The physical differences between plant sexes can correlate with nutritional needs, and directly affect the relationship between the plants and AMF. To examine this idea further we ask the question: **How does AMF association vary across important plant growth phases, and across genders?** We hypothesize that 1) Colonization rates of important AMF structures will vary throughout the year, coinciding with key *G. maculatum* growth phases, and 2) Female and hermaphroditic individuals will differ in AMF colonization.

Methods

Study Species

Geranium maculatum (Spotted Geranium, Geraniaceae) is a perennial, rhizomatous herb common to the eastern half of North America, ranging from Quebec to Florida (USDA 2013). Basal leaves emerge from underground rhizomes in mid-February and flowers are produced on forked stalks from mid-March to early June (Chang 2006) in Georgia, USA. Flower buds are formed in the previous season and lie dormant over winter. The plants are gynodioecious, producing either female flowers with reduced, sterile anthers or larger, protandrous

hermaphroditic flowers. Sex ratios in *G. maculatum* populations in Georgia vary from all hermaphrodites to ~50:50 female:hermaphrodite (Chang 2006).

Population Sampling

To obtain a diverse sampling of *G. maculatum's* southeastern range, a total of nine populations (North Carolina: AZ, TP, TC, Georgia: OT, WT, HP, BH, MP, RL nine in 2011 and six in 2012-13; Figure 1.1) were collected at locations ranging from Northeast Georgia to South West North Carolina. Populations were chosen on availability of flowering individuals (as it is only possible to determine sex of a plant while it is flowering), and only six populations were used in 2012-13 due to a lack of flowering individuals.

During March/April 2011 plants were visually identified in the field as either females or hermaphrodites. Ten female and ten hermaphrodite individuals were chosen haphazardly throughout each of the nine populations (except for AZ where there were only four females flowering at the time of collection) and labeled with small plastic flags. We then collected roots during plant dormancy from each of these individuals in July 2011 (D-'11). For 2012-13, we labeled forty female and forty hermaphrodite plants throughout each of six populations (BH, OT, AZ, WT, HP, TP) in March/April 2012 and collected roots from ten female and ten hermaphrodite individuals in each of the four subsequent sampling rounds (Rounds 4-7). Round 4 (Fl-'12) roots were collected during flowering, Round 5 (Fr-'12) roots at the end of fruit production, Round 6 (V1-'12) roots as the plants were going dormant in July 2012, and Round 7 (V2-'13) roots during leaf emergence in February 2013. Round 8 (Fl-'13) roots was comprised of an additional 10 female and 10 hermaphrodite plants that were identified and roots collected during flowering in late March 2013 (Figure 1.2). We divided the collection rounds a priori

based on the growth phases of the plants into Dormant (D, Round 2), Vegetative post flowering (V1) and pre flowering (V2) (Rounds 6 and 7) and Reproductive, including flowering (Fl) and fruiting (Fr) (Rounds 4, 5 and 8) categories.

Root Collection

To examine the natural mycorrhizal relationship occurring in the field, we sampled roots by first unearthing the rhizomes by hand or with a shovel and removed a mix of new, finer and old, thicker root segments from at least three areas around the rhizome. Sampled roots were placed in coin envelops for transportation. Exposed rhizomes were buried back in their original localities, and the soil around them was moistened to avoid desiccation and to aid in re-establishment. We also recorded the number of leaves, length and width of largest leaf, height of the tallest leaf and flowering stalk, number of flowers, number of fruits and number of seeds during initial plant sex identification and again during root collection. Root samples were placed in a drying oven upon returning from the field, and kept at 65°C until processed to prevent other fungal growth.

Staining for Fungal Structures

Roots were first cleared of all pigments before their fungal structures were stained to quantify colonization. The dried, collected roots were rinsed in tap water to remove excess soil before being placed in Simport Biopsy Cassettes (M510-7) and then left in tap water at 4^{o}C overnight to rehydrate. To clear the roots, ten to fifteen cassettes containing thin roots were then placed in boiling 10% KOH for 7-10minutes (until KOH turned brown), rinsed with tap water and then either placed in tap water at 4^{o}C overnight or set in 3% H_20_2 for one hour. This procedure would be repeated till all roots were cleared. Cassettes with thick roots were incubated in 10% KOH in a water bath at 65^{o}C for an hour (10-20 cassettes in 800mL of 10%KOH) then processed as

described above. Once cleared of other pigments, roots are ready for staining. All roots were placed in 1%HCL before being incubated in 0.005% Direct Blue Stain at 65^{o}C for 50 minutes. Afterwards roots were briefly rinsed with tap water and placed in fresh tap water at 4^{o}C overnight to leach out extra stains (Lankau 2013).

Stained roots were cut into ~1cm sections and placed on glass slides. The specimens were preserved in Poly-vynyl-lacto-glyceral glue under the cover slips. Slides were examined at 200x and scored for percent hyphae, arbuscule and vesicle colonization. Percent of fungal structures was determined via the line intersect method (McGonigle et al. 1990).

Data Analysis

We used two Generalized Linear Models (GLM) to determine whether populations and sexes differed in AMF colonization rates. We first analyzed the three fungal measurements (% hyphal, vesicle and arbuscule colonization rates) with sex (female and hermaphrodite), growth phase (**D-'11**, **Fl-'12**, **Fr-'12**, **V1-'12**, **V2-'13**, **Fl-'13**), and plant population as independent variables to quantify variance between life phases. Interactions between sex and life phase round were examined. Least square means were compared using Tukey-Kramer's Adjustment for Multiple Comparisons.

A second GLM was performed with percent fungal structures as the dependent variables and sex and population as the predicting variables, organized by collection round and including an interaction term between and sex and population. Percent arbuscule and vesicle coverage of the roots were (square root (arcsin)) transformed to improve normalcy for all GLM analyses. Fungal colonization means for each population and round were analyzed using proc means, SAS® statistical software version 3.0 for PC.

Correlation analysis was carried out between colonization rates of the different fungal structures and the various morphological measurements. Data from all rounds were analyzed for fungal correlations with # of leaves, and data only from reproductive life phase rounds (R4-R, R5-F and R8-R) were correlated with # of flowers, # of fruits, and # of seeds using Pearson Correlation Coefficients. Both correlation analyses were run with and without grouping the data into sex (female and hermaphrodite). All analyses were performed using SAS® statistical software version 3.0 for PC.

Results

Hyphal Colonization

Hyphal colonization can represent fungal effectiveness, as higher levels of intracellular hyphae provide predictors of nutrient transfer levels. Hyphae rates in female and hermaphrodite plants were not significantly different (**female**-18±0.8%, **hermaphrodite**-17.5±0.6%) across the span of this study (2011-2013), yet rounds were significantly different from one another (Table 1.1). Populations also varied significantly in their colonization levels across this study with TP and TC populations ($F_{8, 505}$=10.16, p<0.0001, LSmeans 10.1±1.2% and 10.1±2.6%) exhibiting the lowest hyphal colonization levels and BH and WT ($F_{8, 505}$=9.31, p<0.0001, LSmeans 20.6±1% and 22±1.3%) the highest (Figure 1.3a). Even with this significant variation between populations, there were noticeable differences between plant growth phases. Both flowering rounds, **Fl-'12** and **Fl-'13** ($F_{5, 505}$=20.44, p<0.0001, LSmeans 20.7±1.1% and 19±1.2%), exhibited higher hyphal colonization than fruiting ($F_{5, 505}$=20.44, p<0.0001, **Fr-'12**: 7.7±1.2%) and post reproductive vegetative phases ($F_{5, 505}$=20.44, p<0.0001, **V1-'12**: 11.1±1.2%). The pre-

reproductive vegetative phase (**V2-'13**) was not significantly different from either flowering life phase rounds (Figure 1.4a).

Arbuscular Colonization

Arbuscular coverage is typically associated with how effective nutrients are being transferred between AMF and the plant's roots. We found that percent coverage by arbuscules on *G. maculatum's* roots was not significantly different between sexes (**female**-4±0.3%, **hermaphrodite**-2±0.2%). Unlike with hyphal colonization, populations did not differ in arbuscule colonization rates (Figure 1.3b). However, colonization was higher during flowering phases, showing significant differences between fruiting and dormant life phase rounds (Figure 1.4b).

Vesicle Colonization

Rates of vesicle colonization have been used as a proxy for plant carbon allocation to AMF. Similar to hyphae and vesicle colonization, there was no significant difference between sexes (**female**-3.1±0.3% **hermaphrodite**-3.5±0.3%). Populations differed in their vesicle levels, with WT and BH ($F_{5, 505}$=4.11, p=<0.0001, LSmeans 16.6±1.5%, 16.5±1.1%) exhibiting significantly higher rates than RL ($F_{5, 505}$=4.11, p=<0.0001, LSmean 5.2±3.2%, Figure 1.2c). *Geranium maculatum* roots had the lowest vesicle coverage during **Fr-'12** and **V1-'12**, with no discernable difference between other growth phases (Figure 1.4c).

Morphological and Reproductive Traits with Mycorrhizal Traits

Overall, the three fungal traits were all significantly positively correlated with each other, with the Pearson correlation coefficients ranging from 0.54 to 0.70 regardless of the sex of the

plants (Table 1.2). Similarly, all morphological traits were also significantly positively correlated with each other with the correlation coefficients ranging from 0.44 to 0.91 (Table 1.2). Between these two types of traits, we found that the number of fruits and seeds and the hyphae colonization exhibited a significantly negative correlation in hermaphrodite (r=-0.31193 p=0.0193, r=-0.29598) and not significant in females (r=-0.21089 p=0.0.2910, r=-0.21161 p=0.2893). One additional correlation showed an interesting pattern when sexes were not distinguished: vesicles showed a positive correlation with number of leaves (r=0.11283, p=0.0183) but when separated into sex, neither hermaphrodite (r=0.10442, p=0.0728) nor female (r=0.14270, p=0.0914) trends remained significant.

Discussion

We found evidence of AMF colonization rates changing in natural populations of *Geranium maculatum*. We predicted that colonization rates would be highest during the reproductive phases of the plant, particularly because of the high phosphorus demand during flowering and found this to be true in our study. We also expected to see a difference in colonization levels between female and hermaphrodite individuals. However, no significant difference was found between sexes. Here we provide preliminary evidence for a highly dynamic seasonal association between *G. maculatum* and arbuscular mycorrhizal fungi.

Life Phase Trends

AMF colonization rates varied across seasons in *Geranium maculatum*. There was a trend for higher colonization rates of hyphae during flowering compared to both dormant and vegetative phases as well as lower arbuscule colonization during dormancy than during the flowering and vegetative phases. The decrease in arbuscular colonization levels during dormancy support the

idea that AMF colonization is most abundant when the plants are active. Rhizomatous plants become inactive after the aboveground mass senesces (Martin 1965; Brandsaeter et al. 2010), effectively ceasing aboveground plant functions, and potentially inhibiting current AMF association. Unlike arbuscule colonization, hyphal colonization levels did not show to decrease during dormant and vegetative phases, however this may have been an artifact of a higher colonization previous to root collection. Hyphal structures can remain present in a plant after they have died, and staining is not completely selective of live tissues. This may also have obscured true colonization rates.

Vesicle coverage was least affected by growth phases in our results. High levels of coverage were present for all phases except for **Fr-'12** and **V1-'12**. This can potentially be explained by the nature of the vesicles, as they are storage structures and do not always react directly with the plant examined. No direct link has been shown between intraradical hyphae and arbuscule colonization rates and extraradicle hyphal networks, but there is evidence of vesicle colonization rates being altered within the roots of multiple plants due to the shared carbon pool (Fitter et al. 1998; Lekberg et al. 2010). Vesicles are affected by multi-plant hyphal networks (Fitter et al. 1998) where they may increase in density with a net increased allocation of carbon from the plant network, not just from the plant roots they are harbored in (Pfeffer et al. 1999). Extraradicular hyphal networks can connect multiple plant species together, and AMF have been shown to share carbon between species, transferring carbon from one plant to another's vesicles (Fitter et al. 1998). Thus it is hard to draw conclusions from vesicle colonization rates in this study design, as we were examining the affect of AMF association on a single plant, not on the connections and interactions between plants.

