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**The Effect of Phosphite on Mycorrhiza Formation in
American Chestnut (*Castanea dentata*)**

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Departmental Thesis

The University of Tennessee at Chattanooga

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Abstract

The Effect of Phosphite on Mycorrhiza Formation in American Chestnut (*Castanea dentata*)

M. Taylor Perkins

One of the primary hindrances to *Castanea dentata* restoration in the Southeast is the root rot disease caused by the fungus-like microorganism *Phytophthora cinnamomi*. Root rot can be combated by the application of mono- and di-potassium salts of phosphorous acid, which are marketed as phosphite fungicides. Despite its value in preventing infection by *P. cinnamomi* it is also thought that phosphite may impede root colonization by beneficial, ectomycorrhizal fungi. I hypothesized that plants given a routine application of phosphite will display fewer mycorrhizas in the root tips than those plants that were not treated with potassium phosphite. Therefore I attempted to elucidate this potential problem by inoculating *C. dentata* roots with three species of ectomycorrhizal fungi (*Pisolithus tinctorius*, *Scleroderma geaster*, and *Scleroderma citrinum*) in greenhouse and nursery settings. Spores of each species of ectomycorrhizal fungus were inoculated into two groups of twenty plants each. For each fungus species one experimental group was treated with potassium phosphite while the second was given no potassium phosphite, serving as a control. Potassium phosphite was administered in an aqueous solution sprayed directly onto the potting medium in the manufacturer recommended concentration of 2.4 g phosphite L⁻¹. The effect of biweekly potassium phosphite application on mycorrhiza formation was studied by measuring the degree of fungal colonization of root tips. Observably, trees given a routine phosphite treatment exhibited a lesser degree of mycorrhizal formation. Statistical tests supported this observation; mycorrhizas are negatively affected by phosphite when applied in the manufacturer-recommended dosage.

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Introduction

The fungus-like oomycete *Phytophthora cinnamomi* Rands is one of the main hindrances to American chestnut (*Castanea dentata* [Marshall] Borkhausen) breeding programs through its role as the causative agent in root rot disease (Jeffers *et al.*, 2007). The use of mono- and di- potassium salts of phosphorous acid (referred to in the literature as phosphite) has been shown to mitigate *P. cinnamomi* symptoms in chestnuts (*Castanea*; plant family Fagaceae) and is the currently prescribed method for treating the disease (Barilovits, 2009; Gentile *et al.*, 2009). Although phosphite is valuable for its role in preventing *P. cinnamomi* infection, it is thought that it may also hinder colonization of the roots by beneficial, ectomycorrhizal fungi. My experiment sought to elucidate this problem with a study of the effects of phosphite treatments on roots of Chinese-American hybrid chestnut seedlings that were inoculated with three different species of ectomycorrhizal fungi. Using the hypothesis that mycorrhiza formation is negatively affected by phosphite, I tested whether chestnut seedlings given the manufacturer recommended dosage of phosphite would possess fewer mycorrhizas in their root tips. In a simple comparison of averages, I found that trees given a routine phosphite treatment exhibited a lesser degree of mycorrhiza formation, which suggests that mycorrhizas are negatively affected by phosphite in the manufacturer recommended dosage.

I. Literature Review

A. The American Chestnut

Little more than a century ago the American chestnut was one of the most valued trees of North America's sylvia. It is thought to have made up 25% or more of the eastern hardwood forests (Burnham, 1988) and contributed a distinct and important ecological niche to these forests. American chestnut could be found from Maine and southern Ontario to southern Alabama and Mississippi but attained its greatest size in the Appalachian mountains of western North Carolina and eastern Tennessee (Sargent, 1905). On a walk in the Appalachian forest, one could see American chestnut from the lowlands to elevations over 1,500 meters (5,000 feet) on the higher mountains (Kephart, 2008; Woods and Shanks, 1959).

Commonly, mature chestnut trees were between 1 meter (3 feet) to 1.5 meters (5 feet) in diameter and 18 meters (60 feet) to 27 meters (90 feet) in height (Detwiler, 1915). In exceptional cases, trees of 3.7 meters (12 feet) in diameter (Peattie, 2007; Sargent, 1905) and 40 meters (131 feet) in height were recorded (Burnham, 1988).

Due to the many positive qualities of its timber and seeds, the American chestnut may well have been the most important hardwood species in North America (Hardin et al., 2001). Since the wood was straight grained, easy to split, and readily worked with hand tools, it was put to a wide range of uses (Burnham, 1988; Merkle and Brown, 1992). The wood seasoned well and was extremely decay resistant due to the tannins contained in the wood and bark (Anagnostakis, 1987). As a result it was

used where the extremes of weather would compromise a lesser wood. It was utilized for buildings, telegraph and utility poles, crossties, mine props, and fence posts (Merkle and Brown, 1992). Stories abound of the persistence of structures manufactured with chestnut wood. Despite being more than a century old, remnants of many farm structures made of chestnut lumber can still be seen at old homesteads across the mountain and hill country of North Georgia (Merkle and Brown, 1992).

