MYCORRHIZAL FUNGI SPORE ABUNDANCE IN OLD-GROWTH FOREST SOIL

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BY

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ABSTRACT

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Mycorrhizal fungi are known to form symbiotic relationships with approximately 80 percent of plants, appearing on every continent except Antarctica. These fungi are important to ecosystems as they increase nutrient uptake of plants, enhance soil health through the addition of glomalin, and assist with the germination of Orchidaceae seeds. In this study, soil samples were collected from 5cm, 10cm, 20cm, 50cm, 1m, and 5m in each of the cardinal and sub-cardinal directions to assess abundance of mycorrhizal spores. Samples were sieved, and spores were extracted with a sucrose gradient. Spores were placed on microscope slides and stained with melzers reagent. It was hypothesized that mycorrhizal fungi spore abundance would decrease with increasing distance from the orchid symbiont *Aplectrum hymale*. Direction was found to have no significant effect on mycorrhizal fungi dispersal. The hypothesis that mycorrhizal spore abundance would increase with proximity to the plant was not supported; however, there was a significant increase of spores with increasing distance from the *Aplectrum hymale* plants up to one meter. Spore abundance was found to decrease sharply between 1 and 5 meters.

Keywords: Mycorrhizal fungi, *Aplectrum hymale, Glomus*, Spore abundance

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Chapter 1. Literature Review

Indiana Native Habitat

Prehistoric Landforms

Indiana is home to many different ecosystems. The formation of these habitats began millions of years ago through various glaciations and bedrock disturbances. The relevant glaciations occurred 2.6 million years ago during the Pleistocene Epoch (Flemming and Rupp, n.d.; Sturgeon, 2017). Climate during the Pleistocene was both colder and more wet than modern climate, which led to the formation of the expansive Laurentide ice sheet. Over time, this ice sheet formed lobes and flowed southward from what is now Canada into the United States. Three main lobes directed glacial flow into Indiana: the Lake Michigan lobe to the northwest, the Saginaw lobe to the north-northeast, and the Huron-Erie lobe to the east-northeast (Flemming and Rupp, n.d.). This glacial movement transformed the landscape through the formation of kames and eskers, depositional lakes, and many varied sediment deposits. Erosion during this time also played a key role in the formation of this midwestern landscape (Sturgeon, 2017).

The Illinoian glaciation extended from Canada into southern Indiana between 300,000 and 132,000 years ago (Sturgeon, 2017). Deposits from this glaciation can be found throughout the majority of the state. The final major advance of the Laurentide ice sheet occurred approximately 24,000 years ago and is referred to as the Wisconsin glaciation (Sturgeon, 2017).

Land Use

The pre-contact landscapes of Indiana may be broadly categorized into three major habitats: deciduous forests, grasslands, and aquatic areas (Mumford,1997; Southerland, 1993). Indiana was home to approximately 20 million acres of forest land, 2 million acres of prairie, and

1.5 million acres of water and wetlands; however, some sources estimate Indiana wetlands to have once covered as much as 5.6 million acres (Jackson, 1997). Specialized orchids could be found in each of these habitats. Some, such as *Aplectum hymale,* have a rich history of use by both Native Americans and European colonists.

The colonial settlement of Indiana began in the 1790's (Jackson, 1997). As Native Americans were pushed further west, English settlers began altering the landscape. Over time, most native habitats were clear-cut, burned, or drained and altered for farming, mining, and other forms of commerce. By 1850 Indiana was home to approximately 1 million settlers (Jackson, 1997). Throughout the early 1900's, half of the United States population were farmers or lived in rural communities. It was not until 1950 when Erza Taft Benson, the United States Secretary of Agriculture, called for farmers to "get big or get out." Following this, farming began to shift from many small family-owned farms to fewer and larger farms (Johns Hopkins, n.d.) This trend towards large monocrop farming is especially present today as one travels through Indiana. However, as cities grew, farmlands were quickly converted to subdivisions. Although there have been incentives to keep native landscapes intact, be it via ecosystem services or monetary gains from state organizations, many areas to this day are still drained and leveled for urban sprawl.

In recent years, many environmental organizations have shifted their focus away from conservation to the tasks of mitigation and restoration. Environmental mitigation aims to reduce the deleterious effects of industry on existing ecosystems. Meanwhile, environmental restoration aims to take land depleted of natural resources, potentially polluted land, or areas otherwise deemed uninhabitable and restore natural resources through methods of soil, water, air, and habitat remediation. Both actions rely heavily on a thorough understanding of local habitat indices.

Habitat Index

In modern times the majority of native habitats exist within the confines or state and national parks, family-owned woodlots, and small "postage stamp" parcels owned by universities and land trust organizations. Widespread species extirpation and extinction within Indiana was recorded as far back as the early 1800's (Jackson, 1997). The drivers behind this sudden decline in diversity are the same today as they were two centuries ago— habitat loss and over-exploitation.

Habitat suitability indices aim to quantify the amount of anthropogenic degradation in a designated area. Criteria for pristine areas are often based on a comparison to pre-contact landscapes and the relative abundance of characteristic species.

Characteristic species are imperative for habitat loss research. Funding for most habitat research is limited, which often forces scientists to prioritize habitats based on management objectives and characteristic species (Johnson, 2007). To assist in distinguishing habitat quality, the United States Fish and Wildlife Service created Habitat Suitability Index models. These models often rank areas between 0 and 1, with areas closer to 0 being more degraded than areas closer to 1. Although useful, these indices are extremely reductive when it comes to communicating the many factors contributing to habitat quality.

Hobbs and Hanley (1990) note that two main limitations on natality should be considered when studying organism response to habitat restoration—resource quantity and resource quality. For instance, would a smaller habitat with a limited number of pristine resources be more desirable than a degraded habitat with an abundance of poor-quality resources? From an individual standpoint, the pristine environment would be preferable; however, the degraded

habitat with more resources would benefit the population as a whole due to the increased carrying capacity (Johnson, 2007; Hobbs and Hanley, 1990). However, it is important to note that for some species, quantity cannot substitute for quality. Birds in a landscape with inadequate nesting cover cannot compensate by using many different nests, just as the Eastern Hellbender Salamander (*Cryptobranchus alleganiensis*) requires clean silt-free water to survive regardless of the size of the stream.

Hobbs and Hanley (1990) found that animal distribution reflects a habitat's carrying capacity only when data is compiled after equilibrium is obtained, there is a long-term equilibrium between animal populations and limiting resources, and animals are distributed in an ideally free manner, i.e. the aggregation of animals is proportional to the available resources in a given area. Although it is highly improbable that these three criteria will ever be met in nature, the authors note that these models are still important and should be improved upon for future research. Retrospective data, although more accurate, lacks the ability to predict the effects of human impacts on the environment.

The Environmental Protection Agency (1993) states that the following must be taken into consideration when evaluating habitat:

- The full range of interactions among habitat components at an ecosystem level;
- The effectiveness of mitigation measures in conserving habitats and their ecological value; and
- The cumulative effects of habitat degrading activities over space and time.

One important variable to consider when assessing habitat suitability is floristic diversity, which has been shown to be a superior indicator of wildlife distribution as compared with structural

diversity (Southerland,1993). This is why the abundance and diversity of flora comprises such a large component of the Habitat Suitability Index. When surveying for habitat suitability, orchids are often considered an indication of a pristine habitat due to their rarity. However, many species of orchid thrive on disturbance as long as the nearby soil is suitable.

Orchids

Orchidaceae

There are over 30,000 known species of orchids which can be found on every continent except Antarctica. Orchidaceae are perennial flowering monocots with parallel leaf venation. Orchidaceae flower parts occur in cycles of three with the labellum, a differentiated third petal, being the most distinctive characteristic of each flower. In most orchids, the labellum is the largest floral segment. Unlike other plants, the reproductive structures of orchids are not separate, but instead supported with in the column. Orchid seeds are also unique. Unlike many other florae, orchids release thousands of dust-like particles from seed capsules, each lacking an endosperm (Homoya, 1993). This makes germination extremely difficult as many conditions must be optimal for survival of the orchid seed.

The three most important factors for orchid success are light, presence of certain fungi, and substrate. The relative fitness of almost all orchids is highly dependent on the quality of light received. One exception to this rule would be the rare Achlorophyllous individuals known to occur within three genera found in North America which rely on mycorrhizal fungi for nutrient acquisition (Light and MacConaill, 1989). The substrate requirements of orchids vary widely between species, even those closely related (Homoya, 1993). Understanding the substrate requirements of various orchid species is imperative to finding them in the wild.

Although habitat disturbance will be addressed in a somewhat negative tone throughout the remainder of this document, it should be noted that some disturbance can be beneficial to orchids. Habitat disturbance has the ability to reduce light competition, allowing certain species of orchids to recolonize an area. It also has the added benefit of preparing the soil for seed germination (Homoya, 1993). However, annual soil disturbance and conversion of entire ecosystems to developed, urban settings are detrimental.

Orchids of Indiana

Indiana is home to 12 distinct natural regions, each with a unique array of orchids. Northern Indiana has the Lake Michigan, Northwestern Morainal, Grand Prairie, Northern Lakes, and Black Swamp natural regions. Central Indiana hosts the Central Till Plain natural region, and southern Indiana has the Southwestern Lowlands, Southern Bottomlands, Shawnee Hills, Highland Rim, Bluegrass, and Big Rivers natural regions (Homoya, 1993).

