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# Mycorrhizal Fungi Spore Abundance in Old-Growth Forest Soil

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## Abstract

Soil samples were collected from the base of *Aplectrum hymale* individuals to assess mycorrhizal spores abundance. The hypothesis that mycorrhizal spore abundance would increase with proximity to the plant was not supported; however, spores increased significantly with distance from the *Aplectrum hymale* plants up to one meter.

## Keywords

Mycorrhizal Fungi, *Glomus*, Spore Abundance, *Aplectrum hymale*, Orchid

## 1. Introduction

Soil 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 [1]. The increased network of mycelium and roots formed when plants and mycorrhizal fungi interact increases nutrient uptake by plants and reduces soil erosion. In addition to these benefits, mycorrhizal fungi have formed a symbiotoic relationship with most Orchidaceae species, which rely on mycorrhizal fungi to germinate.

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 in relation to an orchid symbiont.

Monitoring fungi populations can be an important part of ongoing ecosystem restoration projects. Successful habitat restoration may even depend on the es-

tablishment of three major fungi guilds—decomposers, mutualists, and parasites [2]. Researchers had identified potential mycorrhizal fungal hotspots in three Indiana counties [3]. 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.* [4], 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.* [5] 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.

The change in biological diversity at 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. 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 [6]. 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.

In the past, distribution of fungi has been inferred from the distribution of fungal fruiting bodies; however, research by McCormick *et al.* [7] 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. 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 [1], 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. Understanding spore abundance in relationship to host plants may help to inform future studies on population movement. It may also assist with the implementation of more efficient restoration practices when it comes to seeding or planting species reliant on mycorrhizal fungi.

## 2. Materials and Methods

### 2.1. Site Description

Soil samples were collected from Ginn Woods, 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. 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.

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).

### 2.2. Sampling Procedure

Each location was sampled via soil probe 49 times, terrain permitting. The initial

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 |

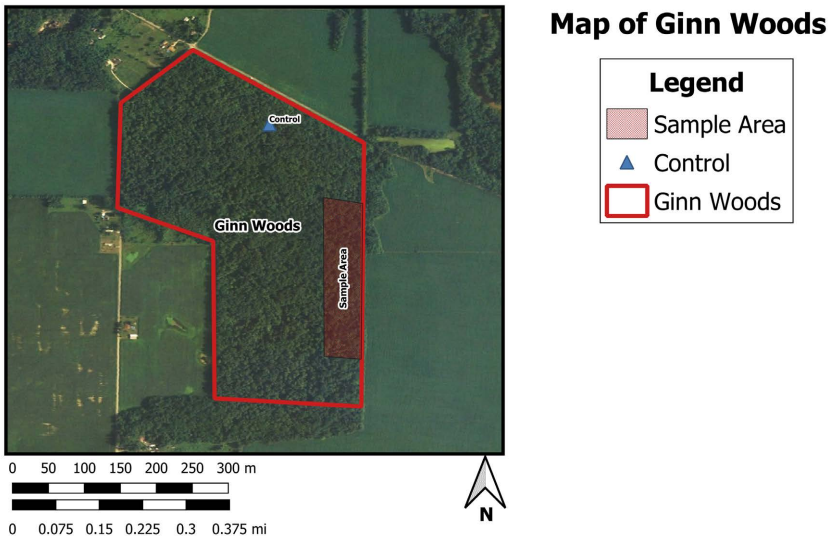


**Figure 1.** Ginn Woods.

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˚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.

## 2.3. Statistical Analysis of Data

ImageJ was used to process images taken from the microscope slides. References images produced by Blaszkowski [8] 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. Data was compiled using Excel, then the programing language R was used to perform the final statistical analysis.

## 3. 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.

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.

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.

Table 2. Spore totals for all test intervals, Ginn Woods.

