Differential colonization by ecto-, arbuscular and ericoid mycorrhizal fungi in forested wetland plants.

By

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Abstract

The roots of most land plants are colonized by mycorrhizal fungi under normal soil conditions, yet the influence of soil moisture on different types of mycorrhizal symbioses is poorly understood. In wet soils, colonization of woody plants by ectomycorrhizal (ECM) fungi tends to be poor, and colonization of herbaceous plants by arbuscular mycorrhizal (AM) is highly variable. However, little information is available on the influence of soil moisture on the colonization of ericaceous roots by ericoid mycorrhizal (ErM) fungi. Colonization was assessed microscopically in the ECM plant *Pinus strobus*, two AM plants (*Cornus canadensis* and *Lysimachia borealis*) and two ErM plants (*Kalmia angustifolia* and *Gaultheria hispidula*) along two upland to wetland gradients in Southwestern Nova Scotia. For the ErM plants, fungal ITS sequencing was used to assess community structure. The data indicate that ErM colonization increases with soil moisture in forested wetlands and is associated with distinctive fungal communities.

August 26th, 2019

For Heidi, my constant companion.

Table of Contents

Figures and Tables

CHAPTER ONE

Literature Review

Introduction

Mycorrhizae are important plant root-fungal symbioses that are active in the majority of ecosystems on earth but are not well understood in some specialized habitats (Read 1991). Wetland soils, which are often anaerobic and limited in nutrient availability, have traditionally been thought of as incapable of hosting mycorrhizal fungi (Bauer et al. 2003). As this idea is challenged by new evidence, more research is required to uncover the patterns of colonization that wetland mycorrhizae follow. Of the three most common mycorrhizal types, ectomycorrhizae are often negatively impacted by increased soil moisture (Jurgensen et al. 1996), while arbuscular mycorrhizae are affected, but less so (Brundrett and Ashwath 2013). Ericoid mycorrhizae, however, have gone nearly unexplored in wetland habitats, despite the fact that the ericaceous plants, whose roots form ericoid mycorrhizae, are common in wetlands (Read 1996; Cairney and Meharg 2003).

In addition to the impacts of soil moisture, other factors in wetlands may be important in determining colonization patterns including season (Bohrer, Friese and Amon 2004), pH (Wetzel and van der Valk 1996), nutrient availability (Clawson, Lockaby and Rummer 2001), plant community (Cornwell, Bedford and Chapin 2001), location on hummocks (Cantelmo and Ehrenfield 1999), and aerenchyma formation in plants (Cooke and Lefor 1998). Inconsistency of methods of mycorrhizal quantification only complicates the task of understanding wetland mycorrhizal colonization (Biermann

and Linderman 1981). Work must be done to compare all three major mycorrhizal types under consistent conditions and with standardized methodologies.

Mycorrhizae

Relationships with fungi are ubiquitous among plants (Saikkonen et al. 1998). Most plants form mycorrhizal symbioses with fungi that grow on and within their roots. Mycorrhizae are non-pathogenic and are beneficial or even essential for plant survival (Smith 1980). Mycorrhizal relationships allow plants better access to water and plant available nutrients in soil, while the fungi gain access to carbon in the form of photosynthesized sugars from the plant (Read 1991). Mycorrhizal hyphae take on the function of root hairs in plants by increasing the surface area for water and nutrient absorption (Beck-Nielsen and Vindbæk Madsen 2001; Bacheler 2014). Mycorrhizae can improve plant growth (Janos 1980), increase uptake of phosphorus and nitrogen in nutrient limited environments (Read 1996; Silvani et al. 2013), reduce toxic heavy metal uptake (Bradley, Burt and Read 1982; Read 1983; Juniper and Abbott 1993), and protect plants from pathogens and disease (Sikes 2010), among other functions (Gianinazzi et al. 2010). Enzymes produced by mycorrhizal fungi can help plants access organically bound nutrients that would be otherwise unavailable (Martino et al. 2018). Plant community structure, particularly in unstable environments, may be heavily influenced by the mycorrhizae that can be established (Kernaghan 2005).

Mycorrhizae can be grouped into three major categories, with the exception of some specialized or multifunctional types (Thormann, Currah and Bayley 1999; Smith

and Read 2008) Ectomycorrhizae (ECM) are mycorrhizae that form mainly on woody plants. They are characterized by the formation of a mantle of fungal hyphae around the root tips of the plant (Figure 1) and a Hartig net that interacts with root cortical cells. ECM are formed by fungi in the phyla Basidiomycota, Ascomycota (Tedersoo, May and Smith 2009). The basidiomycetes, or club fungi, are best known for the reproductive mushrooms that they may form, and they have a large range of ecological functions beyond the production of ectomycorrhizae. Ascomycetes, or cup fungi, also have a broad ecological and geographical range (Smith and Read 2008).

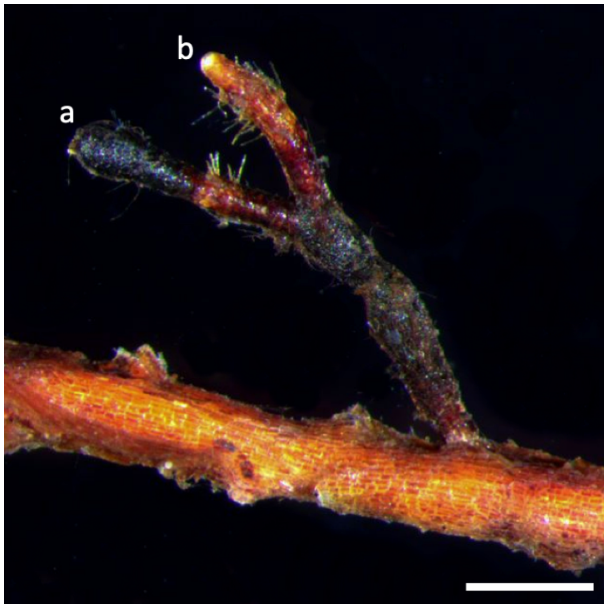


Figure 1. Fine root tips of *Pinus strobus* showing a mycorrhizal tip (a) alongside an uncolonized tip (b) from the forested wetland near Merrymakedge Beach.

The oldest, best-known, and most common type of mycorrhizae are the arbuscular mycorrhizae (AM) (Brundrett 2002). These are formed by fungi in the phylum

Glomeromycota (Brundrett and Ashwath 2013) in a large spatial and geographical range of land plants including herbaceous plants and some deciduous trees (Smith and Read 2008). The Glomeromycetes are obligate mycorrhizal symbionts and are characterized by the formation of tree-shaped structures called “arbuscules” within the cortical cells of the plant’s roots, although they often form storage vesicles as well (Beck-Nielsen and Vindbæk Madsen 2001) (Figure 2). Arbuscular mycorrhizae are the most studied mycorrhizae because of their valuable role in agriculture. They tend to be relatively seasonal and ephemeral, with AM plants being most heavily colonized in the spring, and much of the colonization disappearing by the mid-to-late summer (Bauer et al. 2003).

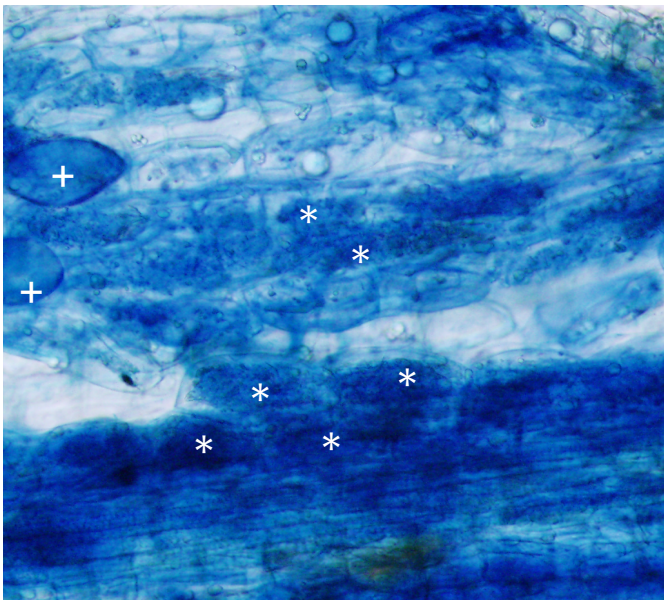


Figure 2. Arbuscules (*) and vesicles (+) in *Cornus canadensis* collected from upland soil near the Mersey Tobeatic Research Institute (200X).

The ericoid mycorrhizae (ErM) comprise the third major group of mycorrhizal fungi but are less studied than the ectomycorrhizae or arbuscular mycorrhizae. Ericoid mycorrhizae form intracellular hyphal coils in the hair roots of ericaceous plants (Smith and Read 2008) (Figure 3). They are known to be more stress tolerant than the other mycorrhizal types and are important in helping ericaceous plants survive in stressful environments such as coastal barrens, heaths, and bogs (Bradley, Burt and Read 1982) likely because of their ability to mobilize organically bound nutrients (Read 1983) and protect against heavy metals in soil (Cairney and Meharg 2003). Ericoid mycorrhizae are often formed by ascomycetes (Allen, Richards and Busso 1989), particularly those in the order Helotiales, although there are basidiomycete fungi that form ericoid mycorrhizae as well, including those in the genera *Serendipita* (formerly *Sebacina*) (Vohník et al. 2016) and *Kurtia* (Kolařík and Vohník 2018). Ericoid mycorrhizae, like AM, tend to vary seasonally in their level of colonization, depending on their habitat (Read 1996).

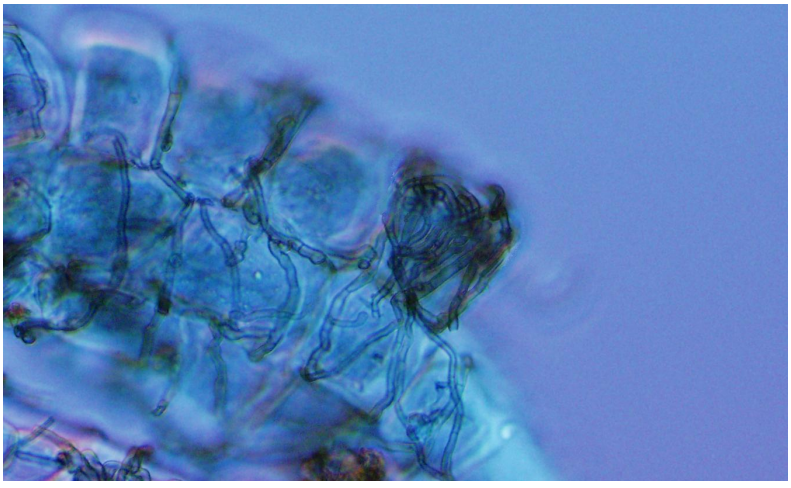


Figure 3. Ericoid mycorrhizal intracellular hyphal coils in *Kalmia angustifolia* collected from the forested wetland near Merrymakedge Beach (200X).

Wetlands

Forested wetlands are an integral part of the Nova Scotian landscape, but their distribution and abundance are not well understood. The Nature Conservancy of Canada has identified Nova Scotian forested wetlands as areas where increased research should be focused. Forested wetlands house numerous species of birds and invertebrates, including the avian species at risk *Cardellina canadensis*, *Contopus cooperi*, and *Euphagus carolinus* (Rice and Harper 2018). The endangered Boreal felt lichen (*Erioderma pedicellatum*) is also found mostly in Atlantic Canadian forested wetlands (Nature Conservancy of Canada 2019). Forested wetlands perform a variety of ecosystem services including flood abatement, water filtration, and carbon sequestration (Zedler and Kercher 2005). Those in Atlantic Canada are particularly unique because they are formed in Acadian forests which are a blended forest type combining deciduous and coniferous trees in a diverse patchwork that is not found anywhere else on earth (Mosseler, Lynds and Major 2003).

All fungi, including those that form mycorrhizae, are aerobic (Tanner and Clayton 1985). As wetland soils are characteristically anaerobic, researchers long-assumed that mycorrhizal relationships would be unable to form there (Clawson, Lockaby and Rummer 2001; Bauer et al. 2003; Dolinar and Gaberščik 2009; Orchard et al. 2016). This conventional wisdom has been challenged as researchers now regularly find evidence of mycorrhizal colonization in wetland plants (Thormann, Currah and Bayley 1999; Cornwell, Bedford and Chapin 2001). However, the mycorrhizal capabilities of wetland plants appear to be somewhat dependent on the type of plants and mycorrhizal fungi

involved (Weishampel and Bedford 2006; Orchard et al. 2016) and soil oxygen and nutrient concentrations (Read, Leake and Perez-Moreno 2004). Some researchers have proposed that wetland plants may form aerenchyma in order to provide oxygen to mycorrhizal fungi, although evidence for this strategy is limited (Kozlowski 1997; Cooke and Lefor 1998). It remains unclear what mechanisms are responsible for determining which mycorrhizae can form in wetlands and what adaptations for survival these fungi may have (Thormann, Currah and Bayley 1999; Cornwell, Bedford and Chapin 2001; Bacheler 2014).

Although researchers once argued that mycorrhizae were unable to survive in wetlands, it now appears that plants in wetlands may be even more reliant on mycorrhizae in wet soils where stressful conditions predominate (Miransari 2009). Mycorrhizae that are adapted to wet soils may currently be facing multiple biodiversity threats including plant community shifts under soil eutrophication (Fagúndez 2012) and suppression of fungal diversity by invasive species (Mummey and Rillig 2006) which could hinder restoration efforts by reducing mycorrhizal potential of soils. Wetland mycorrhizae may also be subjected to new patterns of droughts, fires and floods that could threaten those with niche adaptations (Wessel et al. 2004). Failure to protect forested wetland biodiversity could lead to worsening impacts of climate change through the loss of these services.

Ectomycorrhizae appear to have relatively uncomplicated patterns of colonization in forested wetland soils. ECM researchers have often found decreased colonization

correlated with increasing soil moisture (Jurgensen et al. 1996; Sumorok et al. 2008). In a pot study by Bougher and Malajczuk (1989), ectomycorrhizae were able to improve plant growth, but when soils were waterlogged, the formation of ectomycorrhizae was significantly reduced. Ectomycorrhizal community compositions may also shift in response to soil water saturation (Robertson et al. 2006; Moeller, Peay and Fukami 2013) or the symbioses may be almost entirely inhibited. Inhibition of ectomycorrhizal colonization in wet soils is so reliable that the reduced presence of fungal mantles has been proposed as an indicator for wetland delineation (Vasilas et al. 2004).

The colonization patterns of arbuscular mycorrhizae in wetlands is less straightforward. There is a clear seasonality in the formation of arbuscular mycorrhizae, with colonization peaking in the spring and being lowest in the mid-to-late summer. This trend may overshadow the impacts of other environmental factors, for example, some researchers found that seasonality was correlated with colonization levels, but despite differences, soil moisture and phosphorus content could not be significantly linked (Bohrer, Friese and Amon 2004). Other researchers found much lower colonization in wet soils than in drier ones (Brundrett and Ashwath 2013; Silvani et al. 2013; Orchard et al. 2016), while AM colonization in a growth chamber study was less impacted by increased soil moisture than in an accompanying field study, which showed a correlation between increasing soil moisture and a decrease in arbuscular mycorrhizal colonization (Stevens and Peterson 1996). In arbuscular mycorrhizal plants, root depth may play a role in determining how much colonization can occur in wet soils as longer rooted plants can be much less colonized than plants with shorter roots (Clayton and Bagyaraj 1984).

Communities of AM fungi may vary in composition between wet and dry soils (Miller and Bever 1999), and plant type may be an important factor in mycorrhizal formation in wetland soils, as colonization was present in wetland dicots but not in monocots (Cornwell, Bedford and Chapin 2001). In contrast, others showed high levels of colonization in a variety of wetland plant types (Allen, Richards and Busso 1989; Wetzel and van der Valk 1996; Kandalepas et al. 2010). In wetland hummocks arbuscular mycorrhizal colonization was higher in the uppermost aerobic microsites than in the lower, wetter soils (Cantelmo and Ehrenfield 1999). In general, although the trends in AM colonization in wetlands lack consensus, most researchers have found some degree of inhibition with increased soil moisture.

The literature on mycorrhizal colonization in wetland ectomycorrhizal and arbuscular mycorrhizal plants is incomplete, but they have received more attention than ericoid mycorrhizal plants in wetlands. Bacheler (2014), in one of the few studies on wetland ericoid mycorrhizae, found more intraradical root penetration in wet soils compared with dry soils. While communities of ericoid mycorrhizal fungi do appear to change in structure between wet and dry sites, colonization levels may be less affected than in other mycorrhizal types (Gorzelak, Hambleton and Massicotte 2012).

Ericoid mycorrhizae may be important in wetland habitats because of their ability to reduce heavy metal uptake in plants (Read 1983), as heavy metals are more bioavailable in wet soils (Bradley, Burt and Read 1982). Ericoid mycorrhizae may also be important to wetland plants because of their ability to access a wide range of organically

bound nitrogen and phosphorus sources that are typically unavailable to other mycorrhizal types (Jonasson and Shaver 1999; Read, Leake and Perez-Moreno 2004) Insufficient evidence exists to draw conclusions about the patterns of ericoid mycorrhizal colonization in wetland soils.

Determinants of Colonization

In addition to average soil moisture, there are other factors likely to be important in determining colonization patterns in wetland soils. Colonization levels vary seasonally for AM and ErM fungi. Plant phenology encourages colonization at some times, and restricts it at others (Read 1996; Bohrer, Friese and Amon 2004; Courty et al. 2006). In general, colonization tends to be highest in the late winter into the spring and lowest in the dry parts of the summer (Hutton, Dixon and Sivasithamparam 1994; García and Mendoza 2008). The interactions between mycorrhizal seasonality and soil moisture have not been well studied as researchers have often been unable to separate the impacts of these two competing factors (Bauer et al. 2003). Temperature changes may play an important role in seasonal shifts in mycorrhizal colonization. Arbuscular mycorrhizal colonization levels can be higher when temperatures increase, although the increase is not necessarily linked to improved mycorrhizal effectiveness. The seasonality of arbuscular mycorrhizae could be explained by the preference of their fungal symbionts for a certain temperature range, and these preferences may be favoured as higher temperature periods extend with the changing climate (Rillig et al. 2002). In the ectomycorrhizal fungi seasonality tends to be linked more to community composition than colonization levels, certain species dominating certain seasons, despite relatively constant diversity and

richness between seasons (Walker, Miller and Horton 2008). Ericoid mycorrhizal colonization, although not well characterized, appears to be somewhat positively correlated with higher temperatures (Olsrud et al. 2004).

