GEOGRAPHIC AND SOIL NUTRIENT LINKS IN THE MYCORRHIZAL ASSOCIATION OF A RARE ORCHID, *CYPRIPEDIUM ACAULE*

by

WILLIAM DOUGLAS BUNCH

(Under the Direction of Richard P. Shefferson)

ABSTRACT

Mycorrhizal associations are a requirement for the germination of orchids in nature. Recent studies have shown that the distributions of ectomycorrhizal and arbuscular mycorrhizal fungi are highly correlated with soil nutrient availability. However, it is unclear how soil nutrient availability influences fungal association in the orchid mycorrhiza. This study was conducted with the goal of determining patterns in orchid mycorrhizal host specialization associated with soil nutrient conditions. Seventeen *Cypripedium acaule* populations were sampled across central and northern Georgia. Soil samples were collected at the site of each plant and analyzed for carbon, total nitrogen, ammonium, nitrate, and pH. Mycorrhizal fungal hosts of each plant were identified from root samples using DNA analysis of key fungal barcoding genes. *C. acaule* was found associating with a wide range of fungi, but was most commonly found associating with *Tulasnella* and *Russula* species. We observed a strong association between geography, soil nutrient availability and the fungi colonizing *C. acaule*.

INDEX WORDS: Mycorrhiza; Orchid; Soil nutrients; Geographic mosaic

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CHAPTER 1

INTRODUCTION

Symbioses are common in nature. These intimate ecological relationships play a large role in the organization of biological communities and often produce some degree of specialization (Douglas 1998; Thompson 2005). One of the most widespread symbioses is the mycorrhiza, which occurs between plants and fungi. This symbiosis is approximately 400 million years old and over 90 percent of all angiosperms form mycorrhizal associations (Read, Koucheki & Hodgson 1976; Newsham, Fitter & Watkinson 1995; Brundrett 2009; Bidartando et al. 2011). The mycorrhizal symbiosis involves the reciprocal transfer of carbon and nutrients between a plant and its fungal partner or partners (Smith & Read 2008; Kiers et al. 2011). The two most common mycorrhizae, arbuscular and ectomycorrhiza, are generally mutualistic. Orchids, however, have evolved their own form of mycorrhiza, which may be parasitic to its host fungus. (Rasmussen & Rasmussen 2009).

The orchid mycorrhiza is obligate to the orchid, but not the fungus. Orchid seeds cannot germinate and develop in the wild without the appropriate mycorrhizal fungal host to provide carbon, nitrogen and other nutrition (Rasmussen 1995; Smith & Read 2008). Most orchid species remain reliant on their mycorrhizal host to varying degrees, even after photosynthetic activity has been established (Rasmussen & Whigham 2002; Cameron, Leake & Read 2006; Rasmussen & Rasmussen 2009). The costs and benefits of these associations have been shown to vary depending on the fungal partner; however, on average these associations do appear to be generally parasitic, with the orchid taking nutrition seemingly without returning anything (Otero,

Bayman & Ackerman 2005; Bidartondo & Read 2008).

Most studies of orchid mycorrhizal associations assume that orchids have evolutionarily specialized on the fungi with which they associate in nature. Specialization is common in nature (Brooks & Mclennan 2002), and some orchids have an unusually narrow host breadth, suggesting that this has occurred (Shefferson et al. 2007; McCormick et al. 2004). Central to the Geographic Mosaic Theory of Coevolution is the idea that interacting species specialize on each other at least some of the time (Thompson 2005). Indeed, orchid mycorrhizal fungi may even choose their hosts. In nature, certain plants do detect, discriminate, and reward the best fungal partners with more carbohydrates (Kiers et al. 2011), although it is unclear how these dynamics might change in a more parasitic system like the orchid mycorrhiza. However, host breadth in nature may reflect differing host ranges and ecologies just as readily as specialization, and is really determined by both biological compatibility and ecological/geographic opportunity (Poulin 2006). The choice of fungal host in orchids may actually have less to do with specialization than with what fungi are available and the context of prevailing environmental conditions.

At present, there is little known about how the soil nutrient environment impacts the mycorrhizal host choice of orchids. The distribution of soil fungi is correlated with variations in pH and the availability of nitrogen, carbon and phosphorus in the soil (Batty et al. 2001; Diez 2007). In a nutrient-rich soil environment, it may be more cost efficient for a host plant to obtain its nutrients directly from the soil rather than from a mycorrhizal fungus that has a carbon cost (Tuomi et al. 2001) In nutrient poor sites, an orchid may choose a mycorrhiza that will help it attain a limiting nutrient in the soil. If this were the case then one would expect to see a high

degree of correlation between the identity of the orchid's fungal associate and the availability of carbon, nitrogen, phosphorus and other soil nutrients.

We investigated the geographic and soil nutrient factors influencing the mycorrhizal host breadth of the pink lady's slipper orchid, *Cypripedium acaule* (Orchidaceae). *C. acaule* has a relatively large mycorrhizal host breadth for the genus *Cypripedium*, and initial studies suggest that fungal hosts vary across populations of the species (Shefferson et al. 2007). We hypothesized that *C. acaule's* fungal hosts vary according to soil pH, elevation, percent organic matter and the availability of carbon and nitrogen in the soil.

CHAPTER 2

METHODS

Study Species

C. acaule's distribution ranges from the southeastern United States to northern Canada. Populations are often found growing in low pH soils, under variable conditions of moisture and shade. The main habitat types of *C. acaule* are pine-dominated forests and mixed hardwood-coniferous stands. Blooming in the southern part of *C. acaule's* range typically occurs from late April – May. During this time the plant will produce two opposite basal leaves and a single flower stalk.

Site Description

Root and soil samples were collected from 70 plants in 16 *C. acaule* populations in central to northern Georgia, USA (Figure 1). In each population, individuals were chosen so as to maximize the spatial distribution sampled. A maximum of five plants were sampled from each population, with lower numbers sampled for populations with less than ten individuals.

On average, elevation at the sampling sites was 513 m ± 32.4 m. Forty-nine of the 70 plants were sampled at an elevation ranging from 250 m to 353 m. The site with the highest elevation was 1039 m. Geographic coordinates ranged from 33° 44'00.4" - 34° 54'07.5" W and 83° 25'47.6" - 84° 42'52.9" N. The dominant canopy genera at all sites were *Pinus* and *Quercus*.