The more subtle qualities of chestnut wood made it practical for smaller tasks as well. Chestnut lumber had an attractive grain, and a soft, reddish golden brown luster when properly finished (Merkle and Brown, 1992). This trait made it useful for furniture, interior trim, musical instruments, caskets, and paneling (Burnham et al., 1986; Merkle and Brown, 1992). The abundant tannins mentioned above were the best available for leather tanning, making the chestnut vital to the leather tanning industry (Burnham, 1988).

American chestnut trees yielded a high quality nut crop nearly every year (Burnham et al., 1986; Hebard, 2005). Research by Minser et al. (1995) explored American chestnut's role as the primary mast producer for wildlife in some areas. The abundant nut crop was useful to humans both nutritionally and economically. The annual collection of chestnuts made it an important cash crop to many Appalachian families (Burnham et al., 1986). Boxcar loads were sent to large eastern cities where they were roasted and sold by street vendors. It is thought that the loss of the chestnut crop added to the hardships caused by the Great Depression in the 1930s in eastern North America (Burnham, 1988).

B. The Decline of the American Chestnut

In the 19th and early 20th centuries, the American chestnut was dealt a double blow by introduced pathogens that would drastically affect its role in eastern North American forests (Schlarbaum et al., 1999). The first of these, *Phytophthora cinnamomi*, was reported in American chestnut populations as early as 1824 (Crandall et al., 1945 in Schlarbaum et al., 1999). *Phytophthora cinnamomi* (a fungus-like oomycete) is the causative agent in root rot disease. This introduction resulted in mortality of chestnuts in low, moist areas and reduced its natural range (Schlarbaum et al., 1999).

The next great injury to the American chestnut came in the early 20th century when a new fungal pathogen was introduced inadvertently to North America on Japanese chestnut seedlings (Anagnostakis, 1987). This introduction found a defenseless host in the American chestnut (Kendrick, 2000). In 1904, a chestnut blight disease caused by the exotic fungus *Cryphonectria parasitica* (Murrill) Barr, was discovered in New York Zoological Park by chief forester H.W. Merkel. Two years later, he estimated that 98% of all the American chestnut trees in the Bronx were infected (1906). In less than ten years, the disease had spread throughout New York, Massachusetts, New Jersey and Pennsylvania (Merkle and Brown, 1992).

Within 40 years, the chestnut blight disease was found throughout the entire natural range of the American chestnut (Griffin, 2000). Practically all the mature American chestnut trees had been killed, though living roots of some trees continue to send up sprouts that are almost always killed by the fungus before they begin to bear

seeds (Burnham, 1988; Kendrick, 2000; Merkle, 1992). It is chiefly in this form that American chestnut survives in the wild today (Paillet, 2002).

C. The American Chestnut Restoration Effort

Soon after *Cryphonectria parasitica* began to kill trees in the northeast scientists began the search for ways of saving the remaining American chestnut trees and restoring the populations that had been lost. The methods that are used today come to us after nearly a century of experimentation. Current North American chestnut research is ultimately focused on the restoration of the American chestnut (Burnham et al., 1986). This requires a two-part plan of action that involves exploring biological control of the chestnut blight disease and breeding the trees for resistance to the fungus (Craddock, 1998).

Biological control of the blight relies on the use of hypovirulent strains of *Cryphonectria parasitica*. In these strains, the fungus itself is infected with virus-like double-stranded RNA elements that reduce the pathogenicity (or virulence) towards the plant (Koonin et al., 1991). Like trees fully affected by blight, trees infected with a hypovirulent strain of *Cryphonectria* will still display a canker. However, the canker caused by a hypovirulent strain will be noticeably less severe and slower growing. In this situation the tree has the ability to heal itself (Anagnostakis, 1987).

A great advantage of hypovirulence as a biocontrol is that it can be applied to trees that are already infected with *Cryphonectria parasitica* and it has been shown to assist the tree in healing damage already done (Merkle and Brown, 1982). This method is so effective that it is thought that hypovirulence is responsible for the

waning of chestnut blight in Europe during the second half of the twentieth century (Jaynes and Elliston, 1980). However due to some obstacles, namely vegetative incompatibility, there has been difficulty in utilizing hypovirulent strains with the same success experienced in Europe (Anagnostakis, 1983; Chen and Nuss, 1999).

While breeding has been used in the restoration effort since the early 20th century, it was not until the early 1980s that an approach was implemented that would lay the foundation for restoration success today (Diskin et al., 2006). In 1981 Charles R. Burnham and colleagues began a backcross breeding program that was designed to incorporate the blight resistance genes of Asiatic chestnut species with the desirable morphological characteristics of the American chestnut (Burnham et al., 1986). Up to this point efforts by the U.S. Department of Agriculture, the Connecticut Agricultural Experiment Station, and private nurserymen had met with little success in merging these traits in one viable tree. The backcross breeding plan became the basis of the chestnut breeding program of the non-profit organization The American Chestnut Foundation (TACF; Diskin et al., 2006).