Plants form mycorrhizal relationships with fungi in order to improve their access to soil nutrients. Generally, arbuscular mycorrhizal plants are most common on high nitrogen low phosphorus sites, such as in the tropics, while ectomycorrhizal plants are more common in higher latitude areas with seasonally available nitrogen and phosphorus, and ericoid mycorrhizal plants dominate in very low nutrient zones such as heaths and tundras (Read 1991). While mycorrhizal fungi are generally thought to prefer inorganic nutrient sources, there is evidence for the ability to access organically bound nitrogen and phosphorus in some mycorrhizae, particularly in the ErM fungi (Cairney et al. 2000; Liu et al. 2017). The ability to mobilize organic forms of nitrogen and phosphorus would be an especially beneficial trait of mycorrhizal symbionts in forested wetlands where organically bound nutrients tend to accumulate, as bacterial decomposition is generally low (McLatchey and Reddy 1998).

Another factor that can impact mycorrhizal colonization levels is soil pH. It is a significant determinant of AM (Wetzel and van der Valk 1996; Cooke and Lefor 1998), ericoid (Hambleton and Currah 1997) and ectomycorrhizal (Moeller, Peay and Fukami 2014) colonization levels. Ericoid mycorrhizal relationships are most often characterized by their dominance in low pH soils (Leake, Shaw and Read 1990), while AM and ECM

colonization is limited under very acidic conditions (Danielson and Visser 1989; Postma, Olsson and Falkengren-Grerup 2007).

Aerenchyma are air pockets in root tissue that allow plants to distribute oxygen from aboveground tissues into their roots and the surrounding soil. Some plants form aerenchyma in their roots in response to elevated soil moisture conditions (Kozlowski 1997). They may form in some plants in wet soils while not forming in the same plants in drier soils, while other plants always form aerenchyma. These tissues may have an impact on mycorrhizal colonization in wetland plants in several ways. Plants that can form aerenchyma may be better adapted to wetland soils and, therefore, less reliant on mycorrhizae for nutrient uptake (Cornwell, Bedford and Chapin 2001). Conversely, plants that form aerenchyma may be better able to host mycorrhizae, as they could be able to provide an aerobic environment for the fungi (Cooke and Lefor 1998). The role of aerenchyma in relation to wetland mycorrhizae is not well known, but is deserving of further attention, as it could explain why aerobic fungi are able to survive in oxygen poor wetland soils.

Quantification Methodologies

Methods for quantification of mycorrhizal colonization vary widely between researchers and over time (Giovannetti and Mosse 1979). This can partially be excused because different methods are required for different mycorrhizal types. For arbuscular mycorrhizae, clearing and staining is the most popular method for visualization. Clearing and staining methods vary, but generally involve using a strong base and heat to remove

plant cell contents, followed by the application of a weak acid then fixing a dye with a second heat treatment. Some researchers use autoclaves to clear and stain, while others use an extended time period to allow reagents to act gently, and others still apply heat using a microwave (Dalpé and Séguin 2013). With these variations comes variability in the accuracy of quantification, as some methods may be better at visualizing mycorrhizae than others (Giovannetti and Mosse 1980) Also, some researchers record a variety of fungal structures (Bacheler 2014) while others are more concerned with the presence or absence of mycorrhizal colonization in general (Bierman and Linderman 1981).

In addition to the use of a variety of clearing and staining methods, researchers also use different methods to quantify mycorrhizal colonization. The most common is the gridline-intersect method (Giovannetti and Mosse 1980; McGonigle et al. 1990) but many others exist. Wide variations in methods of quantification make comparisons between studies difficult, if not impossible. Quantification of ericoid mycorrhizae generally follows modified methods of AM visualization and quantification (Hutton, Dixon and Sivasithamparam 1994; Bacheler 2014) although, because ericoid mycorrhizae form different structures, these methods may not be as appropriate. The picture is complicated more by the fact that some plants form dual mycorrhizae or hybrid mycorrhizae which have characteristics of both types (Smith 1980; Smith and Read 2008; Vohník and Albrechtová 2011). Methods for quantifying ectomycorrhizal colonization are different from those for endomycorrhizas (AM and ErM) and generally involves low-power microscopic identification of the fungal mantle or cross sectioning to view the Hartig net (Flores-Rentería et al. 2014).

Molecular analyses are popular in studies of all types of mycorrhizal fungi, but because of the presence of endophytic fungi that occur within plant tissues but do not form mycorrhizae, and the insufficient characterization of mycorrhizal communities in wetland habitats (particularly with respect to ErM), this method is not best for understanding colonization patterns in wet soils. Molecular analysis is, however, more comparable between mycorrhizal types and researchers, and over time (Martin 2007).

Mycorrhiza researchers may assess colonization using cultivation experiments in the field or in growth chambers (Bradley, Burt and Read 1982; Allen, Richards and Busso 1989; Bougher and Malajczuk 1989; Chen, Kahlili and Cairney 2003). While these *in vivo* experiments can provide the opportunity to isolate particular traits without the influence of confounding factors, their results must not be taken as conclusive, as they are not always representative of colonization patterns found in nature.

Conclusions

Mycorrhizal colonization in wetlands was once believed to be negligible. It is now clear that many wetland plants do become colonized by symbiotic fungi, although the factors involved are not well understood. Ectomycorrhizae appear to be negatively impacted by high average soil moisture. Arbuscular mycorrhizae also appear to be somewhat negatively affected by wet soils, although factors such as plant type and season are also important. For ericoid mycorrhizae, colonization patterns in wet soils have gone largely unexplored, although the research that does exist suggests that ericoid

mycorrhizae may be less affected by high average soil moisture. Variations in quantification methods have made comparisons between existing studies difficult. Studies of all three major mycorrhizal types in the same environment, in the same season, and using standard methods of quantification are required. Other factors including pH, aerenchyma formation, temperature, and soil nutrient levels must also be taken into consideration. Only after this research is done can the mycorrhizal colonization patterns of wetland mycorrhizal fungi be better understood.

References

Allen MF, Richards JH, Busso CA. 1989. Influence of clipping and soil-water status on vesicular-arbuscular mycorrhizae of 2 semi-arid tussock grasses. Biology and Fertility of Soils 8(4):285-9.

Bacheler RJ. 2014. Distribution of two ericoid mycorrhizae in a New Jersey pine barrens upland-wetland site and the effect of mycorrhizal presence on ericaceous plants. Camden, New Jersey: Rutgers University - Camden.

Bauer CR, Kellogg CH, Bridgham SD, Lamberti GA. 2003. Mycorrhizal colonization across hydrologic gradients in restored and reference freshwater wetlands. Wetlands 23(4):961-8.

Beck-Nielsen D and Vindbæk Madsen T. 2001. Occurrence of vesicular–arbuscular mycorrhiza in aquatic macrophytes from lakes and streams. Aquatic Botany 71(2):141-8.

Biermann B and Linderman RG. 1981. Quantifying vesicular-arbuscular mycorrhizae: A proposed method towards standardization. New Phytologist 87(1):63-7.

Bohrer KE, Friese CF, Amon JP. 2004. Seasonal dynamics of arbuscular mycorrhizal fungi in differing wetland habitats. Mycorrhiza 14(5):329-37.

Boreal felt lichen [Internet]: Nature Conservancy of Canada; c2019 [cited 2019]. Available from: http://www.natureconservancy.ca/en/what-we-do/resource-centre/featured-species/lichens/boreal-felt-lichen.html

Bougher NL and Malajczuk N. 1990. Effects of high soil moisture on formation of ectomycorrhizas and growth of karri (*Eucalyptus diversicolor*) seedlings inoculated with descolea maculata, pisolithus tinctorius and laccaria laccata. New Phytologist 114(1):87-91.

Bradley R, Burt A, Read D. 1982. The biology of mycorrhiza in the Ericaceae. New Phytologist 91(2):197-209.

Brundrett MC. 2002. Coevolution of roots and mycorrhizas of land plants. New Phytologist 154(2):275-304.

Brundrett MC and Ashwath N. 2013. Glomeromycotan mycorrhizal fungi from tropical Australia III. measuring diversity in natural and disturbed habitats. Plant Soil 370(1-2):419-33.

Cairney JW and Meharg AA. 2003. Ericoid mycorrhiza: A partnership that exploits harsh edaphic conditions. European Journal Soil Science 54(4):735-40.

Cairney J, Sawyer N, Sharples J, Meharg A. 2000. Intraspecific variation in nitrogen source utilisation by isolates of the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf and Kernan. Soil Biology Biochemistry 32(8-9):1319-22.

Cantelmo Jr AJ and Ehrenfeld JG. 1999. Effects of microtopography on mycorrhizal infection in Atlantic white cedar (*Chamaecyparis thyoides* (L.) Mills.). Mycorrhiza 8(4):175-80.

Chen DM, Khalili K, Cairney JW. 2003. Influence of water stress on biomass production by isolates of an ericoid mycorrhizal endophyte of *Woollsia pungens* and *Epacris microphylla* (Ericaceae). Mycorrhiza 13(3):173-6.

Clawson RG, Lockaby BG, Rummer B. 2001. Changes in production and nutrient cycling across a wetness gradient within a floodplain forest. Ecosystems 4(2):126-38.

Clayton J and Bagyaraj D. 1984. Vesicular-arbuscular mycorrhizas in submerged aquatic plants of New Zealand. Aquatic Botany 19(3):251-62.

Cooke JC and Lefor MW. 1998. The mycorrhizal status of selected plant species from Connecticut wetlands and transition zones. Restoration Ecology 6(2):214-22.

Cornwell WK, Bedford BL, Chapin CT. 2001. Occurrence of arbuscular mycorrhizal fungi in a phosphorus-poor wetland and mycorrhizal response to phosphorus fertilization. American Journal of Botany 88(10):1824-9.

Courty P, Pouysegur R, Buée M, Garbaye J. 2006. Laccase and phosphatase activities of the dominant ectomycorrhizal types in a lowland oak forest. Soil Biology Biochemistry 38(6):1219-22.

Dalpé Y and Séguin SM. 2013. Microwave-assisted technology for the clearing and staining of arbuscular mycorrhizal fungi in roots. Mycorrhiza 23(4):333-40.

Danielson R and Visser S. 1989. Effects of forest soil acidification on ectomycorrhizal and vesicular—arbuscular mycorrhizal development. New Phytologist 112(1):41-7.

Dolinar N and Gaberščik A. 2010. Mycorrhizal colonization and growth of *Phragmites australis* in an intermittent wetland. Aquatic Botany 93(2):93-8.

Fagúndez J. 2012. Heathlands confronting global change: Drivers of biodiversity loss from past to future scenarios. Annals of Botany 111(2):151-72.

Flores-Renteria L, Lau MK, Lamit LJ, Gehring CA. 2014. An elusive ectomycorrhizal fungus reveals itself: A new species of *Geopora* (Pyronemataceae) associated with *Pinus edulis*. Mycologia 106(3):553-63.

Garcia IV and Mendoza RE. 2008. Relationships among soil properties, plant nutrition and arbuscular mycorrhizal fungi-plant symbioses in a temperate grassland along hydrologic, saline and sodic gradients. FEMS Microbiology Ecology 63(3):359-71.

Gianinazzi S, Gollotte A, Binet M-, van Tuinen D, Redecker D, Wipf D. 2010. Agroecology: The key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza 20(8):519-30.

Giovannetti M and Mosse B. 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytologist 84(3):489-500.

Gorzelak MA, Hambleton S, Massicotte HB. 2012. Community structure of ericoid mycorrhizas and root-associated fungi of *Vaccinium membranaceum* across an elevation gradient in the Canadian Rocky Mountains. Fungal Ecology 5(1):36-45.

Hambleton S and Currah RS. 1997. Fungal endophytes from the roots of alpine and boreal Ericaceae. Canadian Journal of Botany-Revue Canadienne De Botanique 75(9):1570-81.

Hutton B, Sivasithamparam K, Dixon K, Pate J. 1996. Pectic zymograms and water stress tolerance of endophytic fungi isolated from western Australian heaths (Epacridaceae). Annals of Botany 77(4):399-404.

Janos DP. 1980. Mycorrhizae influence tropical succession. Biotropica 12(2):56-64.

Jonasson S and Shaver GR. 1999. Within-stand nutrient cycling in arctic and boreal wetlands. Ecology 80(7):2139-50.

Juniper S and Abbott L. 1993. Vesicular-arbuscular mycorrhizas and soil salinity. Mycorrhiza 4(2):45-57.

Jurgensen MF, Richter DL, Davis MM, McKevlin MR, Craft MH. 1996. Mycorrhizal relationships in bottomland hardwood forests of the southern United States. Wetlands Ecology and Management 4(4):223-33.

Kandalepas D, Stevens KJ, Shaffer GP, Platt WJ. 2010. How abundant are root-colonizing fungi in southeastern Louisiana's degraded marshes? Wetlands 30(2):189-99.

Kernaghan G. 2005. Mycorrhizal diversity: Cause and effect? Pedobiologia 49(6):511-20.

Kolařík M and Vohník M. 2018. When the ribosomal DNA does not tell the truth: The case of the taxonomic position of *Kurtia argillacea*, an ericoid mycorrhizal fungus residing among hymenochaetales. Fungal Biology 122(1):1-18.

Kozlowski T. 1997. Responses of woody plants to flooding and salinity. Tree Physiology 17(7):490.

Leake J, Shaw C, Read D. 1990. The role of ericoid mycorrhizas in the ecology of Ericaceous plants. Agriculture, Ecosystems and Environment 29(1-4):237-50.

Liu M, Li C, Xu X, Wanek W, Jiang N, Wang H, Yang X. 2017. Organic and inorganic nitrogen uptake by 21 dominant tree species in temperate and tropical forests. Tree Physiology 37(11):1515-26.

Martin KJ. 2007. Introduction to molecular analysis of ectomycorrhizal communities. Soil Science Society of America Journal 71(2):601-10.

Martino E, Morin E, Grelet G, Kuo A, Kohler A, Daghino S, Barry KW, Cichocki N, Clum A, Dockter RB. 2018. Comparative genomics and transcriptomics depict ericoid mycorrhizal fungi as versatile saprotrophs and plant mutualists. New Phytologist 217(3):1213-29.

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA. 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytologist 115(3):495-501.

McLatchey GP and Reddy K. 1998. Regulation of organic matter decomposition and nutrient release in a wetland soil. Journal of Environmental Quality 27(5):1268-74.

Miller SP and Bever JD. 1999. Distribution of arbuscular mycorrhizal fungi in stands of the wetland grass *Panicum hemitomon* along a wide hydrologic gradient. Oecologia 119(4):586-92.

Miransari M. 2010. Contribution of arbuscular mycorrhizal symbiosis to plant growth under different types of soil stress. Plant Biology 12(4):563-9.

Moeller HV, Peay KG, Fukami T. 2014. Ectomycorrhizal fungal traits reflect environmental conditions along a coastal California edaphic gradient. FEMS Microbiology Ecology 87(3):797-806.

Mosseler A, Lynds J, Major J. 2003. Old-growth forests of the acadian forest region. Environmental Reviews 11(S1):S47-77.

Mummey DL and Rillig MC. 2006. The invasive plant species *Centaurea maculosa* alters arbuscular mycorrhizal fungal communities in the field. Plant Soil 288(1-2):81-90.

Olsrud M, Melillo J, Christensen T, Michelsen A, Wallander H, Olsson PA. 2004. Response of ericoid mycorrhizal colonization and functioning to global change factors. New Phytologist 162(2):459-69.

Orchard S, Standish R, Nicol D, Gupta V, Ryan M. 2016. The response of fine root endophyte (*Glomus tenu*e) to waterlogging is dependent on host plant species and soil type. Plant Soil 403(1-2):305-15.

Postma JW, Olsson PA, Falkengren-Grerup U. 2007. Root colonisation by arbuscular mycorrhizal, fine endophytic and dark septate fungi across a pH gradient in acid beech forests. Soil Biology and Biochemistry 39(2):400-8.

Read DJ. 1996. The structure and function of the ericoid mycorrhizal root. Annals of Botany 77(4):365-74.

Read DJ. 1991. Mycorrhizas in ecosystems. Experientia 47(4):376-91.

Read DJ. 1983. The biology of mycorrhiza in the Ericales. Canadian Journal of Botany-Revue Canadienne De Botanique 61(3):985-1004.

Read DJ, Leake J, Perez-Moreno J. 2004. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. Canadian Journal of Botany-Revue Canadienne De Botanique 82(8):1243-63.

Rice M and Harper K. 2018. Forested wetlands; A unique, but poorly understood, ecosystem in Atlantic Canada.

Rillig MC, Wright SF, Shaw MR, Field CB. 2002. Artificial climate warming positively affects arbuscular mycorrhizae but decreases soil aggregate water stability in an annual grassland. Oikos 97(1):52-8.

Robertson SJ, Tackaberry LE, Egger KN, Massicotte HB. 2006. Ectomycorrhizal fungal communities of black spruce differ between wetland and upland forests. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 36(4):972-85.

Saikkonen K, Faeth SH, Helander M, Sullivan TJ. 1998. Fungal endophytes: A continuum of interactions with host plants. Annual Review of Ecology and Systematics 29:319-43.

Sikes BA. 2010. When do arbuscular mycorrhizal fungi protect plant roots from pathogens? Plant Signaling and Behavior 5(6):763-5.

Silvani V, Rothen C, Rodriguez MA, Cisneros G, Godeas A, Aranda-Rickert A, Fracchia S. 2013. Fungal root colonization of *Puccinellia frigida* (phil.) johnston, a dominant grass species inhabiting the margins of high-altitude hypersaline Andean wetlands. Aquatic Botany 108:26-32.

Smith SE and Read DJ. 2008. Mycorrhizal symbiosis. 3rd ed ed. New York, New York: Elsevier.

Smith SSE. 1980. Mycorrhizas of autotrophic higher-plants. Biological Reviews of the Cambridge Philosophical Society 55(NOV):475-510.

Spatafora JW, Chang Y, Benny GL, Lazarus K, Smith ME, Berbee ML, Bonito G, Corradi N, Grigoriev I, Gryganskyi A. 2016. A phylum-level phylogenetic

classification of zygomycete fungi based on genome-scale data. Mycologia 108(5):1028-46.

Stevens KJ and Peterson RL. 1996. The effect of a water gradient on the vesicular-arbuscular mycorrhizal status of *Lythrum salicaria* L.(purple loosestrife). Mycorrhiza 6(2):99-104.

Sumorok B, Kosiński K, Michalska-Hejduk D, Kiedrzyńska E. 2008. Distribution of ectomycorrhizal fungi in periodically inundated plant communities on the Pilica river floodplain. Ecohydrology & Hydrobiology 8(2-4):401-10.

Tanner CC and Clayton JS. 1985. Effects of vesicular—arbuscular mycorrhizas on growth and nutrition of a submerged aquatic plant. Aquatic Botany 22(3):377-86.

Tedersoo L, May TW, Smith ME. 2010. Ectomycorrhizal lifestyle in fungi: Global diversity, distribution, and evolution of phylogenetic lineages. Mycorrhiza 20(4):217-63.

Thormann MN, Currah RS, Bayley SE. 1999. The mycorrhizal status of the dominant vegetation along a peatland gradient in southern boreal Alberta, Canada. Wetlands 19(2):438-50.