Arbuscular mycorrhizal fungi colonization rates varied significantly throughout the year and within similar plant phases. The highest colonization rates came during the reproductive and vegetative phases, including rounds **Fl-'12**, **Fl-'13** and **V2-'13** (Fig. 2.3). Roots from Fr-'12 had significantly lower colonization levels than the other reproductive phases, potentially because fruiting has been shown to not be largely affected by phosphorus acquisition (Conversa et al. 2013). The two vegetative phases measured (**V1-'12** and **V2-'13**) varied significantly in their associations with AMF. Plants in the **V1-'12** showed low colonization rates of all three fungal structures, similar to plants in the previous fruiting round (Fig. 2.3). However, in early spring 2013 when the plants were coming out of dormancy and initiating vegetative growth, **V2-'13**, colonization levels of all AMF structures were high, and not significantly different from those seen during flowering.

We propose two potential speculations for the differences seen between the two vegetative rounds, **V1-'12** and **V2-'13.** First, the plant's allocation of photosynthates to storage structures changes across the seasons, with a potential decrease in dependency for macronutrients via AMF association (Merryweather and Fitter 1998; Mortimer et al. 2005). *G. maculatum* relies heavily on AMF early on in the season to help with reproduction and vegetative growth, though as the plant moves to a more vegetative phase, it focuses more of its resources on rhizome growth and decreases its association with the AMF. Rhizome growth in *Geranium maculatum* typically occurs after floral development later on in the summer (Martin 1965). In early spring, the plant's nutrients are primarily allocated to leaf and flower bud development (Mortimer et al. 2005). At this point phosphorus is in high demand for flower production (Poulton et al. 2001a, b; Poulton et al. 2002). After shoot development and reproduction, few new leaves are produced, and the majority of the growth and carbon allocation has been shown to take place underground (Asaeda

et al. 2008) as the rhizome begins to expand from one of the lateral meristems (Martin 1965). Rhizome production is typically a large carbon sink and rhizome growth has been found to vie with other plant functions (such as fruit production) for photosynthate allocation (Yu et al. 2013).

A second potential explanation for the large variation in colonization rates seen between vegetative rounds could be due to the seasonal variation in thickness of the roots. Early in the spring, while *Geranium maculatum* plants are coming out of dormancy, they develop finer, more numerous roots, often abandoning the majority of the previous year's roots (personal observation). It is easier for AMF to colonize thinner roots as the plant is developing early spring, but as the season progresses many of the thinner roots develop into thicker cuticled roots (pers. obs.) that may be more difficult to colonize. Hartnett et al. (2013) found a significant negative trend between root fibrousness (RFI) and AMF colonization of multiple species of African semi-arid savanna grasses. The more drought tolerant species were found to have a higher RFI index, and significantly lower AMF colonization. The same trend of increased RFI and decreased AMF colonization was found in tall grass prairie forbs (Hetrick et al. 1992). However this relationship was also plastic, depending on environmental conditions (Hetrick et al. 1992). If plant carbon allocation to AMF decreases, this may explain the decrease in AMF colonization on *G. maculatum's* roots post-reproduction and during dormancy. However, this has not been empirically tested, and requires further investigation, as very few studies have looked at correlating RFI and AMF colonization rates. Our explanations are also speculative, as we are only discussing two individual time points in *G. maculatum's* life, and to provide a more concrete explanation, more data would be necessary.

Sex Variation in AMF Association

We predicted that AMF would associate differently between the two sexes of *G. maculatum*, and that these association differences would potentially benefit one sex over the other. Our predictions were founded off of the idea that due to different nutritional needs of the morphologically different sexes of *G. maculatum,* AMF association levels would vary to match the plant's needs (Ashman 1994). Research done by Varga and Kytoviita (2010b) quantifying AMF relationships to plant sex on the European congener, *G. sylvaticum* showed AMF benefits differing between the sexes. However, we found no evidence of a sex specific relationship to AMF in *G. maculatum.* Throughout our study there were no significant differences between AMF colonization levels and sex in *G. maculatum*, but this does not mean that association did not have undetected effects that we could not quantify with the measurements taken.

We examined colonization levels of natural association in the field and compared them to leaf and flower production; however there are many more effects that AMF association may have on a plant that are harder to measure in the field. AMF beneficial effects have been manifested in plant drought resistance (Omirou et al. 2013; Sochacki et al. 2013), increased phosphorus in pollen (Poulton et al. 2001a, b; Poulton et al. 2002) and greater growth and reproductive output in general (Koide 1991; Stanley et al. 1993; Wilson et al. 2001; Siddiqui and Akhtar 2009). Although we measured growth and reproductive output, we neither quantified the phosphorus levels in the field nor phosphorus acquisition by the plants. We also did not look at other reproductive measurements such as pollen production and viability. To provide a more definitive answer as to whether or not association between AMF and gynodioecious sexes varies, a more comprehensive, environmentally controlled experiment concerning phosphorus uptake and reproductive output would be beneficial.

Conclusions

In summary, we found temporal variation in fungal association with *Geranium maculatum* throughout the year, and that there was evidence of higher colonization rates during (and immediately before) the onset of reproduction. However, colonization rates decreased significantly after flowering, and remained low during fruiting and the late summer vegetative phase. Populations also differed significantly in their AMF colonization rates, which could create local environmental adaptation or genotypic differences in these species. There were no strong variances noted within a population in association between female and hermaphrodite plants, yet we did not measure all potential benefits of AMF association, so we can not conclude that association does not matter to reproduction in this gynodioecious system.

More research needs to be done examining the carbon/phosphorus transfer rates in these two sexes under laboratory conditions to tease apart this complex relationship. Also, AMF plants are not typically sterile in the field, and greenhouse manipulation to see how *Geranium maculatum* reacts to sterile treatments, versus various combinations of field fungi may elucidate this intricate symbiosis. However, we do provide evidence that AMF relationships not only vary among populations, but during different important life phases, lending evidence of how dynamic AMF associations can be.

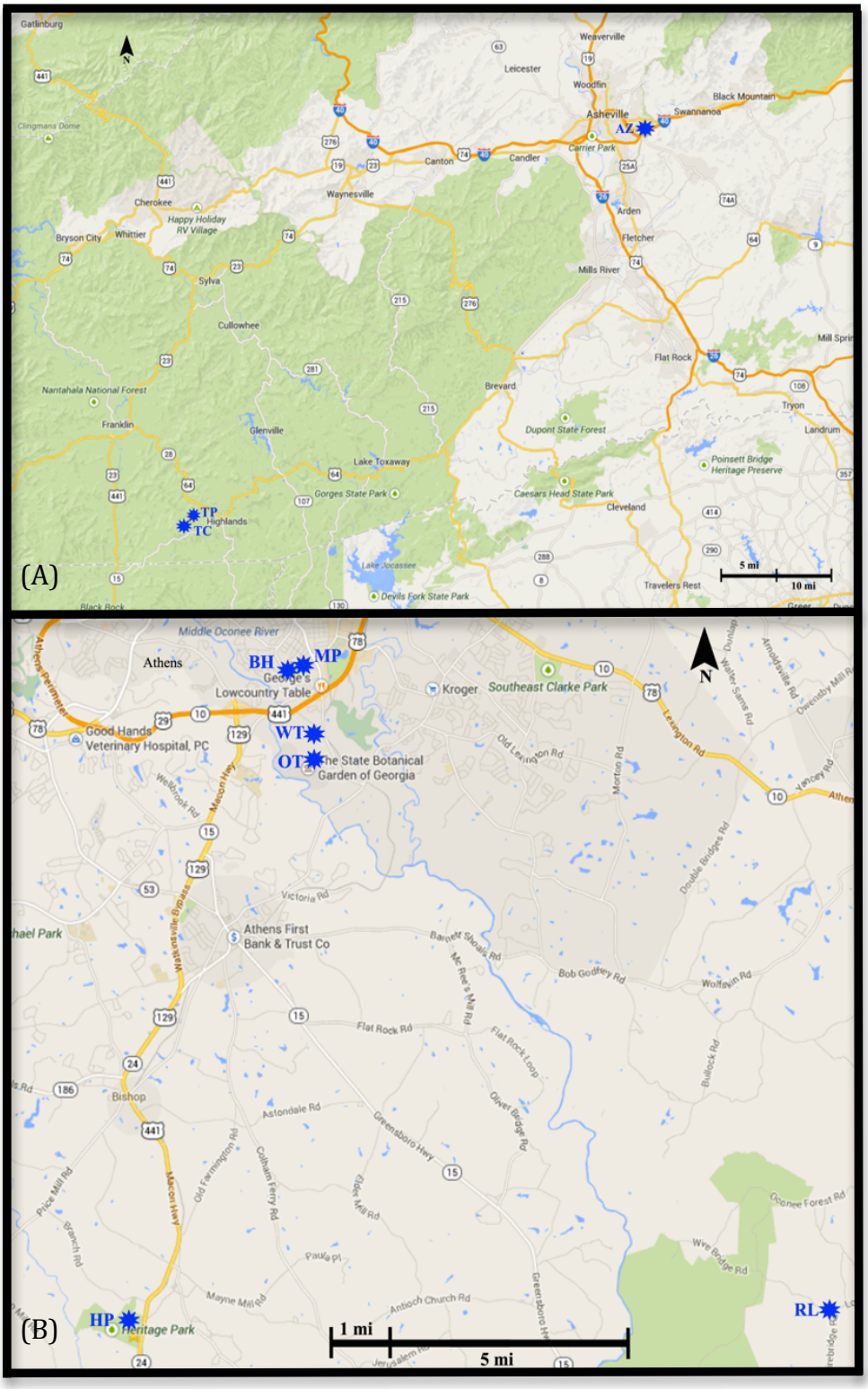


Figure 1.1. Locations of all populations sampled for AMF colonization rates. Maps are located in A) North Carolina and B) Georgia, USA (Google Maps 2013).