The Central Till Plain natural region is the largest in the state. The Central Till Plain is viewed as a 'melting pot' for orchid distribution within the state. There are no known orchid species endemic to this region (Homoya, 1993).

Only 10 percent of known orchids exist within temperate climates. The earliest record of the orchids of Indiana comes from Asahel Clapp, who listed 17 taxa of orchids in the 1830's (Homoya, 1993). In modern times approximately 46 species from approximately 18 genera are documented. There are currently five species of orchid listed as extirpated from Indiana and seven listed as endangered (Department of Natural Resources, 2020; Homoya, 1993). *Arethusa bulbosa, Corallorhiza trifida, Platanthera dilatate, Platanthera hookeri,*and *Platanthera orbiculate* are currently listed as extirpated from the state of Indiana. The state endangered orchids include: *Cypripedium acaule, Malaxis unifolia, Platanthera ciliaris, Platantherea flava*

var. flava, *Spirathes magnicamporum,*and *Spiranthes romanzoffiana. Platanthera leucophaea* was once listed as extirpated from the state; however, the DNR now lists it as state endangered.

Orchid-Fungi Interactions

Orchid seedlings are released without an endosperm, thus relying on nutrients in soil organic matter to germinate. However, orchid seedlings are not strictly saprophytic, *i.e.*, orchid seedlings cannot osmotically absorb nutrients from decaying organic matter on their own. These seedlings require a symbiotic relationship with mycorrhizal fungi to obtain nutrients (Homoya, 1993). Harley and Smith (1983) note that the mycorrhizal interactions of northern temperate orchids are not highly specific -- many different fungal taxa may be involved. However, two researchers have suggested that green-leaved orchids use a 'take now, pay later' scheme which has contributed to mycorrhizal specificity wherein green sporophytes are established to repay mycorrhizal partners for the carbon invested (Cameron et al., 2008; Field et al., 2015). This leads some scientists to believe that rather than exchanging equal amounts of organic carbon for equal amounts of nutrients, there is a form of intergenerational carbon subsidy allowing for prolonged "parent nurture" (Field et al., 2015). Homoya (1993) notes that often these mycorrhizal fungi interactions become parasitic, i.e., the fungus will often overtake the seedling, causing it to perish. He posits that this may be the reason behind so few orchids surviving to adulthood when millions of seeds are broadcast into the environment each year.

Aplectrum hyemale

Aplectrum hyemale, also known as the Putty Root orchid or the Adam and Eve orchid, is common across the state of Indiana. It has a long and colorful history, with accounts of the corms being used as glue, medicine, and for divination purposes. Ideal Putty Root orchid habitat consists of mesic forests, although they are common in most hardwood forests (Homoya, 1993).

This overwintering orchid has a solitary leaf which is present from fall to early spring. The leaf begins to wither and die in mid-May, prior to the emergence of the flowering stalk (Homoya, 1993).

The Putty Root orchid blooms from early May to mid-June. The stalks range in height from 30–35 cm tall with approximately 8–15 flowers per stalk (Homoya, 1993). These light green flowers have a tendency to droop, making them increasingly difficult to spot among the understory flora and leaf litter.

The Putty Root orchid is thought to be both autogamous (self- fertilizing) and agamouspermous, *i.e.,* able to set seed without fertilization (Hogan, 1983; Homoya, 1993). This hypothesis seems to be supported by a study of the pollinators visiting *Aplectrum hyemale.* Researchers found that in a four-day period only one species of pollinator, *Dialictus oblongus,* was found to visit the orchid. These researchers also noted that of the single species to visit *Aplectrum hyemale* only five actually entered the flowers and none were seen to have pollinia upon exiting. Despite this, fruit set in the observed Putty Root orchids was still found to be 82.1 percent (Hogan, 1983). *Aplectrum hyemale* currently has no known specific mycorrhizal associates. Instead, it is believed to be a generalist species, relying on the most abundant mycorrhizal fungi in a given area (Auclair, 1972).

Fungi

Taxonomy

Of the over 75,000 classified species of fungi, only 24 species of mushroom are commonly cultivated (McCoy, 2016). As with many organisms, fungi were first classified by Carl Linnaeus. Linnaeus, however, thought fungi were similar to worms and gave them the genus name "*Chaos fungorum"* (McCoy, 2016). Elias Fries, a Swedish botanist and mycologist, was the next to

contribute to fungal taxonomy. He wrote *Systema Mycologicum*, in which he described known species of fungi using a new classification system (Peterson, 2015). Despite advances in microscopy and DNA sequencing, the system created by Fries is still valid for many groups of fungi to this day. Robert Whittaker developed the five-kingdom system in the 1950's (Hagen, 2012). This system of classification separated fungi from plants, thereby creating the new kingdom of Myceteae.

Within the kingdom of Myceteae are seven phyla – Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Glomeromycota, Microsporidia, and Neocallimastigomycota (McCoy, 2016). Blastocladiomycota, Chytridiomycota, Glomeromycota, Microsporidia, and Neocallimastigomycota each contain soil-dwelling species which often require a microscope for identification. Glomeromycota and Microsporidia, placed in the subkingdom Dykaria, contain yeasts and slime molds (McCoy, 2016). Glomeromycota is the most important phyla when studying endomycorrhizal fungi because it contains Acaulospora and *Glomus*, two of the most globally abundant mycorrhizal fungi.

Glomeromycota contain 169 known morphospecies in 10 genera (McCoy, 2016). These fungi do not form fruiting bodies and must instead be identified to genus by spore morphology. Spores may be found singularly in the soil, in clusters, or aggregated into sporocarps (McCoy, 2016). All species within the Glomeromycota phyla have the ability to form mycorrhizal relationships with plant roots by forming structures known as arbuscules within the cells of the root (McCoy, 2016). Arbuscular mycorrhizal fungi (AMF) use two main colonization patterns within plant roots. The first pattern is linear and is termed Arum-type; the second, Paris-type, is coiled and intracellular (Muthukumar et al., 2016). These mutualistic relationships benefit flora by increasing the overall surface area of root systems and providing the plant with increased

phosphorus, nitrogen, micronutrients and water. The flora then provides carbon, glucose, and fructose to the fungi. These associations are short-lived, as arbuscules tend to break down anywhere from 4 to 15 days after formation (McCoy, 2016).

According to Blaszkowski (2003), mycorrhizal spores are released into the soil from hyphal tips. These spores lack transportation structures such as flagella. Therefore, Glomeromycota spores often rely on other organisms such as insects or animals to transport spores from one location to the next. Researchers in Africa have studied how elephants are a significant vector for spore dispersal (Paugy, 2004).

In terms of fungal spores, mycorrhizal spores are some of the largest, often ranging from 20µm to 800µm in diameter (McCoy, 2016). These spores each contain 800 to 35,000 distinct nuclei, many of which may belong to other microorganisms (Covacevich, 2010; Thangadurai et al., 2010). This is one reason that spore morphology is commonly used to identify mycorrhizal fungi, rather than DNA sequencing (McCoy, 2016). It is thought that the extreme number of nuclei within the spores provide an evolutionary advantage. Some researchers have found epigenetic changes occurring in as little as a few months (McCoy, 2016). This could also explain the genetic difference found between mycorrhizal generations and between ‘wild-caught’ and pot-cultured mycorrhiza (Covacevich, 2010; Ehinger et al., 2012). Although mycorrhizal fungi reproduce asexually, researchers have found that hyphal fusion between two genetically distinct fungi may produce hybrids (Croll et al., 2009). Angelard et al. (2010) found that hybrid spores were both genetically and phenotypically distinct from both the parental lines and other hybrids with the same parental lines. Segregation of mycorrhizal fungi also gave rise to a new range of significantly distinct phenotypes (Ehinger et al., 2012; Angelard, 2010). These differences may

significantly affect the efficiency or ability of mycorrhizal fungi to interact symbiotically with plants (Angelard, 2010).

Acaulospora

Acaulospora distribution along roots is often patchy (Blaszkowski, 2003). The spores develop laterally from the neck of a soporiferous saccule and lack a pedicel (Morton and Benny, 1990). Juvenile *Acaulospora* spores have only one cell wall, but may later differentiate to as many as three cell walls. Following differentiation of cell walls, the formation of two semi-flexible germination walls begins (Blaszkowski, 2003).

Glomus

Blaszkowski (2003) state than *Glomus* hosts 19 species; however, McCoy (2016) lists the number of morphospecies as high as 70. The distinction between the number of species and morphospecies is an important one, especially for mycorrhizal fungi, given that mycorrhizal fungi identification often relies on identification of morphological features of spores. Either way, *Glomus* is regarded as the largest genus within the Glomeromycota phyla (McCoy, 2016; Blaszkowski, 2003).

The majority of *Glomus* species produce spores singularly in the soil (Blaszkowski, 2003). These spores contain only one wall with at least two layers; however, these layers may be shed with age. Different layers of the spore wall will stain red with Melzar's reagent depending on species (Blaszkowski, 2003).

Mycorrhizal Fungi

Mycorrhizal fungi can form symbiotic relationships with up to 80 percent of terrestrial plants (McCormick et al.,; 2016 Cobb et al., 2018). This interaction dates back to 350–460 million years ago, when the order Glomales is thought to have arose. Many scientists have suggested that

the fungal symbiosis we see today was imperative for land colonization by plants (Harrier, 2001; Field et al., 2015). Mycorrhizal fungi benefit the soil by enhancing aggregation, stability, and water-holding capacity through the release of glomalin, making them key components of healthy soils (McCoy, 2016).