Vasilas B, Vasilas L, Thompson J, Rizzo A, Fuhrmann J, Evans T, Pesek J, Kunkle K. 2004. Ectomycorrhizal mantles as indicators of hydrology for jurisdictional wetland determinations. Wetlands 24(4):784-95.

Vohník M, Pánek M, Fehrer J, Selosse M. 2016. Experimental evidence of ericoid mycorrhizal potential within Serendipitaceae (Sebacinales). Mycorrhiza 26(8):831-46.

Vohnik M and Albrechtova J. 2011. The co-occurrence and morphological continuum between ericoid mycorrhiza and dark septate endophytes in roots of six European Rhododendron species. Folia Geobotanica 46(4):373-86.

Walker JF, Miller Jr OK, Horton JL. 2008. Seasonal dynamics of ectomycorrhizal fungus assemblages on oak seedlings in the southeastern Appalachian Mountains. Mycorrhiza 18(3):123-32.

Weishampel PA and Bedford BL. 2006. Wetland dicots and monocots differ in colonization by arbuscular mycorrhizal fungi and dark septate endophytes. Mycorrhiza 16(7):495-502.

Wessel WW, Tietema A, Beier C, Emmett BA, Peñuelas J, Riis–Nielsen T. 2004. A qualitative ecosystem assessment for different shrublands in western Europe under impact of climate change. Ecosystems 7(6):662-71.

Wetzel PR and VanderValk AG. 1996. Vesicular-arbuscular mycorrhizae in prairie pothole wetland vegetation in Iowa and North Dakota. Canadian Journal of Botany-Revue Canadienne De Botanique 74(6):883-90.

Zedler JB and Kercher S. 2005. Wetland resources: Status, trends, ecosystem services, and restorability. Annual Review of Environment and Resources 30:39-74.

CHAPTER TWO

Mycorrhizal Colonization Along an Upland to Forested Wetland Gradient

Abstract

Mycorrhizal symbioses are not well studied in forested wetlands. In wet soils, colonization of woody plants by ectomycorrhizal (ECM) fungi tends to be poor and is highly variable in arbuscular mycorrhizal (AM) herbaceous plants. However, little information is available on the influence of soil moisture on the colonization of ericaceous roots by ericoid mycorrhizal (ErM) fungi. In this study mycorrhizal fungal colonization levels and edaphic factors along two upland to wetland transects in Southwestern Nova Scotia were studied. Colonization was assessed microscopically in *Pinus strobus* (ECM), *Cornus canadensis* and *Lysimachia borealis* (AM) and *Kalmia angustifolia* and *Gaultheria hispidula* (ErM). The data indicate that ErM colonization increases with soil moisture in forested wetlands.

Introduction

Mycorrhizae are ubiquitous symbioses between plant roots and fungi (Brundrett 2004). They are found on most plants around the world but levels of root colonization by mycorrhizal fungi are affected by a variety of factors including seasonality, soil nutrient levels, global distribution, temperature and surrounding plant community (Eom et al. 2000; Bauer et al. 2003; Nilsson et al. 2005; Smith and Read 2008; Soudzilovskaia et al. 2015). Another important factor in the formation of mycorrhizal relationships is the average soil moisture content.

a b c

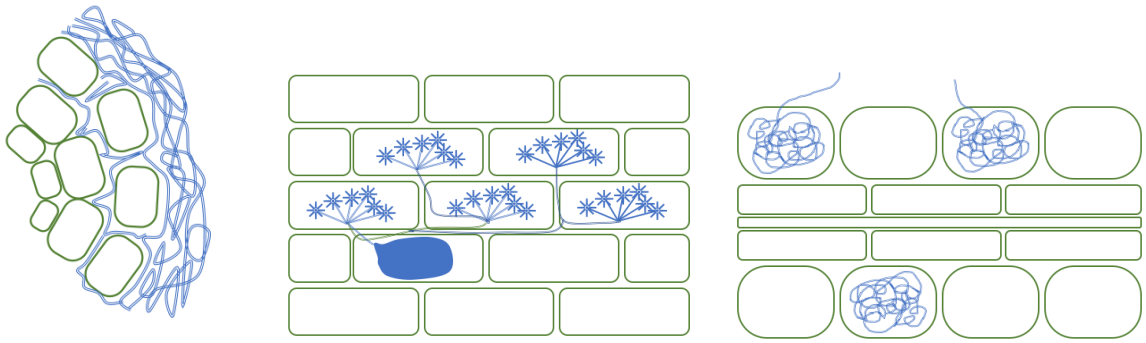


Figure 4 (a-c). Anatomy of mycorrhizal symbioses with green representing plant tissue and fungal tissue in blue. a) Ectomycorrhiza with the fungal mantle surrounding the root tip and the Hartig net extending around the root cortical cells) b) Arbuscular Mycorrhizae with intracellular fungal arbuscules and a storage vesicle c) Ericoid Mycorrhizae with intracellular fungal hyphal coils.

Ectomycorrhizae (ECM) (Figure 4a) form on woody plants and are widely distributed throughout the Acadian forest (Nilsson et al. 2005). Elevated soil moisture conditions are clearly inhibitory to ectomycorrhizae (Vasilas et al. 2004; Robertson et al. 2006). A broad range of plants, from herbaceous understory vegetation to some deciduous trees, form arbuscular mycorrhizae (AM) (Figure 4b). High soil moisture content appears to also be somewhat inhibitory to (AM) colonization, but the confounding factors of seasonality, root length, and plant host species makes this relationship more variable and less predictable (Clayton and Bagyaraj 1984; Cornwell, Bedford and Chapin 2001; Brundrett and Ashwath 2013). In the case of the ericoid mycorrhizae (ErM) of ericaceous plants (Figure 4c; Smith and Read 2008), very few studies have focused on colonization

in wetland habitats, but the research that does exist suggests that they may be relatively tolerant to high soil moisture contents (Bacheler 2014).

In the current study, mycorrhizal colonization patterns from upland forests to forested wetlands were compared among ectomycorrhizal, arbuscular mycorrhizal, and ericoid mycorrhizal plants. Variations in mycorrhizal colonization were related to volumetric water content, soil nutrients, pH, and temperature, as well as foliar nutrient levels in the plant species assessed for mycorrhizal colonization. The objective was to gain an improved understanding of colonization patterns in common mycorrhizal plants in forested wetlands compared to upland forests. Increases in average soil moisture content was predicted to be associated with a decrease in ectomycorrhizal and arbuscular mycorrhizal colonization, but with no decrease in ericoid mycorrhizal colonization.

Materials and Methods

Plant Selection

Two plant species known to form AM, two that form ErM, and one that forms ECM were selected (Malloch and Malloch 1981). AM and ErM plants that are common in Nova Scotian upland forests and forested wetlands were selected using Boland (2014). Availability of the selected plant species was confirmed by a plant survey of the sites conducted by Logan Gray as a part of a larger Atlantic Ecosystems Initiative project on forested wetlands. The AM plant species studied were *Cornus canadensis* L. and *Lysimachia borealis* (Raf.) U. Manns & Anderb. and the ErM plants were *Kalmia angustifolia* L. and *Gaultheria hispidula* (L.) Muhl. ex Bigelow (Figure 5). The ECM

plant was *Pinus strobus* L. Other ECM plant options were considered, including *Picea spp.* and *Abies balsamea*. However, *Picea* was excluded from the study due to the tendency for different species to occur along the moisture gradient, with *Picea rubens* in the uplands and *Picea mariana* in the wetlands, and possible hybrids in between. *Abies balsamea* was also excluded from the study, as it avoided the wettest conditions by growing mainly on hummocks within the forested wetland plots and mycorrhizal colonization levels can be higher on hummocks than in surrounding flooded soil (Cantelmo and Ehrenfeld 1999). Other ECM plant species were not common enough along the transects to be used.

Figure 5. *Cornus canadensis* (AM) and *Gaultheria hispidula* (ErM) growing together in the Mount Merritt upland plot in June 2019.

Site Descriptions

Two forested wetland sites were examined in Southwestern Nova Scotia. One in Kejimkujik National Park (44º23'17N 65º12'21W and 96m elevation) near Merrymakedge Beach (MME) (Figure 6a) and the other (44º25'37N 65 º05'25W and 102m elevation) located near the Mersey Tobeatic Research Institute on Mount Merritt road (MTM) (Figure 6b). Sites were classified using Nova Scotia's Forest Ecosystem Classification (FEC) system (Neily et al. 2013) FEC classifications were conducted by Dr. Kevin Keys of the Nova Scotia Department of Lands and Forestry.

Table 1. Summary of forest and soil FEC classifications.

	Merrymakedge		
	Upland	**Ecotone**	**Wetland**
Forest Type	Red spruce – White pine / Lambkill / Bracken (SH4)	Black spruce / False holly / Ladies' tresses sphagnum (SP7)	Tamarack – Black spruce / Lambkill / Sphagnum (WC7)
Dominant Moss	*Pleurozium schreberi*	*P. schreberi or Hylocomium splendens*	*Sphagnum* spp. (Vonn post of 2-3)
Soil Type	MCT Loamy (2L)	MCT Moist loamy (3L)	Organic (14)

	Mount Merritt		
	Upland	**Ecotone**	**Wetland**
Forest Type	Red spruce – White pine / Lambkill / Bracken (SH4)	Red spruce – Balsam fir / Stair-step moss – Sphagnum (SH6)	Red maple – Balsam fir / Wood aster / Sphagnum (WD6)
Dominant Moss	*Pleurozium schreberi*	*Hylocomium splendens*	*Sphagnum* spp. (Vonn post of 2-3)
Soil Type	Dry shallow MCT loamy (15L)	MCT Moist loamy (3L)	Organic (14)

a b

Figure 6 (a and b). Forested wetland plots on sites a) Merrymakedge and b) Mount Merritt.

Sampling

A 120m transect was established on each site, with the middle of the ecotone at 60m, the forested wetland at 0m and the upland forest at 120m. The transects were divided into three plots labelled "wet", "ecotone", and "dry" and delineated by the ratios of *Sphagnum spp.* (wet), *Hylocomium splendens* (ecotone), and *Pleurozium schreberi* (relatively dry) moss types. The ecotone area, dominated by *Hylocomium splendens* moss, was narrower on site MTM than on MME. Soil, root and leaf samples were collected from as close to the transect possible, not exceeding 15m from the transect line in either direction. Individual plants were visually identified, and leaf, root, and soil samples were collected from each along with pH using an ExStik® Waterproof pH Meter (EXTECH Instruments), and volumetric water content (VWC %) and temperature (˚C) data using a

ProCheck® by Decagon Devices and Hoskin Scientific multimeter. From *Kalmia*, *Cornus,* and *Pinus*, four replicate samples of fifteen mature leaves were collected for foliar nutrient analysis along with 400-500g soil samples from the organic horizon. Roots were located by tracing from the base of the plant and approximately 50cm of ECM and approximately 250-500cm^3 of AM and ErM roots were sampled from organic horizons only. For each plant species four replicate sets of root samples were collected in the spring and fall seasons (Table 2). Samples were transported to the laboratory on ice and then frozen at -18ºC until processing.

Table 2. Study design of roots sampled with four replicates each.

Site	**Season**	**Plot**	**Species (Mycorrhizal Type)**
MME	Spring (June 14th, 2017)	Upland	*Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM) *Cornus canadensis* and *Lysimachia borealis* (AM)
		Ecotone	*Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM) *Cornus canadensis* and *Lysimachia borealis* (AM)
		Wetland	*Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM) *Cornus canadensis* and *Lysimachia borealis* (AM)
MTM	Spring (June 18th, 2017)	Upland	*Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM) *Cornus canadensis* and *Lysimachia borealis* (AM)
		Ecotone	*Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM) *Cornus canadensis* and *Lysimachia borealis* (AM)
		Wetland	*Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM) *Cornus canadensis* and *Lysimachia borealis* (AM)
MME	Fall (November 5th, 2017)	Upland	*Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM) *Cornus canadensis* and *Lysimachia borealis* (AM)
		Ecotone	*Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM)

		Wetland	*Cornus canadensis* and *Lysimachia borealis* (AM) *Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM) *Cornus canadensis* and *Lysimachia borealis* (AM)
MTM	Fall (November 5th 2017)	Upland	*Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM) *Cornus canadensis* and *Lysimachia borealis* (AM)
		Ecotone	*Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM) *Cornus canadensis* and *Lysimachia borealis* (AM)
		Wetland	*Pinus strobus* (ECM) *Kalmia angustifolia* and *Gaultheria hispidula* (ErM) *Cornus canadensis* and *Lysimachia borealis* (AM)

Plant and Soil Nutrient Analysis

Soil bulk densities from the east, west, and center of each plot, within 7 m from either side of the transect line, were calculated by collecting 400cm^2 surface area cubes of organic horizon and measuring the organic horizon depth from each side of the excavation. Soil for bulk density samples was weighed after being dried for three days at 80˚C.

Samples of approximately 15 healthy, mature leaves were dried in plastic cups for five days at room temperature before being sent in Ziploc bags to the University of Guelph Agriculture and Food Laboratory where they were tested for nitrogen, phosphorus, potassium, magnesium, and calcium content (Plant Package 1), according to the Ontario Ministry of Agriculture, Food, and Rural Affairs (OMFRA) accredited method (OMFRA 2019).

For soil nutrient samples, any large roots were removed, and samples were air dried in foil trays for five days at room temperature then sealed in Ziploc bags and sent to the University of Guelph Agriculture and Food Laboratory for soil nutrient analysis. The samples were tested for soil pH (saturated paste and SMP buffer methods), sodium bicarbonate extractable phosphorus, and ammonium acetate extractable potassium and magnesium. The soil ammonium-nitrogen and nitrate and nitrite-nitrogen were also determined by 2M KCl colorimetry.

Clearing and Staining of Root Samples

Root samples were processed over 2mm and 300µm mesh sieves. They were washed free of all visible soil particles in cold water before being rinsed in fine mesh-wrapped plastic filter boxes under cold water for fifteen minutes to remove any remaining soil particles. Clean roots from AM and ErM plants were cleared in 10% potassium hydroxide for twenty hours at 60ºC in a Fisher IsoTemp® Incubator 200 series. After twenty hours the roots were checked for sufficient clearing. Those still containing obvious pigments were cleared for an additional two hours at 60ºC in fresh KOH. After clearing roots were washed with distilled water for five minutes, acidified in 1% glacial acetic acid for five minutes, then stained in a solution of 1% Parker Pen Company Quink® (a dark blue non-toxic ink) in 7% glacial acetic acid for twenty hours at 60ºC. The roots were destained by agitating in a 1:1:1 solution of glacial acetic acid, glycerol, and distilled water for 10 minutes.

Stained roots were sectioned into 1cm pieces of the finest roots (AM) or hair roots (ErM) and randomly selected using a gridded petri dish for microscopic quantification of colonization. Three 1cm long root sections were selected for each ErM and AM plant. Each field of view along the root lengths was photographed using CellSens® standard (Olympus corporation) microscope software and an Olympus BX43 microscope at 200X total magnification. This produced 20 microphotographs per 1cm root segment, each representing 0.5mm of root tissue. The photographs were copied to PowerPoint® (Microsoft®) where they were overlaid with a 7 X 9 (63 intersections) grid, allowing colonization to be assessed at every 70 micrometers vertically and every 55 micrometers horizontally along the length of the root (Figure 7), in a modification of the magnified intersections method (McGonigle et al. 1990). At each of the 63 intersections the colonization was assessed as either absent or present. For arbuscular mycorrhizal roots, each intersection containing fungal tissue was also classified as an arbuscule, vesicle, hypha, or dark septate endophyte hypha. For ericoid mycorrhizal roots, intersections with fungal tissue were classified as a hyphal coil, hypha, or dark septate endophyte hypha. Any intersections that were not occupied by plant or fungal tissue were classified as empty when present so that percent colonization for each root could be determined.

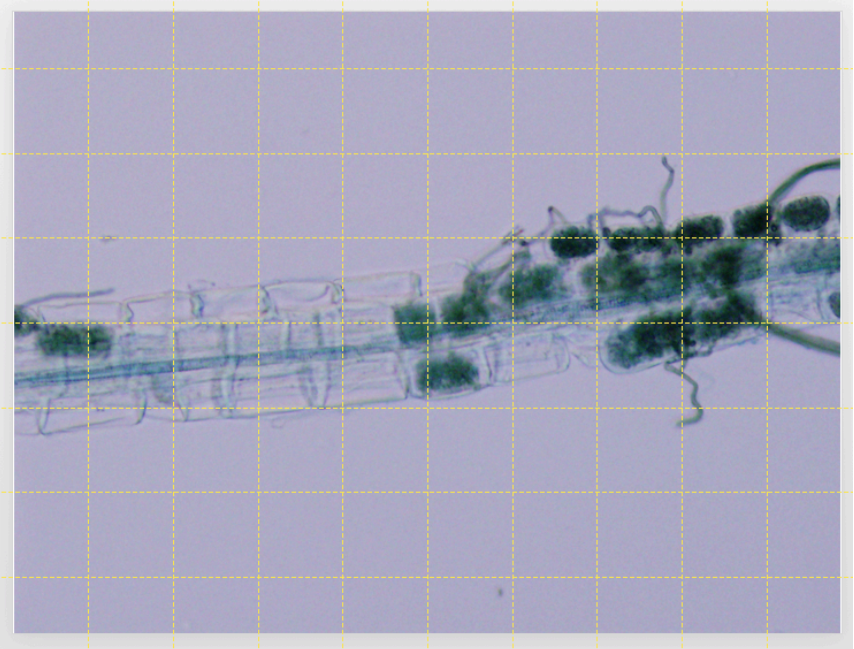


Figure 7. Example of the 7 X 9 grid overlaid on a 0.5mm section of *Kalmia angustifolia* root from Mount Merritt cleared and stained with Quink®.

Ectomycorrhizal colonization was assessed by characterizing the fine root tips collected. Root tips were removed from each washed sample under 10X magnification using a Nikon SMZ800 dissecting microscope. For each root tip, binary presence/absence data was recorded on bifurcation, fungal mantle, apparent root health, root hairs, and apical meristem swelling. In *Pinus* roots bifurcation, fungal mantle, and apical meristem swelling are all indicative of mycorrhizal colonization, while the presence of root hairs indicates a lack of symbiosis (Peterson and Bonfante 1994). Roots tips that appeared unhealthy, shriveled, or decomposed were considered dead and were not classified according to their mycorrhizal status. Root tips exhibiting any of the features of a

mycorrhizal relationship were classified as colonized, those with root hairs or lacking mycorrhizal features were classified as non-mycorrhizal.

Percent colonization represented percent of total gridline intersections covering plant root tissue that also contained mycorrhizal fungal tissue for each 1cm ErM and AM root segment. Segments were averaged to give a per-plant percent colonization. In ECM roots, the proportion of the total fine root tips that were classified as mycorrhizal was used to produce the percent colonization.

Data Analysis

Bulk density was calculated at the east, west, and center of each plot using the formula: average organic soil mass (g)/(soil depth (cm) * 400 (soil sample surface area (cm))). Bulk densities were averaged across each plot and multiplied by 1000 to give the units kg/m^3 for use in calculating the plant available nitrogen and phosphorus.