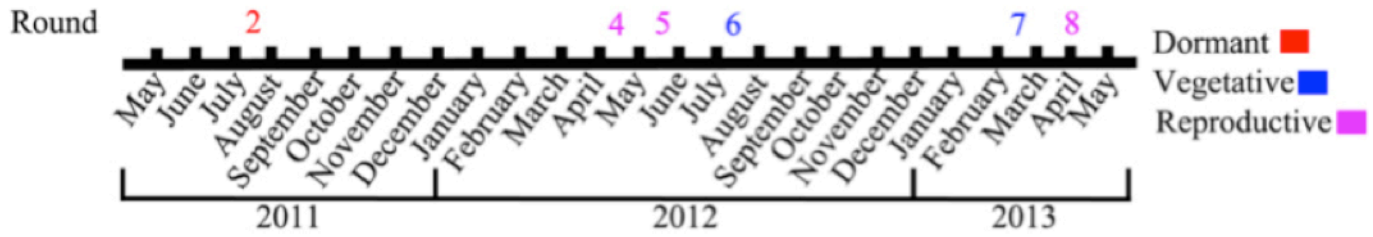
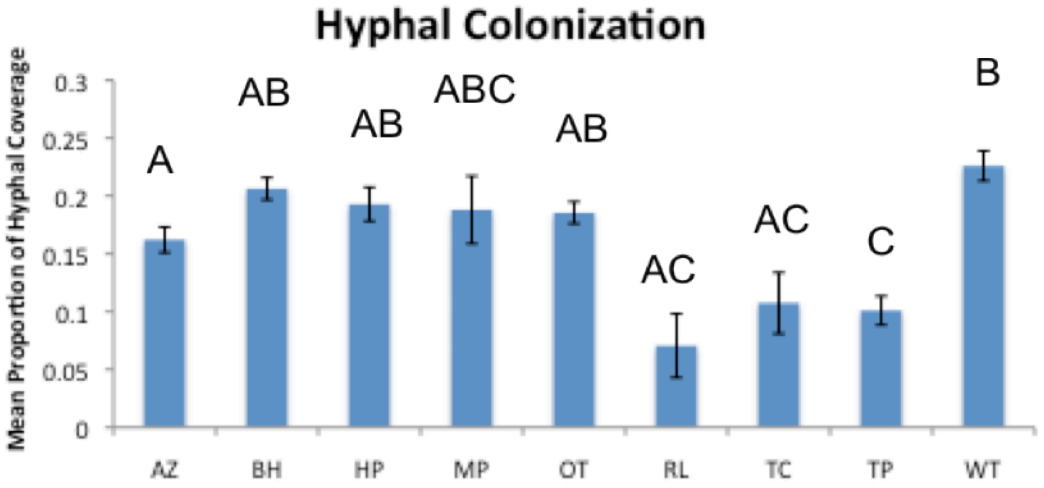
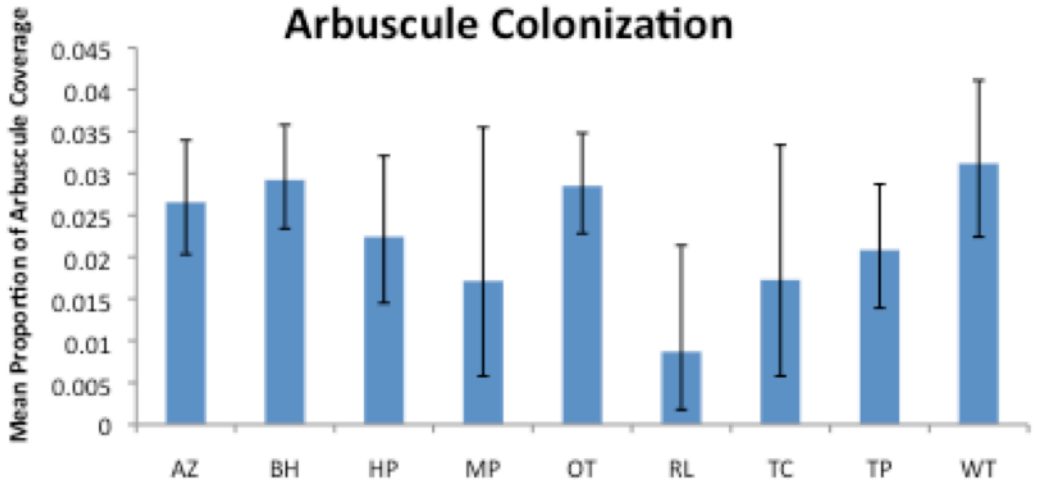


Figure 1.2. Dates of collection for each round of root analysis. The color of each round corresponds with the plant's life phase (Dormant-red, Vegetative-blue, and Reproductive-purple).

3b



3c



3a

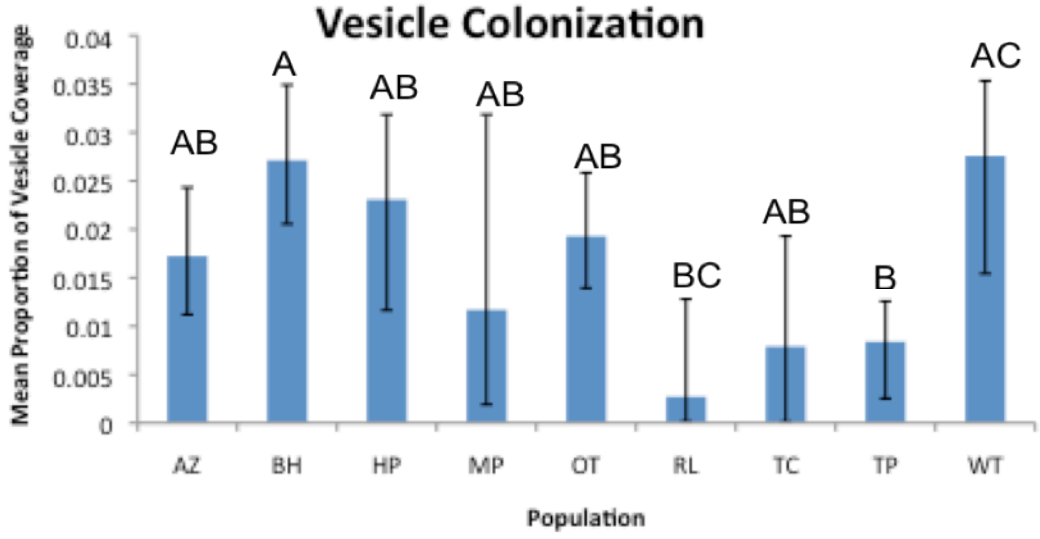
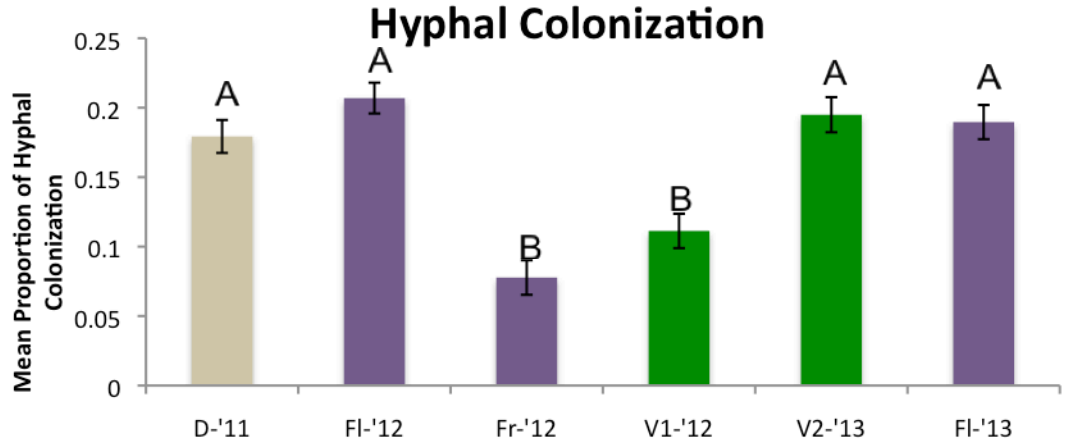
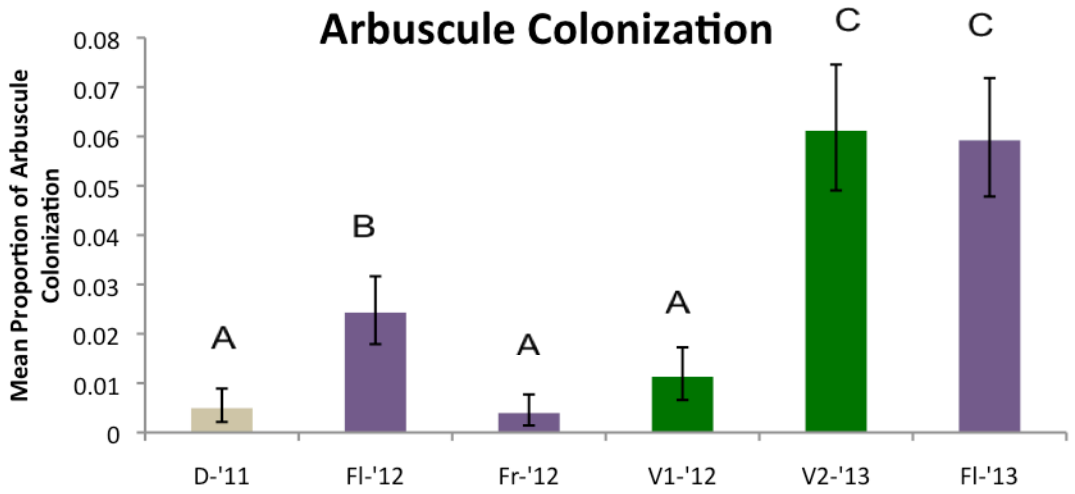


Figure 1.3. Mean a) hyphal, b) arbuscule, and c) vesicle colonization rates between populations. Populations that share a letter are not significantly different from each other. Error bars represent s.e. for 3a, and 95% CI for 3b and 3c.

4a



4b



4c

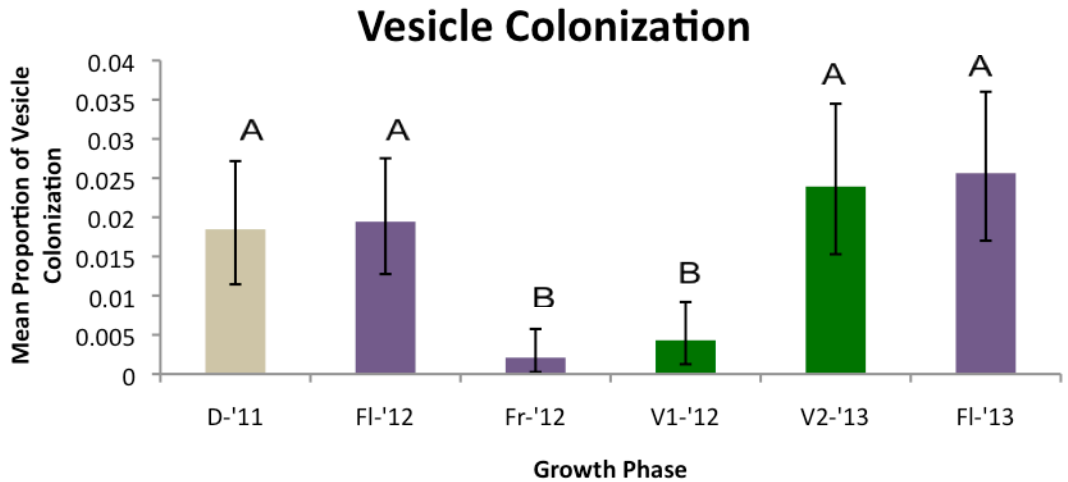


Figure 1.4. Mean a) hyphal, b) arbuscule, and c) vesicle colonization rates across growth phases. Colors represent: Tan= dormant, purple=reproducing, green=vegetative growth phases. Bars that share a letter are not significantly different from each other. Error bars represent s.e. for 4a, and 95% CI for 4b and 4c.

Table 1.1. Comparisons between hyphal colonization levels of the plant growth phases. Top half are p values for comparisons of hermaphrodite plants and bottom half are p values for female comparisons.

		Hermaphrodite					
	Round	D-'11	Fl-'12	Fr-'12	V1-'12	V2-'13	Fl-'13
Female	D-'11		0.638	0.0007*	0.1416	0.9999	0.9944
	Fl-'12	1		<0.0001**	<0.0001**	0.9223	0.9953
	Fr-'12	0.002*	0.0005*		0.8642	<0.0001**	<0.0001**
	V1-'12	0.1479	0.0964	0.9723		0.0043*	0.0005*
	V2-'13	1	0.9991	<0.0001**	0.0127*		1
	Fl-'13	1	1	0.0063	0.3459	0.9765	

Table 1.2. Correlations between fungal structures and plant structures, male and female data analyzed separately, showing r values in white and p values in grey. female correlations are above the line and hermaphrodite correlations are below.