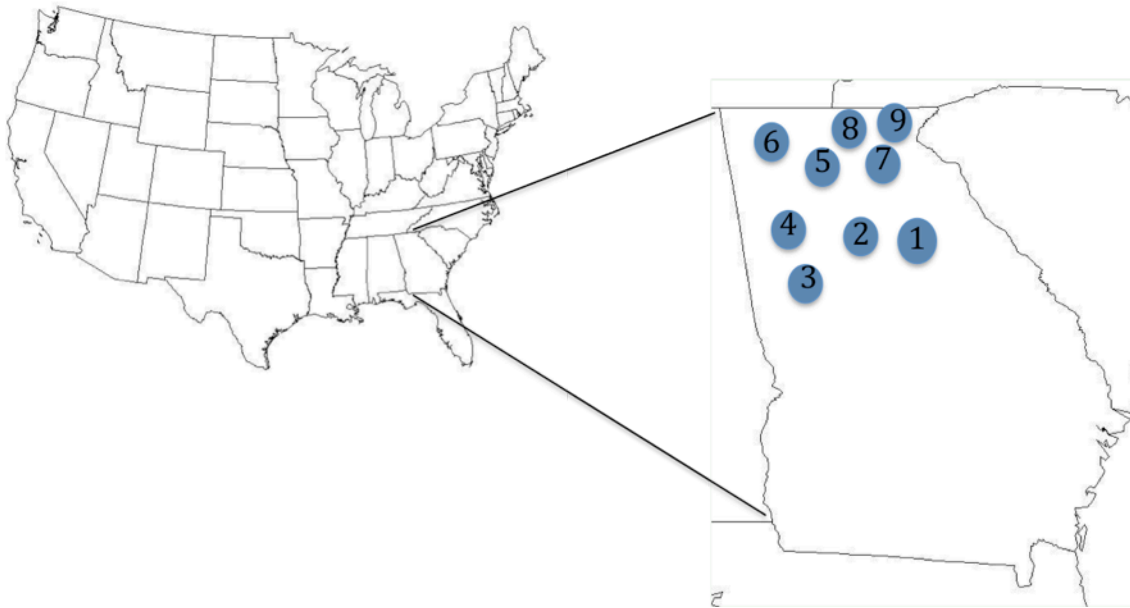

Figure 1: Study Area. Study area in middle and northern Georgia. Numbers correspond to clusters of populations sampled (1= Fort Yargo State Park, 2= Autrey Mill Nature Preserve, 3= Sweetwater Creek State Park, 4= Red Top Mountain State Park, 5= Amicalola Falls State Park, 6= Fort Mountain State Park, 7= Smithgall Woods Conservation Area, 8= Vogel State Park, 9= Black Rock Mountain State Park).

Soil Collection & Analysis

Leaf litter at the field sites was brushed away from the base of each plant before sampling the soil. Soil samples were collected around each plant sampled for fungal identification. This was done by taking 6 soil probes, 3 cm diameter, ~10cm deep, at approximately 5 inches from the base of each plant. The proportion of organic matter to mineral soil was estimated using the first soil probe. The soil samples were then homogenized on a tray for each individual plant. Soil not used for plant-available nitrogen was placed in a Ziploc bag for analysis of moisture content, pH, total carbon, and total nitrogen. Extraction of plant available nitrogen was started in the field, immediately upon homogenizing the soil probes. Plant-available nitrogen was determined by extraction in 2M KCl (Turner & Romero 2009). KCl samples from the field were taken back to the lab and weighed in order to determine the wet-weight of soil added to each sample cup.

The soils were then placed on a shaker table for 6 hours and filtered. The moisture content of the soil samples used for KCl extraction was determined by weighing a 5-gram subsample of soil, contained in the Ziploc bag, from each plant in an aluminum weigh boat within three hours of sampling. These samples were then dried for three days at 60ºC and re-weighed. Moisture content was then subtracted from the soils "wet" weight in order to determine the dry weight of the soil used for extraction of plant-available nitrogen. Nitrate and ammonium concentrations were determined using ion chromatography, at the Analytical Chemistry Lab at the Odum School of Ecology, University of Georgia. Using the dry-weight of the soil we determined the mass of nitrate and ammonium kg^{-1} of soil. Soil pH was determined using an Accument AB15 pH meter according to Robertson et al. (1999).

Total carbon and total nitrogen were determined via micro-dumas combustion at the Analytical Chemistry Laboratory at the Odum School of Ecology, University of Georgia (http://www.ecology.uga.edu/facilities_centers.php?Analytical_Chemistry_Laboratory-3/). Soil samples were oven dried for three days at 60ºC and subsequently ground with a ball mill. Twenty-five to thirty milligrams of the ground soil from each soil sample was then added to individual combustible tin capsules and sent to the Analytical Chemistry Lab, UGA for analysis.

Molecular Methodology

Four roots were removed from each plant and immediately placed on ice in order to determine their fungal associates. Roots were surface sterilized in the laboratory using 10% sodium hypochlorite solution. Each root was cut into 1-inch sections. Four cross sections from each 1-inch section were then examined under a compound microscope for the presence of pelotons, hyphal coils within the cells of the plant root cortex, which are the key evidence of orchid mycorrhizal colonization (Rasmussen 1995). Each section that contained pelotons was

placed in a microcentrifuge tube with 400 μL of AP1 buffer (Qiagen Inc., Valencia, California, USA). All roots were examined for mycorrhizal colonization and stored in an -80°C freezer within four days of collection.

We extracted DNA using the Qiagen DNeasy Plant Mini Kit. To identify the mycorrhizal associates of each plant, we PCR amplified the internal transcribed spacer (ITS) region using the following primer pairs: ITS1f-ITS4 (Gardes & Bruns 1993), ITS1OF-ITS4OF (Taylor & McCormick 2008), and ITS1f-cNL2f (Taylor et al. 2003). PCR samples were then Sanger sequenced via BigDye v3.1 chemistry on a 3730xl sequencer (Applied Biosystems). To corroborate results and potentially find unamplified fungi, we also PCR amplified the mitochondrial large subunit (mtLSU) rDNA using primers ML5-ML6 (Bruns et al. 1998).

A three-enzyme RFLP analysis was then performed on the successful PCRs using restriction enzymes Hinf1, Alu1, and Mbo1, and a number of samples representative of the unique RFLP types were chosen for Sanger sequencing of the ITS region. Fungal identification was performed using BLAST analysis of the resulting sequences. The closest match at the species level was determined using Emerencia, if the BLAST analysis revealed an uncultured fungal species (Nilsson et al. 2005).

Sequences in this project were submitted to GenBank (xxxx-xxxx). If a fungal species was found multiple times in a single plant it was excluded from the data set, because it was unknown if the fungi were from a single individual. Data obtained from a unique fungal sequence has no statistical power, so fungi that only occurred once were also excluded from further analysis.

We used PCR cloning to isolate mixed samples, using Strategene XL-10 Gold Ultracompetent cells (Stratagene Inc., Santa Clara, California, USA) and the pDrive cloning

vector (Qiagen Inc., Valencia, California, USA). Eight colonies per sample were PCR amplified and subject to an RFLP to determine fungal diversity per mixed sample. A sample of colonies representative of the unique RFLP types in each mixed sample was chosen for Sanger sequencing of the ITS region.

Statistical Analysis

A Shapiro-Wilk suggested that the geographic and soil nutrient data was not normally distributed and non-parametric analyses should be used. We tested if soil nutrient availability differed among sites with a Kruskal-Wallis test. We tested each soil component for spatial autocorrelation with a Mantel test with 1000 replications. The mantel test was run using the mantel.rtest function. We conducted a principal components analysis (PCA) to test for links between soil nutrient availability and fungal host. The PCA loadings were used to determine the strength of each component in determining the different environments where the fungi were found associating. The vectors used in the final PCA were elevation, pH, percent organic matter, percent carbon, percent nitrogen, nitrate, and ammonium. All statistical tests were run in R (R Development Core Team).