The backcross method begins by crossing a blight resistant Chinese chestnut (*Castanea mollissima* Blume) with a blight susceptible but otherwise satisfactory American chestnut (Burnham, 1988; Burnham et al., 1986, Diskin et al., 2006). The progeny of this cross are theoretically $\frac{1}{2}$ Chinese chestnut and $\frac{1}{2}$ American chestnut and will exhibit partial blight resistance (Burnham et al., 1986). At this point a series of backcrosses with American parents are carried out to regain desirable American traits such as shape, size, and growth habit while maintaining the blight resistance of

the Chinese ancestor (Diskin et al., 2006). At each step of backcrossing, resistant trees are selected by injecting chestnut blight fungus into the stem of progeny and observing canker symptoms (Hebard, 2005). Trees that show the most resistance are selected for use in subsequent steps. At the end of three backcrosses, the American complement to the genome should average 15/16 (94%) (Diskin, 2006; Hebard, 2005).

Despite their desirable morphological traits, the final backcross progeny [dubbed the Backcross 3 generation (B3)] will show varying levels of resistance; some individuals will be blight susceptible while others will be resistant (Hebard, 2005). To recover trees homozygous (alleles coding for blight resistance are present on all homologous chromosomes) and true-breeding (yielding only progeny that are blight resistant) for blight resistance, the B3s were intercrossed with other B3s and selected for resistance (Hebard, 1994). A second intercross will take place between the B3 progeny to further ensure blight resistance. The resulting tree, a Chinese-American B3F3 hybrid, theoretically possesses 94% of the American chestnut genome while incorporating Chinese chestnut genes that confer the highest blight resistance available from the Chinese ancestor. It is the aim of The American Chestnut Foundation that the B3F3 hybrid will be the vehicle by which American chestnut tree is reintroduced to Appalachian forests (Hebard, 2005).

D. The Mycorrhiza

The term mycorrhiza (which means “fungus-root”) was first coined in 1885 by German biologist A.B. Frank to describe a structure that is a union of two different organisms, plant and fungus, into a single organ (Frank, 2005). In its essence a mycorrhiza is the symbiotic relationship formed by a filamentous fungus that has grown around the roots of a host plant (Kendrick, 2000). It is an organ of nutrient exchange where nutrients absorbed from the soil by the fungus are translocated to the plant and photosynthetic products are passed to the fungus (Kendrick, 2000; Norris et al., 1994). A large number of experiments have determined that fungi assist in the uptake of phosphate and nitrogen compounds from the soil while all or most of the carbon compounds in the mycorrhiza are provided by the plant host (Norris et al., 1994).

The mycorrhizal relationship between plant and fungus is so prevalent, it is thought that over 90% of all higher plant species typically form mycorrhizas (Peterson et al., 1984). Since the discovery of mycorrhizas numerous experiments have been devised to ascertain mycorrhizas’ effect on plant development. The conclusion arrived upon by many is that mycorrhizal plants grow faster than non-mycorrhizal ones (Norris et al., 1994). This has had profound implications in studies of plant physiology and ecology. Researchers have found that some plants experience diminished growth without a fungal symbiont (Kendrick, 2000). Even when plants can function without mycorrhizas, those that formed these organs need less fertilizer, withstand heavy metal and acid rain better, and grow better on infertile soils of

marginal land, mine spoils, and at high elevations (Kendrick, 2000). In addition, some mycorrhizal fungi are thought to function as a biocontrol against root diseases by providing a barrier between root pathogens and the plant root (Pinnix, 2005).

Many types of mycorrhiza have been described in the literature. The type most commonly formed by the American chestnut is the ectomycorrhiza (Palmer et al., 2008; Rhoades et al., 2003). They are distinguished from other types of mycorrhizas by the mantle (or sheath) formed by the fungal hyphae (the tubular architectural module of fungi) that enclose the root and a Hartig net formed by the penetration of hyphae between cortical cells of the root (Kendrick, 2000; Smith and Read, 1997). Other strands of hyphae branch outward from the mantle into the substrate and obtain nutrients (Kendrick, 2000; Smith and Read, 1997). Some of these hyphae will be present in parallel aggregations that make up a mycelium that serves as an agent for the spread of the fungus throughout the soil (Kendrick, 2000).

Compared to non-mycorrhizal roots, ectomycorrhizal roots will be thicker, of a different color, and much more branched (Goodman et al., 1996; Kendrick, 2000). The variation in color and thickness are due to the mantle of hyphae formed around the root while increased branching is caused by plant growth hormones produced by the fungus (Goodman et al., 1996; Kendrick, 2000; Smith and Read, 1997).

Morphological aspects of the mantle, emanating hyphae, and outer root structure are regarded as the most informative features when characterizing ectomycorrhiza, while the Hartig net is ultimately the diagnostic feature for ectomycorrhiza presence (Brundrett, 2008; Kendrick, 2000; Norris et al., 1994).