Two main types of endomycorrhizal fungi exist, i.e., arbuscular mycorrhizal fungi and vesicular mycorrhizal fungi. Simply put, some mycorrhizal fungi specialize in associations with tree roots, while others with vascular plant roots. There is also evidence that some genera of mycorrhizal fungi interact with both trees and vascular plants indiscriminately (Blaszkowski, 2003).

Mycorrhizal associations are not always beneficial to the plant. It was discussed above how mycorrhizal fungi may overtake orchid seedlings during the germination process, ultimately leading to the death of the orchid seed (Homoya, 1993). Another study has shown that colonization by mycorrhizal fungi may have some adverse side effects: Field et al. (2015) showed significant stunting of *Ophiglossum vulgatum* that were colonized by mycorrhizal fungi, which suggests that the carbon required by mycorrhizal fungi may results in uptake of carbon significant for plant growth .

Mycorrhizal fungi are obligatory biotrophs, meaning they depend on carbon from other plants to survive. However, external carbon sources are not required during mycorrhizal spore germination. Instead, lipids, mostly triacylglycerides, become the main source of carbon. Much like the endosperm within a plant seed, mycorrhizal fungi spores rely on carbon from these lipids to germinate. Mycorrhizal fungi can also take up the monosaccharide hexose from host roots (Harrier, 2002).

Agriculture can severely affect fungal diversity within the soil depending on cover crop. Detheridge and Crockatt. (2016) found that two species of clover, *Trifolium pratense* and *T. repens* experienced the most adverse effects in their ability to add nitrogen to the soil. The study showed a negative correlation between soil nitrate levels and mycorrhizal fungi populations. Tillage also has an effect on soil fungal populations. In areas that practiced no-till agriculture, mycelium networks grew larger; soil moisture content remained higher, resulting in increased fungal biomass; and root colonization by AMF increased (Detheridge and Crockatt, 2016). Another more recent anthropogenic phenomenon, i.e., eutrophication, has been recognized as a contributor to mycorrhizal fungi decline as well. As mentioned by Detheridge and Crockatt (2016), the increased nitrogen deposition has had a considerable impact on mycorrhizal mycelial production and ectomycorrhizal root tip abundance. However, according to Cobb et al. (2018) different soil amendments had no significant effect on the AMF colonization of *Vigna unguiculata*.

Burke et al., (2019) studied the effects of invasive garlic mustard removal and deer exclusion on mycorrhizal fungal communities over eight years. The allelopathic chemicals garlic mustard releases into soil are known to negatively affect native plants. The allelopathic chemicals released by garlic mustard may also affect microbial communities in soil. However, these researchers found no significant difference in number of mycorrhizal spores between the different treatments. Helander et al. (2018) focused on the effects of glyphosate on mycorrhizal fungi. They found that glyphosate reduced the mycorrhizal colonization and arbuscules present in the roots of both weeds and crops, suggesting that mycorrhizal spores and external hyphae may be adversely affected by residual glyphosate.

When considering new land for development, many do not consider the impact that borders between landscapes have on the ecosystem. The area between two ecosystems is commonly referred to as an ecotone. The difference in biological diversity at these edges are the result of changes to the microclimate and shifts in the flow of abiotic and biotic materials. Mycorrhizal fungi associations, fungal biomass, and abundance of fruiting bodies are all affected by these edges. Soil at the forests edge is often warmer and drier than soil at the forest core; however, these abiotic and biotic factors are also known to fluctuate more rapidly at the forest edge. These factors are important to fungal life cycles because temperature and moisture regulate fungal spore release, germination, and mycelial extension. Unlike plants, fungal spores can react to these fluctuations on an hourly timescale. Decreased soil respiration and changes in wind patterns may also affect fungi (Crockatt, 2012). More importantly, mycorrhizal fungi abundance influences the suitability of habitats for organisms in other taxa such as late-stage colonizers, fungivores, and beetles. This means that mycorrhizal fungi may cause secondary edge effects for other taxa (Crockatt, 2012).

Monitoring fungal populations can be an important part of ongoing ecosystem restoration projects. Successful habitat restoration may even depend on the establishment of three major fungi guilds— decomposers, parasites, and mutualists (Avis et al., 2017). In the past, researchers identified potential mycorrhizal fungal hotspots in three Indiana counties (Northwest Indiana, n.d.). Although the study focused on macrofungi, researchers called for the use of qPCR to better identify the presence of mycorrhizal fungi. In addition to identifying native mycorrhizal fungi, Torres-Arias et al. (2017) highlighted the potential use of native AMF as a soil inoculum, noting that this form of inoculation has been shown to increase plant resistance to environmental stressors. The researchers identified six common families of fungi in their samples,

Acaulosporacea, Archaeosporacea, Diversisporoacea, Gigasporacea, Glomeracea, and Pacisporacea—noting that the genus *Glomus* was the most common. Similarly, Lopes Leal et al. (2016) studied mycorrhizal fungi after heavy metal remediation had occurred. The most common genera found were *Glomus* and *Acaulospora*. The researchers noted that the glycoprotein glomalin, produced by some AMF, has the ability to retain heavy metals. This finding demonstrates that along with monitoring the abundance of AMF in soils, glomalin may serve as another useful monitoring tool. The researchers determined the frequency of occurrence for each arbuscular mycorrhizal fungal species by dividing the number of soil samples in which the species occurred by the total number of soil samples.

According to Brueseke et al. (2016), only 10 percent of restoration projects are monitored after completion to inform future restoration goals. Few restoration projects have had the benefit of continued monitoring, or any kind of follow-up research to determine if the site has continued to uphold the newly-established ecosystem. By studying mycorrhizal abundance and hotspots in soils, researchers can further ensure future ecological restoration success. Peter McCoy, author of *Radical Mycology* (2016) also notes the importance of using mycorrhizal fungi as a bioindicator. Not only can mycorrhizal fungi be an indicator of heathy, undisturbed soils, but some common species may be indicators of heavy metal pollution.

Soil Inoculation

Mycorrhizal fungi soil amendments such as MYKE, MycoApply, and MycoGrow have been available in commercial markets for years. Many well-established gardeners add them to soil while transplanting anything from vegetables to trees. These products often highlight the mycorrhizal fungi ability to increase nutrient uptake by increasing the surface area of the rhizal

network. Depending on the product, some contain strictly endomycorrhizal or ectomycorrhizal fungi spores while others contain a mixture of both.

Ectomycorrhizal fungi usually form symbiotic relationships with trees and other woody plants. Ectomycorrhizal fungi work by surrounding a root and exchanging nutrients extracellularly. Only ten percent of plant species form symbiotic relationships with ectomycorrhizal fungi. Endomycorrhizal fungi form relationships with approximately 80 percent of terrestrial plants. In this symbiotic relationship, the fungi exchange nutrients intracellularly.

A meta-analysis of 1,994 studies from 1965 to 2006 noted that floral responses to addition of mycorrhizal fungi ranged from positive to negative (Hoeksema et al., 2010). The majority response, however, was positive. The researchers also noted that C4 grasses and non-nitrogen fixing plants had the greatest response to mycorrhizal inoculation. Although the response was smaller, it should be noted that C3 grasses and nitrogen-fixing plants also responded to mycorrhizal fungi inoculation. Hoeksema et al. (2010) also found that forbs inoculated with a single species of AMF did not have as high a response as forbs inoculated with multiple species of mycorrhizal fungi, a mix of mycorrhizal fungi and non-mycorrhizal microbes, or whole soil inoculum.

The meta-analysis by Hoeksema et al. (2010) also noted that plant functional characteristics may be more important to colonization of roots by both ecto- and endo-mycorrhizal fungi than whether the study was performed in the laboratory or in the field. Plant functional characteristics were also more important than inoculum complexity, phosphorus fertilization, or mycorrhizal type. A study on mine reclamation by Johnson (1998) found that phosphorus levels negatively impacted mycorrhizal fungi colonization, and therefore plant nutrient uptake, as level of phosphorus increased. As mentioned previously, researchers have

found that C4 grasses had a higher response to mycorrhizal fungi than did C3 grasses. N-fixing plants were found to exhibit lower response to mycorrhizal colonization; however, Johnson (1998)noted that many studies used phosphorous-rich soils. The use of these soils may have suppressed the total net benefit of mycorrhizal fungi symbiosis.

Mycorrhizal symbiosis was higher between plants and fungi when multiple fungi and microbial species were present within the inoculum. One explanation is that mycorrhizal fungi may protect plants from harmful soil pathogens (Fritter and Garbaye, 1994; Newsham et al., 1995). Furthermore, plant productivity may be stimulated by the increased nitrogen availability from predators within the soil food web (Hoeksema et al., 2010). This demonstrates the importance of holistic ecosystem views when studying plant-fungal interactions.

Future applications of mycorrhizal fungi inoculation are currently being researched across the globe. Much of this research has contributed to our understanding of mycorrhizal fungi and plant interactions, but it has also brought new light into possible solutions for agriculture during an age of rising sea levels, changing climate, and anthropocentric habitat destruction. A study by Al-Karaki (2006) found that tomato plants inoculated with AMF had increased dry matter and fresh fruit yield. Al-Karaki (2006) also found that enhancement of fruit yield was 60 percent higher for tomatoes watered with a saline solution and inoculated with mycorrhizal fungi compared to water with a saline solution and not inoculated. Tomato plants inoculated with mycorrhizal fungi and watered without a saline solution experienced a 29 percent increase in fruit yield (Al-Karaki, 2006).