CHAPTER 3

RESULTS

Soil Nutrient Availability

The soil environment was highly variable between and within sites. Soil nutrient availability varied significantly among populations (Table 2). Percent carbon, percent nitrogen, percent organic matter, and pH were not spatially auto–correlated (Table 1). Soil pH was low at all sites, averaging 4.18 ± 0.05 and varied less than all of the other soil components (coefficient of variation = 0.101) (Figure 2a). Both carbon and nitrogen were present at low levels in soils at the sampled sites, with the former being more abundant than the latter (carbon: 6.45% ± 0.496%, nitrogen: 0.23% ± 0.014%). Carbon was also more variable than nitrogen (CV = 0.64). The coefficient of variation for nitrogen was 0.50. Ammonium was found to be nearly twice as abundant as nitrate on average (ammonium: 1.70 ± 0.177 vs. nitrate: 0.59 ± 0.056). Both ammonium and nitrate were highly variable with coefficients of variation of 0.79 and 0.87, respectively.

Table 1: Mantel Test. Scores correspond to the degree to which each variable is spatially auto-correlated, as determined by a Mantel test.

	Elevation	**% OM**	**pH**	**Total C**	**Total N**	**Ammonium**	**Nitrate**
Score	0.544	0.088	0.033	-0.048	0.03	0.0038	0.044
P-value	0.001	0.005	0.18	0.91	0.23	0.39	0.14

Table 2: Kruskal-Wallis Test. Chi-Squared values were calculated using a Kruskal-Wallis test, which was used to if geographic and soil nutrient factors varied significantly among the locations of each plant.

	Elevation	% OM	pH	Total C	Total N	Ammonium	Nitrate
Chi-squared	65.27	43.51	39.99	39.65	34.95	50.51	39.94
P-value	3.07E-08	0.0001	0.0005	0.0005	0.0025	9.95E-06	0.0005

Percent organic matter and percent nitrogen were the only variables significantly correlated with elevation (Table 3). Percent nitrogen and percent carbon were significantly correlated with one another (0.79). Additionally, percent organic matter, percent carbon, percent nitrogen and ammonium were all significantly and negatively correlated with pH (Table 3). Elevation and nitrate were the only components that were positively associated with pH (elevation: 0.18, nitrate: 0.29). Percent organic matter was significantly correlated with percent nitrogen, percent carbon, elevation and ammonium (Table 3). Nitrate was significantly correlated with percent organic matter, but was far less correlated with (Table 3).

Table 3: Pearson's Correlation Matrix. **(a)** Pearson's correlation matrix of soil and geographic components. **(b)** P-values associated with the correlation matrix.

(a)

	Elevation	% OM	pH	Total C	Total N	Nitrate	Ammonium
Elevation	1	-0.41	0.18	0.1	0.34	0.17	0.11
% OM	-0.41	1	-0.53	0.57	0.4	0.06	0.34
pH	0.18	-0.53	1	-0.51	-0.32	0.29	-0.09
C	0.1	0.57	-0.51	1	0.79	0.17	0.6
N	0.34	0.4	-0.32	0.79	1	0.1	0.45
Nitrate	0.17	0.06	0.29	0.17	0.1	1	0.22
Ammonium	0.11	0.34	-0.09	0.6	0.45	0.22	1

(b)

	Elevation	% OM	pH	Total C	Total N	Nitrate	Ammonium
Elevation	0	3.68E-04	1.40E-01	3.94E-01	3.84E-03	0.16178	3.84E-01
% OM	0.00037	0	1.99E-06	2.80E-07	6.11E-04	0.64656	3.79E-03
pH	0.13996	1.99E-06	0	6.55E-06	6.10E-03	0.01430	4.39E-01
C	0.39426	2.80E-07	6.55E-06	0	4.44E-16	0.15247	3.60E-08
N	0.00384	6.11E-04	6.10E-03	4.44E-16	0	0.41883	8.95E-05
Nitrate	0.16177	6.47E-01	1.43E-02	1.52E-01	4.19E-01	0	6.59E-02
Ammonium	0.38450	3.79E-03	4.39E-01	3.60E-08	8.95E-05	0.06586	0

DNA Sequencing

PCR product was obtained for 51 of the 70 plants sampled and 95 of the 250 root samples. Of the 95 root samples, 35 samples were either repeats of the same fungus in the same plant or a fungus that occurred only once. We found 30 unique fungal species colonizing the roots of *C. acaule* individuals (Table 1A). Only 15 of the 30 species occurred in more than one plant. The main fungal associates were from the genera *Tulasnella* and *Russula*, including *Tulasnella tomaculum* and *Russula crustosa*.

The average number of fungal taxa colonizing orchid individuals at each population was 3.5 ± 0.09. Population 3 at Fort Yargo State Park had the lowest elevation and the second highest diversity of fungal associates (FY: 5, Table 1A). Sweetwater Creek State Park population 1, on the other hand, had the second lowest diversity of fungal associates and was only 17 meters higher in elevation when compared to Fort Yargo population 3 (SWC: 2; Table 1A). There was no significant relationship between elevation and the number of fungal associates, according to a linear regression ($R^2 = 0.032$, p-value = 0.25).

Principal Component Analysis

Nitrate and ammonium did not significantly impact the PCA and were subsequently removed from analysis. The equation for the first principal component was PC1 =

0.243(elevation) + 0.385(percent organic matter) -0.445(pH) + 0.577(percent carbon) +0.512(percent nitrogen). The equation for the second component was PC2 = 0.707(elevation) - 0.526(percent organic matter) + 0.322(pH) + 0.346(percent nitrogen). Principal component analysis revealed that 76 percent of the variance in fungal associate could be explained within the first two components, using elevation, percent carbon, percent nitrogen, percent organic matter and pH as explanatory variables (PC1= 0.49, PC2= 0.27.9; Figure 2).

Certain fungal species are clearly habitat generalists, while others are found only in a particular environment type. *Tulasnella* spp. were found in a broad set of environmental conditions (Figure 3). *Tulasnella tomaculum* occurred in the largest range of environments, while *Tulasnella asymmetrica* was found under a narrow range of environmental factors. *Tulasnella asymmetrica* was typically found in environments where each of the soil nutrients was close to the average across all sites. *Tulasnella tomaculum* was found in sites ranging from high carbon, moderate elevation and high percent organic matter to sites with low carbon, low elevation and high pH (Figure 4). On average however, *Tulasnella tomaculum* was found at moderately low carbon, nitrogen and elevation levels.

Russula species were also found in a range of environments (Figure 5). However the range of environments varied slightly from that found across *Tulasnella* species. *Russula laccata* was typically found across a range of elevation, but with relatively high nitrogen, carbon, and percent organic matter conditions. Environments near the average of each soil nutrient tested had several potential fungal associates. Environments with high nutrient content or elevation tended to have a limited number of fungal associates (Figure 2).

