

Plant size influences mycorrhizal colonization of
Polemonium foliosissimum

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ABSTRACT

The purpose of this experiment was to investigate the relationship between the different genders of the gynodioecious species *P. foliosissimum* and their mycorrhizal associates. Mycorrhizal colonization of roots was quantified, and soil and plant tissue analyses were performed. Further, a full factorial clipping experiment was conducted in which plants of both genders were reduced in size by approximately one half in order to stress the associations by reducing the potential carbon assimilation available to be exchanged with the fungal partner. Colonization of roots of plants involved in the experiment was then quantified and plant tissue analysis will be performed. Gender did not influence overall mycorrhizal colonization or proportional colonization of mycorrhizal structures within roots; however, soil and plant tissue analyses indicate that females may be extracting higher amounts of nutrients from the soil than hermaphrodites, in particular magnesium, though this difference could not be attributed to a mycorrhizal benefit in this study. Further, the size of the plant host (# of stems) was found to significantly influence mycorrhizal colonization prior to and subsequent to the clipping treatment and proportional colonization by mycorrhizal vesicles increased significantly in plants subject to the clipping treatment. It is suggested that a size-specific relationship exists between *P. foliosissimum* and its mycorrhizal symbionts and that when under stress, the mycorrhizal associates reallocate assimilated carbon to storage structures.

INTRODUCTION

Most angiosperms are hermaphroditic – male function is garnered through the production of pollen, and female function through the production of ovules and seeds. In gynodioecious plants, some plants are male-sterile and exhibit only female function, while others are typical hermaphrodites. Having given up male function, females must have an advantage to persist in populations. Several models seek to explain the success of females in populations in which they at first appear to be at a disadvantage; these include higher tolerance to reduced nutrient availability, sex-specific differences in tolerance to plant enemies (i.e., herbivores), and sex-specific interactions with other species in which one sex morph receives a greater benefit from associations with other organisms, including mycorrhizal fungi (as reviewed by Ashman, 2006).

Nearly two-thirds of all plant species maintain a symbiotic relationship with arbuscular mycorrhizal fungi (AMF) (Helgason and Fitter, 2005). AMF are endophytic fungi that penetrate roots of plants and extend thread-like hyphae into the surrounding soil (Allen, 1991). These hyphae act, in effect, as extensions of the root system of plants, gathering inorganic nutrients (i.e. phosphorus) from the surrounding soil and transporting them to the root in exchange for photosynthetic carbon (Allen, 1991). Through providing access to soil nutrients, mycorrhizae affect their plant hosts in other ways as well, e.g., by stimulating photosynthesis, increasing plant resistance to pathogens and herbivores, and stimulating photosynthesis and the rate of nutrient cycling (Newsham et al., 1995, Kula et al., 2005, Hart and Klironomos, 2002). In exchange for these services, AMF acquire all of the carbon used for growth and reproduction from their plant hosts (Smith and Read, 2008). For gynodioecious plant species, AMF have been observed to

increase male function of hermaphrodites and increase the phosphorous uptake of females (Pendleton, 2000, Varga and Kytoviita, 2010). These findings suggest that mycorrhizal associations in gynodioecious species may be sex-specific, conferring differing benefits on their plant hosts, and are also in line with models which suggest that females persist in gynodioecious populations due to differential benefits accrued by mutualistic associations.

Polemonium foliosissimum, is a perennial, non-woody herb found in sub-alpine regions of the southwestern United States (McDonough and Laycock 1975). Some populations of *P. foliosissimum* are gynodioecious, while others are purely hermaphroditic (AK Brody and G Clarke, personal communication). Despite the importance of AMF to plant fitness, surprisingly few studies have been performed to assess the relative influence of AMF on the different sex morphs of gynodioecious species and, to my knowledge, no study has evaluated the relationship in the gynodioecious *P. foliosissimum* and its mycorrhizal partners. In this study, I asked: do females receive increased benefits from their association with mycorrhizae than do their hermaphroditic counterparts? I hypothesized that the mycorrhizal association between *P. foliosissimum* and its mycorrhizal partners is sex-specific and confers greater benefit upon the females of this species. To test this hypothesis, I examined the mycorrhizal associations of both females and hermaphrodites and conducted a clipping experiment to observe whether differences in response to reduction in photosynthetic material (ie., herbivory) between the sexes can be explained by differing relationships with their mycorrhizal associations. This was done by quantifying mycorrhizal root colonization and analyzing the nutrient content of leaf tissue as a proxy for mycorrhizal benefit. Several plant functional traits were also measured in order to characterize the plants involved in the experiment.