E. Phytophthora cinnamomi

Phytophthora is a genus of fungus-like oomycetes that contains many plant pathogenic species (Barilovits, 2009). They are responsible for a large number of plant diseases that have had drastic ecological and economic effects around the world. *Phytophthora* spp. have been known to affect oak forests in Spain and North America, soybean in North America, cacao trees in West Africa, alder trees in western Europe, eucalyptus in Australia, and a number of other plants around the world (Chen and Zentmyer, 1970; Howard, 2001; Howard et al., 2000).

Probably the most famous victim of *Phytophthora* infection was the Irish potato crop in the moist, cool summers of the years 1845-1847. After having been separated from its host 250 years earlier, *Phytophthora infestans* (Montagne) de Bary was unintentionally introduced to Ireland from North America (Solomon, 2008). Conditions were ideal for this water mold and it caused potato tubers to rot in the fields. The destruction of the potato crop resulted in a famine that was responsible for massive emigration and a death total estimated between 250,000 and 1 million people (Solomon, 2008).

One member of this genus, *Phytophthora cinnamomi*, presents one of the most formidable obstacles to American chestnut restoration (Rhoades et al., 2003). Research has shown that *P. cinnamomi*, the causative agent in root rot disease, is close to 100% fatal to pure American chestnuts. Given this, the failure of many plantings has been attributed to this pathogen (Barilovits, 2009). Symptoms include

root and collar rot, branch dieback, and defoliation prior to the ultimate death of the tree (Rhoades et al., 2003).

The difficulties presented by root rot disease have caused researchers to look for a solution in the resistance to root rot carried by Asian chestnut species (Jeffers et al., 2007). Another solution is the use of systemic fungicides, chiefly among these phosphite (Barilovits, 2009). Such fungicides have been used for years to prevent *Phytophthora* infection in other species (Cohen and Coffey, 1986).

F. Phosphite

Solutions containing salts of the anionioic form of phosphonic acid (HPO_3^{2-}) are the currently prescribed remedy for *P. cinnamomi* infection (Barilovits, 2009; Hardy et al., 2001; Howard, 2000). They are marketed as “phosphite fungicides” and referred to in much of the literature as “phosphite” (Brunings et al., 2005). Although structurally different from phosphonate, this is a term used by some researchers when referring to this chemical (Brunings et al., 2005).

Phosphite’s mode of action is a complex process that directly acts on the pathogen while indirectly stimulating the plant’s defenses. After uptake, phosphite is translocated in both the xylem and phloem (Hardy et al., 2001). In the phloem phosphite is trapped and translocated throughout the plant in association with photo-assimilates in a source-sink manner (Hardy et al., 2001). What results is a strong and rapid defense response by the plant that stops pathogen spread (Hardy et al., 2001). Application of phosphite may take the form of trunk injections, foliar spray, and soil drench (Howard et al., 2001).

Despite phosphite's positive traits, there is evidence that it possesses certain phytotoxic characteristics (Hardy et al., 2001; Howard, 2000; Howard et al., 2001). Researchers have found that it accumulates in the area of the root tips colonized by ectomycorrhizal fungi (Howard et al., 2001). In some cases, this results in necrosis of these fine root tips which causes a reduction in sites for mycorrhiza formation (Howard et al., 2001). In addition, damage to the roots can cause changes in root exudates. This can affect the soil microflora, particularly those bacteria that positively interact with mycorrhizas (Howard et al., 2001). With these factors in mind, researchers have recently begun to investigate what problems this may pose for plant growers.

II. Materials and Methods

A. Experimental Groups

My study consisted of eight experimental groups that received various combinations of ectomycorrhizal fungus spores and phosphite treatments (Table 1). The experimental groups were arranged in a way that would allow me to discern what effect the phosphite application would have on mycorrhiza formation in *Castanea dentata* seedlings. The independent variable, phosphite, was applied in a simple manner that would display an effect on the dependent variable, mycorrhizal root tips, if any effect existed. Plants that were given a routine phosphite treatment were expected to possess fewer mycorrhizas than plants that were not given phosphite.

Groups A and B were inoculated with *Pisolithus tinctorius* (Pers.) Coker and Couch, a known ectomycorrhizal associate of American chestnut (Grand, 1976). Group A was given a routine dose of phosphite administered via an aqueous solution. Group B received no phosphite throughout the growing season.

Groups C and D were inoculated with *Scleroderma geaster* Fr. *Scleroderma geaster* has not been documented as an ectomycorrhizal associate of American chestnut but several other species in the *Scleroderma* genus have been documented to form this relationship with American chestnut (Palmer et al., 2008; Pinnix, 2005). Group C was given phosphite while Group D received no phosphite.

Groups E and F were given no fungus inoculum. Group E was given phosphite and Group F was not given phosphite. After these groups were established I discovered that specimens of *Scleroderma citrinum* Pers., a known ectomycorrhizal

symbiont of American chestnut (Palmer, 2006), were also available in the UTC Herbarium. This fungus was used to inoculate groups G and H. Group G was treated with phosphite while Group H received no phosphite.