		Female						
		Hyphae	Arbuscules	Vesicles	Leaves	Flowers	Fruits	Seeds
Hermaphrodite	Hyphae		0.57548	0.68328	0.1154	0.02076	-0.21089	-0.21161
			<.0001**	<.0001**	0.173	0.8707	0.291	0.2893
	Arbuscules	0.66258		0.43862	0.03786	-0.1293	-0.0093	-0.05169
		<.0001**		<.0001**	0.6558	0.3086	0.9633	0.7979
	Vesicles	0.70241	0.58349		0.1427	0.1434	0.01516	0.0596
		<.0001**	<.0001**		0.0914	0.2583	0.9402	0.7678
	Leaves	0.06465	0.02755	0.10442		0.5499	0.00889	0.0312
		0.2675	0.6369	0.0728		<.0001**	0.9649	0.8772
	Flowers	-0.0846	-0.0156	-0.07568	0.46182		.	.
		0.3462	0.8624	0.3996	<.0001		.	.
	Fruits	-0.31193	-0.12145	-0.25222	0.56358	.		0.90391
		0.0193	0.3726	0.0607	<.0001	.		<.0001**
	Seeds	-0.29598	0.12051	-0.22964	0.63858	.	0.9137	
		0.0268	0.3763	0.0887	<.0001**	.	<.0001**	

CHAPTER 3

LOCAL ADAPTATION OF *GERANIUM MACULATUM* TO ARBUSCULAR MYCORRHIZAL FUNGI

Introduction

Organisms in longstanding symbioses have had the opportunity to adapt concurrently to each other such that consistent, mutual benefits evolved due to their close proximity and the past environmental constraints, driving these relationships. Extant evidence for how these symbioses came to be is shown in how environmental conditions drive the strength of symbiotic relationships. For example, low nutrient availability tends to favor stronger associations between plants and their fungal mutualist, while nutrient rich soils effectively decrease this association (Johnson et al. 2010; reviewed in Smith et al. 2010). Arbuscular mycorrhizal fungi (AMF) colonize their plant host's roots with hyphae and arbuscules that facilitate the transfer of limiting nutrients such as phosphorus and nitrogen in exchange for carbon photosynthates produced by the plant (Smith and Smith 2011). Drastic variances in association due to environmental cues are often the norm for this relationship, and can even be manifested in microclimates within a population (reviewed in Bever et al. 2012). Given a potentially strong selective pressure on plants in a nutrient impoverished environment, if variation in the plant-fungal association has a genetic basis, persistent selection could lead to the evolution of a higher dependence of plants on AMF. This selection may eventually lead to plant genotypes adapting to their specific biotic AMF environments in order to gain necessary soil nutrients.

Though the close interaction of plants with their sympatric AMF has been known for a long time, studies of local adaptation in plants have traditionally ignored AMF as a biotic environmental condition but only focused on how plant species may adapt to their abiotic environments (Leimu and Fischer 2008; Espeland 2013). Biotic environmental factors can be equally important in shaping a plant's adaptation and therefore must be considered; symbiotic relationships provide an ideal system to study adaptation to biotic environments. For example, Ji et al. (2013) found that *Adropogon gerardii* individuals gained the greatest benefit from associations with their home site fungi compared to foreign site fungi, even though the former contained lower diversity in their fungal community. They suggested that such improved plant performance was likely due to local adaptation to those specific AMF species' genotypes (Ji et al. 2013). In addition, field conditions, host plant population structure (Graff 1999; Van Etten and Chang 2009) and genotypes of AMF associates can vary even within a population, providing potential for the development of local adaptations not only at the population level, but on a microhabitat scale varying based on types of individuals within the population (Via 1991; Bever et al. 2012).

Populations of polymorphic individuals have been shown to exhibit variable responses to environmental and biological influences (Parker 1991; Stehlik et al. 2006; Lyytinen et al. 2008; Martins et al. 2009; Givnish 2010). If this plastic ability is heritable, there is a potential for the development of phenotype-specific adapted responses (Jablonka 2013). In gynodioecious systems, phenotypic traits have shown to vary between sexes in response to herbivory and insect infestation (Ashman et al. 2004; Cole and Ashman 2005). If the interactions and responses persist overtime, there is potential for these reactions to affect the reproductive success of the plant, making these biotic interactions ultimately heritable (Jablonka 2013). In symbiotic

relationships, such as plant-AMF, there is the potential for polymorphic plants undergoing positive interactions to adapt to AMF positively, yet in different ways. Few studies have examined the interaction between the phenotypic differences of plant sexes and AMF relationships (but see Varga 2010; Varga and Kytoviita 2010a, b) albeit much work has been done examining hermaphroditic species (reviewed in Koide 1991; Smith et al. 2010). Gynodioecious systems will allow us to consider if polymorphic individuals, i.e. different sexes, have developed specific relationships with AMF over time due to potential differences in nutritional needs that are satisfied by the mutualism.

These historical variations in the nutritional needs may alter how plants of different sex currently interacts with their symbiont fungus, primarily because the AMF relationship is largely nutrient deficiency driven (Koide 1991). It has been shown that the sex of a plant may dictate the temporal fluctuation in their resource needs due to their different allocation pattern to different reproductive structures (Poulton et al. 2001a; Poulton et al. 2002). For example, in dioecious systems, males allocate more phosphorus into their flower production, and females invest more biomass into fruit production (Hemborg and Karlsson 1999; Harris and Pannell 2008). In gynodioecious systems, females have been found to allocate more of their biomass to seeds (fertilized ovules), whereas hermaphrodites tend to invest significantly more resources into pollen production, yet still invest in their ovules as well (Ashman 1994; Poulton et al. 2002).

Females are at a disadvantage in mixed populations because they can only transfer their genetic material through one means, ovules, while hermaphrodites can increase their fitness through both pollen and ovules. It is widely accepted that in order to persist in populations, females need to maintain a reproductive advantage through producing more and/or better seeds compared to hermaphrodites (Lewis 1941; Lloyd 1976; Charlesworth and Charlesworth 1978;

Charlesworth 1981), which has been empirically shown in many species (for example Eckhart 1992; Wolfe and Shmida 1997; Nilsson and Agren 2006). Seed production generally requires more carbon than pollen production (Pendleton 2000; Poulton et al. 2001b), whereas pollen production requires high phosphorus levels. These sex-specific needs for carbon and phosphorus could be altering female and hermaphrodite respective relationships with AMF in specific ways.

It is from these lines of thought that we propose the following question: Do plants of different sexes show varying patterns in adaptation to a symbiotic aspect of their biotic environment, the AMF community? To help answer this question we examined the association between AMF and a gynodioecious plant, the spotted leaf geranium, *Geranium maculatum.* We conducted a common garden study using plants from three populations that were treated with AMF treatments of home AMF assemblage, away AMF assemblage and no-AMF. We chose to use *G. maculatum* for two reasons: 1) there is evidence that females produce more seeds than hermaphrodites (Agren and Willson 1991; Chang 2006), and 2) a congener of *G. maculatum, G. sylvaticum,* shows variable relationships in AMF response between hermaphrodites and females (Varga and Kytoviita 2010b). If *G. maculatum* plants are adapted to their home AMF assemblages then we propose to see: I) An increase in plant growth and fitness with fungal inoculation; II) Populations benefiting more from their home fungal assemblages; and III) Sex-driven differences in AMF association and growth response.

Methods

Study Species

Geranium maculatum L. (Geraniaceae) is a perennial, rhizomatous herb common to the eastern half of North America, ranging from Quebec to Florida (USDA 2013). Basal leaves

emerge from underground rhizomes in mid-February and flowers are produced on branched stalks from mid-March to early June in Georgia, USA (Chang 2006). Flower buds are formed in the previous season and lie dormant over winter. The plants are gynodioecious, where individuals produce either female flowers with reduced, sterile anthers or hermaphroditic flowers with larger, protandrous anthers. Sex ratios in *G. maculatum* located in Georgia vary from all hermaphrodites, i.e. zero females, to ~50:50 female:hermaphrodite (Chang 2006).

Population Sampling and Processing

To test local adaptation of *Geranium maculatum* to their AMF communities around the southeastern range of this species, rhizomes of mature flowering individuals were collected haphazardly from two populations in Georgia, USA (BH: Lat=33.92647, Long=-83.388033, EJ: Lat=34.774023, Long=-84.673610) and one population from North Carolina (AZ: Lat=35.575187, Long=-82.489525). Sex of the plants were identified during the flowering seasons of 2011 and 2012 (March – April) and rhizomes along with the soil surrounding the rhizomes' roots were collected in Fall 2011 and again in the spring of 2012. Collected plants were temporarily stored at 4°C until processed within a week of collection.

To remove the AMF that were originally colonizing the plant roots in the field populations, rhizomes were stripped of their roots and surface sterilized with 5% Bleach solution. Root hormone was applied before rhizomes were placed in 4" square pots with sterile Fafard 3B scientific soil mixture. After planting, all rhizomes were sprayed with fungicide and received a tablespoon of Osmocote (NPK: 14-14-14) slow-release fertilizer. The removed field roots and soil from each rhizome were then mixed together into two, 6inch round pots with one randomly chosen hermaphrodite or female from each population to start the trap cultures. These pots did

not receive any fungicide, and were grown up separately from the other AMF-free plants (see fungal spore extraction below).

Note: Plants collected in the fall of 2011 were placed immediately into the cold room for a month after sterilization, and the majority of them contracted a deadly fungal disease that they did not recover from. Plants collected in the spring of 2012 were placed directly into the greenhouse after sterilization and had a much lower mortality rate.

Plant Cloning

Plants were allowed to grow for 3 months under optimal conditions in the greenhouse, receiving water-soluble fertilizer once a week (Jack's Pro, NPK: 20-10-20) before they were vegetatively cloned. In July 2012, plants were removed from their pots and all soil was washed off of the plant roots. The number of meristems on each rhizome was noted, and the largest meristems along with some rhizomes were mechanically removed from the original rhizome and placed in new pots with sterile soil to create a vegetative clone. Roots were left on these new rhizome clones, and all plants received fungicide after planting to reduce the infection of pathogenic fungi through the cut surface of the rhizome and to decrease potential AMF contamination. The new clones were allowed to grow for two more months, under the same optimal conditions. This cloning process was repeated in September 2012 in order to generate enough plants for the common garden study.

Field Soil Collection and Fungal Spore Extraction

We used field soil as the basis to create the growth medium for this study. Field soil from the three home sites was collected, mixed and used as experimental soil to emulate soil conditions in the field. At six random locations throughout each site (BH, EJ, AZ) a total of 50 gallons of soil

from the A and B-horizon was collected haphazardly. Four liters of soil from each site was set aside for spore collection (trap cultures) and the rest of the soil was then all mixed together and autoclaved in a Lindig Media Steamer at ~160-180°C for 45 minutes. The autoclaved field soil was then mixed with Vermiculite (6:1 mixture) and divided into 6inch round pots.

AMF spores were collected from two sources: trap cultures created from the original rhizome collections, and from field soil. Fifty mL of soil and 1.25L of water were placed into a blender, and blended twice at 3 seconds, with a 5 second pause in-between. The slurry was then sifted through a top sieve of 500um and a bottom sieve of 45um and rinsed with DI water thoroughly until the majority of the soil had washed through the bottom sieve. The remaining slurry was then collected in a 250mL beaker. The collected spore slurry was added to two Falcon tubes containing 20mL of 60% sucrose solution, creating a total volume of 50mL in each tube. The tubes were then centrifuged in 10°C at 37,250 rpm for 5 minutes. The supernatant was then poured onto a 20um sieve and rinsed thoroughly with tap water. The spores left on the top of the sieve were collected and stored at 4°C in DI water. Number of spores per mL was quantified, and plants were inoculated within 1 day of spore extraction (protocol modified from Morton 2013).

Plant rhizomes were inoculated by adding 1mL of ~100 spore/mL solution to each pot with their respective treatments, and 1mL of DI water to the control plants. After two weeks, spore extraction and inoculation was repeated and the plants were inoculated with another 1mL of ~100 spores/mL as well as 1mL of the combined (from all three sites) bacterial slurry. Bacterial slurry was created by blending 50mL of field soil in 1.25L of water (same as above) but the fluid that washed through the 40um sieve was collected. The resulting slurry was then vacuum-filtered for an hour through 20um filter paper in a Buchner funnel. Vacuum slurries from each field site

were mixed together and then used to inoculate all plants to control for microbial, fungal interactions.

Prior to being subjected to the fungal inoculate, roots from thirty randomly chosen individuals were collected and dried in coin envelopes at 60°C to later be analyzed for AMF colonization. When observed, no AMF structures were seen on the roots, ensuring that there was no previous AMF colonization before fungal treatments were applied. However evidence of septate (non-AMF) fungus was present in low densities.