METHODS

Assignment of plants to treatments

To examine the relationship between the *P. foliosissimum* sex-morphs and their mycorrhizal symbionts, I performed a full factorial experiment in which females and hermaphrodites were subject to a reduction of approximately 50% of their photosynthetic ability by a clipping treatment, and the effect of this reduction on the mycorrhizal associations of the sex-morphs was characterized by obtaining roots of plants clipped and non-clipped for comparison of fungal colonization. Also, I collected roots of plants not clipped at the time of clipping in order to infer the concurrent status of the mycorrhizal associations of the clipped plants. Further, I used the plant tissue collected at clipping for analysis of nitrogen and phosphorous content for three females and three hermaphrodites, each representative of a different plant size class. And finally, I performed soil nutrient analyses on the soil collected from three females and three hermaphrodites, again representative of different plant size classes. At the end of the experiment, plant material from both clipped and non-clipped plants will be dried and analyzed for nutrient content. This step is currently in process.

The experiment was performed using a population of *P. foliosissimum* located in Gothic, CO. I first censused the population and selected thirty female and thirty hermaphrodite plants which showed little to no signs of previous herbivory or other

damage. Plants ranged in size from four to 31 total stems for both sexes. Plants were matched according to size and then randomly assigned to one of 3 treatments: 1) clipped, 2) cored for mycorrhizal colonization at the time of the clipping event, and 3) controls that were neither clipped nor cored. Because it was necessary to determine the sex of the plants prior to assigning them to treatments, the treatments were assigned and clipping completed within a the first few days of bloom for each plant. Plants were clipped or cored on July 16th and July 27th, 2011.

Collection of root material for analysis of mycorrhizal colonization at the clipping event

Prior to starting the experiment and in order to familiarize myself with *P. foliosissimum* roots, I excised several plants from near the Gothic population and rinsed their roots free of soil. I then took digital images of these known *P. foliosissimum* roots using a dissecting microscope for comparison with field collected roots. Before coring my experimental plants, I took cores at 5, 10 and 20 cms from the base of plants, extracted their roots from the soils and compared these to roots taken from excised plants. From this I determined that a 5 cm core contains more roots identifiable as *P. foliosissimum* roots, and so chose this as the standard sampling distance for all plants involved in the experiment.

Between July 16th and July 27th, 2011, I collected all root samples coinciding with the clipping event. Using a 2.5cm x 20cm soil corer, I extracted and pooled three soil samples 5cm from around the base of each plant in the coring treatment. The cores were taken evenly about the plant unless rocks below the surface of the soil prohibited coring to the full depth; in which case, cores were distributed around the plant as evenly and to as full a depth as possible. Using a dissecting microscope, I anatomically compared root fragments from the pooled samples to my digital images of known *P. foliosissimum* roots and isolated 50cm of roots .20mm-.50mm in diameter from each pooled sample. I then stained these roots with a 5% ink and vinegar solution, (Pelikan Royal Blue Ink 4001; Pelikan, Hannover, Germany) using the methods of Vierheilig et al., (1998). In brief, the roots were cleared in 10% KOH for 24h at room temperature, rinsed with tap water, bleached in 3% hydrogen peroxide for 20min, rinsed again, acidified with 1% HCl for one minute and then placed in 5% ink and vinegar for 24h, after which the stained roots were rinsed and stored in water acidified with a few drops of vinegar until slides were made for quantification of mycorrhizal structures. Stained roots were placed on slides and mycorrhizal structures were observed using a Leica DM E compound light microscope at 200x and 400x magnification and quantified following the methods of McGonigle et al., (1990). Observations indicated that mycorrhizal structures within *P. foliosissimum* roots could represent not only arbuscular mycorrhizae, but also ericoid and possibly arbutoid and/or monotropoid mycorrhizae. Photographs of mycorrhizal structures were taken for comparison with known images and consultation with experts. For this reason, quantification of nutrient exchange sites was not performed as the morphology of these structures are known to vary depending the type of mycorrhizal association observed. During the staining procedure, the stained roots of 4 plants decayed and were rendered un-quantifiable due to the time lapse between preparation and viewing; therefore the data for these plants were not available for analysis, and the total observations for pre-clipping colonization was reduced from 20 to 16.