B. Planting and Inoculation

This study was conducted at the University of Tennessee at Chattanooga greenhouse and nursery beginning in the spring of 2011. On March 10, 2011 I planted 160 fourth-backcross hybrid chestnuts supplied by The American Chestnut Foundation. All pots were washed beforehand to prevent contamination by ambient fungal spores and pests. The seeds were the progeny of a CH297 × TNHAM1 cross. The mother tree, CH297, is an American-Chinese hybrid grown at The American Chestnut Foundation's Meadowview Research Farms. The father tree, TNHAM1, is a surviving naturally-occurring American chestnut found near the town of Signal Mountain in Hamilton County, Tennessee.

The seedlings were grown individually in 7.65-L containers [Stuewe & Sons TP812 Treepots (Corvallis, Oregon)]. The potting medium was Metro Mix Southern Perennial Mix manufactured by Sun Gro Horticulture (Vancouver, British Columbia). The medium consists of pine bark, Canadian Sphagnum peat moss, perlite, starter nutrient charge (with gypsum), slow release nitrogen, and dolomitic limestone.

I inoculated the experimental groups with spores of ectomycorrhizal fungi on March 18, 2011. All spores were isolated from fungus specimens collected locally in oak woodlands and stored in the UTC Herbarium. I used a dry suspension that consisted of spores collected from fungus fruiting bodies mixed into potting medium.

I began this procedure by scraping the spores from the fruiting body of the fungus. The *Pisolithus tinctorius* fruiting body yielded 4.3 g of spores. The *Sclerodera geaster* fruiting body yielded 6.3 g of spores. Two specimens of *Scleroderma citrinum* yielded 1 g of spores. Kendrick (2000) found that less than 1 mg of spores are required to inoculate a plant. Given this, the amount of spores I extracted would be sufficient to ensure successful inoculation.

I thoroughly mixed the spores of each species into 5 L of soil. Next, I mixed approximately 177 mL (0.75 cup) of the each inoculum into the potting medium around each appropriate seed. I watered the seeds to assist spore dispersal throughout the container. I repeated this procedure for all three species of fungus and their respective experimental groups while taking care to avoid contamination of seeds by any unwanted spores. This meant thoroughly cleaning the work area and mixing bucket between applications of each fungus species.

C. Phosphite Treatments

On April 14, 2011 I began the application of phosphite to the appropriate experimental groups. I used Alude Systemic Fungicide manufactured by Cleary Chemical Corporation (Dayton, New Jersey). Alude contains 45.8% mono- and di-potassium salts of phosphorous acid. I diluted this to the manufacturer recommended concentration of 4 tsp Alude/gallon of water (5.21 mL/L). The resulting solution was 2.4 g phosphite L⁻¹. This was sprayed into the potting medium until drenched. I repeated application of this solution every two weeks throughout the growing season until root harvesting began in October.

D. Root Collection

I began root sample collection in October 2011 by severing first-order lateral roots from the area directly below the root crown. Root samples were collected and cleaned using techniques practiced in standard mycorrhiza research (Goodman et al., 1996; Norris et al., 1994; Smith and Read, 1997). From each plant, approximately five roots of at least 10 cm in length were severed using fine tip scissors from just below the root crown collar (epicotyl) region. Next, I gently washed the roots under cold water to remove substrate while taking care to preserve mycelial strands and rhizomorphs. Then the root samples were packed into petri dishes with a moist piece of cardboard, and stored inside a refrigerator until the completion of sampling and the initiation of quantifying. In those plants that were lacking in root vigor, fewer roots were collected to avoid any significant impairment to the plant's survival.

Unfortunately, many of the root samples developed mold and a second root collection had to be taken in February 2012. During this round of sampling, the root collar diameter of each plant was measured. A recent study by Clark et al. (2010) found that root collar diameter has a high correlation to nursery seedling quality and first year field performance in American chestnut plantings. In my experiment, these data were used to explore a potential correlation between mycorrhiza formation and root collar diameter. An analog caliper was used to measure the diameter to the nearest 0.1 mm approximately 2.5 cm (1 inch) above the root collar.

E. Quantification of Ectomycorrhizal Associations

After I completed root harvesting, I evaluated the main and interactive effects of phosphite application and fungal inoculation type on mycorrhizal success. Many techniques have been developed for mycorrhizal quantification. Mycorrhizal research is a rapidly growing field, with new plant-fungus mycorrhizal relationships constantly being discovered. This, combined with a wide range of mycorrhiza morphologies, has spurred the development of a wide range of techniques, each designed to accurately characterize the various types of mycorrhizal relationships present in nature. The method I used was a combination of techniques already utilized by researchers. I sought to use a technique that was easily repeatable, that minimized error in measurements, and that would allow for comparison of results with those of previous research.