Plant available nitrogen (kg/ha) in the soil was calculated using the formula: ((ammonium (mg/kg) + nitrate (mg/kg)) * soil depth (m) * bulk density (kg/m^3))/100. Plant available phosphorus was calculated using the formula: phosphorus (mg/L) * soil depth (m) *10. Edaphic factors pH, N, P, were compared by one-way ANOVA in Past 3.1.0 (Hammer, Harper and Ryan 2001).

Generalized quasi-binomial linear regression models of percent colonization were constructed using R Statistical Software (R Core Team 2013). Individual models were produced for each plant species type for a total of five models (n=36 per model) where

colonization (%) was fitted to the z-transformed variables pH, volumetric water content (%), temperature (˚C), plant available nitrogen (kg/ha) and phosphorus (kg/ha). The variables were tested for multicollinearity using the vif (variance inflation factor) function in the package car with a cut-off of 3. The variables season and site were not included in the final generalized linear models as they each had a vif over 3 with temperature. Instead, temperature was used as a proxy for site and season. Results were assembled into dot and whisker plots with 95% confidence intervals using the package dwplot.

Results

Site Descriptions

Table 3. Edaphic factors (mean ± standard error) by season and site.

	Spring		
	Wetland	**Ecotone**	**Upland**
		Merrymakedge	
pH	3.93 ± 0.07	3.81 ± 0.10	3.67 ± 0.28
VWC (%)	79.60 ± 4.19	39.48 ± 2.58	24.57 ± 1.85
Temperature (˚C)	17.81 ± 0.25	14.99 ± 0.23	18.53 ± 0.12
Nitrogen (kg/ha)	2.52 ± 0.19	3.69 ± 0.31	5.55 ± 0.61
Phosphorus (kg/ha)	2.89 ± 0.14	3.20 ± 0.20	5.16 ± 0.30
		Mount Merritt	
pH	3.73 ± 0.03	3.68 ± 0.11	3.29 ± 0.10
VWC (%)	86.49 ± 1.03	52.33 ± 0.93	13.49 ± 1.72
Temperature (˚C)	21.85 ± 0.19	20.01 ± 0.48	27.24 ± 1.11
Nitrogen (kg/ha)	1.39 ± 0.12	1.76 ± 0.19	1.09 ± 0.77
Phosphorus (kg/ha)	3.75 ± 0.20	3.86 ± 0.44	2.42 ± 0.41

	Fall		
	Wetland	**Ecotone**	**Upland**
		Merrymakedge	

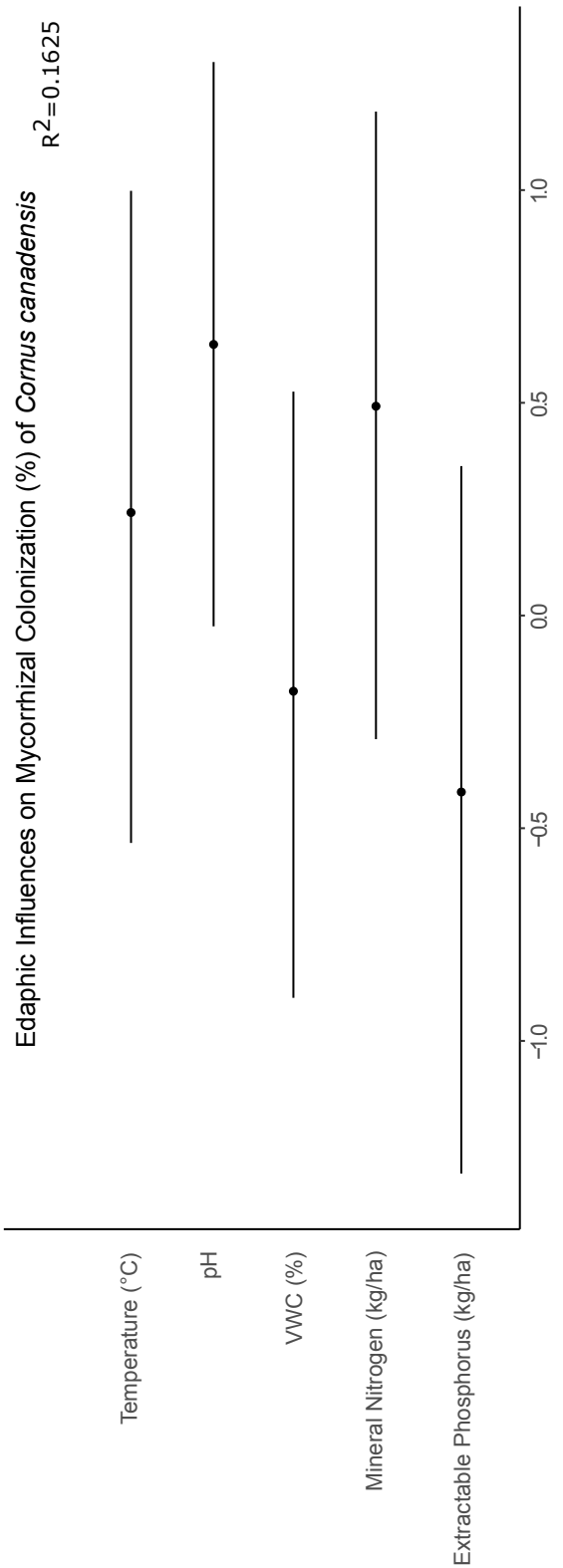
pH	3.51 ± 0.03	3.46 ± 0.04	3.50 ± 0.06
VWC (%)	49.85 ± 5.16	32.68 ± 1.59	28.90 ± 1.23
Temperature (˚C)	11.02 ± 0.10	8.75 ± 0.32	5.63 ± 0.11
		Mount Merritt	
pH	4.96 ± 0.29	3.65 ± 0.06	3.88 ± 0.05
VWC (%)	58.87 ± 3.53	45.89 ± 3.67	25.14 ± 0.61
Temperature (˚C)	17.30 ± 0.14	16.51 ± 0.21	11.47 ± 0.14

Edaphic Factors

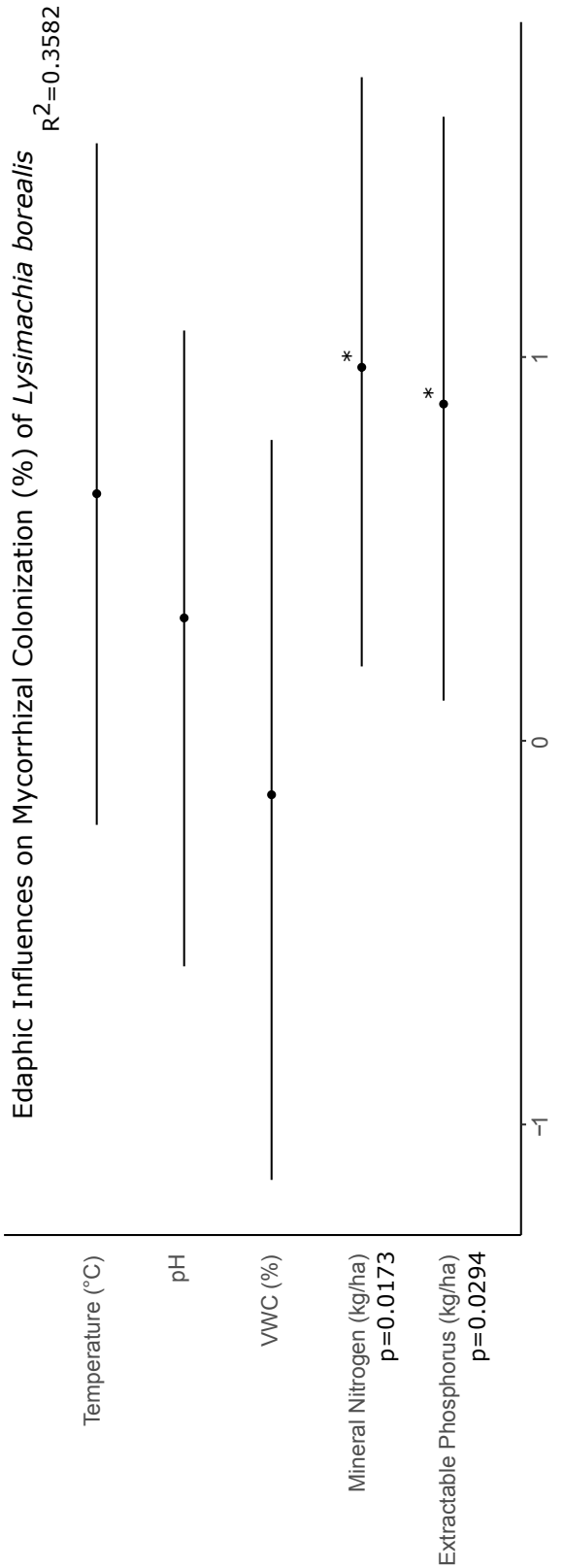
Soil pH, plant available nitrogen, and phosphorus did not vary significantly between the sites or plots ($p>0.05$) and pH was not significantly different between the seasons (soil nutrients were measured only in the spring) (Table 3). Volumetric water content increased significantly along the transects from upland to wetland, but was not significantly different between sites. The average soil moisture in the spring was 24.57% in the upland to 79.60% in the wetland at Merrymakedge, while the transect at Mount Merritt had a greater range, from 13.49% to 86.49% from the upland to the wetland. The wetlands on both sites were drier in the fall, with an average soil moisture of 49.85% at Merrymakedge and 58.87% at Mount Merritt. Merrymakedge was a cooler, shadier site, with an average spring soil temperature of between 14.99˚C to 18.53˚C compared to spring soil temperatures between 20.01˚C to 27.24˚C at Mount Merritt. The same trend was observed in the fall, with soil temperatures of 5.63-11.02˚C and 11.47-17.30˚C at Merrymakedge and Mount Merritt, respectively. No significant relationships were observed between plant foliar and soil nutrient levels (not shown).

Percent Mycorrhizal Colonization in Forested Wetlands

a


Edaphic Influences on Mycorrhizal Colonization (%) of Cornus canadensis
R2=0.1625
Temperature (°C)
pH
VWC (%)
Mineral Nitrogen (kg/ha)
Extractable Phosphorus (kg/ha)
−1.0
−0.5
0.0
0.5
1.0

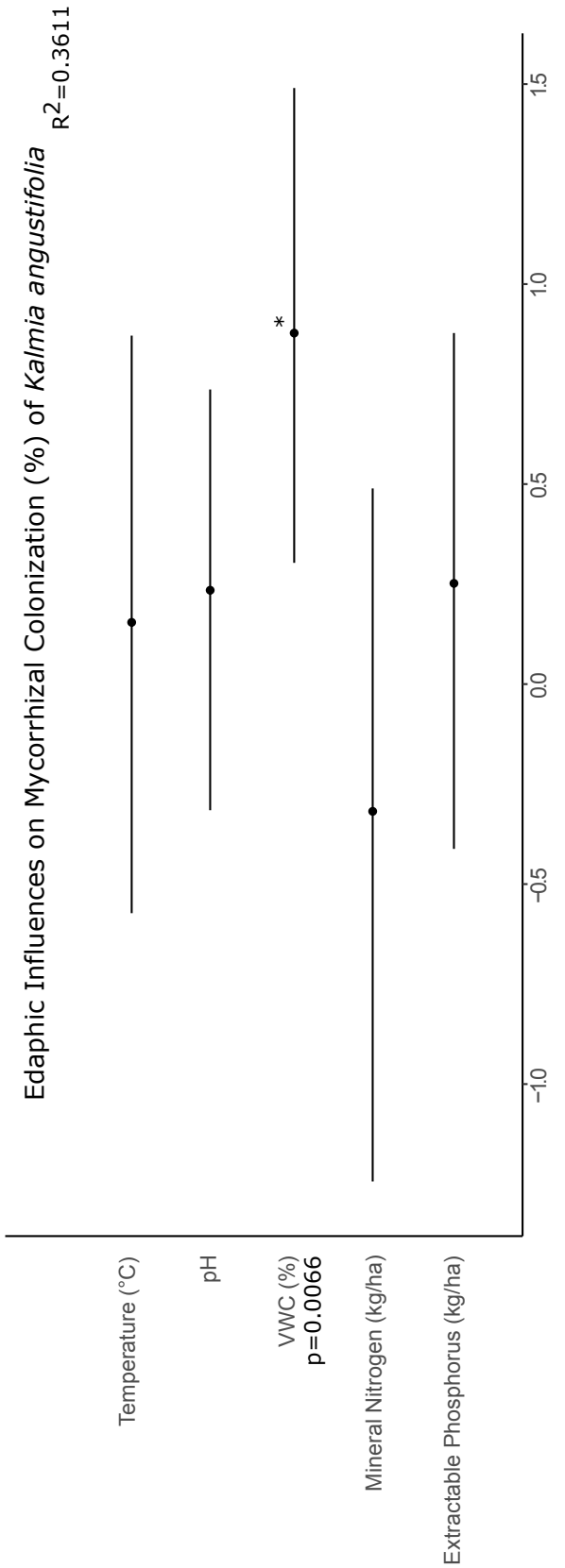
b


Edaphic Influences on Mycorrhizal Colonization (%) of Lysimachia borealis
R2=0.3582
Temperature (°C)
pH
VWC (%)
Mineral Nitrogen (kg/ha)
p=0.0173
Extractable Phosphorus (kg/ha)
p=0.0294
*
*
−1
0
1

c

Edaphic Influences on Mycorrhizal Colonization (%) of Pinus strobus
R2=0.1419
Temperature (°C)
pH
VWC (%)
Mineral Nitrogen (kg/ha)
Extractable Phosphorus (kg/ha)
−1.5
−1.0
−0.5
0.0
0.5
1.0

d


Edaphic Influences on Mycorrhizal Colonization (%) of *Kalmia angustifolia*
R^2=0.3611
Temperature (°C)
pH
VWC (%)
p=0.0066
Mineral Nitrogen (kg/ha)
Extractable Phosphorus (kg/ha)
*
−1.0
−0.5
0.0
0.5
1.0
1.5

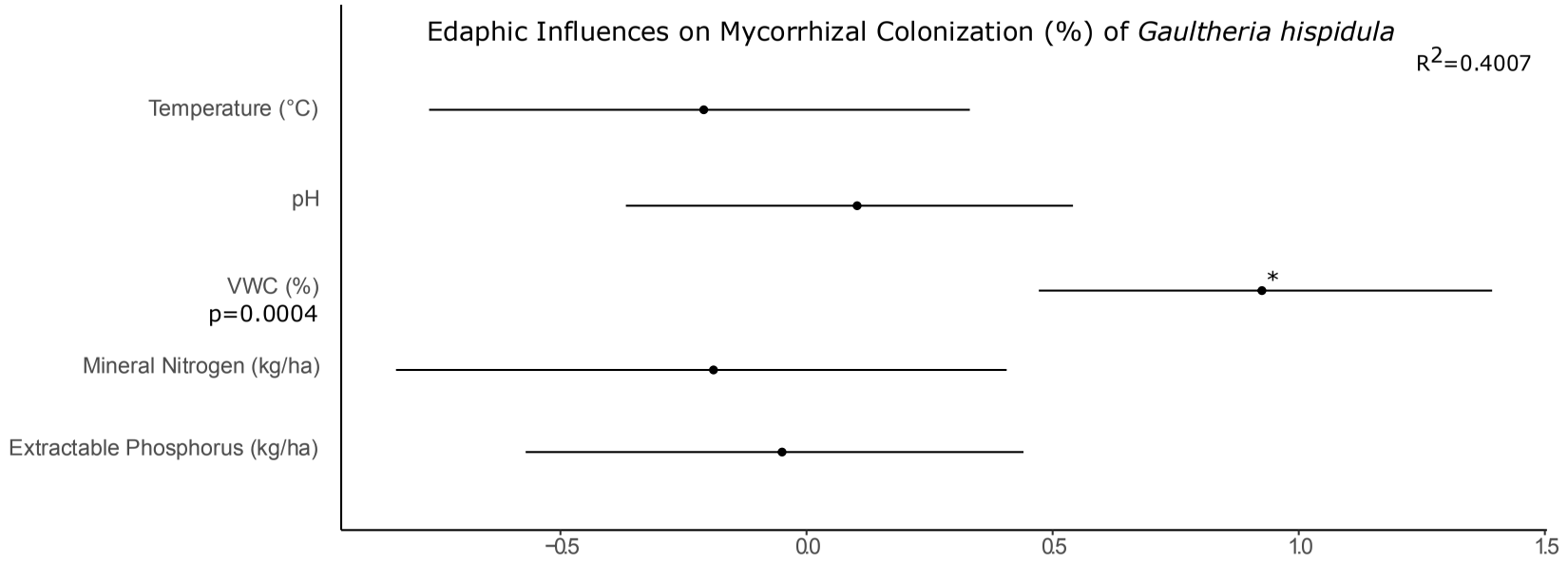

Edaphic Influences on Mycorrhizal Colonization (%) of *Gaultheria hispidula*
R^2=0.4007
Temperature (°C)
pH
VWC (%)
p=0.0004
*
Mineral Nitrogen (kg/ha)
Extractable Phosphorus (kg/ha)
−0.5
0.0
0.5
1.0
1.5

Figure 8 (a-b) (pages 48-52). Dot and whisker plots of edaphic influences on mycorrhizal colonization (%) of *Pinus strobus* (a), *Cornus canadensis* (b), *Lysimachia borealis* (c), *Kalmia angustifolia* (d), and *Gaultheria hispidula* (e). Each dot represents the quasi-binomial generalized linear model coefficient value for each variable (Temperature (˚C), VWC (%), pH, plant available nitrogen (mg/ha), and phosphorus (mg/ha)), with the associated whiskers representing the 95% confidence interval. Significant factors (*) listed at α=0.05 or smaller. Non-significant p-values are not shown.