Collection and analysis of soil at the clipping event

Soil cores were taken evenly about the site, one for each 750 ft² such that 9 cores were taken. These cores were homogenized and analyzed for nutrient content. Three hermaphrodites and three females were also selected to have their soil cores analyzed for nutrient content. The three individuals of each sex were matched in size such that each of the three plants per gender represented one of three size classes, 1-9 stems, 10-18 stems, and 19+ stems. Three soil cores were taken about the plant as described previously and homogenized prior to analysis. Samples were dried at room temperature for 96h and shipped to the University of Maine Analytical Laboratory. Organic matter was determined by loss on ignition, nutrients were extracted using the modified Morgan method, phosphorous content was determined colorimetrically; all other nutrients were determined by ICP-OES.

Collection and analysis of plant material at the clipping event

To reduce the photosynthetic ability of the plant in an attempt to stress the mycorrhizal association, plants assigned to the clipping treatment had stems removed at the clipping event. Stems were clipped 4 inches from the ground. In all cases, I selected stems in a haphazard manner with the determination on which stems to remove being made by subjectively attempting to reduce the plants above-ground mass by one half. Exactly half the stems were clipped except in cases where the total number of stems was uneven; in these cases, care was taken to remove a number of stems that would reduce the plant's above-ground mass by approximately one half.

The base of the clipped stems were wrapped in a moist paper towel and placed in an unclosed plastic ziplock bag. Plants were thus placed in a fridge at 6°C until processing. I then dried the clipped plant material for 84h at 55°C to a constant weight. Several of the leaves of one female plant were damaged while refrigerated prior to weighing; this plant has therefore been excluded from leaf measurements and tissue nutrient analysis. Three hermaphrodites and three females were selected to have their leaf tissue analyzed for nutrient content. These individuals were selected in the same manner as those for soil nutrient analysis, and according to the plant size classes described previously. Leaf tissue used for analysis was selected according to Cornelissen et al, (2003) in that only the youngest, fully expanded leaves were used, with petiole and rachis removed. Leaf nutrient content was analyzed by the University of Maine Analytical Laboratory, and determined by dry ashing, acid digestion, and inductively coupled plasma spectrometry.

Measurement of Whole-Leaf Size (WLS) and Specific Leaf Area (SLA)

Using the clipped plant material, I chose two of the youngest, fully expanded leaves for analysis of WLS and SLA and measured them according to Cornelissen et al. (2003) by digitally scanning the leaves and using ImageJ software (ImageJ version 1.42q, National Institute of Health, 2009) to calculate the area of the leaves. SLA was calculated by dividing the area of the leaf by its oven-dry mass, expressed in mm²mg⁻¹.

Collection of root and plant material at the end of the season

To observe for effects of clipping on the mycorrhizal associations of the two sex-morphs, I quantified colonization of the roots of clipped plants as well as the unmodified controls using the methods described previously. Data were arcsine square root transformed for normalization. Further, I selected plant tissue from all control and non-clipped plants for nutrient analysis as described previously. Plant tissue is currently drying and will be sent to the University of Maine Analytical Laboratory for analysis.

Results

Assignment of plants to treatments

A three-way ANOVA revealed no significant difference in plant size or previous damage between plants of each gender within assigned treatments ($n=60$, $F_{(2,54)}=.2328$, $p=0.79$; $F_{(2,54)}=.1327$, $p=0.88$ respectively).