According to Brundrett (2008; 2009), most researchers quantify ectomycorrhizal associations by counting the short root tips that have formed ectomycorrhizas (using superficial dissecting microscope examinations). As recommended by Goodman et al. (1996), I immersed the root in a petri dish filled with water. In root tips that were ectomycorrhizal, I noted a characteristic thickening caused by the fungal mantle that enveloped the root tip. In addition, ectomycorrhizal root tips could be noted by a mantle that was markedly different in color than nonmycorrhizal root tips. In many cases, the mantles of ectomycorrhizal root tips would possess other features such as emanating hyphae, rhizomorphs, and mycelial strands that could be used to make a positive diagnosis for ectomycorrhizas.

I counted the number of ectomycorrhizal second-order lateral roots per length of first-order lateral root. A particular second order lateral root may have possessed one or a number of ectomycorrhizal third order root tips but in the interest of efficiency a second order root that was mycorrhizal was simply given a value of 1 in my calculations. For each root, my observations were characterized in units of the number of 2° lateral roots that possess ectomycorrhiza / centimeters of 1° lateral root length. An average for each plant was calculated using the values of each root. An average value per treatment was assigned using the individual plant values.

Because many plants were host to more than one type of fungus, I performed this procedure a number of times on each root. Each repetition would focus on one fungus species. This process was made easier by the fact that ectomycorrhizas of most fungus species displayed sharply contrasting morphological characteristics. Current mycorrhizal researchers cite the mantle (in surface view), rhizomorphs/mycelial strands, and emanating hyphae as the primary features used to characterize ectomycorrhizas (Norris et al., 1994; Smith and Read, 1997). Many times the color and texture of the ectomycorrhizal mantle could be used to quickly characterize an ectomycorrhiza of interest. In spite of an extensive search of the primary literature and databases, I could find no photographs of ectomycorrhizas formed between American chestnut and the three species of fungus inocula. This led me to assign a morphotype code to each distinct species of fungus observed. Assigning a morphotype code allowed me to differentiate between the types of

ectomycorrhiza while removing the time consuming task of species identification that is not within the scope of my project.

After counting mycorrhizal 2° lateral roots I calculated the average number of mycorrhizal 2° roots per 10 cm of 1° lateral root in each experimental group. I performed an analysis of variance (ANOVA) to determine the main and interactive effects of phosphite treatment and fungal inoculants on ectomycorrhizal development. Dr. Boyd used SPSS (IBM, Armonk, New York) to perform the analysis of variance (ANOVA). Dr. Boyd, Dr. Shaw, and Dr. Craddock assisted me in interpreting the results of the various ANOVA tests.

III. Results

A. Overview

Two ectomycorrhizal fungus species of distinctly different morphologies were commonly observed in the experimental groups. The first species was given the designation Morphotype I (Figure 1). Roots displaying Morphotype I colonization possessed a thick mantle of lustrous white fungal hyphae encasing the root tips. Using criteria outlined by Goodman et al. (1996), I classified the mantle branching pattern as unbranched (the mycorrhiza is confined to a single root tip) on some roots or irregular (the mycorrhiza encompasses multiple root tips and is without a main axis) on more extensively colonized roots. In many cases, hyphae and mycelial strands could be seen emanating from the mantle. The mycelial strands displayed a color similar to that of the mantle and were made up of densely packed hyphae growing perpendicular from the mantle.

The second type of ectomycorrhiza, Morphotype II, displayed a mantle that was chocolate brown to black in color (Figure 2). Hyphae that make up the mantle were more densely woven around the root than those of Morphotype I. The mantle branching pattern was unbranched or monopodial pyramidal (a mycorrhiza with an axis from which branches originate that are shorter than the axis and lie in 3 or more planes). Emanating hyphae and mycelial strands were observed but less common in this morphotype.

B. Degree of Mycorrhiza Formation

The average number of mycorrhizal 2^o roots per 10cm of 1^o lateral root for Morphotype I, Morphotype II, and total mycorrhizas are given in Table 2. The standard error for both morphotypes and total mycorrhizas in each experimental group are given in Table 3. The significance of differences between groups treated with phosphite and groups given no phosphite can be found in Table 4. In tests of between-subjects effects fungus inoculum type had no significant effect on total mycorrhizas ($P = .630$). Phosphite had a significant effect on total mycorrhizas ($P \leq .001$). The results of an ANOVA test used to calculate the significance of differences between groups treated with phosphite and groups given no phosphite are given in Table 4. The difference between phosphite and no phosphite groups in the *Scleroderma citrinum* and no fungus inoculum groups was significant ($P \leq 0.001$ in both cases). The difference in groups inoculated with *Scleroderma geaster* was not significant ($P = 0.152$).

C. Root collar diameter

The average root collar diameter of each treatment group can be found in Figure 5. The effect of phosphite on root collar diameter was not statistically significant ($P = 0.206$). The effect of fungus inoculum on root collar diameter was highly significant ($P \leq 0.001$).

D. *Pisolithus tinctorius*

For the reasons mentioned above, the number of replicates in groups inoculated with *Pisolithus tinctorius* (Groups A and B) was greatly reduced. As a

result they were of no statistical value. It is worth noting however that the remaining plants from groups A and B possessed ectomycorrhizas of a distinctly different morphotype than those observed in the other experimental groups (C, D, E, F, and G).