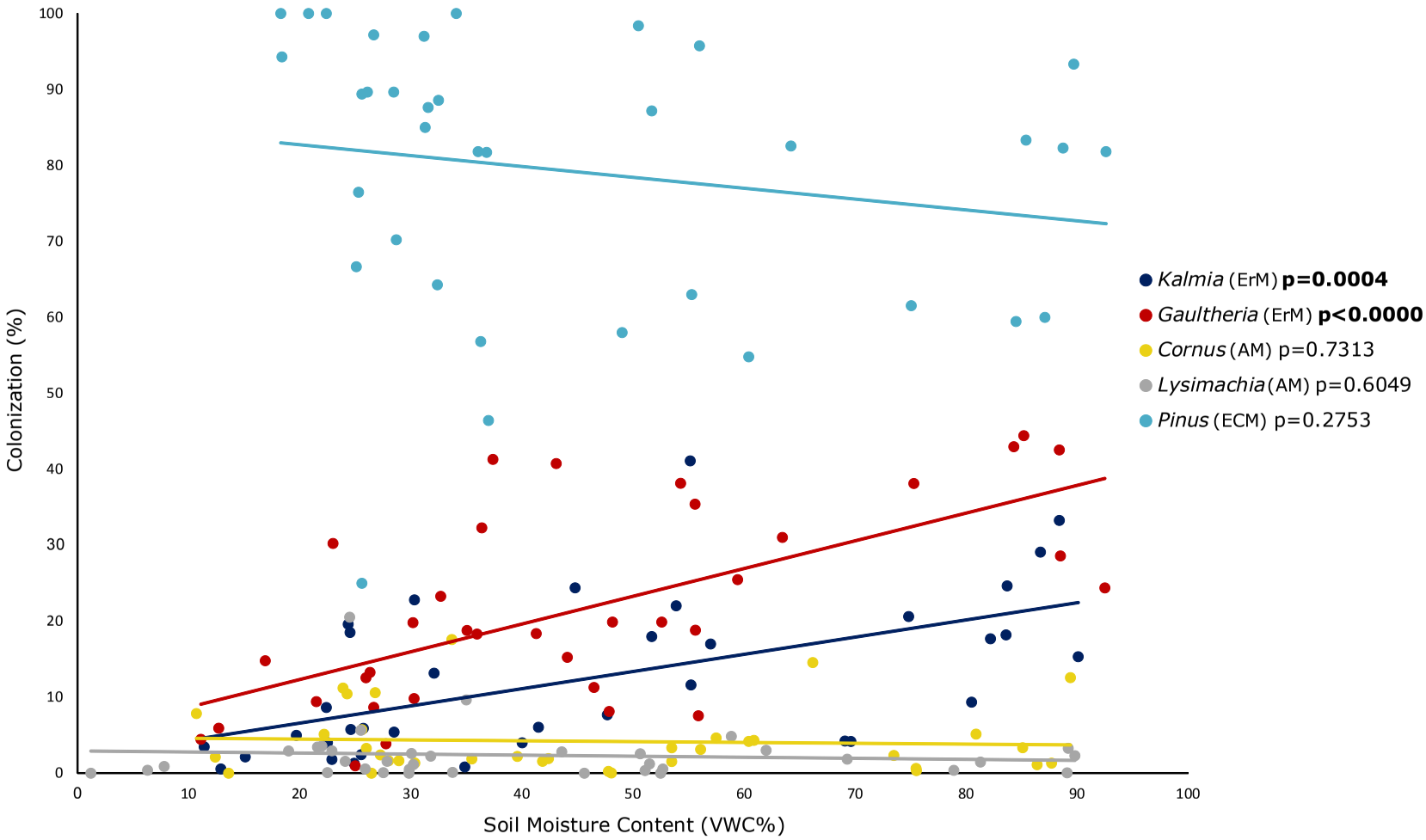
Average soil moisture, temperature, and pH were not significant factors in predicting colonization level in either arbuscular mycorrhizal plant. In the *Lysimachia borealis* model both plant available nitrogen and phosphorus were significant variables. None of the factors measured were significant in predicting colonization level in *Cornus canadensis*. In *Cornus canadens*is (Figure 8a), 16.25% of the variation in colonization level could be explained by the variables observed, while in *Lysimachia borealis*, 35.82% of the variation in could be explained (Figure 8b).

Ectomycorrhizal colonization was not significantly related to changes in VWC (%) along the forested upland to wetland transects (Figure 8c). Using the measured variables, only 14.19% of the variation in percent colonization could be explained. None of the variables had a significant influence on the colonization level. However, at the plot level, ectomycorrhizal colonization was lower in the wetland than in the upland (One-way ANOVA; p=0.005).

A strong, significant positive relationship between percent colonization and soil volumetric water content was detected for both ericoid mycorrhizal plants. The trend was more pronounced in *Gaultheria* (Figure 8d) than in *Kalmia* (Figure 8e), but was observed in both host plants. None of the other factors measured were significant in predicting the mycorrhizal colonization level of the ericaceous plants studied.

When compared to volumetric water content directly, colonization levels of ectomycorrhizal and arbuscular mycorrhizal fungi did not significantly change across the transects, while the colonization level of ericoid mycorrhizal fungi increased significantly with increasing soil moisture content (Figure 9).

Figure 9 (Next page). Percent colonization compared to volumetric water content by plant species with mycorrhizal type in parentheses. Significant p-values at $\alpha < 0.05$ are bold.


Colonization (%)
Soil Moisture Content (VWC%)
0
10
20
30
40
50
60
70
80
90
100
Kalmia (ErM) **p=0.0004**
Gaultheria (ErM) **p<0.0000**
Cornus (AM) p=0.7313
Lysimachia (AM) p=0.6049
Pinus (ECM) p=0.2753

Discussion and Conclusions

In the five plants studied, average soil moisture content was a significant predictor of mycorrhizal colonization level in only the two ericoid mycorrhizal species. Plant available phosphorus and nitrogen were significantly linked to *Lysimachia borealis* percent colonization, while none of the other factors were significant in any of the other plants. Soil temperature and VWC were higher in the spring than in the fall, but no seasonal effect on pH was observed. There were no significant differences in pH or soil nutrients on either transect. Merrymakedge was shadier and more nutrient rich than Mount Merritt, yet similar colonization patterns were observed on both sites.

Although arbuscular mycorrhizal colonization was not well explained by the measured variables, plant available nitrogen and phosphorus were the best predictors of AM colonization level. This is in agreement with the characterization of AM as important associates for phosphorus and nitrogen acquisition (Read 1991). It is somewhat surprising that AM colonization was not significantly different between seasons (as represented by temperature in the model), but it is possible that the between site differences in temperature obscured a seasonal shift in AM colonization level in the model. Nonetheless, the lack of significant differences in colonization between upland and wetland sites is also in agreement with other recent studies (Allen, Richards and Busso 1989; Wetzel and van der Valk 1996; Kandalepas et al. 2010).

Ectomycorrhizal colonization levels were not well predicted by any of the variables measured in this study. Surprisingly, average soil moisture content was not a

significant predictor of ectomycorrhizal colonization as it has been in previous studies. This could be explained by the fact that I excluded any inactive fine root tips and used multiple indicators of mycorrhizal status for quantification. Other studies including partially senesced root tips and/or fewer mycorrhizal indicators may have underestimated colonization levels in forested wetlands. Another possible explanation for this observation is that some of the *Pinus strobus* plants were growing on hummocks and therefore not subjected to wet enough conditions to produce a steep decline in mycorrhizal status. Ectomycorrhizal colonization was lower in the wetland than in the upland when considered at the plot level, suggesting that there is another variable that was not measured that is responsible for the decreased colonization.

Ericoid mycorrhizal colonization, although somewhat different between the two ericaceous species, increased significantly from the upland forest through the ecotone to the forested wetlands. Conversely, season, pH, site, temperature, and soil nitrogen and phosphorus were not significant factors in predicting ericoid mycorrhizal colonization.

One possible limitation of this study is the number of segments used to assess mycorrhizal colonization level in each plant type. Some studies in this discipline use up to 100 root segments per plant to determine percent colonization, while my calculations were based on 3 root segments from 3 replicate plant samples per plot studied. While this number of root segments studied quickly multiplied through the inclusion of 5 plant species, 3 plot types, 2 sites, and 2 seasons, a study on forested wetland ericoid

mycorrhizal plants alone might benefit from the examination of more root segments per sample.

The sodium bicarbonate extractable (Olsen 1954) method used for measurement of plant available phosphorus in the studied soils may not have been optimal, as the Olsen method is best used for neutral or alkaline soils, and the sites studied ranged in pH from (2.71 to 7.33). The Olsen method was selected as a component of a package of plant available soil parameters but absolute extractable phosphorus in these soils might have been better described by the Bray- 1 or 2 methods (Radwan, Kraft and Shumway 1985). Despite these limitations, the soil mean pH was reasonably consistent between sites and along the transects, meaning that the relative extractable soil phosphorus is informative, even if the absolute levels of soil phosphorus may have been underestimated.

Of the three mycorrhizal types studied, only ericoid mycorrhizal colonization could be significantly linked to soil moisture content, while ectomycorrhizal colonization decreased at the plot level between the upland and wetland, and arbuscular mycorrhizal colonization was not significantly correlated to VWC or plot. As the major mycorrhizal types are formed by different groups of fungi, with ECM formed by homobasidiomycetes and ascomycetes, AM by Glomeromycota, and ErM by heterobasidiomycetes and ascomycetes, it can be inferred that differences in their abilities to colonize wetlands are linked to water tolerance in the different groups of fungi. Ectomycorrhizal fungi, which are typical of woody plants in temperate forests, may not have been evolutionarily pressured to evolve wetland tolerance, or perhaps only certain species of ECM fungi can

survive in wetlands. This speculation is supported by the fact that wetland tolerant trees in the genus *Acer* are arbuscular mycorrhizal (Kessler 1966). Ericaceous plants are known for being broadly stress tolerant, thus it is not surprising that their symbionts are also tolerant of a range of edaphic conditions. This may be as a result of higher water tolerance in ErM fungi, or perhaps there are a range of potential ErM fungi that are specialized for survival in different stressful habitats.

This study shows that ericoid mycorrhizal colonization can be significantly higher in forested wetlands than in nearby upland forests. This capability is important to consider when planning conservation efforts for forested wetlands. Ericaceous plants in their native habitats are prone to displacement by invasive grasses under eutrophic conditions in normal soils, where their competitive abilities to liberate organically bound nutrients are not as advantageous (Fagúndez 2012). If eutrophication occurs in wetlands, ErM plants might be less prone to displacement because of their higher levels of fungal colonization, or, if they are displaced in the same way, their highly specialized symbioses may be lost. This would be problematic as native species are more valuable than invasive ones for promoting mycorrhizal fungal biodiversity, and non-native plant species can reduce mycorrhizal biodiversity and lower the inoculum potential of soil (Mummey and Rillig 2006). The mycorrhizal inoculum potential of wetland soils may be particularly delicate, as species that thrive there are likely to be highly specialized. If ericaceous plants with wetland-specialized ericoid mycorrhizal fungal symbionts are displaced by invasive (likely AM) grasses, they may be difficult, if not impossible, to re-establish.

References

Allen MF, Richards JH, Busso CA. 1989. Influence of clipping and soil-water status on vesicular-arbuscular mycorrhizae of 2 semi-arid tussock grasses. Biology and Fertility of Soils 8(4):285-9.

Bacheler RJ. 2014. Distribution of two ericoid mycorrhizae in a New Jersey pine barrens upland-wetland site and the effect of mycorrhizal presence on Ericaceous plants. Camden, New Jersey: Rutgers University - Camden.

Bauer CR, Kellogg CH, Bridgham SD, Lamberti GA. 2003. Mycorrhizal colonization across hydrologic gradients in restored and reference freshwater wetlands. Wetlands 23(4):961-8.

Brundrett M. 2004. Diversity and classification of mycorrhizal associations. Biological Reviews 79(3):473-95.

Brundrett MC and Ashwath N. 2013. Glomeromycotan mycorrhizal fungi from tropical Australia III. measuring diversity in natural and disturbed habitats. Plant Soil 370(1-2):419-33.

Cantelmo Jr AJ and Ehrenfeld JG. 1999. Effects of microtopography on mycorrhizal infection in Atlantic white cedar (*Chamaecyparis thyoides* (L.) mills.). Mycorrhiza 8(4):175-80.

Clayton J and Bagyaraj D. 1984. Vesicular-arbuscular mycorrhizas in submerged aquatic plants of New Zealand. Aquatic Botany 19(3):251-62.

Cornwell WK, Bedford BL, Chapin CT. 2001. Occurrence of arbuscular mycorrhizal fungi in a phosphorus-poor wetland and mycorrhizal response to phosphorus fertilization. American Journal of Botany 88(10):1824-9.

Eom A, Hartnett DC, Wilson GW. 2000. Host plant species effects on arbuscular mycorrhizal fungal communities in tallgrass prairie. Oecologia 122(3):435-44.

Fagúndez J. 2012. Heathlands confronting global change: Drivers of biodiversity loss from past to future scenarios. Annals of Botany 111(2):151-72.

Hammer Ø, Harper DA, Ryan PD. 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4(1):9.

Kandalepas D, Stevens KJ, Shaffer GP, Platt WJ. 2010. How abundant are root-colonizing fungi in southeastern Louisiana's degraded marshes? Wetlands 30(2):189-99.

Kessler KJ. 1966. Growth and development of mycorrhizae of sugar maple (*Acer saccharum mars*h.). Canadian Journal of Botany 44(10):1413-25.

Malloch D and Malloch B. 1981. The mycorrhizal status of boreal plants: Species from northeastern Ontario. Canadian Journal of Botany 59(11):2167-72.

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA. 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytologist 115(3):495-501.

Mummey DL and Rillig MC. 2006. The invasive plant species *Centaurea maculosa* alters arbuscular mycorrhizal fungal communities in the field. Plant Soil 288(1-2):81-90.

Neily P, Basquill S, Quigley E, Keys K. 2017. Ecological land classification for Nova Scotia. Nova Scotia: Nova Scotia Department of Natural Resources, Renewable Resources Branch. Report nr 13.

Nilsson LO, Giesler R, Bååth E, Wallander H. 2005. Growth and biomass of mycorrhizal mycelia in coniferous forests along short natural nutrient gradients. New Phytologist 165(2):613-22.

Olsen SR. 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. United States Department of Agriculture; Washington.

[Internet]. Ontario, Canada: Government of Ontario; c2019 [cited 2019 June 5]. Available from: http://www.omafra.gov.on.ca/english/nm/regs/sampro/sampro03-12.htm.

Peterson RL and Bonfante P. 1994. Comparative structure of vesicular-arbuscular mycorrhizas and ectomycorrhizas. Plant Soil 159(1):79-88.

Radwan M, Kraft J, Shumway J. 1985. Evaluation of different extractants for phosphorus in western hemlock soils. Soil Science 140(6):429-35.

Read DJ. 1991. Mycorrhizas in ecosystems. Experientia 47(4):376-91.

Robertson SJ, Tackaberry LE, Egger KN, Massicotte HB. 2006. Ectomycorrhizal fungal communities of black spruce differ between wetland and upland forests. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 36(4):972-85.

Smith SE and Read DJ. 2008. Mycorrhizal symbiosis. 3rd ed ed. New York, New York: Elsevier.

Soudzilovskaia NA, Douma JC, Akhmetzhanova AA, van Bodegom PM, Cornwell WK, Moens EJ, Treseder KK, Tibbett M, Wang Y, Cornelissen JH. 2015. Global patterns of plant root colonization intensity by mycorrhizal fungi explained by climate and soil chemistry. Global Ecology and Biogeography 24(3):371-82.

Team RC. 2013. R: A language and environment for statistical computing.

Vasilas B, Vasilas L, Thompson J, Rizzo A, Fuhrmann J, Evans T, Pesek J, Kunkle K. 2004. Ectomycorrhizal mantles as indicators of hydrology for jurisdictional wetland determinations. Wetlands 24(4):784-95.

Wetzel PR and VanderValk AG. 1996. Vesicular-arbuscular mycorrhizae in prairie pothole wetland vegetation in Iowa and North Dakota. Canadian Journal of Botany-Revue Canadienne De Botanique 74(6):883-90.

CHAPTER THREE

Ericoid Mycorrhizal Communities Along Forest to Wetland Gradients

Abstract

There has recently been a large increase in the understanding of ericoid mycorrhizal (ErM) fungal communities. The symbiosis was long thought to take place between Ericaceous plants and only one species of fungus, *Rhyzoscyphus ericae*. However, evidence has arisen in the past two decades for the existence of other ErM fungi. Now, thanks to advanced molecular techniques, there are more than ten known species of fungi that form ErM. Still, studies on ErM communities from natural habitats are rare. In this study the fungal root inhabitants of two Ericaceous plants were characterized along forest to wetland gradients by DNA cloning and sequencing. *Serendipita* was the dominant putative ErM fungal species observed while *Pezoloma (Rhizoscyphus)*, *Hyaloscypha (Meliniomyces)*, and *Oideodendron* were also observed. Distinct Sebacinales OTUs were found in the wetland and upland sites, suggesting habitat specialization in this order in which species have recently been characterized as ErM.

Introduction

The classic conception of the ericoid mycorrhizal (ErM) symbiosis came from the easily cultured helotialean *Pezoloma ericae* aggregate (formerly *Rhizoscyphus ericae* aggregate and *Hymenoscyphus ericae*). However, culture-based studies of mycorrhizal communities are highly biased, as there are many symbiotic fungi that are not easily cultured (Allen et al. 2003). Early molecular studies of ErM communities that sequenced

DNA from fungi cultured from roots are also subject to the same biases (Chambers, Liu and Cairney 2000; Sharples et al. 2000). Culture-independent molecular methods have vastly expanded the understanding of ericoid mycorrhizal symbioses (Hazard et al. 2014), which are now known to be formed by the ascomycetes *Hyaloscypha* (*Meliniomyces*) *variabilis*, *Oideodendron maius* (Walker et al. 2011), *Gamarada delbrueckii* (Midgley et al. 2018), *Cairneyella variabilis* (Palmer et al. 2007), heterobasidiomycetes in the genus *Serendipita* (Vohník et al. 2016) and the homobasidiomycete *Kurtia argillacea* (Kolařík and Vohník 2018).

Ericoid mycorrhizal relationships have received very little research attention, as they are relatively uncommon in agriculture, except in the cultivation of blueberry or cranberry or some ornamentals and are seen as relatively unimportant in forestry.

Very few studies of ErM fungi have been conducted directly on Ericaceous hair roots (culture independent). Instead, many have opted for the culture dependent studies, isolating fungi from roots and then sequencing the isolates. While this allows for the option of investigating the mycorrhizal status of isolates through re-synthesis experiments, this approach inevitably underestimates diversity due to the resistance of many fungi to growth in culture (Tedersoo et al. 2010).

Studies of bacterial and fungal communities in soil provide evidence for structural shifts across natural gradients, including changes in pH, soil moisture content, and nutrients (Li et al. 2018). Similarly, mycorrhizal communities appear to be influenced by

edaphic shifts, including higher ECM and ErM biomass in low nutrient soils, while AM biomass can be higher in low phosphorus, higher pH microsites (Nilsson et al. 2005). The same trend has been recorded on a global scale, with higher ErM colonization toward the poles, ECM at intermediate latitudes, and AM at the tropics (Read 1991). Shifts have also been observed along a successional gradient (Huusko, Ruotsalainen and Markkola 2017) with AM fungi dominating in the youngest zones with a shift toward dark septate endophytes in later successional ages. Kohout and Tedersoo (2017) found changes in fungal community composition along a soil moisture gradient in the ericoid mycorrhizal plant *Erica dominans*, with more operational taxonomic units (OTUs) in the Helotiales in wet sites, although their next generation sequences could not be identified to the species level.