Pre-clipping mycorrhizal root colonization

I used a one-way ANOVA to examine the influence of gender on mycorrhizal colonization for plants sampled at the time of the clipping event. I found that gender does not significantly influence mycorrhizal colonization of *P. foliosissimum* ($n=16$, $F_{(1,14)}=0.0027$, $p=.9589$). However, though not statistically significant, a two-way ANOVA examining the influence of the interaction between gender and plant size (gender*plant size) on mycorrhizal colonization revealed a trend indicating that larger hermaphrodites (with more stems) were more highly colonized than their female counterparts ($n=16$, $F_{(1,12)}=2.0623$, $p=0.1765$). I combined the colonization data for both genders and used a one-way ANOVA to examine the influence of plant size on mycorrhizal colonization, and found a significant difference in colonization based on plant size, with larger plants having higher colonization ($n=16$, $F_{(1,14)}=5.2428$, $p=.0381$) (Fig. 1).

Dry weight of the clipped material

I used a one-way ANOVA to compare the dry weight of the clipped material between the two genders of *P. foliosissimum*, and found that gender did not significantly influence the dry weight of the clipped material ($n=20$, $F_{(1,18)}=0.4656$, $p=0.5037$). However, a two-way ANOVA examining the interaction between gender*plant size revealed that the clipped material of larger hermaphrodites weighed significantly more than that of their female counterparts ($n=20$, $F_{(1,16)}=4.9971$, $p=0.0400$) (Fig. 2).

Whole leaf size and Specific Leaf Area of clipped material

Using a one-way ANOVA, I examined the influence of gender on both whole leaf size (WLS) and specific leaf area (SLA) of the clipped material. Gender did not significantly influence either WLS or SLA of the plants involved in this experiment ($n=19$, $F_{(1,17)}=.6432$, $p=.4336$; $F_{(1,17)}=.7646$, $p=.3941$ respectively); however, a two-way ANOVA examining the influence of the interaction between gender*plant size revealed that leaves of large hermaphrodites were significantly larger than their female counterparts ($n=19$, $F_{(1,15)}=4.7075$, $p=.0465$) (Fig. 3), while this same interaction was altogether insignificant in regards to SLA ($n=19$, $F_{(1,15)}=0.2361$, $p=0.6340$).

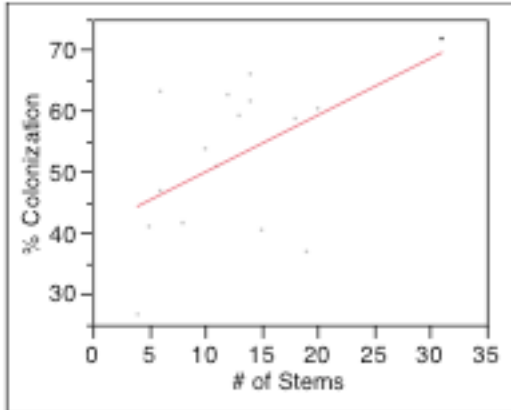


Fig. 1. Percent colonization of *Polemonium foliosissimum* roots by mycorrhizal fungi across plant sizes differed significantly prior to the clipping event ($n=16$, $p=0.0381$).

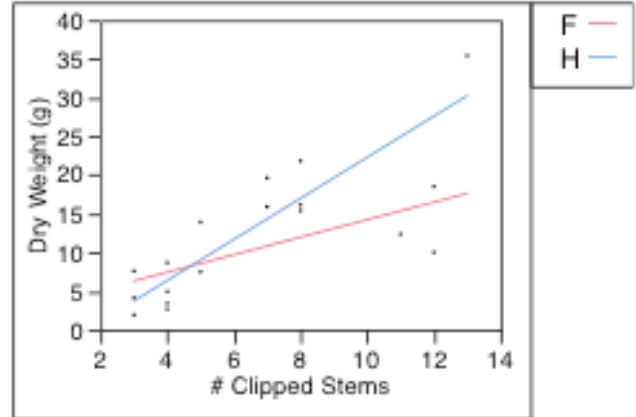


Fig. 2. Dry weight of clipped material differed significantly between the genders as plant size increased ($n=20$, $p=0.0400$). The number of clipped stems represents approximately half the total number of stems per plant (see methods).