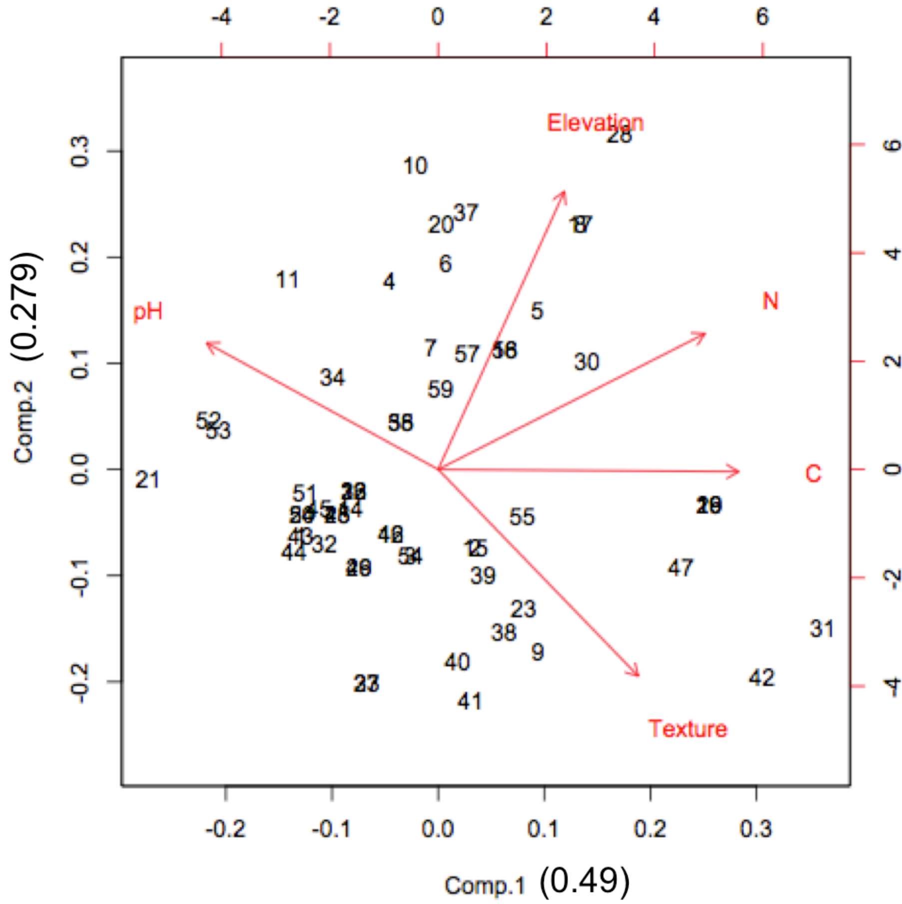

Figure 2: Principal Component Bi-plot. Principal component analysis using elevation, percent nitrogen, percent carbon, percent organic matter and pH as explanatory variables. Numbers correspond to the individuals sampled. The length of the vectors represents the strength of that variable on the PCA. The proportion of variance explained is listed beside each component.

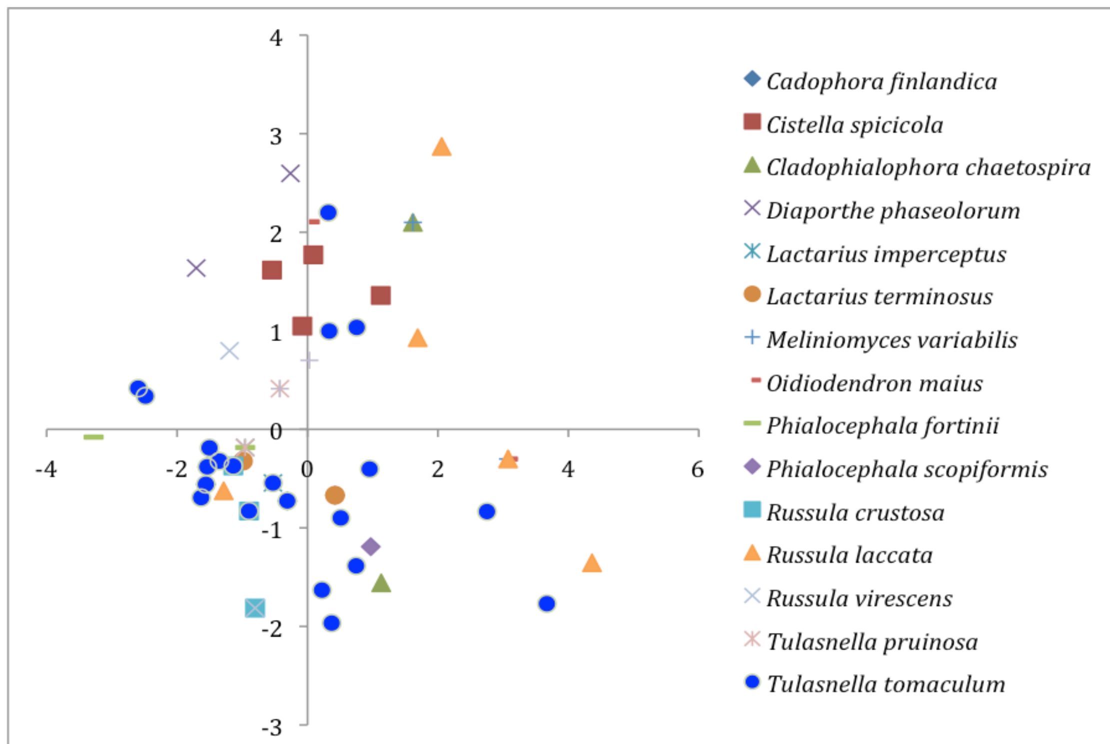

Figure 3: Fungal Soil Environment. This graph was produced using the first two components of the PCA. Each type of symbol corresponds to a different fungal host. Each point on the graph corresponds to a different host plant. The separation in points represents the unique soil environment found at each site. The soil components used in the PCA were elevation, pH, total nitrogen, total carbon, and soil texture.

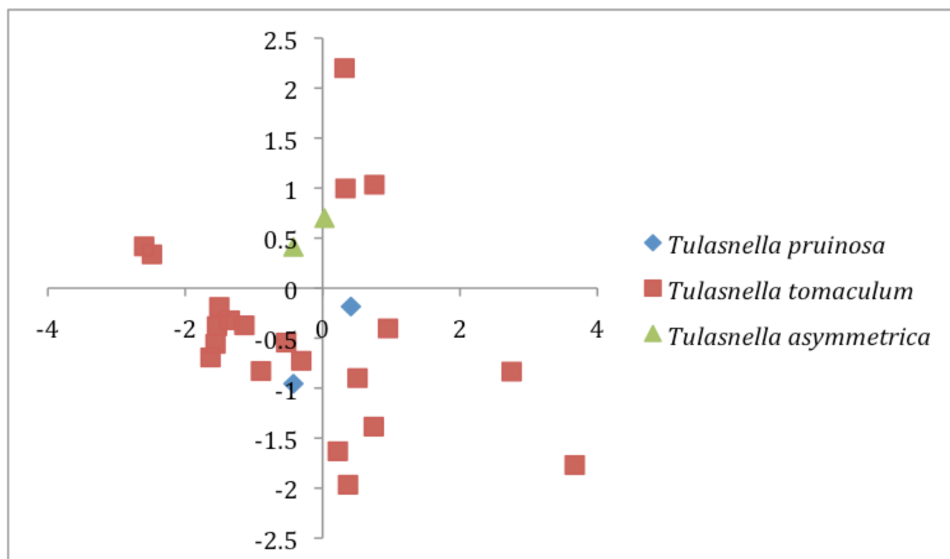

Figure 4: *Tulasnella* Soil Environment. Graphical representation of the environments where *Tulasnella* species were found. This graph was produced using the first two principal components of the PCA. The soil components used in the PCA were elevation, pH, total nitrogen, total carbon, and soil texture.