Remediation

Researchers are studying the efficacy of mycorrhizal fungi in soil remediation. With the ability of mycorrhizal fungi to increase nutrient uptake by plants, it is hypothesized that it may have

limited applications for phytoremediation. Phytoremediation is more than just using plants to uptake heavy metals from the soil, i.e. phytoaccumulation. This term also encompasses phytodegradation, phytostabilization, rhizofiltration, and biodegradation (Khan, 2000).

Phytoremediation is not without its limitations. The list of known metal hyperaccumulators is short, and the process of phytoremediation often takes much longer than other more technology-dependent techniques. Another concern among researchers is the potential for these contaminated hyperaccumulators to enter the food chain via grazing animals (Khan et al., 2000). A study by Stahl et al. (1988) noted that native mycorrhizal fungi had a negative response to disturbed soils, specifically, coal mine spoil. They found that after the soil was disturbed the native mycorrhizal fungi was unable to form effective symbiotic relationships and unable to assist in the establishment, growth, or survival of native *Artemisia tridentata* subsp. *wyomingensis*. Researchers continue to seek ways to improve the efficacy of phytoremediation.

Understanding the interactions between mycorrhizal fungi and hyperaccumulators is one way researchers are looking to improve phytoremediation. Although mycorrhizal fungi associations and heavy metal uptake are highly dependent on plant characteristics, many researchers have reported the presence of mycorrhizae on hyperaccumulators growing in contaminated ecosystems (Khan et al., 2000). This finding is not entirely unexpected, as it is known that mycorrhizal fungi have the ability to form some level of symbiotic relationships with almost all plants. One study noted that although infectivity of mycorrhizal fungi on contaminated plots with a low density of plant cover decreased the second year after inoculation, the overall efficacy of reclamation increased over a three-year period with an increasing proportion of mycorrhizal-dependent grasses (Noyd et al., 1996).

The most common mycorrhizal fungi found on contaminated soils belong to the genera *Glomus* and *Gigaspora* (Khan et al., 2000; Mathur et al., 2007). Little is known about mycorrhizal fungi and heavy metal interactions, although it is suggested that the outcome of these interactions depends heavily on the species of mycorrhizal fungi and the metal contaminant. Mycorrhizal fungi are known to increase the uptake of metals such as copper, zinc, nickel, cadmium, and lead (Mathur et al., 2007). One study found that although mycorrhizal fungi assisted in uptake of cadmium by *Trifolium subterraneum,* the cadmium was stored only in roots. Fungal immobilization by *Glomus mosseae* prevented the transfer of cadmium from mycorrhizae to the plant (Joner and Leyval, 1997).

As Khan et al. (2000) notes, there are many conflicting studies when it comes to the efficacy of mycorrhizal fungi and remediation. Some reports indicate higher allocations of heavy metal in plants due to the addition of mycorrhizal fungi, while others note no significant difference in metal allocation or even a reduced concentration of metals in plant tissue. Similar conflicting reports were noted by Stahl et al. (1988) as they discussed the disparities between the negative response of native mycorrhizal fungi to soil disturbance in their study, and the many other studies in which positive results were determined for colonization of non-native mycorrhizal fungi. Future research should aim to further understand the interactions between specific genera of mycorrhizal fungi and specific heavy metal contaminants. Also important, however, is the understanding of interactions between mycorrhizal fungi and the plants used in phytoremediation and the native abundance of mycorrhizal fungi in ecosystems around the globe. Without the ability to establish a baseline of healthy soil ecosystems prior to remediation, we may never know the true cost of cleanup.

Conclusion

A thorough and holistic understanding of mycorrhizal fungi is imperative for the understanding of ecosystems both above and below ground. The unique history and geomorphology of Indiana lends itself to many unique habitats across the state; however, our history of deforestation, agriculture, and urbanization of these lands has led to an increased need for environmental remediation. Thus far, the use of habitat suitability indices has been extremely helpful to researchers and restoration professionals, not only by quantifying current habitat disturbance, but also by providing a baseline for restoration goals.

Habitat indices often rely on the use of bioindicators, such as plants found within Orchidaceae. Terrestrial orchids are often a unique indicator of below-ground health, as they rely solely on a symbiotic relationship with mycorrhizal fungi for seed germination.

Although reports are conflicting, it is hypothesized that soil inoculation with mycorrhizal fungi could have applications for both agriculture and heavy metal remediation. Still, further research is needed to understand the complex dynamics between mycorrhizal fungi, host plants, and the microbiome found within soils.

Chapter 2- Research

INTRODUCTION

Across North America, urban sprawl has extended further into rural areas. Restoration ecologists, environmental consulting firms, and conservation-minded laypeople are pushing to have brownfields assessed, in part to slow sprawl but also to improve the economic sustainability of the affected area. Occasionally, phase one assessments of brownfields recommend remediation. Hazardous waste remediation often requires treating soil by removing toxic contaminants. In some recovered sites, however, the soil has become somewhat sterile and is not conducive to creation of a functioning ecosystem. Soil itself is a microcosm teeming with often unseen and overlooked biotic interactions. The symbiotic relationship between mycorrhizal fungi and root systems is one such interaction. Mycorrhizal fungi are imperative for soil formation. The glomalin produced by the mycelium of *Glomeromycota* — one of the most abundant phyla — binds soil particles and improves overall soil structure (McCoy, 2016). The increased network of mycelium and roots formed when plants and mycorrhizal fungi interact increases nutrient uptake by plants and reduces soil erosion. Ecosystem restoration projects benefit from mycorrhizal fungi by encouraging the growth of healthier plants and ensuring a more diverse soil biome.

By understanding the natural distribution of soil mycorrhizal fungi spores, we can move towards better remediation practices and increase our knowledge of the extra steps that may be needed for effective future restoration efforts. This research aims to provide restoration ecologists and enhance understanding of mycorrhizal fungi response to various environmental disturbances by answering the question: How does the density of mycorrhizal fungi spores change in response to distance from Orchidaceae symbiotes. We hypothesized that mycorrhizal spore abundance will decrease with increasing distance from *Aplectrum hyemale.* The objective

of this study was to measure spore abundance at selected distances in each of the cardinal and sub-cardinal directions. These measurements will indicate whether distance, direction, or both play a role in spore abundance within soil.

The lack of macroscopic fruiting bodies makes researching AMF extremely difficult and is one of the main reasons for the knowledge gap between fungal orders. In fact, much, if not all, of the research involving mycorrhizal fungi begins with a green plant. Most researchers writing about mycorrhizal fungi are not mycologists, but instead botanists or geneticists trying to understand the interactions between mycorrhizal fungi and plants. It is common knowledge that mycorrhizal associations benefit plants; but beyond that, our knowledge of mycorrhizal fungi is scant.

Mycorrhizal fungi can form symbiotic relationships with up to 80 percent of terrestrial plants, making them a highly important factor in habitat restoration efforts (Cobb et al., 2018; McCormick et al., 2016). This interaction between fungi and plants dates back 350–460 million years, when the order Glomales is thought to have evolved. It has been suggested that the fungal symbiosis we see today was imperative for land colonization by plants (Harrier, 2001; Field et al., 2015). Some researchers have even shown increasing efficiency of phosphorous exchange pathways between mycorrhizal fungi and plants with increasing complexity. Two researchers have suggested that green-leaved orchids use a ‘take now, pay later’ scheme which has contributed to mycorrhizal specificity wherein green sporophytes are established to repay mycorrhizal partners for the carbon invested (Cameron et al., 2008; Field et al., 2015). This leads some scientists to believe that rather than exchanging equal amounts of organic carbon for equal amounts of nutrients, there is a form of intergenerational carbon subsidy allowing for prolonged ‘parent nurture’ (Field et al., 2015).

AMF use two main colonization patterns within plant roots. The first pattern is linear and is termed *Arum*-type; the second, *Paris*-type, is coiled and intracellular (Muthukumar et al., 2016). These mutualistic relationships benefit flora by increasing the overall surface area of systems and allowing the plant to absorb more nutrients than previously possible. One study, however, has shown that colonization by mycorrhizal fungi may have some negative side-effects; Field et al. (2015) has shown significant stunting of *Ophioglossum vulgatum* that have been colonized by mycorrhizal fungi, which suggests that the carbon required by mycorrhizal fungi may take up a significant portion of plant carbon.

In addition to benefiting flora, mycorrhizal fungi benefit soil by enhancing aggregation, stability, and water-holding capacity through the release of glomalin. Mycorrhizal fungi are key components of healthy soils. Unfortunately, they are commonly overlooked when considering best management practices and plant cultivation. One study (Detheridge et al., 2016) has shown that agriculture can severely affect fungal diversity within the soil depending on the cover crop. Two species of clover, *Trifolium pratense* and *T. repens*, had the most adverse effect. The study showed a notable negative correlation between soil nitrate levels and mycorrhizal fungi populations (Detheridge et al., 2016). Soil tillage also has an effect on soil fungi populations. In areas that practiced no-till agriculture, mycelium networks were able to grow larger; soil moisture content remained higher, leading to increased fungal biomass; and root colonization by AMF increased (Detheridge et al., 2016). A more recent anthropogenic phenomenon, eutrophication, has been recognized as a contributor to mycorrhizal fungi decline. As mentioned by Detheridge et al. (2016) and Crockatt (2016), increased nitrogen deposition has had a considerable impact on mycorrhizal mycelial production and endomycorrhizal root tip abundance.