| | Direction | Spore count | Distance | Direction | Spore count |
|---|---|---|---|---|---|
| 5 | N | 4 | 50 cm | N | 2 |
| 5 | NE | 16 | 50 cm | NE | 15 |
| 5 | E | 7 | 50 cm | E | 9 |
| 5 | SE | 1 | 50 cm | SE | 3 |
| 5 | S | 1 | 50 cm | S | 10 |
| 5 | SW | 12 | 50 cm | SW | 1 |
| 5 | W | 1 | 50 cm | W | 9 |
| 5 | NW | 1 | 50 cm | NW | 8 |
| 10 | N | 25 | 1 m | N | 12 |
| 10 | NE | 1 | 1 m | NE | 0 |
| 10 | E | 1 | 1 m | E | 12 |
| 10 | SE | 3 | 1 m | SE | 12 |
| 10 | S | 2 | 1 m | S | 7 |
| 10 | SW | 2 | 1 m | SW | 5 |
| 10 | W | 6 | 1 m | W | 6 |
| 10 | NW | 7 | 1 m | NW | 5 |
| 20 | N | 12 | 5 m | N | 6 |
| 20 | NE | 0 | 5 m | NE | 0 |
| 20 | E | 2 | 5 m | E | 5 |
| 20 | SE | 5 | 5 m | SE | 3 |
| 20 | S | 5 | 5 m | S | 0 |
| 20 | SW | 9 | 5 m | SW | 3 |
| 20 | W | 10 | 5 m | W | 3 |
| 20 | NW | 2 | 5 m | NW | 3 |

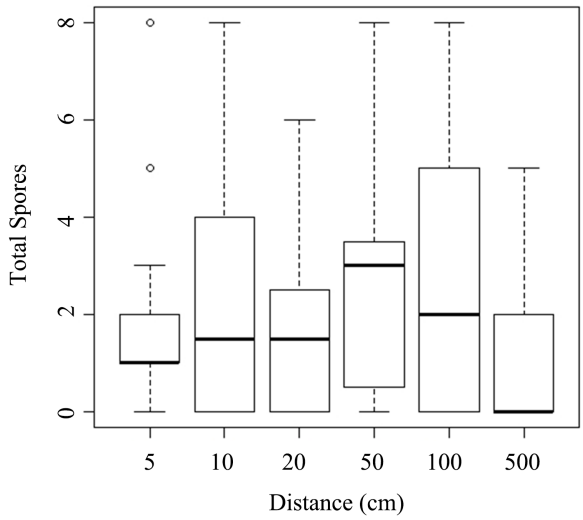


**Figure 2.** Total spores at each distance from the Putty Root orchid.

**Table 3.** Spore total at each control test interval.

| 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 |

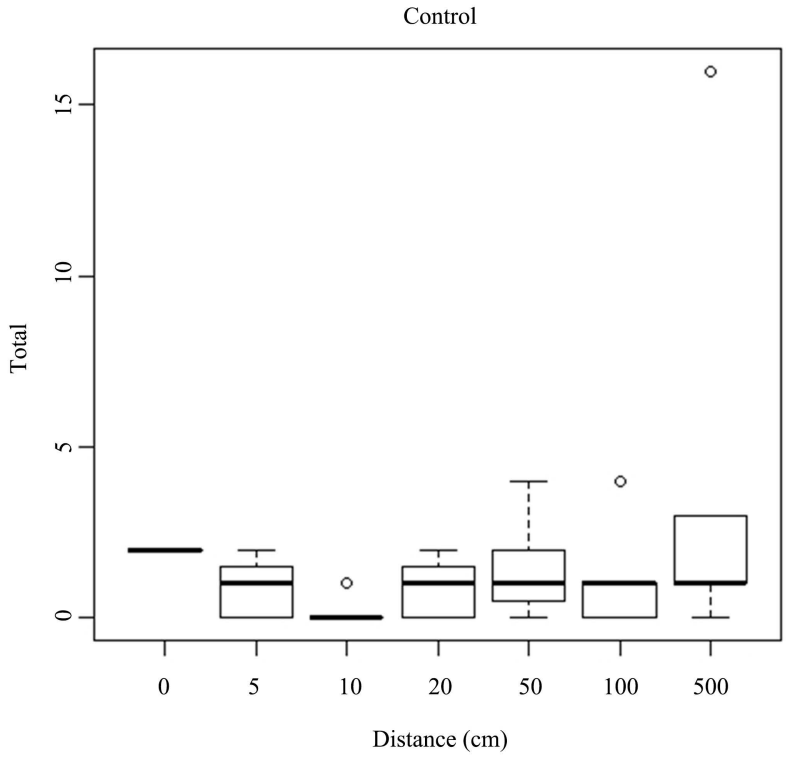


**Figure 3.** Total spores at each distance for the control sample.

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 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.