IV. Conclusion

In every case, those plants treated with phosphite exhibited fewer mycorrhizas per root than plants that were not given phosphite (Figure 3). This difference in groups inoculated with *Scleroderma geaster*, groups C and D, is not statistically significant however ($P = 0.152$). This does not support the assertion that phosphite had an effect on the difference in mycorrhizas in groups inoculated with *Scleroderma geaster*.

The difference between groups inoculated with *Scleroderma citrinum* (groups G and H) was statistically significant ($P \leq 0.001$). This suggests that phosphite had an effect on the number of mycorrhizas present in groups inoculated with *Scleroderma citrinum*. The greatest difference in mycorrhizas was seen in the groups that were given no fungus inoculum, groups E and F. A P -value less than 0.001 supports the assertion that phosphite had an effect on the difference seen here.

The drastic difference in mycorrhizas between groups E and F is interesting in that it reveals the phosphite effect on plants that naturally acquired fungus in the nursery. Unlike groups intentionally inoculated with spores (groups C, D, G, and H) groups E and F came into contact with presumably naturally-occurring fungi or they were contaminated during inoculation of the other groups in the greenhouse. These results show a strong phosphite effect on ambient fungal colonization of the root tips. In groups C, D, G, and H it is possible that the high number of spores introduced to the potting medium may have “overpowered” phosphite’s effect on fungus colonization. We might conclude that groups E and F provide a more accurate

representation of phosphite's effect on mycorrhiza formation in typical greenhouse grown seedlings.

When examining the root collar diameters amongst experimental groups, the statistical tests suggest that the phosphite treatments had no effect on root collar diameter ($P > 0.05$). However a highly significant P -value for the effect of fungus inoculum on root collar diameter was observed ($P \leq 0.001$). Specifically, plants inoculated with *Scleroderma geaster* had greater root collar diameter than plants inoculated with *Scleroderma citrinum* or no fungus (Figure 5). This suggests a relationship between fungus inoculum and root collar diameter. This is especially interesting when viewed in light of the work done by Clark et al. (2010) on root collar diameter as the single most important predictor for outplanting success in American chestnut. Considering this, more research on mycorrhizal inoculum and seedling root collar diameter is warranted.

Overall, the data above suggest that phosphite affects the amount of mycorrhiza formation in greenhouse grown Chinese-American hybrid chestnut seedlings. Greater numbers of mycorrhizas were seen in the groups that were not given phosphite treatment (groups D, F, and H), and this was statistically significant for groups F and H. In conclusion, we can say that the hypothesis is supported by the results.

Ideas for future research in this area may include examining phosphite's effect on already existing mycorrhizal systems. The effect of different phosphite concentrations should also be analyzed. This type of information could provide

valuable information regarding phosphite effects to chestnut growers that use phosphite to combat *Phytophthora* root rot.

V. Tables and Figures

A. Tables

Table 1. Experimental Groups with Treatment Codes, Corresponding Fungi Inoculum, and Phosphite Application.

| Experimental Group | Treatment Code | Fungus Inoculum | Routine Phosphite Application |
|--------------------|----------------|------------------------------|-------------------------------|
| A | A: Pt + P | <i>Pisolithus tinctorius</i> | yes |
| B | B: Pt no P | <i>Pisolithus tinctorius</i> | no |
| C | C: Sg + P | <i>Scleroderma geaster</i> | yes |
| D | D: Sg no P | <i>Scleroderma geaster</i> | no |
| E | E: + P | None | yes |
| F | F: no P | None | no |
| G | G: Sc + P | <i>Scleroderma citrinum</i> | yes |
| H | H: Sc no P | <i>Scleroderma citrinum</i> | no |

Table 2. Average number of mycorrhizal 2^o roots per 10cm of 1^o lateral root length.

| Treatment | Morphotype I | Morphotype II | Total |
|---------------|--------------|---------------|-------|
| C (S.g. + P) | 2.8 | 3.11 | 5.91 |
| D (S.g. no P) | 7.38 | 0.85 | 8.23 |
| E (+P) | 0.87 | 0.64 | 1.51 |
| F (no P) | 2.44 | 9.37 | 11.81 |
| G (S.c. + P) | 0.05 | 5.21 | 5.26 |
| H (S.c. no P) | 0 | 10.11 | 10.11 |

Table 3. Standard error for average number of mycorrhizal 2^o lateral roots per 10cm of 1^o lateral root length. Standard error is provided for Morphotypes I and II as well as the total mycorrhizas. N represents the number of roots observed in each experimental group.

| | Standard Error | | |
|--------------|-----------------------|---------------|-----------------|
| | Morphotype I | Morphotype II | All morphotypes |
| C: S.g. + P | 1.106 (N=35) | 0.758 (N=35) | 1.192 (N=35) |
| D: S.g. no P | 1.155 (N=48) | 0.347 (N=48) | 1.146 (N=48) |
| E: + P | 0.404 (N=39) | 0.304 (N=39) | 0.525 (N=39) |
| F: no P | 1.199 (N=27) | 2.819 (N=27) | 2.761 (N=27) |
| G: S.c. + P | 0.045 (N=66) | 0.628 (N=66) | 0.625 (N=66) |
| H: S.c. no P | 0 (N=61) | 0.759 (N=61) | 0.759 (N=61) |