Mycorrhizal fungi depend on carbon from other plants to survive, making them obligatory biotrophs. However, external carbon sources are not required during spore germination; instead, lipids, mostly triacylglycerides, are the main source of carbon. Mycorrhizal fungi can also absorb hexose from host roots. In other words, although mycorrhizal fungi are known to survive by utilizing carbon provided by a host plant, it may also use lipid stores as a source of carbon during the germination phase (Harrier, 2001).

Monitoring fungi populations can be an important part of ongoing ecosystem restoration projects. Successful habitat restoration may even depend on the establishment of three major fungi guilds — decomposers, mutualists, and parasites (Avis et al., 2017). Researchers had identified potential mycorrhizal fungal hotspots in three Indiana counties (NIRMI, n.d.). Although the study focused on macrofungi, the researchers call for the use of qPCR to better identify the presence of mycorrhizal fungi. In addition to identifying native mycorrhizal fungi, Torres-Arias et al (2017) highlighted the potential use of native AMF as a soil inoculum, noting that this form of inoculation has been shown to increase plant resistance to environmental stressors. They identified six common families of fungi in their samples, Acaulosporaceae, Archaeosporaceae, Diversisporoaceae, Gigasporaceae, Glomeraceae, and Pacisporaceae—noting that the genus *Glomus* was the most common. Similarly, Lopes Leal et al. (2016) studied mycorrhizal fungi present in soil following remediation of a heavy metal-contaminated site. The most common genera found were *Glomus* and *Acaulospora*. The researchers note that the glycoprotein glomalin, produced by some AMF, has the ability to bind heavy metals. This finding demonstrates that along with monitoring the abundance of AMF in soils, glomalin may be another useful monitoring tool. The researchers determined the frequency of occurrence for

each AMF species by dividing the number of soil samples in which the species occurred by the total number of soil samples.

Different soil amendments impart no significant effect on the AMF colonization of *Vigna unguiculata* (Cobb et al., 2018). Similarly, Burke et al. (2019) studied the effects of invasive garlic mustard (*Alliaria petiolata*) removal and deer exclusion on mycorrhizal fungal communities over eight years. Garlic mustard is important to the current study because it is pervasive in Indiana, and the secondary allelopathic chemicals it releases into the soil are known to negatively affect many native plant species. According to Burke et al. (2019), the allelopathic chemicals released by garlic mustard may also affect microbial communities within the soil. The lowest number of spores occurred in areas where deer had access and garlic mustard was present; however, no significant difference was found in the number of mycorrhizal spores between different plots. The most common mycorrhizal fungi identified was *Funneliformis mosseae*. Helander et al. (2018) focused on the effects of glyphosate on mycorrhizal fungi. They found that glyphosate had the ability to reduce mycorrhizal colonization and arbuscules present in the roots of both weeds and crops, thus suggesting that mycorrhizal spores and external hyphae may be adversely affected by residual glyphosate in the soil.

When considering new land for development, many land managers and contractors do not consider the impact that borders between landscapes have on adjacent ecosystems. The change in biological diversity at these edges, known as ecotones, are the result of changes to the microclimate and shifts in the flow of abiotic and biotic materials. Mycorrhizal fungi associations, fungal biomass, and the abundance of fruiting bodies are all affected by edges. Soil at the forest edge is often warmer and drier than soil at the forest core; furthermore, these variables are also known to fluctuate more rapidly at the forest edge. These two factors, soil

temperature and soil moisture, are important to fungal life cycles because temperature and moisture regulate fungal spore release, germination, and mycelial extension. Unlike plants, fungal spores can react to these fluctuations on an hourly timescale. Decreased soil respiration and changes in wind patterns may also affect fungi (Crockatt, 2012). Changes in horizontal permeability within an ecotone affect wind and other horizontally-vectored processes. Wind velocity tends to be higher in transitional zones, which in turn increases transpiration thus decreasing soil moisture (Schmidt et al., 2016). More importantly, mycorrhizal fungal abundance influences the suitability of habitats for organisms in other taxa such as late-stage colonizers, fungivores, and beetles. This means that mycorrhizal fungi may cause secondary edge effects for other taxa (Crockatt, 2012).

Edge effects are important when it comes to understanding restoration projects. Many restoration projects are small, referred to as 'postage-stamp' parcels by local restoration ecologist and land-trust employee Bob Easter (Bob Easter, Pers. Comm., 2015). When considering the success of Indiana restoration projects, one must understand that many wooded habitats are not technically forests due to the limited distance between property edges. While one long-term goal for these areas may be to increase overall space while restoring habitats, a thorough understanding of the edge effects caused by such borders is imperative to their success.

Few studies have focused on mycorrhizal fungi density in anthropogenically-disturbed soils; however, the topic has been gaining traction in recent years due to the increased use of scanning electron microscopy and DNA analysis. In the past, distribution of fungi has been inferred from the distribution of fungal fruiting bodies; however, research by McCormick et al. (2016) demonstrates that DNA assays may be a more accurate indicator of distribution. Spore baiting is another method to measure the mycorrhizal fungi present in soils. This process can

take years to complete, however, and was considered too inefficient for the scope of the current study. Instead, this study used soil sieves and sucrose to extract mycorrhizal fungi spores from soils with a history of low anthropogenic impact. These methods were considered the most cost-effective and efficient way to establish a baseline for future studies.

A minority of restoration projects, ten percent according to Brueseke et al. (2016), are monitored after completion to inform future restoration goals. Very few restoration projects have had continued monitoring or any kind of follow-up research to determine whether the site has continued to sustain the established ecosystem. More importantly, the flora of these restoration projects depends on a healthy soil. By studying mycorrhizal abundance and hotspots in soils researchers can further ensure future ecological restoration success. Peter McCoy, author of *Radical Mycology* (2016), notes the importance of using mycorrhizal fungi as a bioindicator. Not only can mycorrhizal fungi be an indicator of heathy, undisturbed soils, but some common species may be indicators of heavy metal pollution.

This research will assess mycorrhizal abundance in Ginn Woods, the second largest undisturbed hardwood forest in Indiana, to establish a baseline for future research. We hypothesize that spore abundance will decrease with increasing distance from the Putty Root (*Aplectrum hyemale* (Muhl. ex Willd.) Torr.) orchid.

RESEARCH METHODS

Site Description

Soil samples were collected from Ginn Woods (lat. 40.353772, long. -85.439648),a property managed by the Ball State University Field Station. Totaling 65 ha (161 ac), Ginn Woods is the second largest old growth forest in Indiana (Badger et al., 2000). Although the main parcel of land was known to have been settled, the owners chose not to farm, graze, or log it, creating a

large area of undisturbed forest acquired by the university in 1971 (History, n.d.). Efforts to remove populations of invasive species such as *Alliaria petiolata* and *Lonicera maackii* over the last decade have contributed to maintaining the continued health and rare diversity of this ecosystem. Here *Aplectrum hyemale* is known to blossom in late May.

Site Selection

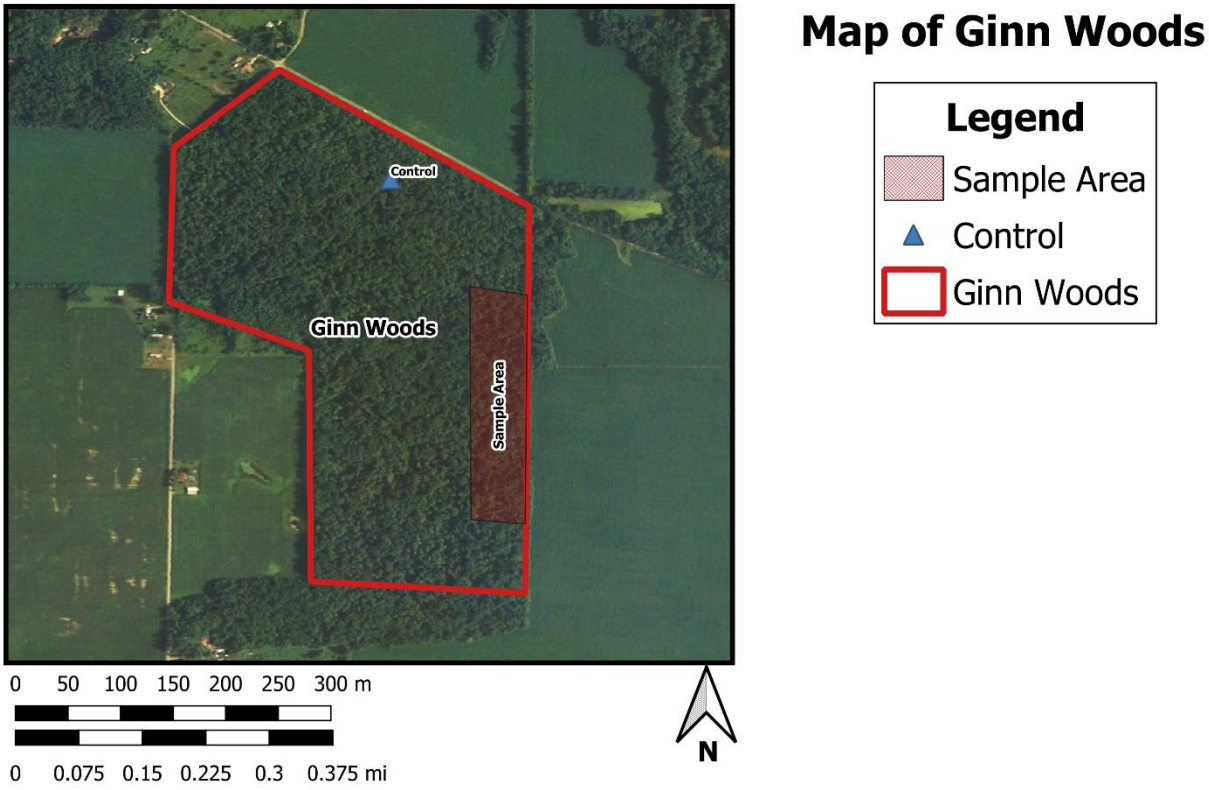

Fig. 1 Map of Ginn Woods

Sample sites were selected based on the location of one or more *Aplectrum hyemale* plants (Table 1). All plants were found near the eastern border of Ginn woods (Figure 1).