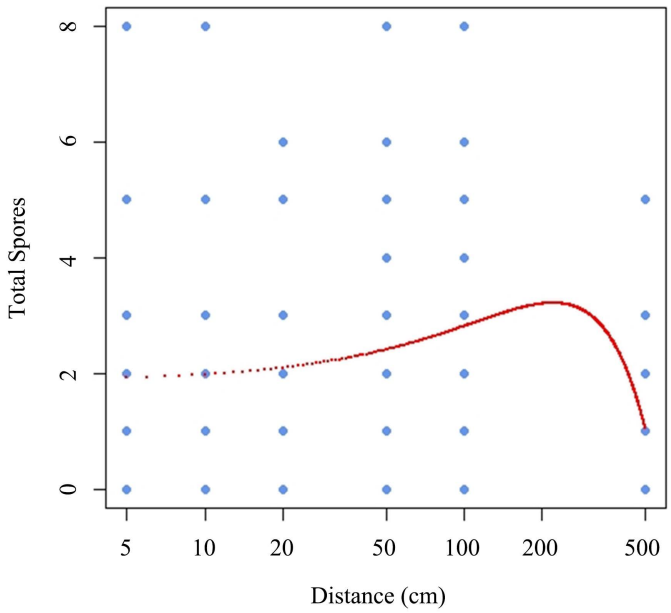


**Figure 4.** Total spores at each distance for the Putty Root orchid

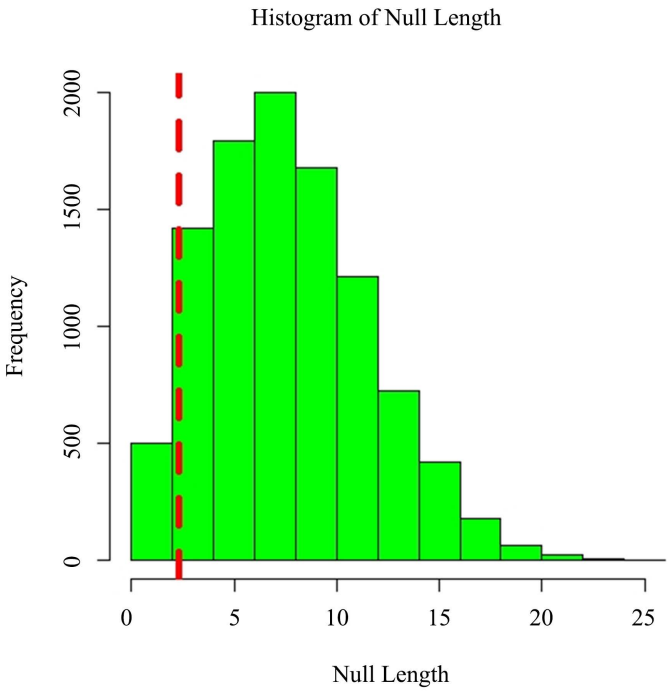


**Figure 5.** Histogram of null length.

## 4. 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 [8]. 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 [1]. 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.* [9] noted that spore density ranged from 5 to 69 spores per gram of soil, with significant temporal and site-specific differences. Alexio *et al.* [10] 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 [10]. This could also explain the low number of spores determined in the current study within the old growth forest of Ginn Woods.

McCoy [1] 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. Toprak *et al.* [11], 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 [12] 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.* [13] 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.* [13] concluded that heavy metal contamination could have a significant impact on mycorrhizal fungal colonization and spore germination, potentially eliminating it.

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, the relative age of the forest stand, or the season samples were collect. Different ecosystems have been known to support different spore densities within soil. For instance, cultivation has been found to reduce mycorrhizal spore diversity [12]. In this study, the genus *Glomus* was the most abundant, with *Acaulospora* being the second most abundant (**Figure 6** and **Figure 7** respectively). Others have noted the high abundance of *Glomus* spores when studying spore density and dispersal in soil [4] [5] [11] [13] [14].