Garden Set Up

Three garden arrays were established around Athens, GA, allowing for wild pollination of experimental plants. Each garden contained 20 female and 20 hermaphroditic individuals from each of the three populations, totaling 120 plants. Four treatments, including fungal inoculate from BH, EJ, AZ or no fungus, were randomly and evenly assigned to each population's individuals so that 5 female and 5 hermaphrodites received each treatment. These individuals were then randomly assigned a location in an 11 x 11 grid and using a two-pot system, placed into the field with a 10cm air barrier between the experimental pot and the ground. Of the three garden locations, two were located in Athens, GA (GRE—Lat=33.929774, Lon=-83.362198, BOT—Lat=33.904569, Lon=-83.383226) and the third in Farmington, GA (DOR—Lat=33.759021, Lon=-83.448930). Note that ramets of the originally collected genets were used in multiple gardens. However, the clones in different garden arrays did not always receive the same treatment because enough clones from every genet could not be harvested to allow complete replication of the same genets in all three of our garden arrays. Also, no genotypes were repeated <u>within</u> a garden plot.

Growth Measurements

Weekly measurements were taken for eleven weeks starting in March and ending in June 2012. Measurements collected included number of leaves present, leaf area (length x width of leaf), leaf height, inflorescence height, number of flowers present (buds and opened flowers), number of flowers open, length of flower petals (one randomly chosen petal per flower, from carpel to petal tip), number of fruits, and number of seeds.

Biomass was collected at two time points. The first collection took place on June 4th and 5th, 2013, and was taken from the plants who had either not flowered, or were done with their reproductive phase. The second collection was taken from the remaining reproductive plants after they had finished (either produced fruit, or stopped flowering) on June 21st. Vegetative and reproductive tissues were separated upon collection, and fruits containing mature seeds were also separated from reproductive tissue. Masses of all tissues were measured separately after being dried for at least 3 days at 60°C.

Flowering Measurements

The reproductive variables of the plants were measured three times a week to gain a more detailed profile of the number of flowers open per day, and flower size. Colored threads were tied around each open flower to identify the date that they flowered and also their potential mates.

Phosphorus Analysis

Percent leaf phosphorus can be used as a proxy for fungal effectiveness of phosphorus transfer. One mature leaf per plant was collected and dried in coin envelopes for at least three

days at 60°C. Leaves were then ground in a tissualizer at 25000rpm for 3 minutes to prepare them for the phosphorous analysis procedures. Between 0.025 and 0.05g of ground leaf tissue was placed in 15mL plastic Falcon tubes and the precise leaf tissue weight added was recorded. Twelve and a half mL of 2% acetic acid was placed into each tube, which was then shaken for ten minutes on a table shaker. The resulting slurry was then filtered through 90mm No. 40 Whatman filter paper into a 125mL Erlenmeyer flask. After extraction, 15mL of distilled water was added to 5mL of the extractant in a 25mL Erlenmeyer flask and 1mL of the dilution was then transferred to clear test tubes (functioning as cuvettes). Four mL of molybdate-absorbic acid solution was added to the dilute sample and then allowed to stand for 20 minutes at room temperature. Afterwards each sample was measured for absorbance at a wavelength of 880nm on a spectrophotometer. These readings were then compared to a standard %P curve (Varvel et al. 1976).

Fungal Colonization

To determine the effectiveness of the fungal treatments, fungal colonization was quantified for the BOT garden and twenty randomly selected individuals from each of the other two gardens (ten flowering and ten non-flowering). Plants were uprooted, and roots were collected from at least three locations around the rhizome. Roots were dried at 60°C in coin envelops for at least a day before being washed and placed in individually marked cassettes. Cassettes were left to sit in tap water overnight (4°C) and then ~40 cassettes were boiled at a time in 3500mL of 10% KOH solution for 7-10 minutes. Roots were then rinsed and placed in 3% H_2O_2 for an hour. This was followed by another round of boiling and soaking in H_2O_2. Roots were then prepared for staining in 1% HCL (1 hr) and stained with .05% Direct Blue in a 65°C water-bath for 50 minutes. Roots were then rinsed and placed on slides, sealed with Poly-vynyl-lacto-glyceral glue

until examined (Lankau 2013). Following McGonigle et al. (1990), roots were scored for percent colonization of the following fungal structure: hyphae, arbuscules and vesicles.

Data Analysis

We used separate Generalized Linear Models to analyze the following traits: Hyphal, Vesicle and Arbuscule colonization rates, percent leaf phosphorus, vegetative mass, total reproductive mass, fruit mass, and total biomass, as well as number of fruits and seeds. Predicting variables in these models included population source for the plants (AZ, BH, EJ), garden array (DOR, GRE, BOT), treatment (**H**-home, **A**-away, **No-AMF** (C)), and sex (female, hermaphrodite) . In addition, initial rhizome size was included as a covariate for all measurements to account for any variation that may have been caused by the initial rhizome size. Fungal measurements and percent leaf phosphorus were (square root (arcsin)) transformed to improve normalcy for all analyses. All possible interactions were initially included for each trait, but were removed in the final analysis if they were not significant. Hence, in the final models, interactions between array*population and array*sex were examined for all fungal traits, and the interaction between sex*population was examined for percent of leaf phosphorus. Least square means obtained from these analyses were compared using Tukey-Kramer's Adjustment for Multiple Comparisons. These analyses were generated using SAS® software, Version 3.0 of the SAS system for Windows.

Number of leaves and number of flowers were examined using an Analyses of Variance (ANOVA) (using JMP Pro 10 (JMP® 1989-2013)), with predicting variables including sex (F-female, H-hermaphrodite), treatment and population and interactions between sex*treatment,

sex*population and treatment*population. Initial rhizome size was also included as a covariate for number of flowers only, as it was shown to have an affect on the variable.

Time to first leaf and first flower were analyzed using a Survival Analysis, with "mortality" being replaced with time of first emergence. Time intervals were analyzed on a weekly (Leaves, 1-11 weeks) and daily (Flowers, 1-77days) scale. First flower emergence was analyzed with days until appearance because flower measurements were taken three times a week, providing a more fine scale look at flowering. Time intervals were grouped under the independent variables; treatment (**H, A, No-AMF**(C)), sex, population, and fungal treatment (EJ, BH, AZ, no-AMF (C)). Model significance was assessed with Log-Rank tests between groups and individual treatments were compared using a One-way Analysis of Variance (ANOVA). Least square means were compared using Tukey-Kramer's HSD (JMP® 1989-2013).

Results

AMF Colonization Rates

When examining population source, hyphal colonization varied significantly between populations, with population EJ exhibiting the highest colonization rates (LSmean 18.5±1.9%). The highest rates of hyphal colonization were observed in plants, regardless of population, planted in the BOT garden array (Table 2.1). There was no significant difference in colonization rates between **Home**, **Away,** and **No-AMF** treatments, however there did seem to be lower colonization on **No-AMF** plants (Table 2.1).

For arbuscule colonization rates, populations were not significantly different from each other (p values ranging from 0.52-0.78). However, garden arrays did differ significantly, with higher

colonization occurring in BOT (Table 2.1). Treatment had no effect on colonization rates, although **No-AMF** plants did have lower colonization (Table 2.1).

Trends seen in hyphal colonization were very similar to vesicle colonization rates. Plants from population EJ exhibited the highest vesicle colonization rates of all populations (LSmean 7.7±1.1%). Plants located in garden array BOT showed the highest rates of colonization of the three garden arrays (LSmeans **female**-8.4±1.6%, **hermaphrodite**-11.5±1.4%). Again, there was no significant difference between treatments, but **C** plants showed lower vesicle colonization (Table 2.1).

Plant Growth

Inoculation treatments with AMF (H and A) had a significant effect on number of fruits and number of seeds, with inoculated plants having higher counts compared to plants with **No-AMF** treatment (Figure 2.1). All other morphological variables including vegetative, reproductive, total dry biomass, and number of flowers produced were not significantly affected by fungal inoculation (Figure 2.2).

Other growth trends of note include the plant home population effect on plant growth. Morphological measurements showed a clear trend of plants from BH producing more vegetative structures than the other populations (EJ, AZ). Plants from the BH population had the highest vegetative biomass ($F_{2, 246}$=69.82, p<0.0001, LSmeans 1.0±0.04g), reproductive biomass ($F_{2, 246}$=11.76, p<0.0001, 0.38±0.03g) and total biomass ($F_{2, 246}$=59.08, p<0.0001, 1.2±0.05g) of all populations, regardless of treatment (Figure 2.3). Plants originally from BH also produced on average a significantly higher number of leaves ($F_{2, 334}$=58.19, p<0.0001, LSmean =17.19 ±0.91) than AZ ($F_{2, 334}$=58.19, p<0.0001, LSmean= 5.46±1.17, p<0.0001) and EJ plants ($F_{2, 334}$=58.19,

$p<0.0001$, LSmean= 7.67±0.82, $p<0.0001$). Flower production did not differ with the effect of plant home populations ($F_{2, 336}=2.56$, $p=0.0786$).

Local Adaptation

Though treatment (plants with their home fungus, and with away and No-AMF treatments) did not affect plants' total biomass, vegetative and reproductive biomass, they significantly affected plants' reproductive output. Specifically, a greater number of fruits ($F_{2, 25}=2.75$, $p=0.0833$) and number of seeds ($F_{2, 25}=2.54$, $p=0.0994$) were produced by **Home** fungal treatments (LSmeans 2.92±0.51 fruits, 9.06±1.62 seeds) compared to **Away** (LSmeans 1.92±0.48 fruits, 5.55±1.52 seeds) and **No-AMF** (Lsmeans 0.75±0.86 fruits, 2.74±2.72 seeds) treatments (Fig. 2.1).

Leaf Phosphorus

The average phosphorus level for our study plants was 0.099%. Interestingly, plants with **Home** (AMF) treatments (LSmean 0.284±0.012%) showed significantly lower leaf P than ones receiving treatment **Away** (LSmean 0.316±0.009, $p=0.0304$), but not significantly different from **No-AMF** (LSmean 0.314±0.012%, $p=0.0762$). Population AZ had the highest leaf phosphorus ($F_{2, 234}=3.87$, $p=0.0223$, LSmean 0.326±0.012%).

Time to First Leaf and Flower

Although the differences between time to first leaf of **Home**, **Away**, and **No-AMF** treatments was not significant (($\chi^2=0.2415$, $p=0.8862$), fungal inoculation from population BH produced leaves on plants, regardless of home population, earlier than AZ and EJ AMF treatments ($p=0.0091$, 0.0292, Figure 2.4c). The home population of the plant significantly affected leaf

emergence also (χ^2= 43.18, p<0.0001), with plants originally from BH producing leaves the earliest (mean 3±0.13weeks, Figure 2.4b).

Plants subjected to **Home** and **Away** treatments flowered earlier than control plants (χ^2=6.6707, p=0.0356, Figure 2.5a). However, there was no difference between **Home** and **Away** treatments (means **H**-54.0±1.7 days, **A**-55.0±1.6 days, p=0.9245). Source population of the plant had a significant effect on first flower time (χ^2=18.2547, p=0.0001, Figure 2.5b). Plants originally from BH (mean 52.4±1.3 days) produced flowers earlier than plants from both EJ (mean 60.4±1.6 days, p=0.0026) and AZ (mean 57.3±2.3 days, p=0.1047) populations (Figure 2.5b). Similar to leaf time, flowering time was affected by fungal treatment as plants inoculated with EJ AMF (49.25±2.89 days) flowered earlier than both the no-AMF (mean 61.2±1.6 days, p=0.0006) and AZ treatments (mean 58.5±2.0 days, p=0.0124) although no difference was observed between plants inoculated with EJ and BH fungi (mean 55.03±1.6 days, p=0.1819, Figure 2.5c).

Effect of Sex

Sex had no significant effect on all fungal measurements (hyphae, arubuscules, and vesicles). There was also no effect of sex on the morphological traits measured (dry vegetative, reproductive and total biomass, # seeds, # of fruits, fruit mass). Total number of leaves and flowers were also not affected by sex, along with time to first leaf and flower.