Sampling Locations

Table 1. Degrees Minute Second Coordinates of sampling locations		
Site	**Latitude**	**Longitude**
1	040°20’2”N	0-85°-24’-24”W
2	040°21’21”N	0-85°-26’-26”W
3	040°20’2”N	0-85°-24’-24”W
4	040°20’20”N	0-85°-26’-26”W
5	040°20’20”N	0-85°-26’-26”W
6	040°21’21”N	0-85°-26’-26”W
Control	040°21’14”N	0-85°-26’-22”W

Sampling Procedure

Each location was sampled via soil probe 49 times, terrain permitting. The initial soil core, denoted as sample # 0, was taken at the base of the orchid to a depth of 30 cm. Next, soil cores were taken at distances of 5 cm, 10 cm, 20 cm, 50 cm, 1 m, and 5 m in each of the cardinal and intermediate directions. Samples were placed into plastic bags and subsequently stored at 4^{o}C while waiting further processing.

In the lab, soil from the core was homogenized manually with a mortar and pestle and then a 5 ± 0.5 g sample was weighed and washed through a set of two sieves with 50 ml of d’H_2O. The first sieve was a no. 60 (250 µm mesh), and the second was a no. 230 (63 µm mesh). The filtrate was transferred to a 50 ml centrifuge tube and centrifuged for 5 min at 3600 rpm and 16° C. Water was decanted from the tubes and discarded, and 5 ml of a 60 percent sucrose solution was added and the pellet resuspended. The tubes were then centrifuged for 1 min at 3600 rpm. The sucrose solution was decanted and filtered through a mesh with a pore size of 25 µm. The spores were then moved from the mesh to a microspore slide via pipette and stained with 10 µl

of Melzer's reagent. The coverslip was sealed with clear nail polish and slides were stored at 4°C until examined.

Slides were viewed on an Invitrogen EVOS XL Core Cell Imaging System at 40× and 100× magnification, scanning from top to bottom and left to right. Images of relevant structures were taken for later assessment.

Statistical Analysis of Data

ImageJ was used to process images taken from the microscope slides. References images produced by Blaszkowski (2003) were used in conjunction with the particle analysis function in ImageJ. Four morphological characteristics, i.e., length, width, color, and circularity were used to identify spores to the lowest order (Robertshaw, 2015). Data was compiled using Excel, then R was used to perform the final statistical analysis.

RESULTS

The results shown in figure 2 are from 121 randomly selected samples, stratified to include representative samples sizes from eight directions and six distance intervals.

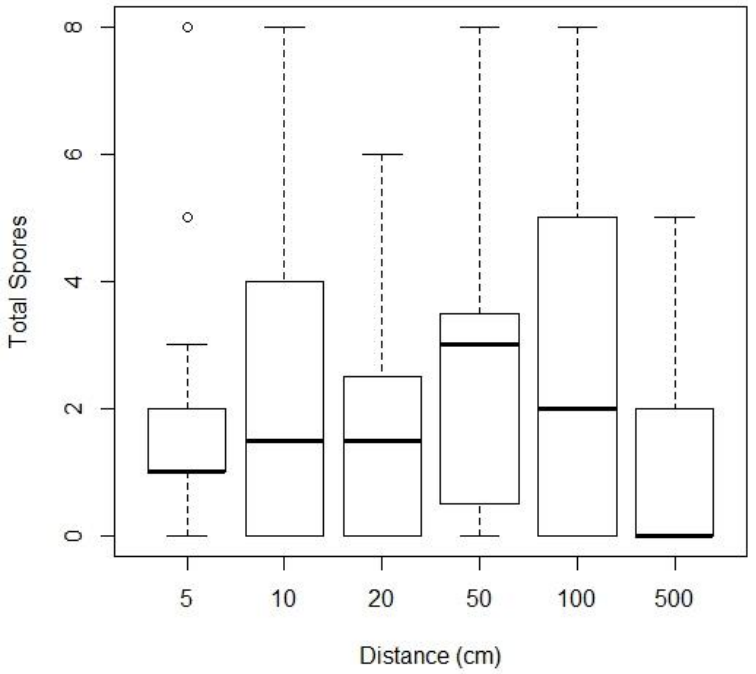

Fig. 2 Quantile data of total spores at each distance interval

A total of 274 spores was identified among the 121 samples reviewed (Table 2). Site 5 had the highest number of spores (n = 63), while site 3 had the lowest (n =18). The genus *Glomus* accounted for the majority of spores

found (n = 224). Thirty-seven samples contained no spores. The average number of spores found per sample was two, with eight being the highest number found per sample. All 1-m lengths combined had the highest number of total spores with n = 59, while all 5-m lengths combined had the lowest (n =23).

Table 2. Spore totals for all test intervals, Ginn Woods.					
Distance, cm	**Direction**	**Spore count**	**Distance**	**Direction**	**Spore count**
5	N	4	50cm	N	2
5	NE	16	50cm	NE	15
5	E	7	50cm	E	9
5	SE	1	50cm	SE	3
5	S	1	50cm	S	10
5	SW	12	50cm	SW	1
5	W	1	50cm	W	9
5	NW	1	50cm	NW	8
10	N	25	1m	N	12
10	NE	1	1m	NE	0
10	E	1	1m	E	12
10	SE	3	1m	SE	12
10	S	2	1m	S	7
10	SW	2	1m	SW	5
10	W	6	1m	W	6
10	NW	7	1m	NW	5
20	N	12	5m	N	6
20	NE	0	5m	NE	0
20	E	2	5m	E	5
20	SE	5	5m	SE	3
20	S	5	5m	S	0
20	SW	9	5m	SW	3
20	W	10	5m	W	3
20	NW	2	5m	NW	3

Table 3. Spore totals for control test intervals, Ginn Woods.

Distance (cm)	Direction	Spore Count	Distance (cm)	Direction	Spore Count
5	N	0	50	N	0
5	NE	0	50	NE	1
5	E	1	50	E	1
5	SE	0	50	SE	2
5	S	1	50	S	2
5	SW	2	50	SW	0
5	W	2	50	W	1
5	NW	N/A	50	NW	4
10	N	0	100	N	2
10	NE	0	100	NE	3
10	E	0	100	E	1
10	SE	0	100	SE	0
10	S	1	100	S	N/A
10	SW	0	100	SW	0
10	W	0	100	W	0
10	NW	0	100	NW	5
20	N	2	500	N	1
20	NE	2	500	NE	4
20	E	1	500	E	0
20	SE	1	500	SE	N/A
20	S	0	500	S	N/A
20	SW	0	500	SW	1
20	W	2	500	W	16
20	NW	2	500	NW	N/A

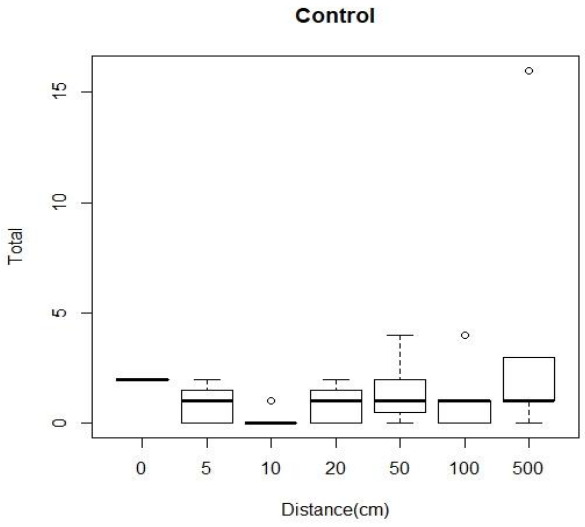

Fig. 3 Quantile data of the control sample

A control site containing 45 samples was also reviewed (Table 3). The majority of slides had no spores (*n* =19). The average number of spores found per sample was 1.25, with 16 being the highest number found (Figure 3). This high spore count may be attributed to mold growth on the slide and not spores found in soil.

The quantile data for distance and spore total is shown in Figure 1. Distance was shown to have a significant effect on spore total ($p = 0.02$); however, spore density did not increase with proximity to *Aplectrum hyemale*; instead, the data shows an upward trend as spores move away from *Aplectrum hyemale,* followed by a sharp decrease in density after 2 m (Figure 4). Distance did not have a significant effect on spore abundance within the control sample ($p = 0.46$).

The hypothesis that a greater number of spores would be found closer to the base of *Aplectrum hyemale* was not supported. In contrast, spore density in the soil increased with increasing distance from the plant, peaking at 1 m. A predictive curve estimates a peak at 2 m, with an abrupt decline at 2 to 5 m.