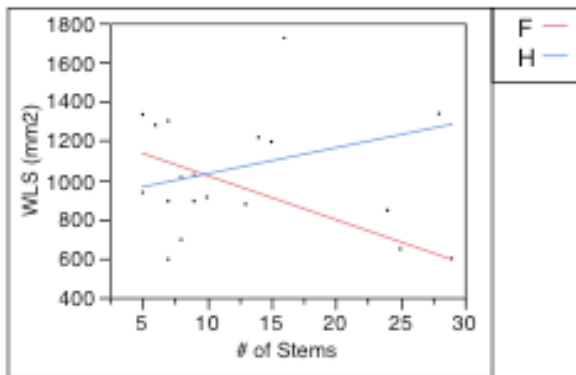


Fig. 3. Whole leaf size of clipped plants differed significantly between the genders as plant size increased ($n=19$, $p=0.0465$).

Soil nutrient analysis

I found no significant differences in the analyses among soils taken from females and hermaphrodites, and no significant difference between those samples and the

homogenized sample; therefore, for convenience, only data from the homogenized site analysis is reported in Table 1.

Analysis of plant tissue at clipping

Females had significantly higher magnesium content in their plant tissue than did hermaphrodites ($n=7$, $F_{(1,5)}=102.7450$, $p=0.0002$), and though not statistically significant, females had slightly higher concentrations of 8 of the 9 remaining nutrients analyzed, with boron being the exception (Table 2).

Table 1. Results of soil nutrient analysis

Soil Analysis	
pH	6.4
Org %	7.8
P (lb/A)	5.6
K (% Sat)	8.0
Ca (% Sat)	77.1
Mg (% Sat)	14.9
S (ppm)	8.0
B (ppm)	0.6
Cu (ppm)	0.27
Fe (ppm)	2.7
Mn (ppm)	3.6
Zn (ppm)	20.0

Table 2. Results of leaf tissue analysis at clipping, Mg content proved significantly higher in females ($n=7$, $p=0.0002$); 8 of 9 remaining nutrients analyzed were higher in females than hermaphrodites.

Ave Leaf Tissue content ($\pm$ SD)		
	Females	Hermaphrodites
P (% Mass)	0.43 ± 0.11	0.32 ± 0.09
K (% Mass)	2.73 ± 0.30	2.49 ± 0.32
Ca (% Mass)	0.90 ± 0.20	0.76 ± 0.14
Mg (% Mass)	0.20 ± 0.01	0.15 ± 0.01
B (ppm)	27.30 ± 3.90	27.70 ± 4.16
Cu (ppm)	8.63 ± 2.53	5.61 ± 1.67
Fe (ppm)	218.25 ± 45.65	160.33 ± 32.04
Mn (ppm)	44.88 ± 24.30	34.90 ± 22.44
Zn (ppm)	99.05 ± 31.53	54.50 ± 30.54
Al (ppm)	189.75 ± 56.48	121.43 ± 86.50

Post-clipping mycorrhizal root colonization

I used a two-way ANOVA to examine main effects and interactions of gender and clipping on mycorrhizal root colonization. The clipping treatment did not significantly influence overall mycorrhizal colonization ($n=38$, $F_{(1,36)}=0.1586$, $p=0.6928$); likewise, it did not differentially influence the overall mycorrhizal colonization between genders of clipped and unclipped plants ($n=38$, $F_{(1,34)}=0.0153$, $p=0.9023$). Further, I found that overall mycorrhizal colonization was significantly influenced by the interaction of treatment*plant size ($n=38$, $F_{(1,34)}=5.1746$, $p=0.0293$) in that smaller clipped plants experienced a reduction in colonization whereas larger clipped plants experienced an increase in colonization when compared to the control plants (Fig. 4).