There was a significant effect of sex in phosphorus acquisition. However, this effect was only seen in plants from population EJ ($F_{3, 234}$=2.84, p=0.0387, Hermaphrodite v Female p=0.0405) where hermaphrodites (Lsmean 0.326±0.014%) exhibited significantly higher percent leaf-P than females (LSmeans 0.28±0.017%) regardless of treatment.

Discussion

Local adaptation is generally considered as a process that describes populations' evolutionary changes that lead to a higher average fitness under the specific abiotic conditions in its environment, yet biotic factors may also have profound influences on plant adaptation (see examples in Grondahl and Ehlers 2008; Johnson et al. 2010; Sherrard and Maherali 2012). Here we provide evidence of local adaptation of the plant, *G. maculatum*, to arbuscular mycorrhizal fungi. We expected to see an increase in growth with AMF association, and more specifically a greater increase with home AMF associates as compared to away, or foreign AMF communities. We found support for faster leaf and flower time and greater biomass with AMF association compared to no-AMF treatments. Although **Home** treatments tended to produce more biomass and plant structures, **Home** was only significantly different from **Away** in percent leaf phosphorus**.** In addition, we expect that because of the phenotypic and physiological differences between female and hermaphrodite plants, they would form different levels of association with AMF, and we found evidence that leaf phosphorus content did differ between sexes in one population (EJ).

Plant Benefit from AMF Association

We found some support for our hypothesis that fungal inoculation will increase plant growth and reproduction, as plants with fungus produced higher numbers of fruits, seeds and more fruit mass. Though, overall mass of plants was not significantly benefitted by fungal inoculation. The lack of a strong trend may be due to fungal contamination in our control group. Prior to receiving their treatments, plants were sterile and did not show signs of AMF growth on their roots (data not shown). Once moved to the field, there were potential contaminating events that may have

taken place throughout the experiment. Although plants were separated from the soil by an air barrier of ~10cm, ant colonies settled in some of the plants over the growing season, rabbits and other herbivores also grazed some of the plants (despite fencing and scent deterrents), and heavy spring rains could have transferred spores from the surroundings through splash-back. We understand that with control contamination it is more difficult to draw accurate conclusions between AMF and no-AMF treatments. Yet, even with potential contamination, there was still a significant increase in reproduction and decrease in time to leaf and flower with fungal treatments, lending some validity to the support of our first hypothesis.

Local Adaptation

We also found some evidence that the performance of plants with their home fungus differed from those with away or non-AMF treatments, however the relationship was negative for home treatments. This support is mainly founded in differences between percent leaf phosphorus. Phosphorus analysis showed that plants associated with **Home** fungal treatment had a significantly lower percent of phosphorus in their leaves than plants with **Away** fungus. This suggests that *G. maculatum* plants are receiving more phosphorus when associated with fungal suites that they are not accustomed to, hinting at a benefit from foreign AMF. The typical trend is that a plant with AMF association has increased phosphorus uptake (Koide 1991; Lu and Koide 1994; Johnson et al. 2010) and fungal inoculants from local sites often increase the total phosphorus in a plant (Sanders and Fitter 1992; Johnson et al. 2010; Johnson et al. 2012; Sherrard and Maherali 2012). However, the opposite has also been shown, where plants with home fungal inoculation produced less phosphorus than with away fungus (Bohrer et al. 2003; Ji et al. 2013). In this scenario, plants potentially gain more phosphorus from the foreign fungi because the refined efficiency that developed between the co-adapted plant/AMF populations has

yet to develop between a foreign AMF population and the host plants. We also saw a trend for higher reproductive biomass in **Home** treatments, implying some benefit from home AMF communities. Plants with **Home** fungus produced more seeds, fruits and fruit biomass than **Away** and **No-AMF** treatments (Fig. 2.1) and flowered earlier (Fig. 2.5a). Although there was less phosphorus in the leaves of plants with H treatments, there may have been more phosphorus allocated to reproduction. In this study we did not measure phosphorus content in flowers or fruits, and in the future this may help tease out this relationship.

Even though we tried to control for environmental factors such as water regime, soil type, soil microbial communities and light availability, there seemed to be a potential reaction in the plants from the vicinity of the garden arrays to the environment. However, there is also a population effect, as plants from BH produce on average significantly more leaves and flowers than AZ (personal observation, unpublished data). These natural morphological differences may have carried over to this study. Plants from BH (which was located within 15 miles of all three garden arrays) produced significantly more average leaves than those from other populations. They also showed to have higher overall biomass than the other populations (Fig. 2.3). Plants from population BH produced, on average, significantly more flowers than plants from population AZ, although total reproductive biomass mass was not significantly different. To elucidate the mechanism behind this variance between populations, it would be beneficial to measure plant growth under controlled conditions and expand this experimental design to each of the plant home sites. Planting three garden arrays at each of the three collection sites would help control for any unseen environmental effect, however due to low population densities of *G. maculatum* scaling up may not be functionally possible without extensive cloning. Greenhouse

growth experiments would help quantify any population genetic effect on the clones' sizes and flower timing/production.

One interesting result from this study was a significant effect of AMF community location on time to first flower and leaf. Plants inoculated with AMF from the BH population produced leaves sooner than any other fungal treatment, and AMF inoculation from EJ decreased time to flower. Evidence from other studies suggests that not only are AMF genotypes important in predicting plant growth (Johnson et al. 2010) but a more complex reaction between plant, home soil and AMF may also have an effect on plant growth (Pankova et al. 2011; Schechter and Bruns 2013). Pankova et al. (2011) found that AMF were affected greatly by the soil environment they were extracted from, typically performing worse in foreign conditions. Having homogenized the soil treatment for our plants, our intent was to eliminate the soil effect so as to see only plant/fungal interactions. However, we still found evidence of certain AMF species compositions affecting plants from different locations in a similar way. Further study of this potential AMF community effect should identify the species associated with this decreased leaf/flower time. Isolation of these species and further testing of their effectiveness could potentially lead to the discovery of more beneficial AMF morphotypes.

Effect of Plant Sex

Contrary to our predictions, we found very little direct evidence that sexes differ in their relationships with AMF. There was no difference between sexes in all measures of fungal association, including percent colonization of hyphae and arbuscules, as well as all the morphological measurements, including biomass and time to leaf and flower. However, hermaphrodites and female plants from population EJ differed significantly in their mean percent

leaf phosphorus. This was unexpected considering the report by Varga and Kytoviita (2010b) on the differences in fungal benefit of the two sexes of *Geranium sylvaticum*, a congener to *G. maculatum*. Varga and Kytoviita (2010b) showed that female plants had higher percent phosphorus compared to hermaphrodites, and that without AM, hermaphrodites did worse than females. We found that hermaphrodites had higher leaf-P than females, and that this effect was only present within one population.

We found no difference in plant benefit from AMF between sexes, and it is possible that we did not see any trend because, unlike Varga and Kytoviita (2010b), we were not examining the effect of only two isolated fungal species, but a whole suite of field extracted AMF associates. Benefit has been shown to increase with the diversity of fungal associates (Koomen et al. 1987), but it has also been shown to vary depending on the species composition of the AMF (Dhillion 1992; Gavito and Varela 1995; Johnson et al. 1997; Collin and Ashman 2010). Mycorrhizal fungi often compete and interact with one another to associate with a plant's roots, and these interactions can alter the effectiveness of the symbiont (Kennedy et al. 2007; Jin et al. 2013). To really develop a clear idea of the association between AMF and different sexes in *G. maculatum*, individual AMF species need to be isolated from the field and grown up separately, and in combination, with both females and hermaphrodites. Additionally, more in depth measurements, such as pollen viability and flower, fruit and pollen phosphorus levels will help better describe this interaction between AMF and a plant's sex.

Conclusions

To summarize, we provide preliminary evidence for local adaptation of *Geranium maculatum* plants to their home site fungus, and evidence of increased growth with AMF

association. Plants with home AMF inoculants did not exhibit vegetative biomass differences, but showed increased biomass for fruits, and a higher number of fruits and seeds. Leaves tended to come out earlier and flowers were also produced earlier under home AMF treatments. However, since there was contamination in the control treatment, these patterns may not have been as clear as if the plants had stayed sterile. There also appeared to be a population phenotype artifact, as plants from BH produced more biomass at a faster pace than other populations.

Our findings have broad scale implications for the local adaptation literature. We provide evidence for local adaptation to home fungi, but we also provide some support for fungal specificity to their environment. Plants inoculated with fungus from populations BH and EJ showed a decrease in time to leaf and time to flower, respectively, and this unique interaction deserves to be examined more closely. There is evidence that the home soil environment of AMF can functionally affect their capability (Pankova et al. 2011), and there is the potential for this relationship to be affected by other factors in the environment. It would be interesting to see what effect growing plants in various soil and environmental factors, emulating a fungus's home conditions, would have on the effectiveness of the fungal suite. Another interesting future direction for this work would be to quantifying any differences in AMF species compositions that exist between species and populations of *Geranium* plants. The discovery of common AMF species could be used to describe a beneficial suit of AMF for *Geranium maculatum*, and other native congeners, potentially being used for conservation purposes.

Local adaptation to biotic influences is just as important as studying adaptation to abiotic factors. This area of study provides a unique look at species adaptations and can offer an interesting perspective on how populations interact with their home environments. Understanding adaptation on such a fine scale will benefit research on plant-AMF relationships

in the future, providing evidence for an even more specialized relationship between host and fungus. Continued exploration of AMF interactions with plant sexes will be important for the understanding of this complex relationship. This interaction provides potential for the development of an inoculant specific to plant sexes, which would greatly benefit crop species that share these unique mating systems.

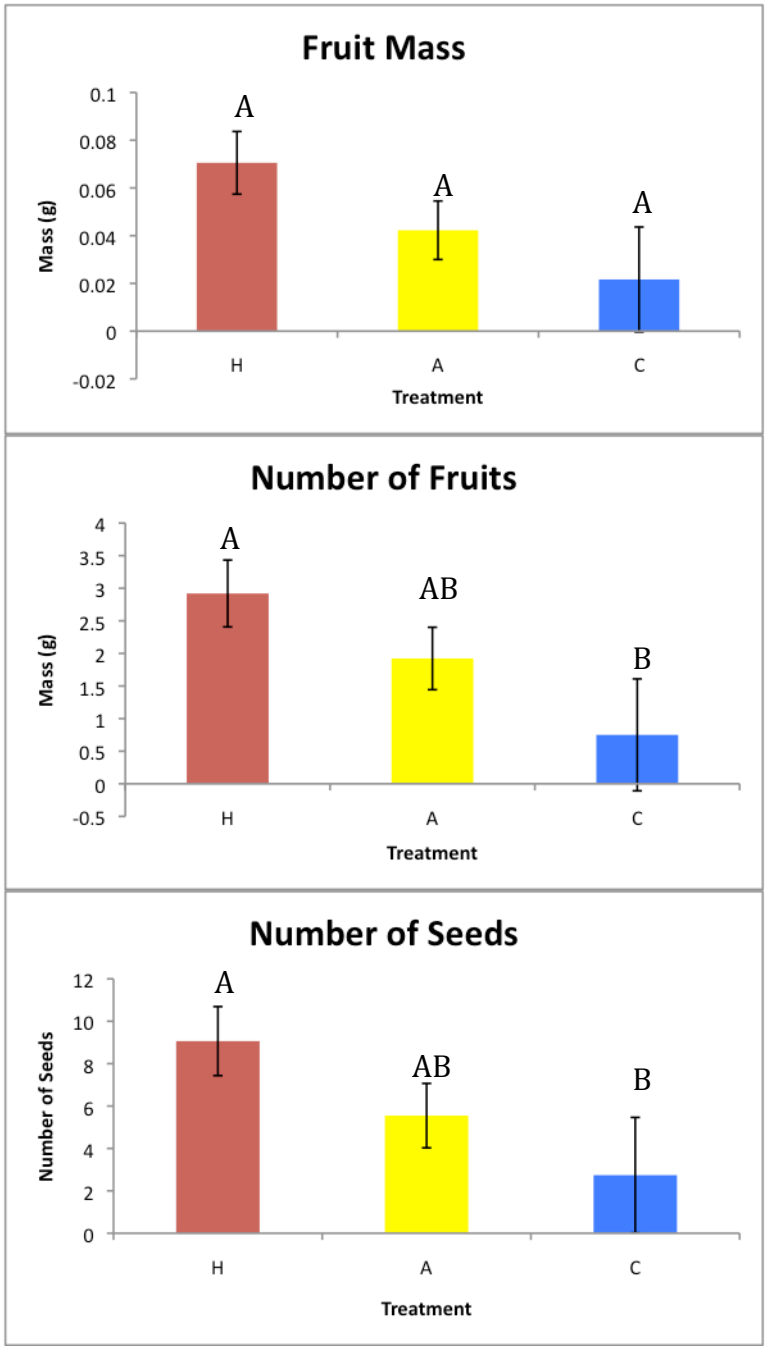


Figure 2.1. Reproductive output under A=away AMF, H=home AMF, and C=control non-AMF treatments. Letters denote significance, with shared letters implicating no significant different between treatments.