Overall, the mean angle of spore direction was found to be 46.75 degrees— approximately NE. However, the length of the mean vector was only 2.32 cm. This length was tested by creating a null distribution to randomize existing directional data, thus providing a more accurate *p*-value. Spore density in relation to both distance and direction was not found to be significant with a *p*-value of 0.09. Figure 5 shows the distribution of null length of the sample, randomized and replicated 10,000

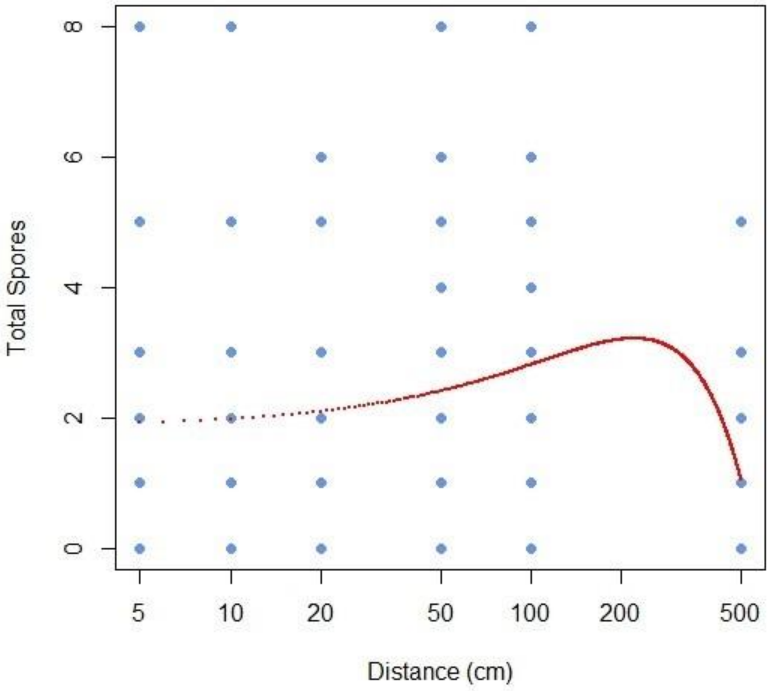

Fig. 4 A predictive curve shows the current trend of spore density over distance from Aplectrum hyemale

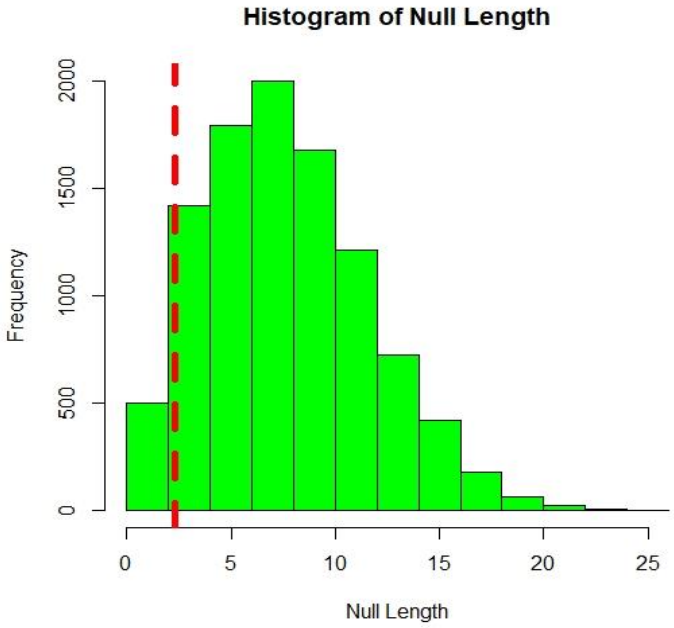

Fig. 5 Null distribution from randomizing current data. Value of recorded data is shown in red

times. A vector length of 15 or higher could have been significant, but the reported vector length of 2.32, shown in red, could be due to random chance.

This result seems to support the theory that spores and sporocarps are released from hyphal tips, as roots and mycelia will extend in every direction unless obstructed.

Figure 6 shows the distance and direction in which samples were collected, with direction shown in radians. The 5-m distance was excluded from this figure to increase the visibility of other samples collected.

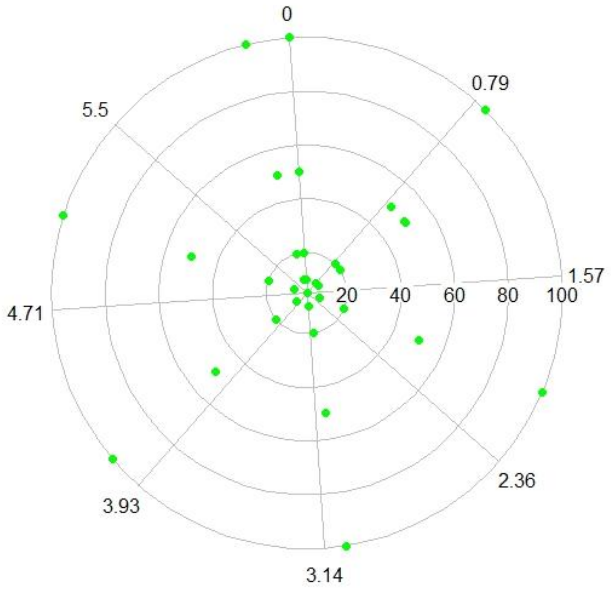

Fig. 6 Distance and direction of samples one through six with direction shown in radians

DISCUSSION

To date, little research had been conducted to assess the dispersal of mycorrhizal fungi in soil, especially in relationship to Orchidaceae symbiotes. Many external factors, abiotic, biotic, and others, should be considered when reviewing the results of this study. First, little was known about mycorrhizal fungi reproduction. Although scientists had suggested that spores were released from hyphal tips, it had yet to be extensively supported by scientific studies (Blaszkowski, 2003). Secondly, the samples for this study were collected in late spring, in conjunction with the flowering of *Aplectrum hyemale;* however, fungi commonly release spores in the fall (McCoy 2016). This could have resulted in a lower number of collected spores. Finally, resources for mycorrhizal fungi spore identification were severely lacking which could have led to the misidentification of a spore's species or genus.

When studying soils from low arctic ecosystems, Varga et al. (2014) noted that spore density ranged from 5 to 69 spores/g of soil, with significant temporal and site-specific differences. Alexio et al. (2014) recorded that dry agricultural soils in Brazil had 9-13 spores per gram of soil; however, they noted that this is higher than values normally recorded for this area. They also found no significant difference between abundance of spores in native forests and abundance of spores in sugarcane fields. They hypothesized that this could be due to the late stage forest stands being less dependent on mycorrhizal fungi than pioneer species (Alexio, 2014). This could also explain the low number of spores determined in the current study within the old growth forest of Ginn Woods.

McCoy (2016) states that woodland ecosystems should contain 1-5 mycorrhizal fungi spores per gram of soil. These estimates are higher than the 2 spores per 5 grams of soil average found within Ginn Woods. Mafaziya and Madawala. (2014) found significantly higher densities

of large and medium-sized spores in the forests of Sri Lanka. They also noted that forest soils had the highest species richness of mycorrhizal fungi. Their results suggested that historic land use and vegetation were key factors in the community structures of mycorrhizal fungi (Mafaziya and Madawala, 2014; Kumar Ietal., 2011). Toprak et al. (2010), also found the highest abundance of mycorrhizal fungi in Florida at a site with least soil disturbance. The mean number of spores found at the less disturbed site was 300 spores per 50 grams of soil and only 84 spores per 50 grams of soil at the more disturbed site. Chandrashekar and Khan (2011) noted that understanding spore density in soil is important for evaluating the affects land use has on soil health. They also found a higher concentration of mycorrhizal fungi spores in forests when compared to agricultural lands.

Kumar et al. (2011) recorded approximately 360 spores per 5 grams of soil near coal field areas in India. They suggested that an understanding of mycorrhizal fungi is essential to understanding the sustainability of reclamation projects devoted to restoring ecosystems disturbed by mining practices. The absence of mycorrhizal fungi from highly disturbed areas may have accounted for the poor survival of plants used in stabilization processes. Kumar et al. (2011) concluded that heavy metal contamination could have a significant impact on mycorrhizal fungal colonization and spore germination, potentially eliminating it.

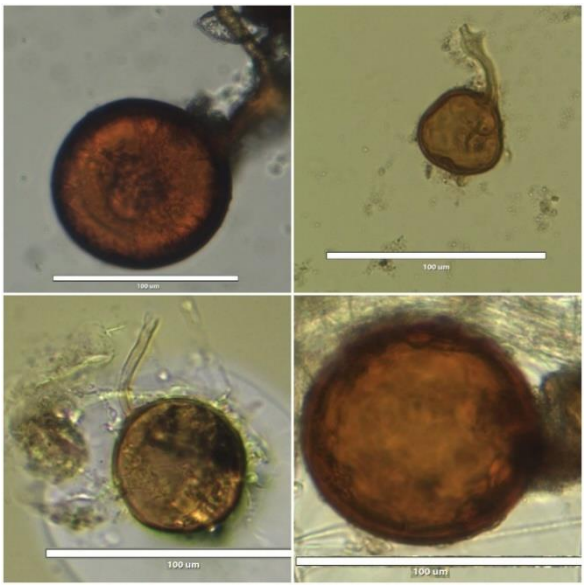

Fig. 7 *Glomus sp*

Unlike other undisturbed locations, spore abundance in Ginn Woods was found to be relatively low. As mentioned earlier, this could be due to nutrient abundance or the relative age of the forest stand. Different ecosystems have been known to support different spore densities within soil. For instance, cultivation has been found to reduce mycorrhizal spore diversity (Chandrashekar and Khan, 2011). In this study, the genus *Glomus* was the most abundant, with *Acaulospora* being the second most abundant (Figures 7 and 8 respectively). Others have noted the high abundance of *Glomus* spores when studying spore density and dispersal in soil (Paugy et al., 2004; Toprak et al., 2010; Kumar et al., 2011; Mafaziya and Madawala, 2014; Lopez Leal, 2016; Torres-Arias et al.,2017).