Forested wetlands are important resources for carbon storage and water filtration and provide important habitats for numerous bird and invertebrate species (Conner 1998). They currently face multiple threats including urbanization (Faulkner 2004), hydrological destabilization (Burkett and Kusler 2000), and displacement by invasive species (Zedler and Kercher 2004). Some evidence suggests that microbial community richness may be reduced in wetlands (Li et al. 2018), but those that can survive in wetland habitats are potentially more sensitive to environmental conditions outside of their ecological niche (Thuiller et al. 2005). Others have suggested that soil fungal species richness may be relatively high in wetlands, but with a different community structure than upland forests (Wolfe et al. 2007). The fungal root-associated communities in forested wetlands require increased attention in order to mitigate the threats to their continuance.

Soil nutrients are an important factor in all soil fungal communities and ericoid mycorrhizae are no exception. Hazard et al. (2014) found a significant correlation between ErM fungal community structure and soil nitrogen. Fungal communities are also impacted by season, with evidence for peaks in diversity in the summer or fall, depending on the functional guild (Voříšková et al. 2014).

The majority of studies on ericoid mycorrhizal communities are limited to a single plant species at a single sampling time, often from a limited geographic range and without the consideration of linked edaphic factors (Bougoure et al. 2007). The inclusion of multiple, potentially linked factors in a single study may prove useful in defining which common covariant factors may be responsible for the observed variations in ericoid mycorrhizal communities. In this study, ericoid mycorrhizal communities associated with two common Acadian forest Ericaceous plants, *Kalmia angustifolia* and *Gaultheria hispidula*, were examined along upland forest to forested wetland transects and fungal DNA sequences from clone libraries were compared between sites, seasons and host species. The objective was to gain an understanding of shifts in root-associated community structure along upland to wetland gradient. Changes in the root-associated fungal community in both ericoid mycorrhizal plant species along the upland to wetland gradient were predicted.

Materials and Methods

DNA Extraction and PCR

Hair roots from *Kalmia angustifolia* and *Gaultheria hispidula* plant samples (see Chapter 2 methods) were sampled for fungal community analysis. Root samples were washed free of all visible soil particles through 2mm and 300µm mesh sieves followed by 15 minutes of washing in fine mesh wrapped plastic filter boxes. The roots of the four plant samples of each plant species on each plot were pooled, resulting in one sample per species, plot, site, and season for a total of 24 pooled samples. From each sample 40 1mm hair roots (Figure 10) were randomly selected for DNA extraction using a gridded Petri dish under 10X magnification using a Nikon SMZ800 dissecting microscope. The roots were frozen in AP1 buffer (Qiagen©) before the DNA was extracted using a Qiagen© DNeasy Plant Mini Kit according to the manufacturer's instructions. The extraction produced two 100µL elutions of DNA. Both elutions of the DNA were diluted by 1/10, 1/20 and 1/50 in nuclease free water. Both the diluted DNA and undiluted samples, were amplified by PCR using the primers ITS1F (Gardes and Bruns 1993) and NL6C2 (Kernaghan, Mayerhofer and Griffin 2017) and the following thermocycler parameters: initialization at 95˚C followed by 35 cycles of 1 minute for denaturation at 95˚C, annealing for 1 minute at 53˚C and 2 minutes of extension at 72˚C followed by an additional 10 minutes of elongation at 72˚C. The PCR products were resolved on a 1.5% agarose gel in 1% sodium boric acid electrophoresis buffer at 122v for 90 minutes. The least dilute elution with a clear band was selected for molecular cloning.

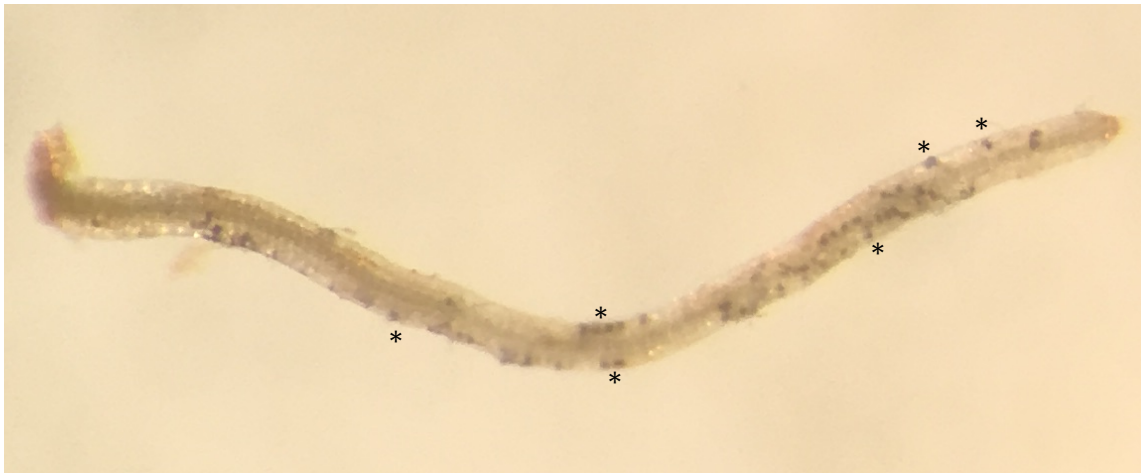


Figure 10. Section (1mm) of *Gaultheria hispidula* hair root collected in the fall from Mount Merritt (20X, unstained) with intracellular hyphal coils (*) symptomatic of colonization by ericoid mycorrhizal fungi.

Cloning and Sequencing

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen©) according to the manufacturer's instructions. Purified PCR products were cloned using the p-GEM®-T Easy Vector System with an overnight ligation for increased number of transformants. Transformed competent cells were inoculated on solid lysogeny broth media with ampicillin, X-Gal, and IPTG, and incubated overnight. Two replicate plates were created for each sample. From each plate 12 white colonies were selected using an autoclaved toothpick and transferred to 50µL of nuclease free water. A total of 24 clone libraries, each containing 24 cloned colonies, were produced. The libraries were frozen at -18˚C until amplification by PCR. Bacterial colonies were amplified using the nested primers ITS1 and ITS4 (White et al. 1990) and the colony PCR thermocycler program: 7 minutes at 94˚C for cell lysis followed by 30 cycles of 1 minute for denaturation at 94˚C,

annealing for 1 minute at 60˚C and 2 minutes of extension at 72˚C followed by an additional 10 minutes of elongation at 72˚C. The PCR products were resolved on a 1.5% agarose gel in 1% sodium boric acid electrophoresis buffer at 122v for 90 minutes. All PCR products produced were then analyzed by restriction fragment length polymorphism (RFLP) using CutSmart buffer and Taq α1 restriction enzyme (New England Biolabs) at 65ºC for 2 hours. RFLP products were resolved on a 1.5% agarose gel in 1% sodium boric acid electrophoresis buffer at 122v for 90 minutes (Figure 11) and restriction fragment patterns were compared to select clones for sequencing. One colony was selected to represent each unique RFLP pattern in each clone library and amplified again for Sanger sequencing by Nanuq - McGill University and Génome Québec Innovation Centre using the primers ITS1 and ITS4 (White et al. 1990) and the colony PCR parameters listed above.

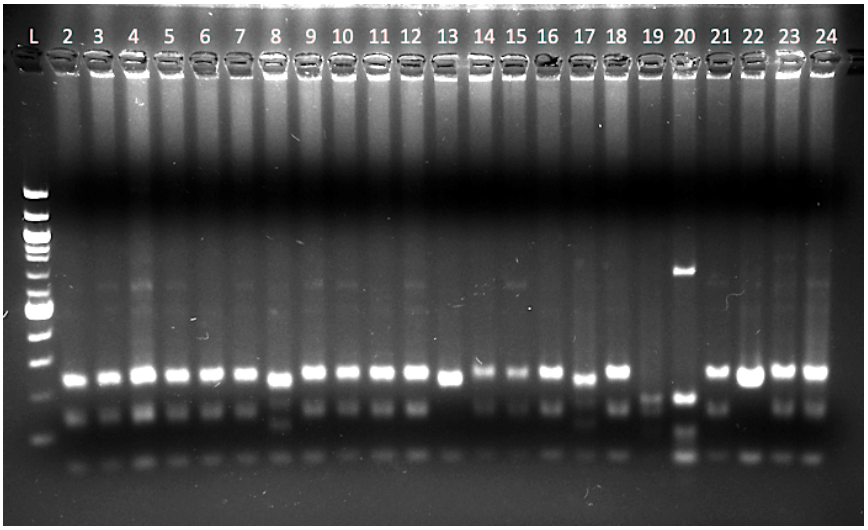


Figure 11. Restriction fragment length polymorphism run on 1.5% agarose electrophoresis gel of a clone library produced from spring harvested *Kalmia angustifolia* roots from the Mount Merritt forested wetland.

Analysis

Consensus reads were assembled in Sequencher 5.3 (Gene Codes Corporation, Ann Arbor, MI) and multiple alignments were prepared using MUSCLE (EMBL-EBI Hixton) for editing and trimming in BioEdit (Ibis Therapeutics). A multiple sequence comparison produced by BioEdit, along with NCBI BLAST, and CD-HIT (Li and Godzik 2006; Fu et al. 2012) was used to bin sequences into OTUs of 97% similarity.

When possible, each OTU was assigned a functional guild using the FUNGuild database (Nguyen et al. 2016) query tool or available literature (Vohník et al. 2016). The total number of samples of each OTU in each guild were assembled into a stacked percentage bar plot in R Statistical Software (R Core Team 2013) with the package ggplot2.

Fungal OTUs and the edaphic variables: volumetric water content, pH, temperature, plant available nitrogen, and phosphorus (Olsen-P) were compared by canonical correspondence analysis (CCA) in Past 3.1.0 (Hammer, Harper and Ryan 2001). Fungal species richness, Fisher's alpha diversity indices and Whittaker's beta diversity indices were also calculated for each sampling plot in Past.

To further investigate the taxonomic positions of OTUs identified as representing members of the Sebacinales, a midpoint rooted maximum parsimony tree comparing all cloned Sebacinales sequences to key reference sequences from GenBank (Clark et al. 2015) was generated in PAUP 4.0a165(X86) (Swofford 2003).

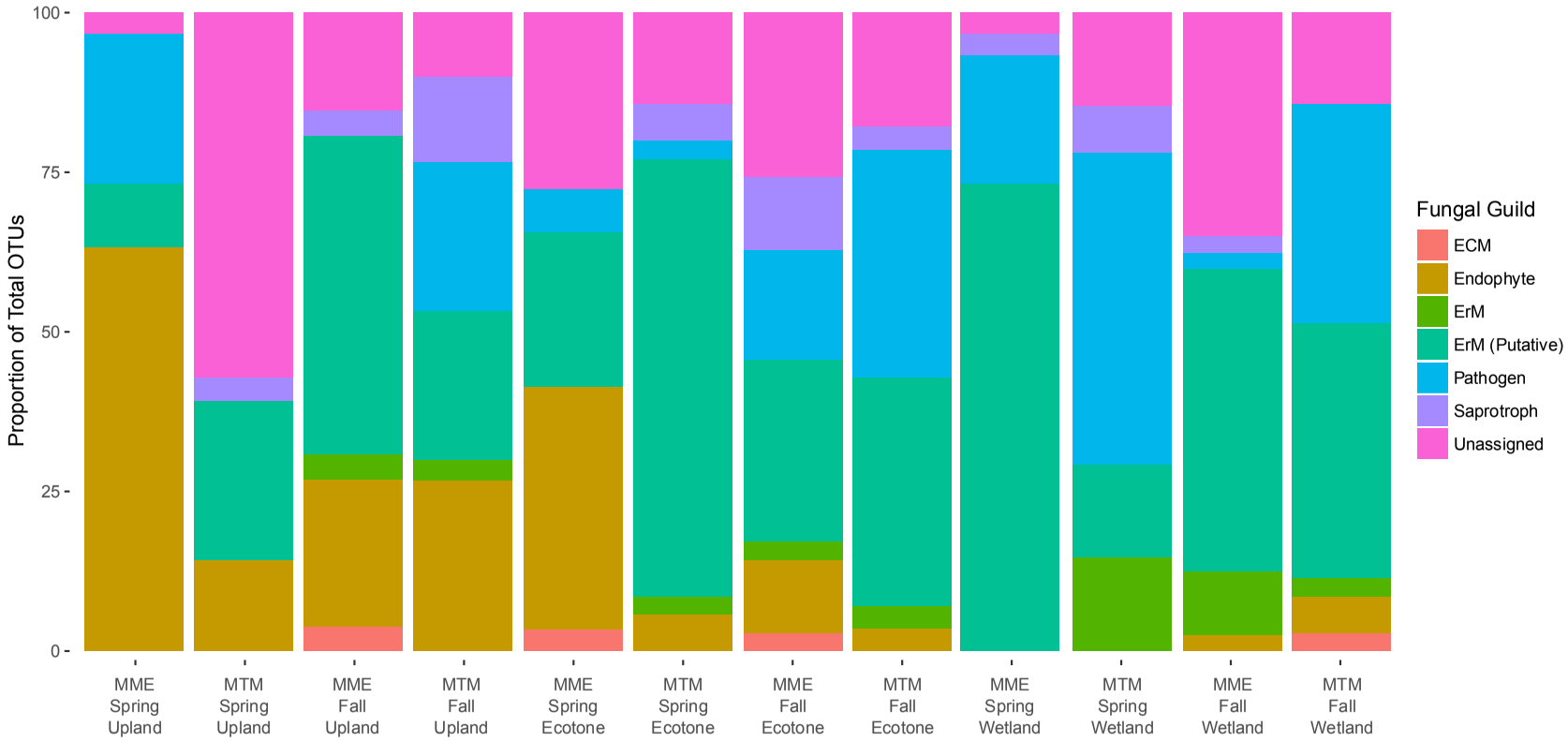
Results

A total of 200 fungal ITS clones were sequenced, uncovering 73 unique OTUs (Table Appendix 1). From these sequences, 12 ErM or putative ErM fungal OTUs were identified. Endophytes, ectomycorrhizal fungi, pathogens, and saprotrophs comprised the other guilds (Figure 12). Twenty-eight OTUs could not be identified to a level sufficient to characterize their functionality and were given the designation "unassigned". In both seasons root endophytes were most common on the upland plots and least common in the forested wetlands, while ErM and putative ErM OTUs were most common in the forested wetlands.

Table 4. Species richness, Fisher's alpha diversity indices and Whittaker's beta diversity indices, means averaged by plot across seasons and sites (mean ± SE for Fisher's α).

	Wetland	**Ecotone**	**Upland**
Richness	44	44	45
Fisher's alpha	24.39 ± 0.78	32.11 ± 1.15	31.34 ± 1.07
Whittaker's beta	4.27	5.54	5.22

Figure 12 (Next page). Stacked percentage bar plot of fungal OTUs in each guild. Bars are arranged by plot from upland to wetland.


Proportion of Total OTUs
100
75
50
25
0
MME Spring Upland
MTM Spring Upland
MME Fall Upland
MTM Fall Upland
MME Spring Ecotone
MTM Spring Ecotone
MME Fall Ecotone
MTM Fall Ecotone
MME Spring Wetland
MTM Spring Wetland
MME Fall Wetland
MTM Fall Wetland
Fungal Guild
ECM
Endophyte
ErM
ErM (Putative)
Pathogen
Saprotroph
Unassigned

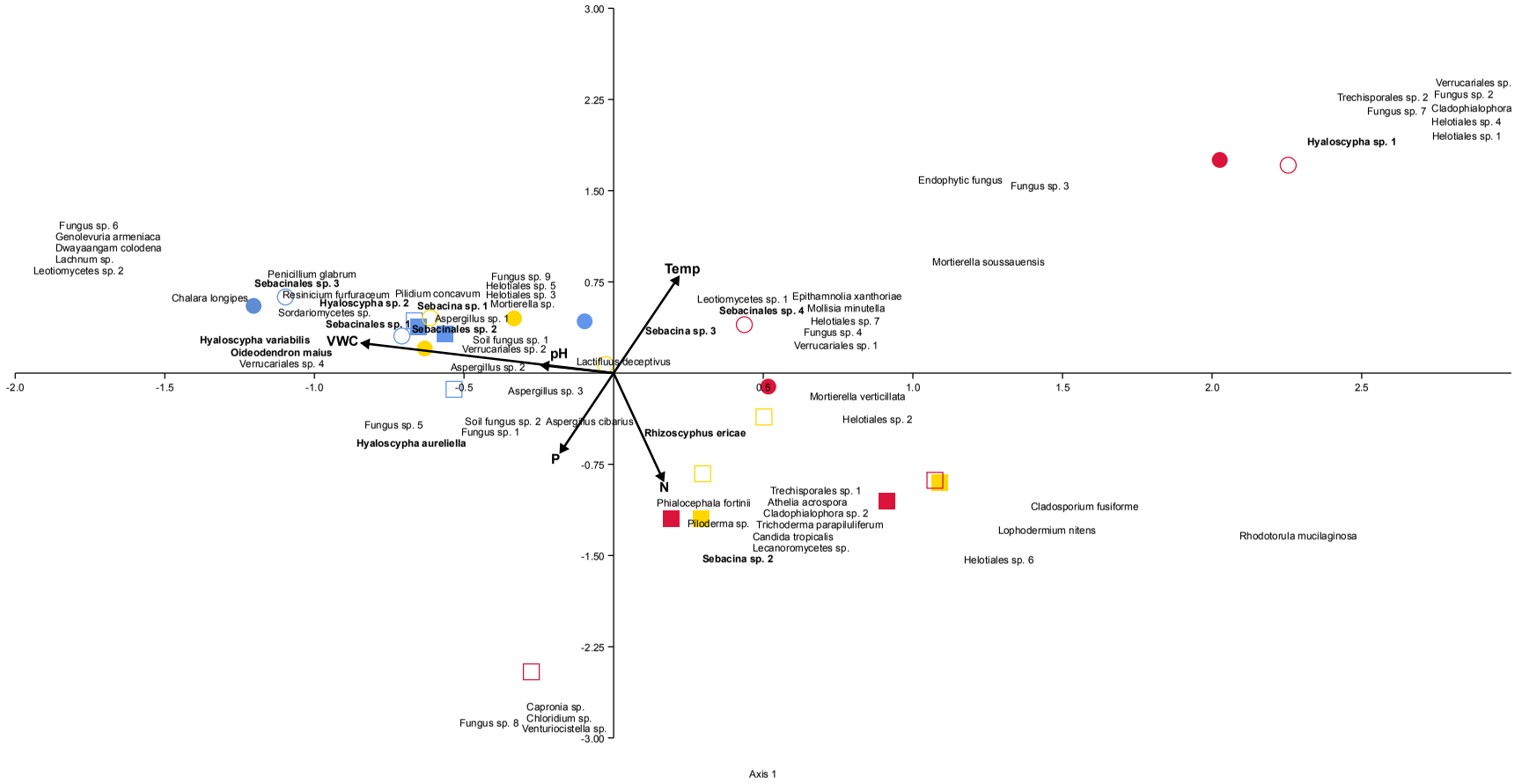
Species richness was greatest in the upland plots, ranging between 1 and 11 OTUs per sample. Of the Merrymakedge plots, the ecotone had the greatest richness, while the wetland had the least. The pattern at Mount Merritt was different, with the upland displaying the greatest richness and the wetland showing the least. In contrast to the richness, Fisher's alpha and Whittaker's beta diversity were highest on both sites in the ecotone, while the wetlands had the lowest alpha and beta diversity (Table 4).