*Table 2.1. Fungal colonization data from all BOT plants and twenty (10-flowerin 10-non-flowering) plants from DOR and GRE garden arrays. LSmeans of percent colonization for all population and garden array combinations, as well as LSmeans of **H**, **A**, and **C** treatments are shown. Treatments within a population are not significantly different if a letter is shared.*

		Source Population								
Hyphae	Garden Array	AZ	s.e.	Significance	BH	s.e.	Significance	EJ	s.e.	Significance
	BOT	0.22870951	0.03318681	A	0.35879083	0.02842522	A	0.32687294	0.03383196	A
	DOR	0.07017424	0.03540917	B	0.06485055	0.03067408	B	0.11694408	0.03306491	B
	GRE	0.08942274	0.03263305	B	0.03466864	0.02958355	B	0.11202546	0.03189124	B
	Adaptation		s.e.	Significance						
	H	0.16097032	0.02106191	A						
	A	0.15703599	0.01526606	A						
	C	0.14948001	0.0205022	A						
Arbuscules	Array	AZ	s.e.	Significance	BH	s.e.	Significance	EJ	s.e.	Significance
	BOT	0.12868688	0.0204957	A	0.1979065	0.01755501	A	0.16001544	0.02089413	A
	DOR	0.043439	0.02186819	B	0.03282567	0.01894387	B	0.06107679	0.02042042	B
	GRE	0.06070472	0.0201537	B	0.02133156	0.01827037	B	0.0444545	0.01969557	B
	Adaptation		s.e.	Significance						
	H	0.09273929	0.01300753	A						
	A	0.08314783	0.0094281	A						
	C	0.07425989	0.01266186	A						
Vesicles	Array	AZ	s.e.	Significance	BH	s.e.	Significance	EJ	s.e.	Significance
	BOT	0.04301419	0.01906004	A	0.12396011	0.01632534	A	0.13165588	0.01943056	A
	DOR	0.02054328	0.0203364	A	0.02416697	0.01761691	B	0.04780746	0.01899003	B
	GRE	0.03701724	0.018742	A	0.00873441	0.01699059	B	0.05083695	0.01831596	B
	Adaptation		s.e.	Significance						
	H	0.05518291	0.0120964	A						
	A	0.05785905	0.00876769	A						
	C	0.04953687	0.01177494	A						

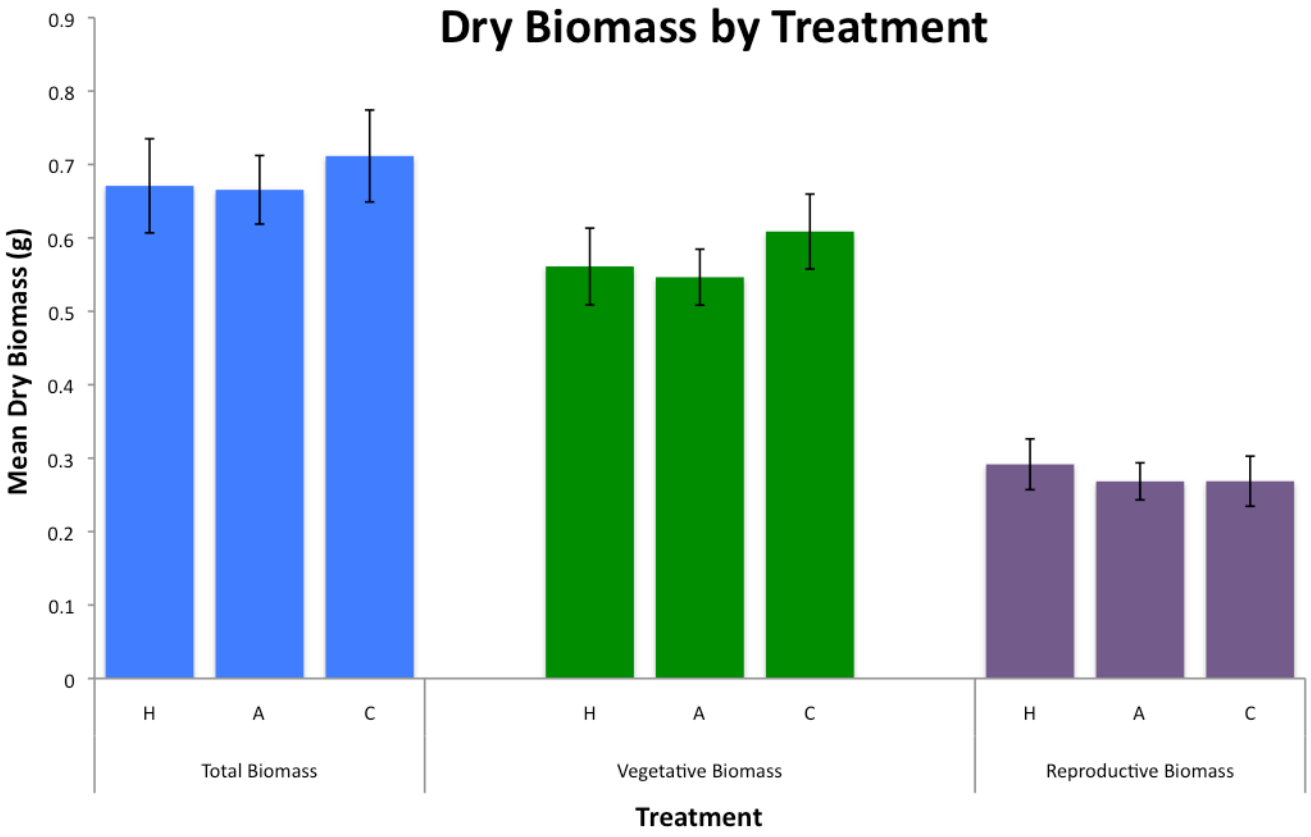


Figure 2.2. Dry masses (g) for vegetative, reproductive and total biomass measurements across all three treatments, H-home, A-away, and C-control. There were no significant differences within a trait measured.

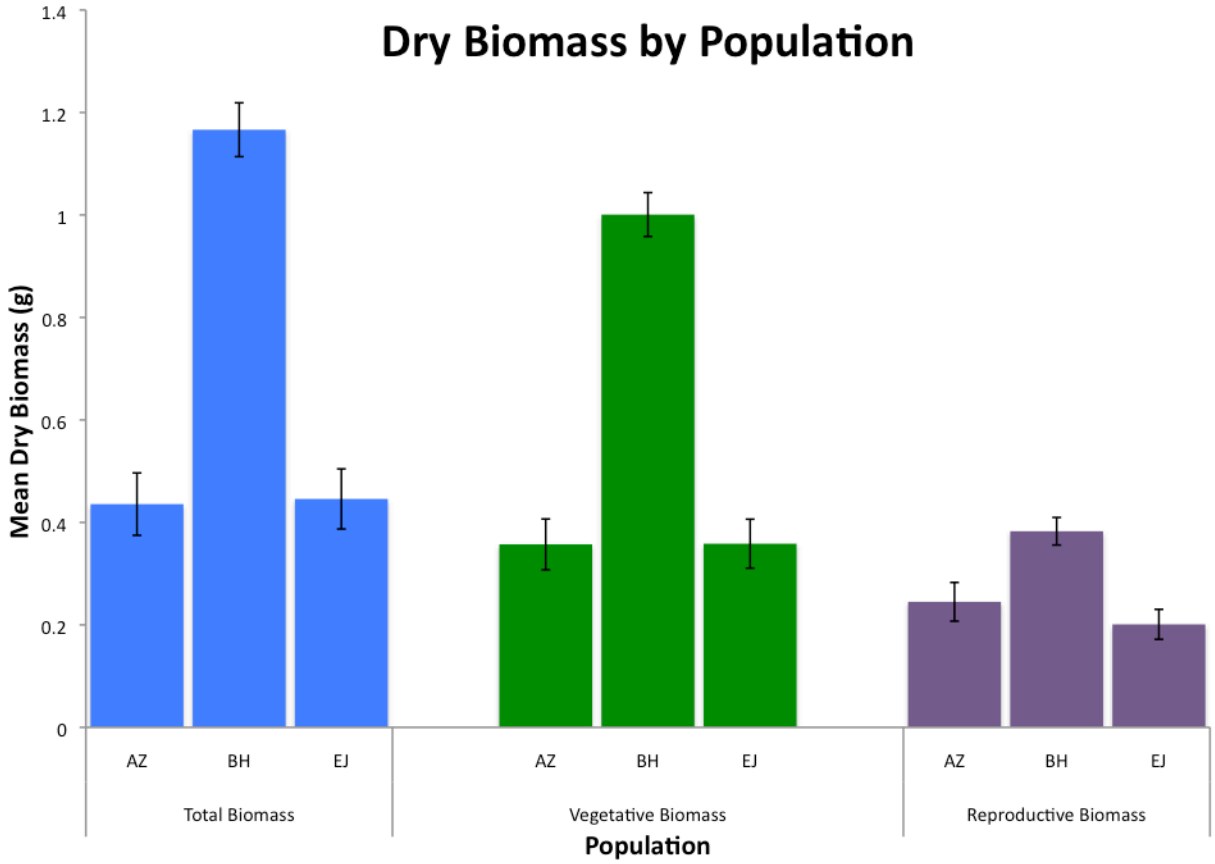


Figure 2.3. Dry Biomass measurements (g) of each population. BH has significantly more biomass than either AZ or EJ for vegetative ($p<0.0001$), reproductive ($p=0.0037$, <0.0001) and total biomass ($p<0.0001$).