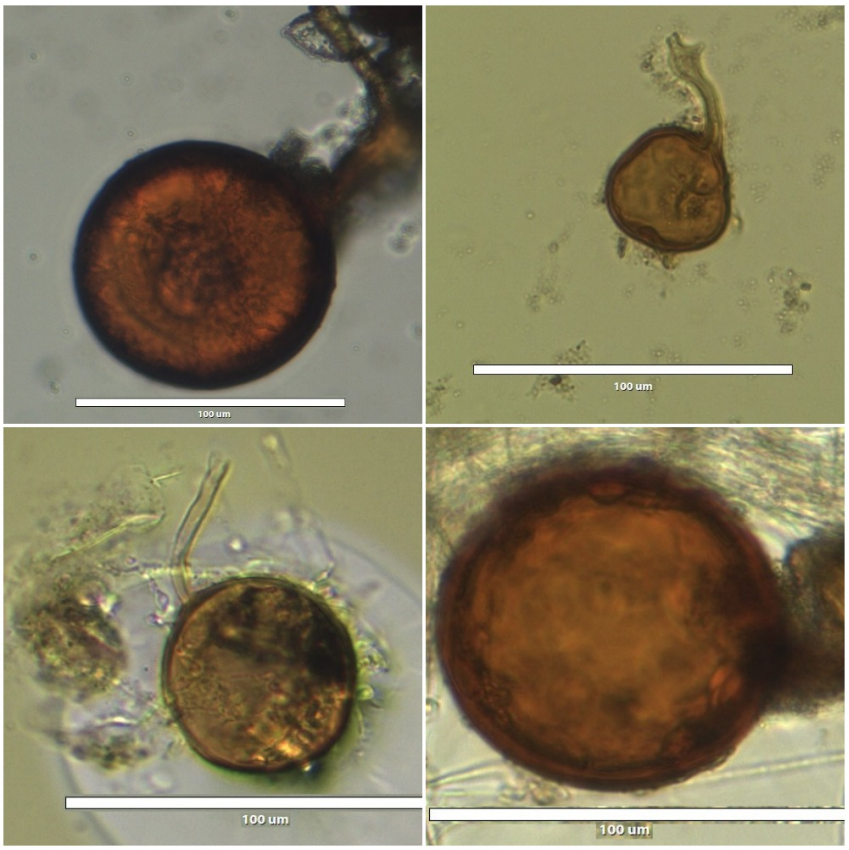


**Figure 6.** Glomus spores.

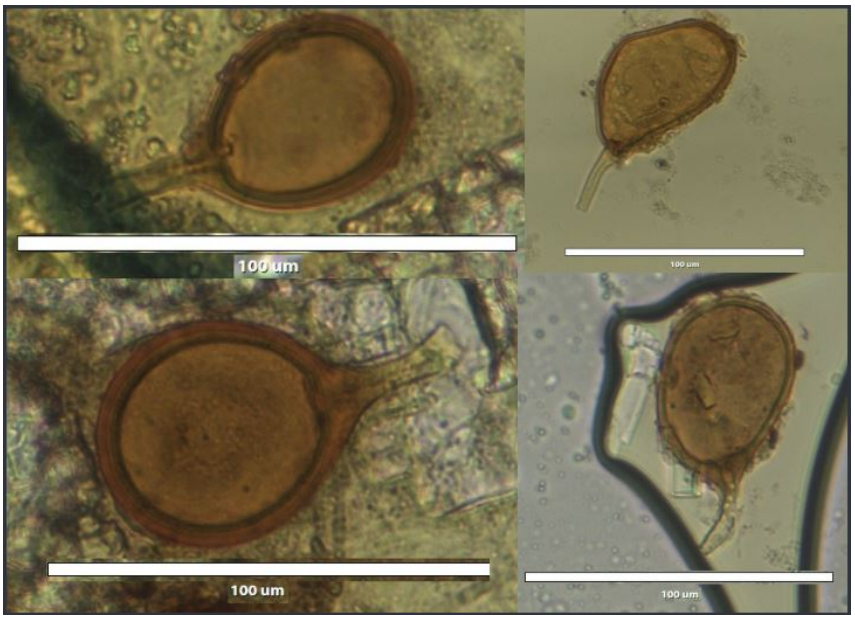


**Figure 7.** Aculaspora spores.

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 8**). Although more spores occurred at the 1-m interval, it is common to find them displaced outside of this parameter.

Researchers have noted that there is little to no correlation between spore abundance in soil and the colonization of roots by mycorrhizal fungi [11]. Paugy *et al.* [14] and Toprak *et al.* [11], 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 [12].

**Figure 8.** Spore dispersion around the Putty Root Orchid.

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 [8]. 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 smaller, local scale of distribution [9]. However, researchers in Africa have found that elephants, or elephant dung more specifically, can act as dispersal agents for mycorrhizal fungi spores [14]. 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 [12].

## 5. Conclusions

The hypothesis that mycorrhizal spore abundance would be highest at the base of the host plant was not supported. Instead, spore abundance was the highest at 1 meter away from the base of the host plant. This could indicate that spores are being released from the hyphal tip of the host plant.

When assessed, directionality of spore abundance was non-existent. Dispersal of spores was shown to be fairly even regardless of cardinal direction.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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