In the canonical correspondence analysis, axis 1 essentially mirrors volumetric water content, with some influence of pH (Figure 13). Axis 2 is related to plant available nitrogen and phosphorus in the negative direction, while temperature is related to axis 2 in the positive direction. However, none of pH, nitrogen, phosphorus, and temperature are related to an axis as strongly as VWC is to axis 1. The wetland plots all cluster to the left side of the diagram (following increasing VWC) and the upland plots cluster to the right, with ecotone plots distributed between the two, showing clear fungal community grouping along the moisture gradient. The two replicate sites do not separate along either axis.

Most of the ErM and putative ErM OTUs clustered in the wetland, with another distinct assemblage in the upland forest and ecotone-associated ErM OTUs distributed throughout, suggesting strong habitat adaptation in ErM fungi. Species in the Sebacinales were common throughout the samples studied, with distinctive OTUs characteristic of either wetland or upland plots. Species in the Helotiales and Verrucariales were also characteristic of the wetlands. In contrast, *Rhizoscyphus* and *Mortierella* species were

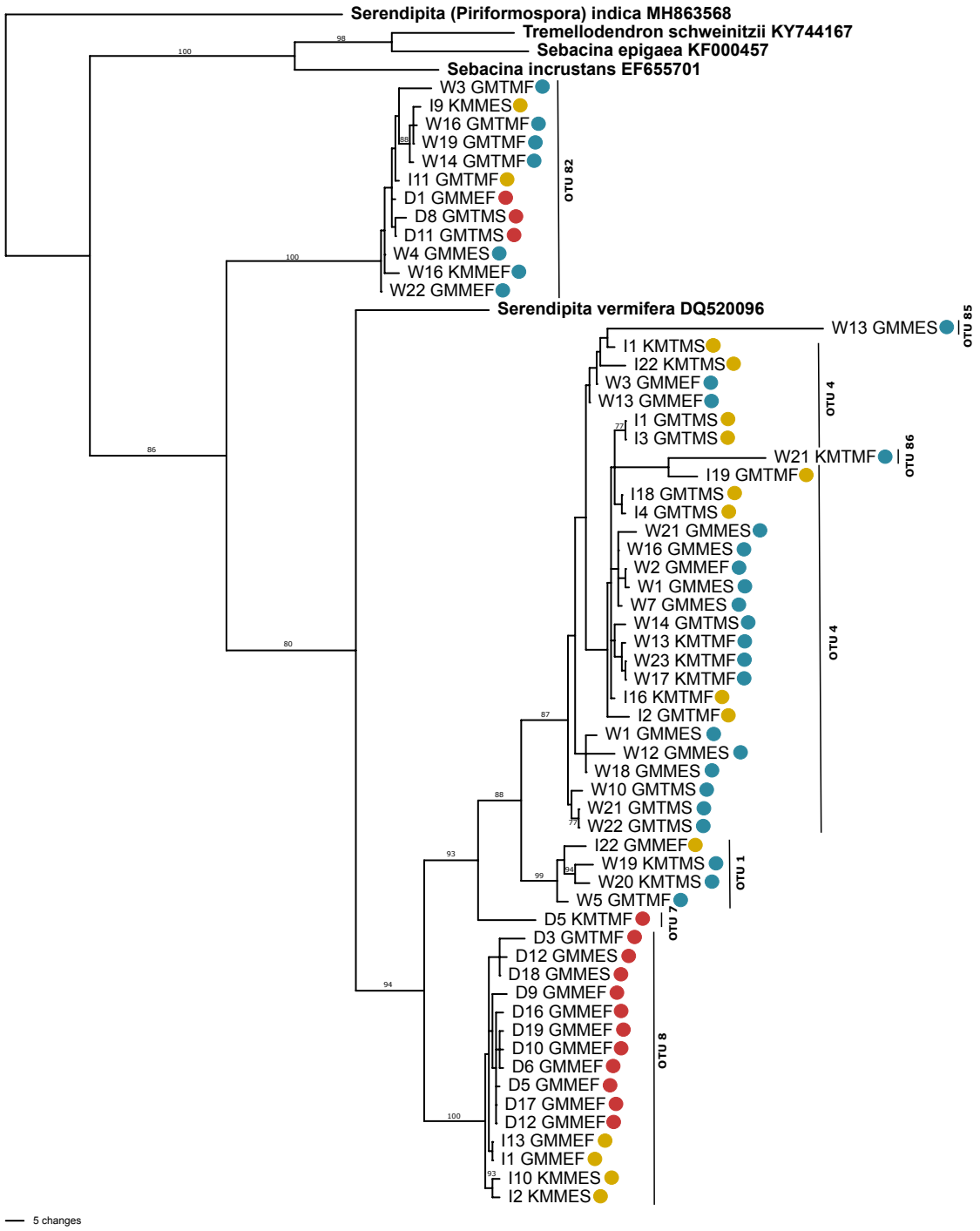
more common in the upland forest. The genera *Capronium* and *Chloridium* were most commonly associated with nutrient rich plots, and Trichisporales and *Cladophialophora* were only seen in the spring

Figure 13. (Next page) Canonical correspondence analysis of fungal associates with *Kalmia angustifolia* (circles) and *Gaultheria hispidula* (squares) along two upland (red) to ecotone (yellow) to wetland (blue) transects on sites Merrymakedge (filled) and Mount Merritt (hollow). ErM and putative ErM OTUs are bold. Axis 1 explains 6.30% of the variation in species distribution and axis 2 explains 5.92%.


3.00
2.25
1.50
0.75
-0.75
-1.50
-2.25
-3.00
-2.0
-1.5
-1.0
-0.5
0.5
1.0
1.5
2.0
2.5
Axis 1
Temp
VWC
pH
P
N
Verrucariales sp.
Trechisporales sp. 2
Fungus sp. 2
Fungus sp. 7
Cladophialophora
Helotiales sp. 4
Helotiales sp. 1
Hyaloscypha sp. 1
Endophytic fungus
Fungus sp. 3
Mortierella soussauensis
Fungus sp. 6
Genolevuria armeniaca
Dwayaangam colodena
Lachnum sp.
Leotiomycetes sp. 2
Penicillium glabrum
Sebacinales sp. 3
Resinicium furfuraceum
Chalara longipes
Pilidium concavum
Hyaloscypha sp. 2
Sebacina sp. 1
Sordariomycetes sp.
Sebacinales sp. 1
Hyaloscypha variabilis
Oideodendron maius
Verrucariales sp. 4
Fungus sp. 9
Helotiales sp. 5
Helotiales sp. 3
Mortierella sp.
Aspergillus sp. 1
Sebacinales sp. 2
Soil fungus sp. 1
Verrucariales sp. 2
Aspergillus sp. 2
Lactifluus deceptivus
Aspergillus sp. 3
Leotiomycetes sp. 1
Sebacinales sp. 4
Epithamnolia xanthoriae
Mollisia minutella
Helotiales sp. 7
Fungus sp. 4
Verrucariales sp. 1
Sebacina sp. 3
Mortierella verticillata
Helotiales sp. 2
Fungus sp. 5
Soil fungus sp. 2
Fungus sp. 1
Hyaloscypha aureliella
Aspergillus cibarius
Rhizoscyphus ericae
Trechisporales sp. 1
Athelia acrospora
Cladophialophora sp. 2
Trichoderma parapiluliferum
Candida tropicalis
Lecanoromycetes sp.
Sebacina sp. 2
Phialocephala fortinii
Piloderma sp.
Cladosporium fusiforme
Lophodermium nitens
Rhodotorula mucilaginosa
Helotiales sp. 6
Capronia sp.
Chloridium sp.
Fungus sp. 8
Venturiocistella sp.

Of all of the clones sequenced, those representing members of Sebacinales were the most common. There was a total of 63 clones in the Sebacinales, belonging to seven distinct OTUs (Figure 14). Of the seven Sebacinales OTUs, only one was observed all along the upland forest to forested wetland transects. All of the other OTUs were observed either only in the ecotone and wetland or only in the ecotone and the upland forest. In six of the seven Sebacinales OTUs, there was no overlap in habitat between the upland associated OTUs and the wetland associated OTUs. When compared with key GenBank reference sequences, the Sebacinales sequences appear to be most closely *Serendipita vermifera*, which has been recently classified as ErM (Vohník et al. 2016). However, the sequence similarity indicates that the fungi detected in the present study likely represent different species within the genus *Serendipita*.

Figure 14. (Next page) Midpoint rooted maximum parsimony tree of sequences representing Sebacinales compared to reference sequences in the Sebacinales from GenBank. Clones from wetland (blue), ecotone (yellow), and upland (red) forest plots are indicated with coloured dots. Bars indicate OTUs as chosen by multiple sequence comparisons in BioEdit and BLAST. Bootstrap values below 75 are not shown.


Serendipita (Piriformospora) indica MH863568
Tremellodendron schweinitzii KY744167
Sebacina epigaea KF000457
Sebacina incrustans EF655701
W3 GMTMF
I9 KMMES
W16 GMTMF
W19 GMTMF
W14 GMTMF
I11 GMTMF
D1 GMMEF
D8 GMTMS
D11 GMTMS
W4 GMMES
W16 KMMEF
W22 GMMEF
OTU 82
Serendipita vermifera DQ520096
W13 GMMES
OTU 85
I1 KMTMS
I22 KMTMS
W3 GMMEF
W13 GMMEF
I1 GMTMS
I3 GMTMS
OTU 4
W21 KMTMF
OTU 86
I19 GMTMF
I18 GMTMS
I4 GMTMS
W21 GMMES
W16 GMMES
W2 GMMEF
W1 GMMES
W7 GMMES
W14 GMTMS
W13 KMTMF
W23 KMTMF
W17 KMTMF
I16 KMTMF
I2 GMTMF
OTU 4
W1 GMMES
W12 GMMES
W18 GMMES
W10 GMTMS
W21 GMTMS
W22 GMTMS
I22 GMMEF
W19 KMTMS
W20 KMTMS
W5 GMTMF
OTU 1
D5 KMTMF
OTU 7
D3 GMTMF
D12 GMMES
D18 GMMES
D9 GMMEF
D16 GMMEF
D19 GMMEF
D10 GMMEF
D6 GMMEF
D5 GMMEF
D17 GMMEF
D12 GMMEF
I13 GMMEF
I1 GMMEF
I10 KMMES
I2 KMMES
OTU 8
5 changes

Discussion and Conclusions

In this study of ErM fungi along a forest to wetland gradient, 12 ErM or putative ErM fungal OTUs were observed. Although the overall fungal species richness did not change as average soil moisture increased, the richness of the OTUs designated as ErM and putative ErM increased along the same transects. Differences in ErM fungal communities were not as strong between seasons, sites, and plant hosts, although some non-ErM species were driven by changes in plant available nitrogen and phosphorus in surface organic horizons. This data supports the hypothesis that ErM fungal communities shift along a wetland to upland gradient.

In this study 40 randomly selected pooled hair roots were used for DNA extraction and subsequent techniques. Spatially distributed studies avoiding sample pooling could provide even more detailed information on the exact soil moisture preferences of symbiotic fungal communities.

Many recent studies of mycorrhizal fungal communities have used advanced molecular platforms such as Pyrosequencing, Illumina, or PacBio. While these techniques are capable of producing impressively large data sets, only the most expensive (such as PacBio) are capable of producing long, high quality reads required to identify often cryptic mycorrhizal fungi to the species level. As only a few species of fungi are known to be involved in ericoid mycorrhizal symbioses, it was important in this study to obtain large, clean sequences. Thus, the more traditional method of DNA cloning and sequencing was determined to be the most appropriate. Future studies of ErM fungal

communities in natural habitats may benefit from more intensive inspection, especially as next generation sequencing technologies improve and become more affordable.

Recent studies of ericoid mycorrhizal communities in wetlands have found high putative ErM species richness, yet only two ErM fungal species were identified (Kohout and Tedersoo 2017). One concern with sequencing ErM fungal communities is that many of these plant symbionts have not been well characterized. Thus, many BLAST matches are to environmental samples. They often originate from Ericaceous plants, but without an identified isolate in culture it is impossible to confirm the mycorrhizal status of a species. All of the species designated as putative ErM in this study were chosen based on evidence from FUNGuild (Nguyen et al. 2016) and literature, but none can be confirmed as ErM without proof via re-synthesis, and they cannot be identified without a pure culture reference. More work is required to isolate and identify ericoid mycorrhizal fungal species so that their biology can truly be understood.

While ErM species richness was higher overall in forested wetland sites, the trend in the Sebacinales was most striking. Certain OTUs were observed only on wetland or ecotone plots, while others were found only in the ecotone or the upland. This finding strongly supports the idea that ErM fungal communities are driven in large part by soil moisture content. Sebacinales have recently been split into two functionally distinct clades, Sebacinales Group A, which forms basidiomes and can be endophytic, ectomycorrhizal and orchid mycorrhizal; and Sebacinales Group B, which is not known to produce spores and is orchid and ericoid mycorrhizal, endophytic, and occasionally

ectomycorrhizal (Weiß et al. 2016). Many of the sequences in this study were most closely related to sequences in the genus *Serendipita (Sebacina)* which is in Sebacinales Group B. The species *Serendipita vermifera* was recently demonstrated to be ericoid mycorrhizal (Vohník et al. 2016). The distinct Sebacinales OTUs identified in this study may represent different species of *Serendipita* with wetland and upland habitat specificity. However, the high level of variability in the ITS region of Sebacinales challenges the delineation of species (Selosse et al. 2007) and may indicate that the Sebacinales OTUs observed are distinct strains, rather than species.

References

Allen TR, Millar T, Berch SM, Berbee ML. 2003. Culturing and direct DNA extraction find different fungi from the same ericoid mycorrhizal roots. New Phytologist 160(1):255-72.

Bougoure DS, Parkin PI, Cairney JW, Alexander IJ, Anderson IC. 2007. Diversity of fungi in hair roots of Ericaceae varies along a vegetation gradient. Molecular Ecology 16(21):4624-36.

Burkett V and Kusler J. 2000. Climate change: Potential impacts and interactions in wetlands of the United States 1. JAWRA Journal of the American Water Resources Association 36(2):313-20.

Chambers S, Liu G, Cairney J. 2000. ITS rDNA sequence comparison of ericoid mycorrhizal endophytes from *Woollsia pungens*. Mycological Research 104(2):168-74.

Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. 2015. Genbank. Nucleic Acids Research 44(D1):D67-72.

Conner WH. 1998. Southern deepwater swamps. In: Messina, Michael G.; Conner H., Eds.Southern Forested Wetlands Ecology and Management.Boca Raton, FL: CRC Press: 261-287.

Faulkner S. 2004. Urbanization impacts on the structure and function of forested wetlands. Urban Ecosystems 7(2):89-106.

Fu L, Niu B, Zhu Z, Wu S, Li W. 2012. CD-HIT: Accelerated for clustering the next-generation sequencing data. Bioinformatics 28(23):3150-2.

Gardes M and Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular Ecology 2(2):113-8.

Hazard C, Gosling P, Mitchell DT, Doohan FM, Bending GD. 2014. Diversity of fungi associated with hair roots of Ericaceous plants is affected by land use. FEMS Microbiology Ecology 87(3):586-600.

Huusko K, Ruotsalainen A, Markkola A. 2017. A shift from arbuscular mycorrhizal to dark septate endophytic colonization in *Deschampsia flexuosa* roots occurs along primary successional gradient. Mycorrhiza 27(2):129-38.

Kernaghan G, Mayerhofer M, Griffin A. 2017. Fungal endophytes of wild and hybrid *Vitis* leaves and their potential for vineyard biocontrol. Canadian Journal of Microbiology 63(7):583-95.

Kohout P and Tedersoo L. 2017. Effect of soil moisture on root-associated fungal communities of *Erica dominans* in Drakensberg mountains in South Africa. Mycorrhiza 27(4):397-406.

Kolařík M and Vohník M. 2018. When the ribosomal DNA does not tell the truth: The case of the taxonomic position of *Kurtia argillacea*, an ericoid mycorrhizal fungus residing among hymenochaetales. Fungal Biology 122(1):1-18.

Li J, Hu H, Cai Z, Lei Y, Zhang M, Li W, Wang J. 2018. Contrasting soil bacterial and fungal communities between the swamp and upland in the boreal forest and their biogeographic distribution patterns. Wetlands :1-11.

Li W and Godzik A. 2006. Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22(13):1658-9.

Midgley DJ, Sutcliffe B, Greenfield P, Tran-Dinh N. 2018. *Gamarada debralockiae* gen. nov. sp. nov.—the genome of the most widespread Australian ericoid mycorrhizal fungus. Mycorrhiza :1-11.

Nguyen NH, Song Z, Bates ST, Branco S, Tedersoo L, Menke J, Schilling JS, Kennedy PG. 2016. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecology 20:241-8.

Nilsson LO, Giesler R, Bååth E, Wallander H. 2005. Growth and biomass of mycorrhizal mycelia in coniferous forests along short natural nutrient gradients. New Phytologist 165(2):613-22.

Palmer J, Horton B, Allaway W, Ashford A. 2007. Growth stimulation of *Woollsia pungens* by a natural ericoid mycorrhizal fungal endophyte. Australasian Mycologist 26:1-8.

Read DJ. 1991. Mycorrhizas in ecosystems. Experientia 47(4):376-91.

Selosse M, Setaro S, Glatard F, Richard F, Urcelay C, Weiß M. 2007. Sebacinales are common mycorrhizal associates of Ericaceae. New Phytologist 174(4):864-78.

Sharples J, Chambers S, Meharg A, Cairney J. 2000. Genetic diversity of root-associated fungal endophytes from *Calluna vulgaris* at contrasting field sites. The New Phytologist 148(1):153-62.

Swofford DL. 2003. PAUP*. Phylogenetic analysis using parsimony (* and other methods) [computer program]. Version 4. Sunderland: Sinauer Associates.

Tedersoo L, May TW, Smith ME. 2010. Ectomycorrhizal lifestyle in fungi: Global diversity, distribution, and evolution of phylogenetic lineages. Mycorrhiza 20(4):217-63.

Thuiller W, Lavorel S, Araujo MB, Sykes MT, Prentice IC. 2005. Climate change threats to plant diversity in Europe. PNAS 102(23):8245-50.

Vohník M, Pánek M, Fehrer J, Selosse M. 2016. Experimental evidence of ericoid mycorrhizal potential within Serendipitaceae (Sebacinales). Mycorrhiza 26(8):831-46.

Voříšková J, Brabcová V, Cajthaml T, Baldrian P. 2014. Seasonal dynamics of fungal communities in a temperate oak forest soil. New Phytologist 201(1):269-78.