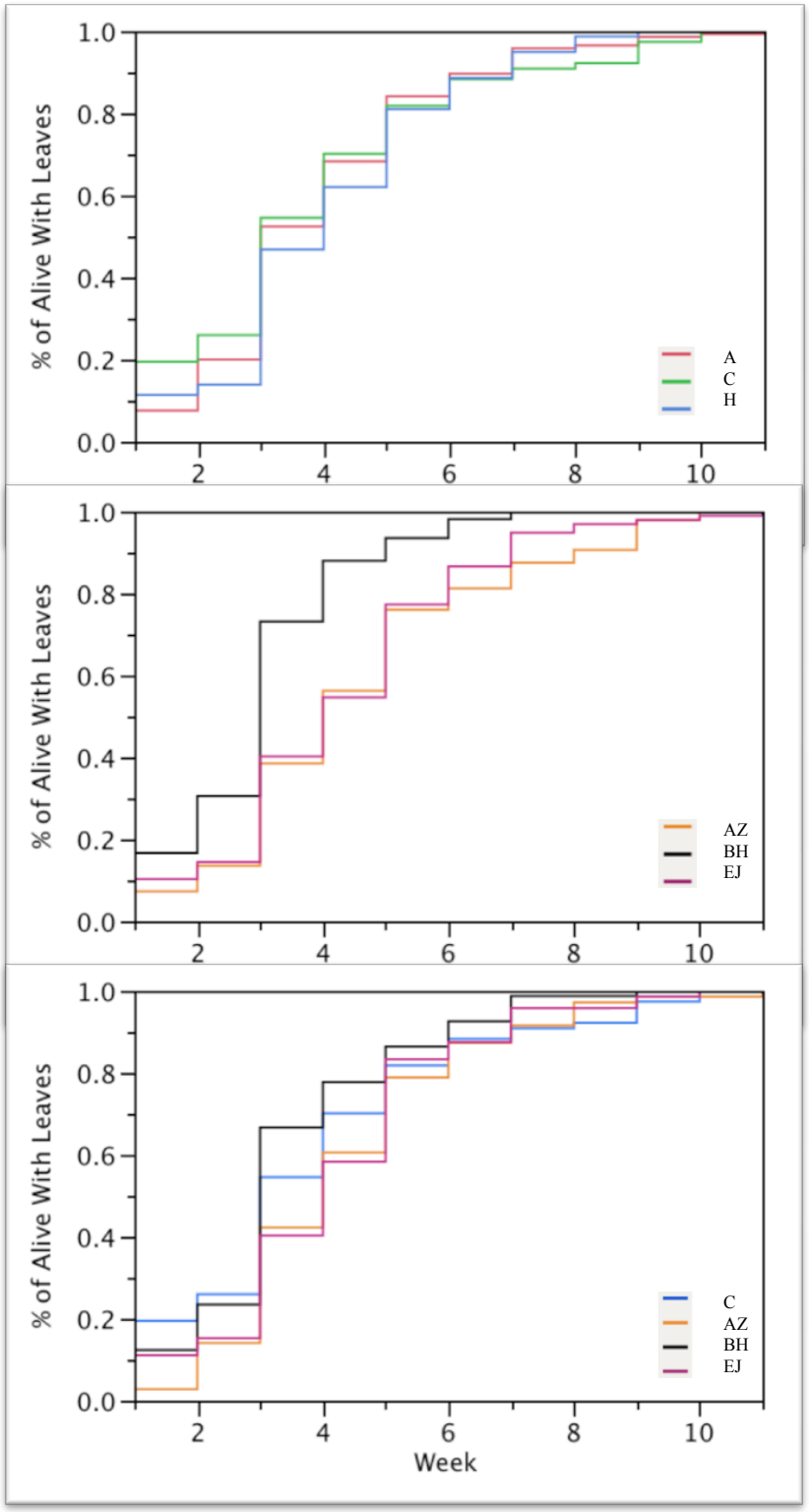


Figure 2.4. Survival analysis of time to first leaf emergence by week. Grouped by a) Treatment, b) Population and c) Fungal treatment.

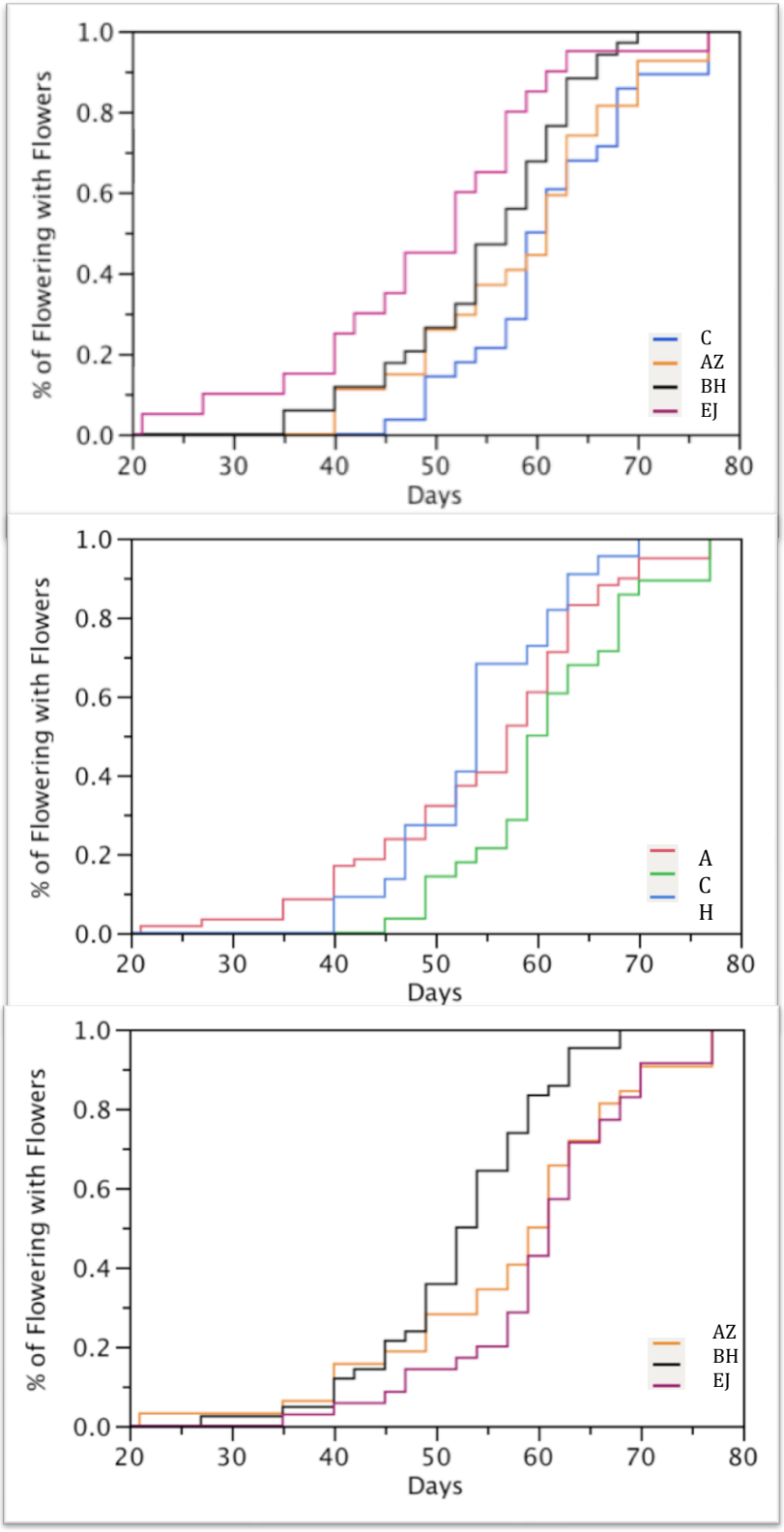


Figure 2.5. Survival analyses of time to first flower (days) grouped by a) fungal treatment, b) treatment and c) plant population. All three groupings show significance (a-p=0.0024, b-p=0.0024, c-p=0.0001).

CHAPTER 4

CONCLUSIONS

Here we summarize our findings as they pertain to the symbiotic relationship between arbuscular mycorrhizal fungi (AMF) and the gynodioecious plant, *Geranium maculatum*. We also place our findings in a broader context of their implications for the gynodioecious literature and provide direction toward future research.

Root Colonization at Important Plant Growth Phases

Specific nutritional needs of plant species vary temporally, depending on the biological stage of the plant (Poulton et al. 2001a; Poulton et al. 2002). Phosphorus is a vital nutrient in flower production, whereas nitrogen and carbon are more important during vegetative growth; it is from this line of thought that we expected to find evidence of varying levels of association between AMF and their plant hosts. Due to differences in tissue production, we also expected AMF association to vary between sexes in the gynodioecious plant, *Geranium maculatum*. There was variation in colonization rates, with higher association near times of reproductive effort, and a decline post reproduction. There was no significant evidence of differences in association between the different sexes and AMF.

Not only were there significant differences between phases, but also between populations within a phase. These variances noted could potentially be due to population environment affects on association levels. Specific plant/AMF historical associations in those populations could also have influenced the colonization. However, a more complex interaction between the plant, AMF

and environment, may account for these variations (Vega-Frutis et al. 2013). It would be interesting to examine the role fungal association has with population dynamics, and what role, if any environment is playing on shaping these populations. Complex abiotic and biotic interactions may drive adaptation between *G. maculatum* and AMF, forming unique associations between species' heritable traits in each environment (Sherrard and Maherali 2012; Jablonka 2013; Vega-Frutis et al. 2013).

Plant Local Adaptation to AMF Communities

Organisms in long standing symbiosis have the potential to locally adapt, but not in the traditional sense of to their environment, but to those biological factors that are influencing that environment. We strove to provide evidence of local adaptation with the spotted geranium, *Geranium maculatum*, and the AMF communities that it associates with. We hypothesized that plants would benefit from fungal association, and specifically benefit more from their home suite of AMF than from foreign site fungus. We also thought that due to the physiological and morphological differences between sexes in *G. maculatum*, colonization levels and plant benefit from association may differ accordingly. We believed that the differences in plant response seen between home AMF and away AMF treatments would likely be due to genotypes (plant and fungus) having adapted concurrently, so that both players have developed to benefit the most from their interaction. Potential adverse effects of associations that are not adapted to each other may manifest as decreased plant growth, possibly due to a less efficient mycorrhizal relationship and thus phosphorus transfer (Koide 1991; Johnson et al. 2012).

With our common garden study, we provide preliminary evidence for local adaptation to AMF in *G. maculatum.* Home AMF inoculation increased plant reproductive output and a

decreased time to flower. Although this relationship was significant between home **(H)** and control **(C)**, there was no significant difference between fungal treatments (home and away). There was also a strong significant trend between plant morphological measurements and plant home population, with plants from BH consistently having higher biomass and earlier leaf emergence than other populations. From these trends we can conclude that although plant growth was not significantly affected by AMF community composition, overall AMF inoculation did affect reproduction in *G. maculatum*. This affect was not well defined, thus we discuss further implications and the future direction of this work below.

AMF Associations in Gynodioecious Plants

There have been few studies examining the relationship between AMF and gynodioecious plants, comparatively (Pendleton 2000; Varga and Kytoviita 2010a, b; Varga et al. 2013; reviewed in Vega-Frutis et al. 2013). Information concerning these relationships may be important for crop species that exhibit this unique mating system, such as beets and strawberries (Botham et al. 2009; Collin and Ashman 2010). Furthermore, gynodioecy has been historically thought of as a transitional stage between hermaphroditism and dioecy (Charlesworth and Charlesworth 1978; Charlesworth 1981), and close associations like AMF have the potential to influence adaptation through constant close contact. Evidence of beneficial, detrimental, and no advantage have been found in gynodioecious species, with no clear trend arising (Varga 2010; Varga and Kytoviita 2010a, b; Varga et al. 2013). Because of the importance of this system to the evolution of mating systems and the lack of knowledge in this area we chose to examine this relationship.

Our results from the aforementioned studies did not provide any conclusive evidence of the benefit or detrimental roles that AMF may play in the growth of the two sexes of *Geranium maculatum* plants. Colonization levels in the field were similar between females and hermaphrodites, and correlations between fungal structures and plant morphology did not differ. General AMF colonization increased during plant reproduction, but no difference was seen between sexes. We also observed evidence of plant adaptation to local fungal inoculants, yet again, with no difference between sexes. However, in one population we found higher percent P in hermaphrodites than in females (regardless of treatment). This preliminary evidence provides some support for sex differences in AMF benefit that needs to be explored more in this system.

Future Directions Concerning Geranium maculatum and AMF

The effects of AMF associations on gynodioecious plants are still largely uncharacterized in the literature, and there is much left to be understood about this relationship. More long-term studies examining the specific fungal players in association with *Geranium maculatum* will be beneficial in quantifying this symbiosis. It is important to better understand symbioses occurring in the field, as these natural interactions may have profound affects on the evolution of mating systems (Varga 2010; Vega-Frutis et al. 2013). In the case of *G. maculatum*, greenhouse experiments with supportive field components will be useful, as quantifying the benefits of certain AMF isolates on growth, at least partially, in an artificial setting will reduce variation provided from field work and allow for more informative comparisons. Lastly, more research focused on the local adaptation of AMF to *G. maculatum* through large scale garden studies with multiple fungal and soil treatments would be beneficial in answering how plants and fungi have adapted to their environment throughout their symbiotic history.

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