Proportional colonization of mycorrhizal vesicles

Using a two-way ANOVA examining the influence and interaction of gender and clipping on the proportional colonization of mycorrhizal vesicles, I found no difference in vesicle colonization between genders in clipped or unclipped plants ($n=38$, $F_{(1,34)}=0.5762$,

p=0.4530). I therefore combined the data for the two genders and using a one-way ANOVA to examine the influence of clipping on vesicle colonization found that clipped plants had significantly higher percent vesicle colonization than unclipped plants (n=38, $F_{(1,36)} = 4.7404$, $p=0.0361$) **NEED BAR GRAPH HERE with SE or SD**. Further, there was a marginally significant influence of the interaction of treatment*plant size on vesicle colonization (n=38, $F_{(1,34)}=4.0071$, $p=0.0533$) in that larger plants experienced a greater difference in vesicle colonization than small plants (Fig. 5.).

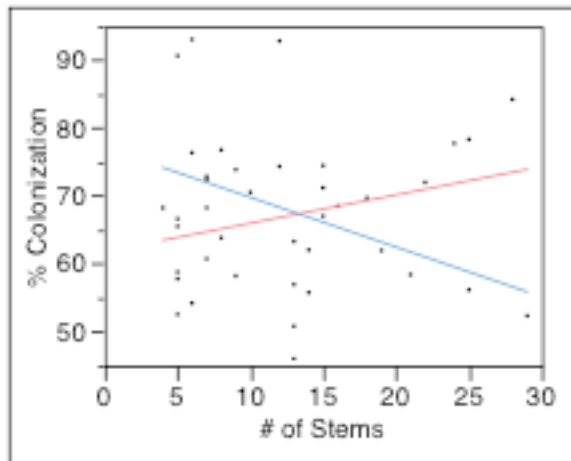


Fig. 4. Mycorrhizal colonization was significantly influenced by the interaction of treatment and plant size (n=38, $p=0.0293$)

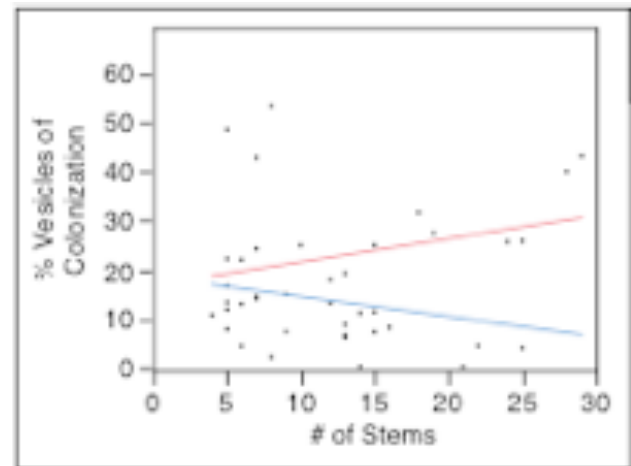


Fig. 5. Proportion of vesicle colonization was marginally influenced by the interaction of treatment and plant size (n=38, $p=0.0533$).



DISCUSSION

Sex-specificity in mycorrhizal colonization

The purpose of this experiment was to investigate the relationship between the different genders of *P. foliosissimum* and their mycorrhizal associates. I found no significant difference in mycorrhizal colonization of the roots of either gender; however, soil analysis results indicating that there were no significant differences in nutrient content of the soil whereas there were differences in the nutrient content of plant tissue indicates that females may be extracting higher amounts of nutrients from the soil than hermaphrodites, in particular magnesium, which was found in significantly higher concentrations in the leaf tissue of females prior to the clipping event and which is the coordinating element of chlorophyll *a* molecules. Further analysis of leaf tissue is required in order to determine if chlorophyll contents differ between the genders. Whether differences in nutrient content can be attributed to mycorrhizal colonization could not be determined from this study. In order to determine the extent to which

mycorrhizal colonization contributes to nutrient uptake by the two genders, an experiment including non-mycorrhizal plants is required. Further, analysis of plant tissue and quantification of seed set subsequent to the clipping event has not yet been completed, and so therefore it has yet to be determined whether mycorrhizal colonization in circumstances of reduced photosynthetic capability provides differing benefits to either females or hermaphrodites in regards to nutrient uptake and seed set.