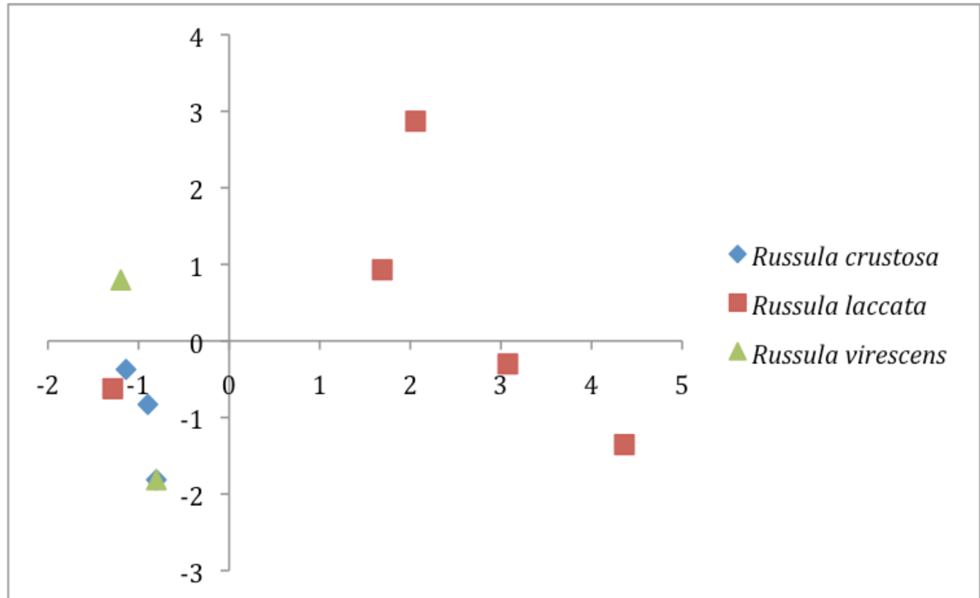

Figure 5: *Russula* Soil Environment. Graphical representation of the environments where *Russula* species were found. This graph was produced using the first two principal components of the PCA. The soil components used in the PCA were elevation, pH, total nitrogen, total carbon, and soil texture.

CHAPTER 4

DISCUSSION

Mycorrhizal specificity varies widely among orchid species and there are multiple hypotheses as to what causes this variation. Recent studies have shown that the distribution of soil fungi is linked to soil nutrient availability (Batty et al. 2001; Diez 2007); however, it is unclear how soil nutrient availability influences the orchid mycorrhiza, which is in many ways unique both ecologically and evolutionarily. We hypothesized that *C. acaule's* fungal hosts would vary according to soil pH, elevation, percent organic matter and the availability of carbon and nitrogen in the soil. Here we observed a strong association between geography, soil nutrient availability and the fungal host of *C. acaule*. Some fungal associates, such as *Cistella spicicola*, were found in relatively discrete environments, while other fungal associates, such as *Tulasnella tomaculum*, were found in a variety of soil nutrient conditions.

Overall levels of soil nutrients likely strongly determine the identities of plants and fungi entering a mycorrhizal relationship. In soils where nutrients are low the orchid may be more reliant on the mycorrhiza for nutrient foraging. Terrestrial orchids typically lack root hairs and are particularly poor at attaining soil nutrients without mycorrhizal associations. In high nutrient environments, however, mycorrhizal fungi are more likely to be parasitic, because it is easier for the plant to uptake nutrients directly, rather than having to pay a carbon cost to the fungi (Tuomi et al. 2001). The limited number of mycorrhizal hosts in environments with high nutrient availability is most likely due to less reliance on the fungus from the orchid after germination. Due to the carbon cost of the interaction, it could be more efficient for the orchid to obtain its

nutrients directly from the soil. There are probably few mycorrhizal relationships that are beneficial for the orchid in high nutrient environments. Fungal distribution could also be used to explain the occurrence of less diversity in fungal associates in relatively high nutrient environments. The habitat may be suitable for only a select number of appropriate mycorrhiza for the orchid. In this way fungal distribution, influenced by soil nutrient availability, may be driving the distribution or orchid host choice across landscapes.

Nitrogen availability is one of the most well known links between mycorrhizal associations and the soil environment. Plants can obtain inorganic forms of nitrogen from the soil without the assistance of mycorrhiza. In nitrogen-limited sites, mycorrhizal associations allow plants to obtain organic nitrogen. Our measures of plant-available nitrogen were not important in determining host environment across our low nitrogen environments, while total nitrogen was found to be significant. At first, this result might appear to suggest that the orchid is not specializing on particular fungi in order to obtain plant-available nitrogen from the soil and that fungal association may be primarily driven by fungal distribution in the soil. However, plant-available nitrogen has a fast turnover rate and a single sampling will most likely not give an accurate picture of the dynamic changes in ammonium and nitrate. Total nitrogen, on the other hand, is more stable than ammonium and nitrate alone. So while plant-available nitrogen appears to not play a large role, this is most likely due to the fast turnover of ammonium and nitrate. Nitrogen is highly correlated with fungal associate and further testing would need to be conducted to determine whether or plant-available nitrogen is significantly correlated with fungal association in *C. acaule*.

Mycorrhizal associations may vary in nature over wide areas, and so studies should be conducted over a wide area and should include the sampling of many populations. Such

sampling has the potential to reveal a greater breadth of host diversity. Variation in soil nutrient availability, however, was not spatially auto-correlated, thus investigating the relationship between orchid mycorrhiza and the soil environment likely does not require the same landscape level sampling as investigating diversity in fungal hosts. This has important implications for studies concerning rare orchids, which often occur in localized habitat types. There may be few populations at the landscape level and even fewer that can be accessed for sampling.

Although we did not measure natural selection in the field, either due to environment or to potential coevolution in the mycorrhiza, we may still speculate as to the evolutionary nature of this relationship in *C. acaule*. Since the identity of the fungal partner varied with soil characteristics and geography, selection imposed by the soil environment may differ across sites. Further, since the fungi differ across populations, any selection imposed on the orchid by the fungus is likely to differ across populations as well, and vice versa. This is consistent with the evolutionary history of the mycorrhiza in *Cypripedium*, which suggests a great deal of change and potential adaptation in *C. acaule*'s lineage relative to most of the genus (Shefferson et al. 2007, Li et al. 2011). Further, since fungi varied due more to soil characteristics than to geographic distance, genetic mixing among populations with differing mycorrhizal fungi may occur. In this circumstance, the genetic re-mixing that is required for geographic mosaics to lead to a fixed coevolutionary response (Gomulkiewicz et al. 2000) in *C. acaule* may already be occurring.

CHAPTER 5

CONCLUSION

We have shown that the soil environment heavily influences fungal association in *C. acaule*. Plant available nitrogen in the form of ammonium and nitrate was found to have very little influence on fungal host, while pH and elevation had a strong influence. Soil nutrient availability appears to be driving a mosaic of fungal associations, with the most common fungal associates being habitat generalists.

Though there is clearly a relationship between the soil environment and fungal associate, it is unclear whether this relationship is determined by fungal distribution or selection on the part of the orchid. Next generation genomic methods have the potential provide enough detail about the suite of suitable fungi in the soil to address this question. Using this methodology, the myriad of potential fungal associates at a particular site can be identified and an estimation of abundance can be made between sites. In order to explicitly show selection on the part of the orchid it may, however, be necessary to conduct a pot-design experiment with specific nutrient additions. These experiments have the potential to describe the interaction on a much finer scale than field sampling. This strategy could also be used to determine whether changes in the nutrient environment can trigger host switching and under what time scale this might happen.