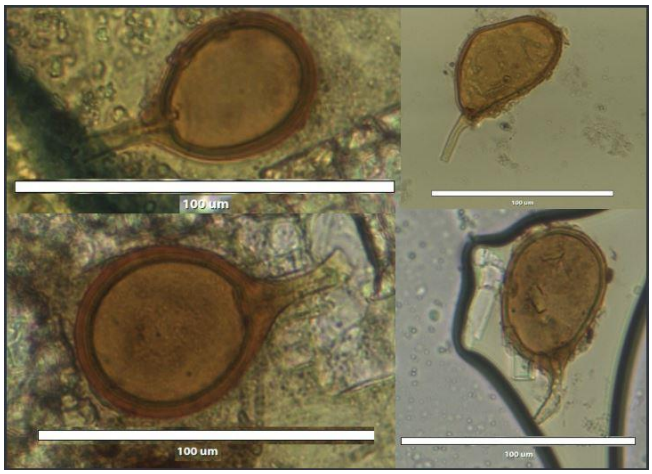

Fig. 8 *Acaulospora sp*

It is possible that low spore density at 5 cm could be because mutualistic relationships had already formed between the fungi and roots of the nearby orchid. As mentioned earlier, samples were collected in late spring near the end of the Putty Root orchid life-cycle, and long after the mycorrhizal spores would have been released.

If correct, the commonly-held belief that spores and sporocarps are released from the hyphal tips could have explained the increase in spores at the 1 m distance. High spore density at 1 m could also have occurred because spores were dispersed too far from symbiotes to form mutualistic relationships. Low spore density at 5 m could have occurred due to a shorter extent of symbiotic root systems. These spores could have been moved farther from host plants by external factors such as wind, water, erosion, or other organisms.

There was no evidence in this study to show that spore density and direction may be correlated. Although no previous research on spore dispersal in temperate soils in relation to orchid symbionts could be found, there are similarities between these findings and those reported in the literature. Direction was found to be insignificant; however, this randomized dispersal still has an interesting effect. When distance and direction, or lack thereof, are both taken into consideration a 'doughnut' of density occurs around the Putty Root Orchid (Figure 9). Although more spores occurred at the 1-m interval, it is common to find them displaced outside of this parameter.

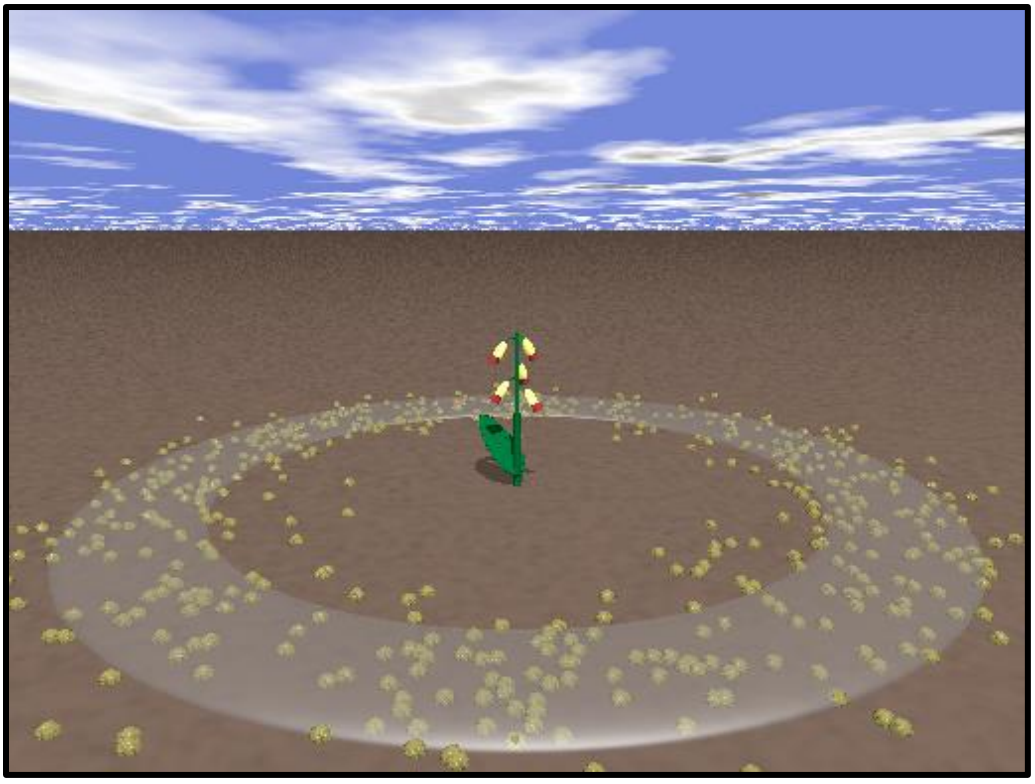

Fig. 9 Spore density around *Aplectrum hyemale*

Researchers have noted that there is little to no correlation between spore abundance in soil and the colonization of roots by mycorrhizal fungi (Toprak et al., 2010). Paugy et al. (2004) and Toprak et al. (2010) have suggested the onus of colonization falls on the plant species, that is, how receptive they are to forming mutualism based on how mycotrophic they are and the nutrient status of the soil. Spores found in soil not only reflected the relative abundance of mycorrhiza, but they also reflected the previous history of a mycorrhizal community (Chandrashekar and Khan, 2011).

As mentioned previously, mycorrhizal fungi reproduction and spore dispersal are not well studied. Many researchers believe that spores are released into soil from hyphal tips (Blaszkowski, 2003). Some have noted global similarities between mycorrhizal fungal taxa which may support the concept that dispersal of mycorrhizal fungi took place over geologic time, making ecologic dynamics important only to the smaller, local scale of distribution (Varga et al., 2014). However, researchers in Africa have found that elephants, or elephant dung more specifically, can act as dispersal agents for mycorrhizal fungi spores (Paugy et al., 2004). Perhaps this example could be expanded to the smaller herbivores commonly found in North America. For instance, earthworms are known to act as distribution vectors of mycorrhizal spores by concentrating propagules within their casts (Chandrashekar and Khan, 2011).

CONCLUSION

Mycorrhizal fungi are extremely important to soil and plant health. Without the mutualistic relationships formed between roots and mycelia, many plants would not survive. Little else is known about mycorrhizal fungi due to the relative lack of fruiting bodies most; however, previous research takes advantage of the obligate mutualism commonly associated with mycorrhizal fungi.

The hypothesis that mycorrhizal fungal spore abundance will decrease with increasing distance from Orchidaceae symbionts was not supported; however, this study did show that spore abundance was highest at a 1-m distance from the symbiont which supports the theory that mycorrhizal spores are released from hyphal tips. Further research on mycorrhizal spore abundance should be conducted to establish a more concise baseline for ecologists, soil scientists, and restoration technicians.

SUGGESTIONS FOR FUTURE RESEARCH

The extent of mycorrhizal fungal mycelium from orchidaceae roots is not well studied; therefore, future researchers may want to focus on mapping mycorrhizal networks in hardwood forest soils to further understand spore dispersal. Although research following the same methodologies has yet to be performed, a similar study conducted on other flora species may hold similar results. Future research should also focus on AMF spore dispersal from native mycotrophic trees and shrubs.

Future research should be extended to the roles that deer, wild boar, and small rodents play in mycorrhizal fungi dispersal. Other research should expand on this study, and studies of other mycotrophic species. Researchers should attempt to enhance our current understanding of mycorrhizal fungi reproduction, movement, and abundance in all habitats. Currently, advances are being made in mycorrhizal fungi identification through DNA sequencing; however, these studies are unable to quantify mycorrhizal spore abundance in soil.

Once researchers have established a baseline for mycorrhizal fungi abundance in various landscapes, scientists should further review the efficacy of mycorrhizal fungi inoculation in soils with a history of disturbance. Special note should be made of the interactions between soil

composition, mycorrhizal fungi, and mycotrophic plant species for use in future habitat restoration projects.

In more urban settings, research should focus on the efficacy of mycorrhizal fungi inoculation in remediation of wastes and brownfield soils. Potentials in mycoremediation are endless; however, future research should focus on glomalin production and the binding of heavy metals in soil. Mycologist Paul Stamets has made invaluable contributions to the study of mycoremediation, even coining the term himself; however, many species of fungi have yet to be tested for their potential benefits to environmental restoration.

Mycologists, conservation ecologists, anthropologists, historians, and biomedical researchers are needed to expand the field of medicinal mycology as well. Often, medicinal studies focus solely on mushrooms—the fruiting bodies of fungi. However, some biomedical labs have been working on ways to extract medicinal compounds from the mycelia rather than fruiting bodies of fungi. These methods are often more efficient and the controlled laboratory environment lends itself to mass production for market use. Understanding any potential ecosystem services these fungi could provide lends itself to further study and conservation.

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