Plant size influences mycorrhizal colonization

Interestingly, mycorrhizal colonization was found to increase significantly with plant size. Mycorrhizal fungi are considered obligate biotrophs in that they receive all of their required carbon from their plant hosts (Franken, 2010). This carbon is received in exchange for nutrients acquired from the soil by the fungus and may be contingent upon the relative cost of allocation to the fungus for nutrient acquisition versus the cost the plant would incur to develop a root system with comparable nutrient absorption capabilities (Neumann and George, 2010). Smaller plants, with less ability to assimilate carbon, may limit colonization if the benefit does not justify the cost; likewise, larger plants may be in a position to provide more carbon to mycorrhizal associates. Further, differences in relative root length may play a part in the difference in colonization between the plant sizes. Parsons showed that some perennial herbs reduce root:shoot ratios with age as they develop roots intended for longterm survival vice short term exploitation, though this study considered only root growth over the course of a single season (1967). To date, I have not located a life history record of *P. foliosissimum* in which this functional trait has been explored. It is possible that there is a significant decrease in the root:shoot ratio of *P. foliosissimum* as plants increase in size, such that larger plants have relatively less root length than smaller plants, while requiring more access to nutrients to support increased above-ground biomass. These larger plants may therefore make available more of their root length to mycorrhizal colonization in order to acquire sufficient nutrients.

Mycorrhizal association in response to clipping treatment

Previous studies have demonstrated varying effects of herbivory on mycorrhizal colonization, both in field and laboratory scenarios to include changes in colonization rates as well as changes in mycorrhizal species diversity (Ghering and Whitham, 2002). In the face of reduced photosynthetic capability, plants may redirect assimilated carbon from their mycorrhizal partners to the restoration of above ground structure (stems), unless there are below-ground factors that limit the growth of those structures, i.e. reduced nutrient uptake, in which case carbon may be allocated to roots in order to increase nutrient uptake (Ghering and Whitham, 2002, Wearn and Gange, 2007). Plant below-ground response to clipping can thus vary from reduction in root growth to increase in root growth. Mycorrhizal colonization subsequent to clipping is thus related to the host plant's response to herbivory (Ghering and Whitham, 2002). In this study, mycorrhizal colonization in response to clipping was seen to be differentially influenced by the size of the host plant, indicating that a size-specific relationship exists between

P.foliosissimum and its mycorrhizal symbionts whereby smaller plants experienced a reduction in colonization while large plants showed an increase in colonization.

Change in vesicular colonization subsequent to clipping treatment

Interestingly, the proportion of vesicles of the overall colonization increased significantly subsequent to the clipping event. Plant stress and reduced carbon assimilation capability may cause the fungus to reallocate carbon to storage structures in anticipation of future needs. Similar changes in vesicular colonization due to plant stress have been seen in clipping experiments, experiments modifying pH, as well as modifications of ozone (Klironomos et al. 2004, Titus and Leps, 2000, van Aarle et al., 2002, Duckmanton and Widden, 1994).

In conclusion, no gender-specific relationship between the sex-morphs of *P.foliosissimum* and mycorrhizal fungi has been detected thus far, though nutrient contents prior to the clipping experiment appear to differ in favor of females; and plant tissue analyses subsequent to treatment are still pending. An interesting effect of plant size on the mycorrhizal association was seen in plants prior to and subsequent to the clipping treatments, indicating that a size-specific relationship exists whereby plant carbon assimilation and allocation to fungal partners may differ over plant size. Further study into the patterns of root:shoot ratios, as well as comparisons of nutrient requirements and relative photosynthetic ability between small and large plants are required in order to ascertain the mechanism by which colonization increases with plant size as well as the mechanism by which plant size influences mycorrhizal colonization subsequent to simulated herbivory in *P. foliosissimum*.

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