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APPENDIX

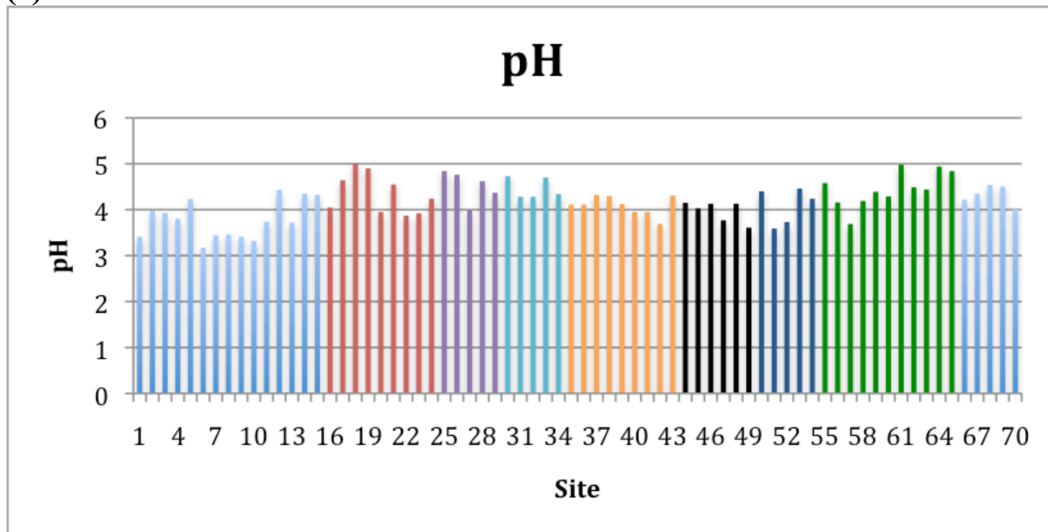
Table 1A: GenBank Accession numbers and BLAST search results for fungal taxa identified in this study. The species identification is based on the nearest BLAST match in GenBank (February 2012).

GenBank #	Species ID	Ref. Sample	Sequence	Nearest Match	% Match
	UC Ectomycorrhiza (Helotiales)	AMI_1_1_1	ITS	DQ497943	99%
	UC Ectomycorrhiza (Helotiales)	AMI_1_1_7	ITS	DQ497943	99%
	Cryptoporiopsis species	AMI_1_2_1	ITS	JF519423	95%
	Cryptosporiopsis melanigena	AMI_1_2_2	ITS	AF141196	97%
	UC Ectomycorrhiza (Helotiales)	AMI_1_3_5	ITS	DQ497943	99%
	UC Ectomycorrhiza (Helotiales)	AMI_1_3_8	ITS	DQ497943	99%
	UC Ectomycorrhiza (Helotiales)	AMI_1_4_1	ITS	DQ497943	99%
	Ilyonectria cyclaminicola	AMI_1_4_4	ITS	JF735304	100%
	Lactarius quietus	AMI_2_1_8	ITS	JF908289	99%
	UC Pezizomycotina	BRM_1_1_1	ITS	FJ554360	96%
	UC Pezizomycotina	BRM_1_1_8	ITS	FJ554360	96%
	Diaporthe sp.	BRM_1_3_4	ITS	EF488448	99%
	UC Tulasnellaceae clone	BRM_1_5_1	ITS	JF691406	87%
	UC Tulasnellaceae clone	BRM_1_5_10	ITS	JF691406	87%
	UC Tulasnellaceae clone	BRM_1_5_2	ITS	JF691406	88%
	UC Tulasnellaceae clone	BRM_1_5_6	ITS	JF691406	88%
	UC Tulasnellaceae clone	BRM_1_5_8	ITS	JF691406	88%
	UC Ectomycorrhiza (Russulaceae)	FM_1_1_1	ITS	DQ777999	99%
	Oidiodendron maius	FM_1_1_7	ITS	JN882306	95%
	UC Ectomycorrhiza (Russulaceae)	FM_1_1_8	ITS	DQ777999	99%
	Cladophialophora chaetospira	FM_1_3_3	ITS	EU035404	91%
	Meliniomyces variabilis	FM_1_3_7	ITS	HM190128	99%
	UC Ectomycorrhiza (Russulaceae)	FM_1_4_2	ITS	DQ777999	99%
	UC Ectomycorrhiza (Russulaceae)	FM_1_4_4	ITS	DQ777999	99%
	Oidiodendron maius	FM_1_5_6	ITS	HQ608115	99%
	Meliniomyces variabilis	FM_1_7_7	ITS	HM190128	100%
	UC Tulasnellaceae clone	FY_1_1_2	ITS	JF691406	89%
	UC Tulasnellaceae clone	FY_1_1_5	ITS	JF691406	89%

	UC Tulasnellaceae clone	FY_1_1_8	ITS	JF691406	89%
	Tulasnella tomaculum	FY_1_2_11	ITS	AY373296	87%
	Tulasnella tomaculum	FY_1_2_6	ITS	AY373296	86%
	Mycosphaerella sp.	FY_1_3_5	ITS	JN225926	95%
	UC Tulasnellaceae clone	FY_1_4_6	ITS	JF691406	90%
	UC Tulasnellaceae clone	FY_1_4_8	ITS	JF691406	81%
	Tulasnella sp.	FY_2_3_3	ITS	JN655634	87%
	Tulasnella sp.	FY_2_3_3	ITS	JN655634	95%
	Tulasnella sp.	FY_2_3_4	ITS	JN655634	94%
	UC Ectomycorrhiza (Russulaceae)	FY_2_4_2	ITS	DQ777999	99%
	Tulasnella tomaculum	FY_2_5_11	ITS	AY373296	86%
	UC Tulasnellaceae clone	FY_2_5_9	ITS	JF691406	88%
	Cladophialophora chaetospira	FY_3_1_1	ITS	EU137333	89%
	UC Helotiales	FY_3_2_4	ITS	FN565266	97%
	UC Phialocephala	FY_3_3_8	ITS	FJ378719	100%
	Tulasnella tomaculum	FY_3_4_3	ITS	AY373296	86%
	Tulasnella sp.	FY_3_5_2	ITS	JN655634	89%
	Tulasnella sp.	FY_3_5_3	ITS	JN655639	96%
	Tulasnella tomaculum	FY_3_5_4	ITS	AY373296	80%
	Tulasnella tomaculum	FY_3_5_5	ITS	AY373296	80%
	Tulasnella tomaculum	JC_1_1_1	ITS	AY373296	79%
	Phialocephala fortinii	JC_1_2_2	ITS	AY394921	99%
	Tulasnella tomaculum	JC_1_3_11	ITS	AY373296	89%
	Tulasnella tomaculum	JC_1_3_5	ITS	AY373296	91%
	Tulasnella tomaculum	JC_1_4_6	ITS	AY373296	80%
	Russula crustosa	JC_1_4_8	ITS	EU598193	98%
	UC Tulasnellaceae clone	JC_1_4_8	ITS	JF691406	82%
	Russula crustosa	JC_1_5_11	ITS	EU598193	99%
	UC Helotiales	RTM_2_1_2	ITS	FN565266	97%
	UC Ectomycorrhiza	RTM_2_1_4	ITS	AJ633589	98%
	Russula vesca	RTM_2_2_1	ITS	HM189956	100%
	Russula vesca	RTM_2_2_9	ITS	HM189955	99%
	UC Ectomycorrhiza (Russulaceae)	RTM_2_4_9	ITS	DQ777999	99%
	UC Leotiomycetes	RTM_3_1_2	ITS	FJ152529	97%
	Meliniomyce sp.	RTM_3_2_1	ITS	EF093175	99%
	Russula crustosa	RTM_3_3_2	ITS	EU598193	99%
	Russula crustosa	RTM_3_3_4	ITS	EU598193	99%
	UC Cantharellales	SGW_1_2_1	ITS	HM451551	85%
	UC Cantharellales	SGW_1_2_4	ITS	HM451551	86%
	UC Cantharellales	SGW_1_3_7	ITS	HM451551	87%
	Russula virescens	SGW_1_4_1	ITS	EU819437	99%
	Russula virescens	SGW_1_4_3	ITS	EU819437	99%
	Russula virescens	SGW_1_4_6	ITS	EU819437	99%