Walker JF, Aldrich-Wolfe L, Riffel A, Barbare H, Simpson NB, Trowbridge J, Jumpponen A. 2011. Diverse Helotiales associated with the roots of three species of arctic Ericaceae provide no evidence for host specificity. New Phytologist 191(2):515-27.

Weiß M, Waller F, Zuccaro A, Selosse M. 2016. Sebacinales–one thousand and one interactions with land plants. New Phytologist 211(1):20-40.

White T, Bruns T, Lee S, Taylor J, Innis M, Gelfand D, Sninsky J. 1990. PCR protocols: A guide to methods and applications.

Wolfe BE, Mummey DL, Rillig MC, Klironomos JN. 2007. Small-scale spatial heterogeneity of arbuscular mycorrhizal fungal abundance and community composition in a wetland plant community. Mycorrhiza 17(3):175-83.

Zedler JB and Kercher S. 2004. Causes and consequences of invasive plants in wetlands: Opportunities, opportunists, and outcomes. Critical Reviews in Plant Sciences 23(5):431-52.

CHAPTER FOUR

General Conclusions

Introduction

The mycorrhizal fungi of forested wetlands are an enigmatic group. As obligate aerobes, it is surprising that fungi are found in waterlogged habitats where oxygen levels are often limited. I found a decrease in ectomycorrhizal (ECM) colonization (although only statistically significant at the plot level) which is in agreement with the suppression of ECM seen along a moisture gradient in a spruce forest (Robertson et al. 2006). My results regarding arbuscular mycorrhizae (AM) are also in line with other studies that have found little or no suppression of arbuscular mycorrhizal (AM) colonization by elevated soil moisture. (Allen, Richards and Busso 1989; Wetzel and van der Valk 1996; Kandalepas et al. 2010). However, in contrast to these other mycorrhizal types, ericoid mycorrhizal (ErM) colonization was higher in the forested wetlands studied than in adjacent upland forest. This increase appears to be linked to an increase in ErM and putative ErM diversity and richness in wetland plots. Furthermore, fungi in the Sebacinales were common in all plots, but with distinct OTUs occurring in the wetland and upland plots. Thus, there is strong evidence for habitat specific adaptations in the ErM associated Sebacinales.

Ectomycorrhizal Colonization

Ectomycorrhizal fungi are easily observed without preparation under the dissecting microscope, making them much easier to quantify than other mycorrhizal

types. This is an advantage, but it is easy to underestimate ECM colonization levels by treating inactive and senescing root tips as uncolonized. Truly uncolonized ECM roots should have visible root hairs and lack other characteristic features, whereas many senescing root tips were likely colonized when younger. Even root tips that lack an apparent fungal mantle may still have an active Hartig net (Downes, Alexander and Cairney 1992). Estimates of ECM fungal OTUs in soil samples by DNA sequencing are also informative but may overestimate colonization because of loosely associated ECM hyphae in soils. In the present study, the decreases in ECM colonization of *Pinus strobus* along the soil moisture gradients were not statistically significant, while decreases found in other similar studies were more dramatic. This may be due to other authors classifying senescing roots as uncolonized, or because some *Pinus* plants avoided the wettest conditions by growing on hummocks. Despite these reasons, it is still surprising that the aerobic fungi associated with ECM plants are able to survive in wetland soils. Perhaps the specific fungi involved are stress-tolerant generalists, or perhaps *Pinus* distributes oxygen to its mycorrhizal associates via aerenchyma.

Arbuscular Mycorrhizal Colonization

AM colonization was not significantly different along the forest to wetland gradients in either plant studied. This is not surprising, as other researchers have found both high and low levels of AM colonization in forested wetlands. It appears that other factors, such as seasonality, soil nutrients, or plant type play a greater role in determining AM colonization rates than soil moisture. This study supports that notion, as it uncovered differences in colonization levels between the plant types, and the edaphic variables

measured better described colonization in *Lysimachia borealis* than in *Cornus canadensis*. While none of the variables were strong predictors of colonization in *Cornus*, colonization in *Lysimachia* was positively associated with plant available nitrogen and phosphorus in surface organic horizons. It was expected that the arbuscular mycorrhizal plants might have higher colonization levels in richer habitats (Egerton-Warburton and Allen 2000), but the finding that *Cornus* was not significantly associated with the soil nutrient factors studied is in line with the finding of other researchers (Bohrer, Friese and Amon 2004).

Ericoid Mycorrhizal Colonization

Colonization levels in the ericoid mycorrhizal plants studied were significantly higher in forested wetland plots than in the adjacent upland forests, regardless of site or season. In the generalized linear regression models describing colonization levels for *Kalmia* and *Gaultheria,* volumetric water content was a more important predictor of percent colonization than pH, seasonal soil temperature, and soil nutrient level.

While a broader examination including more hair roots per sample may have uncovered more fine scale differences in colonization levels, VWC was significantly associated with ErM colonization success in general. Ericaceous plants have numerous adaptations likely to protect them in stressful habitats, including thick, waxy evergreen leaves, small stature, and long, thin hair roots that provide a high surface area for nutrient absorption with low resource cost of production (Small 1972; Grime 1977). As associates of stress tolerant plants, ErM fungi must either be broadly stress tolerant themselves,

dependent on their plant hosts for protection, or highly adapted to specific habitats. The discovery of distinct assemblages of fungi in the Sebacinales between the wetland and upland habitats provides evidence for the latter of these strategies.

Mycorrhizal Adaptations

Almost all land plants form mycorrhizal relationships. The major types, ectomycorrhiza (ECM), arbuscular mycorrhiza (AM), ericoid mycorrhiza (ErM), and sometimes orchid mycorrhiza (ORM), have become increasingly less delineated in recent years. While researchers once believed that ECM are formed mostly by basidiomycetes, AM by glomeromycetes, and ErM by ascomycetes (and for a long time just *Rhizoscyphus ericae*), it is now clear that there is often significant overlap between the species of fungi involved in some of the mycorrhizal types. For example, some ericoid mycorrhizal fungi form ECM-like mantles on the root tips of ectomycorrhizal plants (Bergero et al. 2000), and the basidiomycete *Serendipita* is an endophyte, an orchid mycorrhizal fungus, and forms ericoid mycorrhizal structures in Ericaceous plants (Vohník et al. 2016). A plethora of new mycorrhizal types have also been recently proposed (Brundrett 2004). With all of these complications, it is reasonable to expect that there is something beyond the plant and fungal lineage that determines mycorrhizal status. Soil fungal communities play a role in determining plant community structure (Van der Heijden et al 1998; Kernaghan 2005) so it is possible that the fungi capable of surviving wetland habitats are determinants of the plants that will thrive there.

While mycorrhizal associations are generally viewed as mutualistic, it is possible that one of the partners may become less beneficial under stressful conditions (Johnson, Graham and Smith 1997). There is evidence that mycorrhizal fungi may be exploited, or may exploit their plant partners, when the conditions are not optimal (Brundrett 2004). This could explain the increased wetland colonization levels in ErM plants and may also be the reason why AM and ECM colonization did not decrease in the wetland as much as expected. The increased abundance in putatively pathogenic OTUs in the wetland plots also points to this explanation.

Mycorrhizal colonization patterns in wetland plants may be partially explained by the variation in the ability of the host plants to maintain a healthy symbiosis under stressful conditions. Plants growing in flooded soils experience myriad stresses, including reduced stomatal aperture and photosynthesis (Kozlowski 1984) and physical injuries to their roots, leading to leaf epinasty (Crawford 1982). Wetland specialist plants may have adaptations for surviving flood conditions, but the plants in this study were specifically chosen for their generalist growth habit. When a plant experiences edaphic stress, it may terminate symbiotic relationships which can be energetically expensive to maintain (Zheng et al. 2015). While this preservationist strategy may conserve energy, it could also cut plants off from valuable soil nutrients. Perhaps, to maintain access to nutrients under edaphic stress, some stressed plants may develop the ability to support their mycorrhizal fungi, possibly through oxygen allocation, or by association with wetland adapted mycorrhizal fungi.

Root-Associated Fungal Communities

In the examination of fungal communities of Ericaceous plants along the forest to wetland gradients, different assemblages of fungi were associated with different soil conditions. While Fisher's alpha and Whittaker's beta diversity, as well as the richness of OTUs classified as endophytes was higher in upland plots, ericoid and putative ericoid mycorrhizal fungal richness was highest in the wetland. Beyond this, specific OTUs within the same order (Sebacinales) were distinctly associated with different habitats. Several Sebacinales OTUs were restricted to the wetland or ecotone, while another was observed only in the upland or ecotone. Only one of the seven Sebacinales OTUs sequenced was found in all three habitats. Mycorrhizal plants that are able to associate with highly adapted symbiotic fungi may have the advantage of continued access to soil nutrients even under stressful conditions.

Conclusions and Future Directions

Ericoid mycorrhizal colonization levels in forested wetlands are higher than previously observed. The cause of this increased colonization level is not yet known but is associated with a distinct community of wetland-dwelling ericoid mycorrhizal fungi. Some of the OTUs in the Sebacinales were restricted to wetland and ecotone plots, while others were restricted to the ecotone and the upland. This is the first indication of habitat specialization in potentially ErM Sebacinales and deserves further investigation. As the OTUs sequenced did not match with any identified fungus, the potential exists for the identification of a new, wetland specialist species in the Sebacinales, likely in the genus *Serendipita*. The increased colonization level of ericoid mycorrhizal plants also deserves

further attention. Specifically, it should be clarified if this increase in colonization level is associated with an increase in effectiveness of the mycorrhizal symbiosis, or if it is a symptom of a more exploitative symbiosis. From a conservation standpoint, this research shows a complexity and level of adaptation in forested wetland fungal communities that must be better understood if they are to be protected.

References

Allen MF, Richards JH, Busso CA. 1989. Influence of clipping and soil-water status on vesicular-arbuscular mycorrhizae of 2 semi-arid tussock grasses. Biology and Fertility of Soils 8(4):285-9.

Bergero R, Perotto S, Girlanda M, Vidano G, Luppi A. 2000. Ericoid mycorrhizal fungi are common root associates of a Mediterranean ectomycorrhizal plant (*Quercus ilex*). Mol Ecol 9(10):1639-49.

Bohrer KE, Friese CF, Amon JP. 2004. Seasonal dynamics of arbuscular mycorrhizal fungi in differing wetland habitats. Mycorrhiza 14(5):329-37.

Brundrett M. 2004. Diversity and classification of mycorrhizal associations. Biological Reviews 79(3):473-95.

Crawford R. 1982. Physiological responses to flooding. In: Physiological plant ecology II. Springer. 453 p.

Downes G, Alexander I, Cairney J. 1992. A study of ageing of spruce [*Picea sitchensis* (bong.) carr.] ectomycorrhizas. I. morphological and cellular changes in mycorrhizas formed by *Tylospora fibrillosa* (burt.) donk and *Paxillus involutus* (batsch. ex fr.) fr. New Phytologist 122(1):141-52.

Egerton-Warburton LM and Allen EB. 2000. Shifts in arbuscular mycorrhizal communities along an anthropogenic nitrogen deposition gradient. Ecological Applications 10(2):484-96.

Grime JP. 1977. Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory. American Naturalist 111(982):1169-94.

Johnson NC, Graham J, Smith FA. 1997. Functioning of mycorrhizal associations along the mutualism–parasitism continuum*. New Phytologist 135(4):575-85.

Kandalepas D, Stevens KJ, Shaffer GP, Platt WJ. 2010. How abundant are root-colonizing fungi in southeastern Louisiana's degraded marshes? Wetlands 30(2):189-99.

Kernaghan G. 2005. Mycorrhizal diversity: Cause and effect? Pedobiologia 49(6):511-20.

Kozlowski T. 1984. Plant responses to flooding of soil. Bioscience 34(3):162-7.

Robertson SJ, Tackaberry LE, Egger KN, Massicotte HB. 2006. Ectomycorrhizal fungal communities of black spruce differ between wetland and upland forests. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 36(4):972-85.

Small E. 1972. Water relations of plants in raised sphagnum peat bogs. Ecology 53(4):726-8.

Van der Heijden, Marcel GA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396(6706):69-72.

Vohník M, Pánek M, Fehrer J, Selosse M. 2016. Experimental evidence of ericoid mycorrhizal potential within Serendipitaceae (Sebacinales). Mycorrhiza 26(8):831-46.

Wetzel PR and VanderValk AG. 1996. Vesicular-arbuscular mycorrhizae in prairie pothole wetland vegetation in Iowa and North Dakota. Canadian Journal of Botany-Revue Canadienne De Botanique 74(6):883-90.

Zheng C, Ji B, Zhang J, Zhang F, Bever JD. 2015. Shading decreases plant carbon preferential allocation towards the most beneficial mycorrhizal mutualist. New Phytologist 205(1):361-8.

Appendix Table 1. OTUs, taxonomies, and GenBank accession numbers of root associates of ericoid mycorrhizal plants *Kalmia angustifolia* and *Gaultheria hispidula*.

OTU	Phylum	Order	Genus	Species	GenBank Match	% Cover	% Identity
52	Ascomycota				JQ272389.1	100	97.33
59	Ascomycota				KF359573.1	100	90.89
19	Ascomycota				HQ022256.1	95	93.97
77	Ascomycota				FJ475651.1	100	93.26
69	Ascomycota	Capnodiales	Cladosporium	fusiforme	MF077225.1	100	100
16	Ascomycota	Chaetomellales	Epithamnolia	xanthoriae	KY814539.1	97	97.71
5	Ascomycota	Chaetomellales	Pilidium	concavatum	KF646103.1	100	99.16
50	Ascomycota	Chaetosphaeriales	Chloridium		MF671833.1	93	98.43
56	Ascomycota	Chaetothyriales	Capronia		EU139148.1	98	97.47
57	Ascomycota	Chaetothyriales	Cladophialophora	minutissima	MH863155.1	97	95.67
58	Ascomycota	Chaetothyriales	Cladophialophora	minutissima	MH487545.1	100	93.87
75	Ascomycota	Eurotiales	Aspergillus	cibarius	MK267410.1	100	99.82
64	Ascomycota	Eurotiales	Aspergillus	sydowii	MH707094.1	100	99.3
80	Ascomycota	Eurotiales	Aspergillus	sydowii	MH707094.1	100	99.3
24	Ascomycota	Eurotiales	Aspergillus	versicolor	MG845255.1	100	100
25	Ascomycota	Eurotiales	Penicillium	glabrum	MK910051.1	98	99.8
17	Ascomycota	Helotiales			HF947859.1	99	97.85
18	Ascomycota	Helotiales			HQ021977.1	96	99.81
20	Ascomycota	Helotiales			KC019908.1	97	91.06
36	Ascomycota	Helotiales			FJ440902.1	95	98.7
72	Ascomycota	Helotiales			KX609411.1	91	98.81
35	Ascomycota	Helotiales			JQ272327.1	100	99.12

37	Ascomycota	Helotiales			EF093150.1	100	99.1
31	Ascomycota	Helotiales	Chalara	longipes	FR717230.1	98	97.98
22	Ascomycota	Helotiales	Dwayaangam	colodena	KT289617.1	96	91.71
66	Ascomycota	Helotiales	Hyaloscypha	aureliella	MH018926.1	96	97.16
30	Ascomycota	Helotiales	Hyaloscypha		JN943606.1	92	97.92
73	Ascomycota	Helotiales	Hyaloscypha	variabilis	EF093178.1	100	97.73
29	Ascomycota	Helotiales	Hyaloscypha		FM172789.1	92	99.42
26	Ascomycota	Helotiales	Lachnum		FJ378855.1	97	97.24
14	Ascomycota	Helotiales	Mollisia	minutella	KJ817294.1	96	99.37
27	Ascomycota	Helotiales	Pezoloma	ericae	JQ711893.1	100	98.79
13	Ascomycota	Helotiales	Phialocephala	fortinii	MK356722.1	100	99.65
33	Ascomycota	Helotiales	Venturiocistella		JN033391.1	99	96.04
48	Ascomycota	Hypocreales	Trichoderma	parapiluliferum	NR_134341.1	98	99.34
23	Ascomycota	Onyginales	Oidiodendron	maius	KF359579.1	100	98.39
76	Ascomycota	Rhytismatales	Lophodermium	nitens	MG877529.1	98	99.41
44	Ascomycota	Saccharomycetales	Candida	tropicalis	MK752669.1	100	99.05
40	Ascomycota	Trechisporales	Trechispora		JX392820.1	93	94.75
43	Ascomycota	Trechisporales			MK131687.1	99	98.35
70	Ascomycota	Trechisporales			MK131687.1	98	99.55
3	Ascomycota	Verrucariales			FJ475710.1	96	99.22
63	Ascomycota	Verrucariales			FJ475710.1	100	97.27
71	Ascomycota	Verrucariales			HQ022024.1	97	99.12
81	Ascomycota	Verrucariales			FJ475710.1	98	98.61
42	Basidiomycota	Atheliales	Athelia	acrospora	KP814332.1	98	99.22
41	Basidiomycota	Atheliales	Piloderma		KP403081.1	100	98.45
51	Basidiomycota	Hymenochaetales	Resinicium	furfuraceum	KP814421.1	94	97.54
74	Basidiomycota	Russulales	Lactifluus	deceptivus	MK069517.1	100	98.59

1	Basidiomycota	Sebacinales	Serendipita		JQ420983.1	100	96.66
8	Basidiomycota	Sebacinales	Serendipita		JQ420992.1	100	98.94
82	Basidiomycota	Sebacinales	Serendipita		JQ420981.1	100	98.93
85	Basidiomycota	Sebacinales			HF947904.1	98	89.59
4	Basidiomycota	Sebacinales			HF947910.1	100	95.8
86	Basidiomycota	Sebacinales			HF947911.1	90	94.04
7	Basidiomycota	Sebacinales			JQ272430.1	99	94.52
38	Basidiomycota	Sporidiobolales	Rhodotorula	mucilaginosa	MG241534.1	100	100
39	Basidiomycota	Tremellales	Genolevuria	bromeliarum	NR_137811.1	95	91.06
47	Mortierellomycota	Mortierellales	Mortierella	soussauensis	JX976063.1	92	100
45	Mortierellomycota	Mortierellales	Mortierella		KP714556.1	87	98.74
46	Mortierellomycota	Mortierellales	Mortierella	verticillata	MH844766.1	100	98.91
2	Unassigned				KC978008.1	100	95.71
32	Unassigned				AM260809.1	100	98.93
34	Unassigned				KT334701.1	87	96.57
9	Unassigned				KP889896.1	99	97.61
21	Unassigned				JN890147.1	87	87.03
55	Unassigned				KF800339.1	97	84.04
60	Unassigned				KP889686.1	100	98.47
61	Unassigned				JF300546.1	100	95.27
65	Unassigned				KF617317.1	100	95.89
62	Unassigned				DQ309235.1	94	98
10	Unassigned				HQ022090.1	89	96.09
15	Unassigned				HQ022294.1	98	98.71