	Paraconiothyrium sporulosum	SGW_1_5_7	ITS	JF340257	96%
	UC Phialocephala	SWC_1_1_2	ITS	FJ378719	100%
	Tulasnella tomaculum	SWC_1_2_6	ITS	AY373296	90%
	Tulasnella tomaculum	SWC_1_2_7	ITS	AY373296	89%
	Tulasnella tomaculum	SWC_1_3_1	ITS	AY373296	88%
	Tulasnella tomaculum	SWC_1_4_9	ITS	AY373296	89%
	UC Fungus	SWC_2_1_3	ITS	GQ160036	99%
	Meliniomyces variabilis	SWC_2_1_8	ITS	HM190128	94%
	Lactarius imperceptus	SWC_2_2_1	ITS	EU819485	99%
	UC Helotiales	SWC_2_2_6	ITS	FJ475771	99%
	UC Cantharellales	SWC_2_2_7	ITS	HM451551	84%
	UC Helotiales	SWC_2_4_1	ITS	FN565266	97%
	UC Tulasnellaceae clone	SWC_2_4_5	ITS	JF691398	80%
	UC Tulasnellaceae clone	SWC_2_5_1	ITS	JF691398	80%
	UC Tulasnellaceae clone	SWC_2_5_2	ITS	JF691398	79%
	UC Tulasnellaceae clone	SWC_2_5_7	ITS	JF691398	79%
	UC Tulasnellaceae clone	SWC_2_5_7	ITS	JF691398	79%
	Tulasnella tomaculum	VOG_1_1_1	ITS	AY373296	87%
	Meliniomyces variabilis	VOG_1_1_6	ITS	HM190128	100%
	UC Fungus	VOG_1_2_2	ITS	EU754982	95%
	UC Fungus	VOG_1_2_6	ITS	EU754982	95%
	UC Ectomycorrhiza (Helotiales)	VOG_1_4_3	ITS	DQ497943	99%
	UC Cantharellales	VOG_1_5_2	ITS	HM451853	85%
	Russula sp.	VOG_2_3_3	ITS	DQ778002	99%
	Russula sp.	VOG_2_3_6	ITS	DQ778002	100%
	Russula sp.	VOG_2_3_8	ITS	DQ778002	99%
	Diaporthe sp.	VOG_2_5_1	ITS	EF488448	99%

(a)



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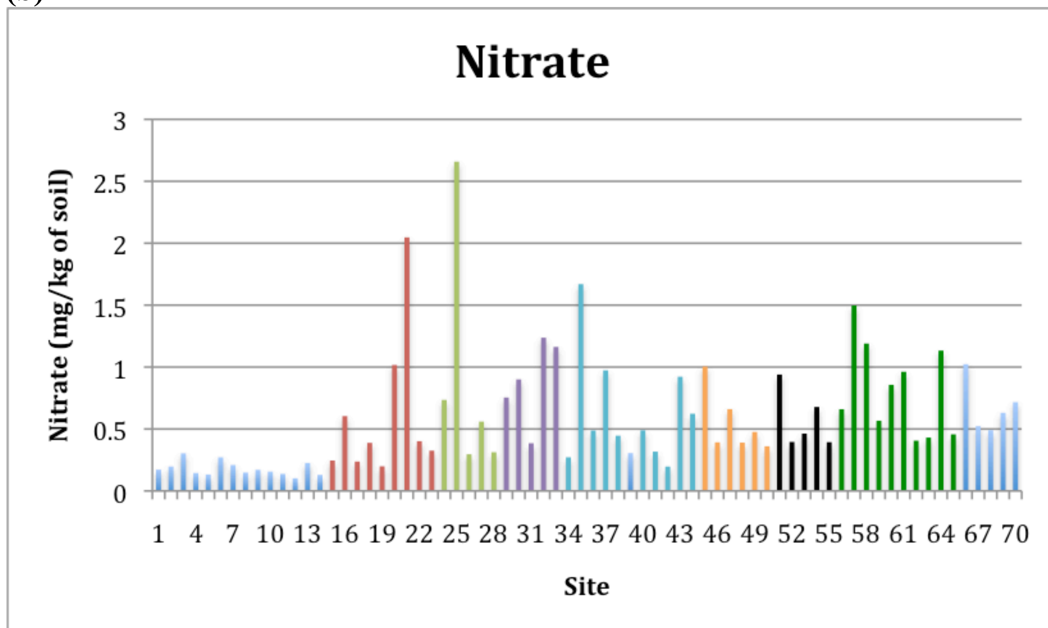

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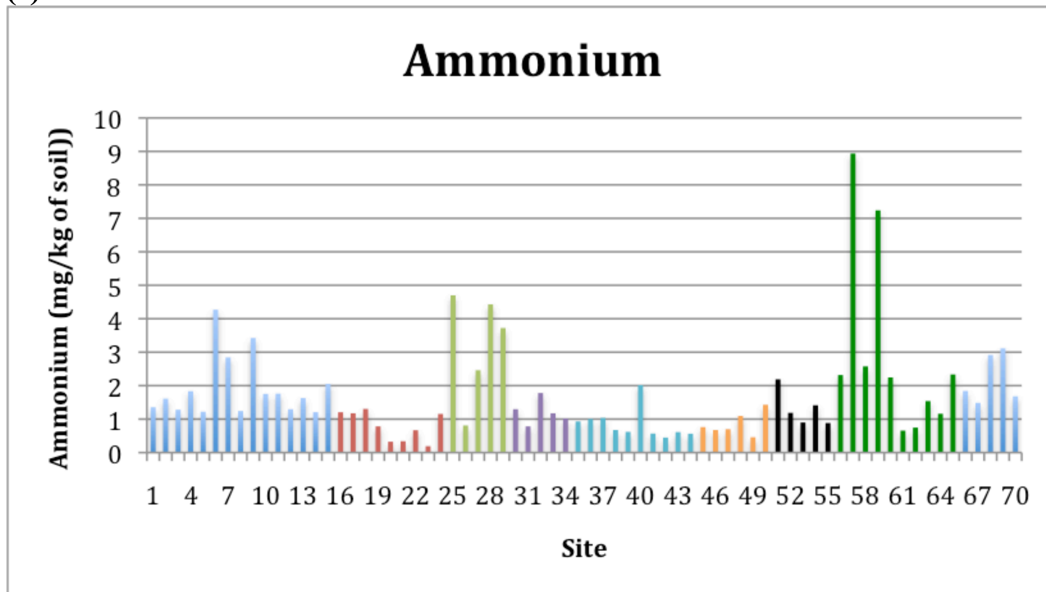

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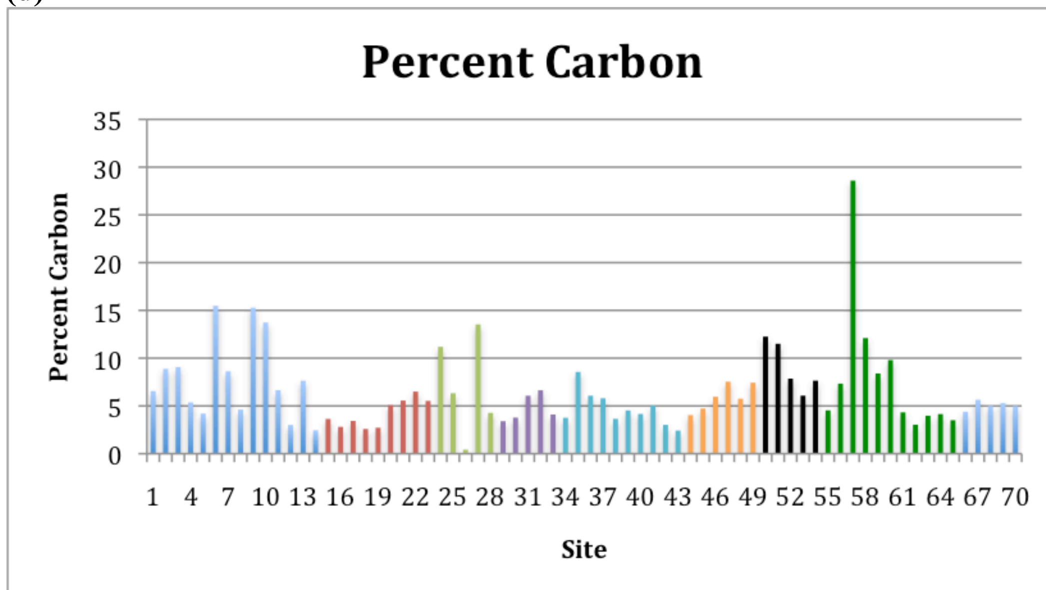

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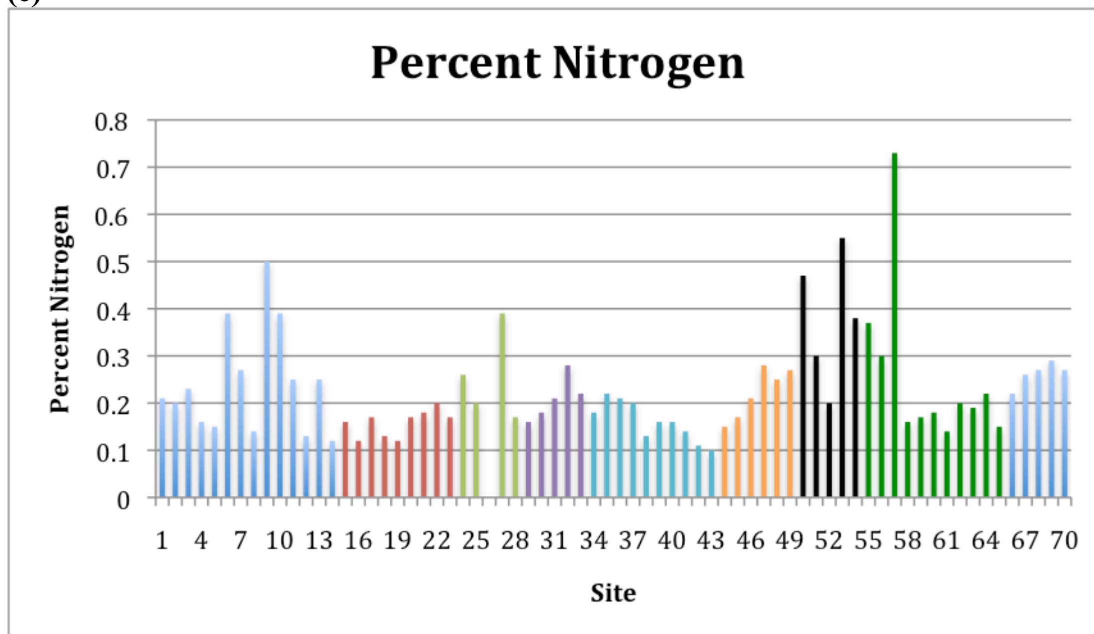

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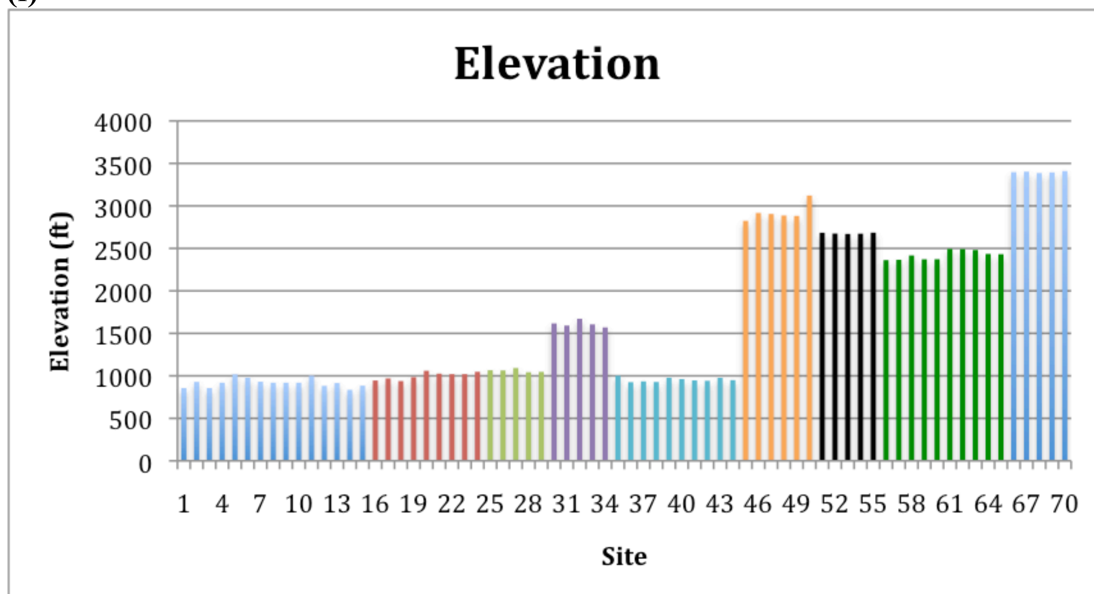

Figure 2A: (a-f) Graphical representation of the variation in soil nutrients and elevation. Each bar represents the site of a single plant. Populations are represented by different colors.