Table 4. A comparison of mean differences between phosphite treatments within each fungus inoculum. A *P*-value cutoff of 0.05 was used to determine significance.

| Fungus Inoculum | Experimental Groups | Mean difference | <i>P</i> - value |
|-----------------------------|----------------------------|------------------------|-------------------------|
| <i>Scleroderma geaster</i> | C and D | ±2.31 | 0.152 |
| none | E and F | ±10.3 | ≤0.001 |
| <i>Scleroderma citrinum</i> | G and H | ±4.86 | ≤0.001 |

B. Figures

Figure 1. Morphotype I. Thick white, lustrous mantle with mycelial strands (surface view through dissecting microscope). Mycelial strand is denoted by an asterisk.

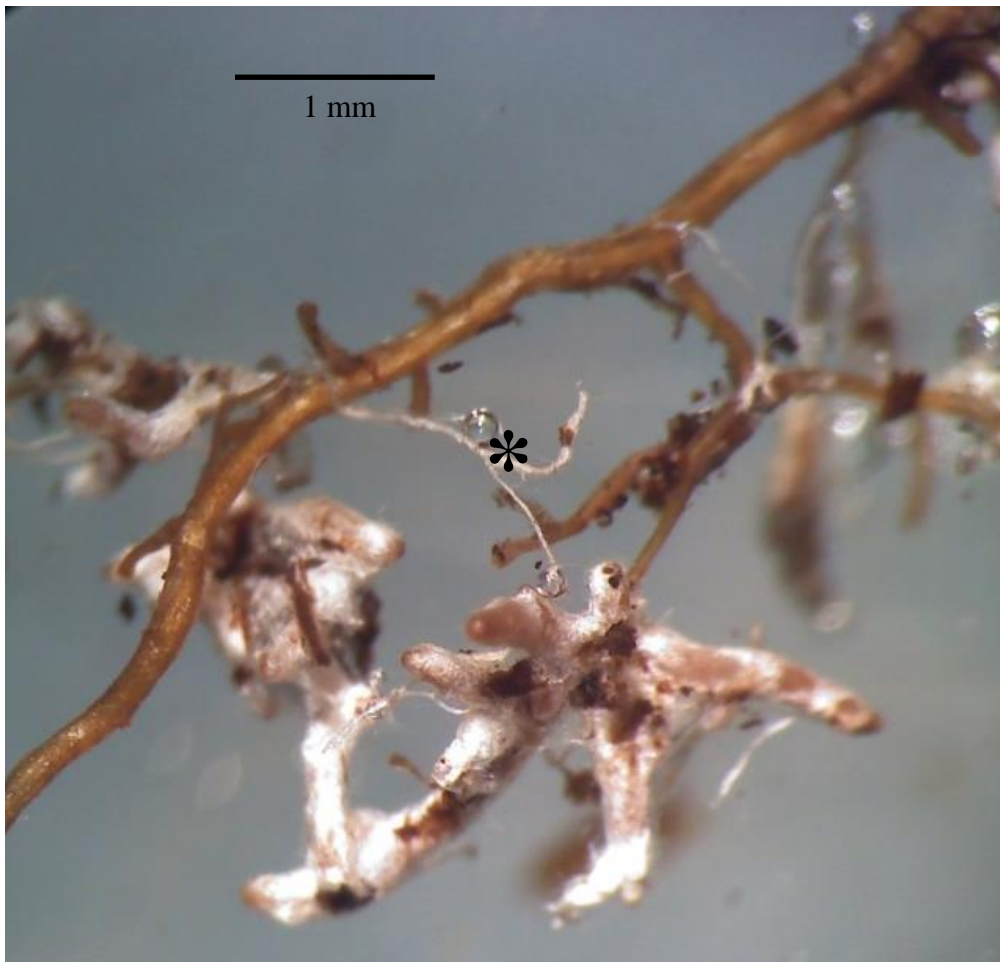


Figure 2. Morphotype II. Thick brown/black mantle with mycelial strand and emanating hyphae (surface view through dissecting microscope). Emanating hypha is denoted by an arrow.



Figure 3. Average number of mycorrhizal 2° lateral roots per 10cm of 1° lateral root length. Bars indicate \pm Standard Error. Columns having the same letter indicate no significant difference ($P \leq 0.05$). Experimental treatments:
 C: S.g. + P = *Scleroderma geaster* plus phosphite; D: S.g. no P = *S. geaster* without phosphite; E: + P = no fungus inoculum plus phosphite;
 F: no P = no fungus inoculum and no phosphite;
 G: S.c. + P = *Scleroderma citrinum* plus phosphite; H: S.c. no P = *S. citrinum* without phosphite.

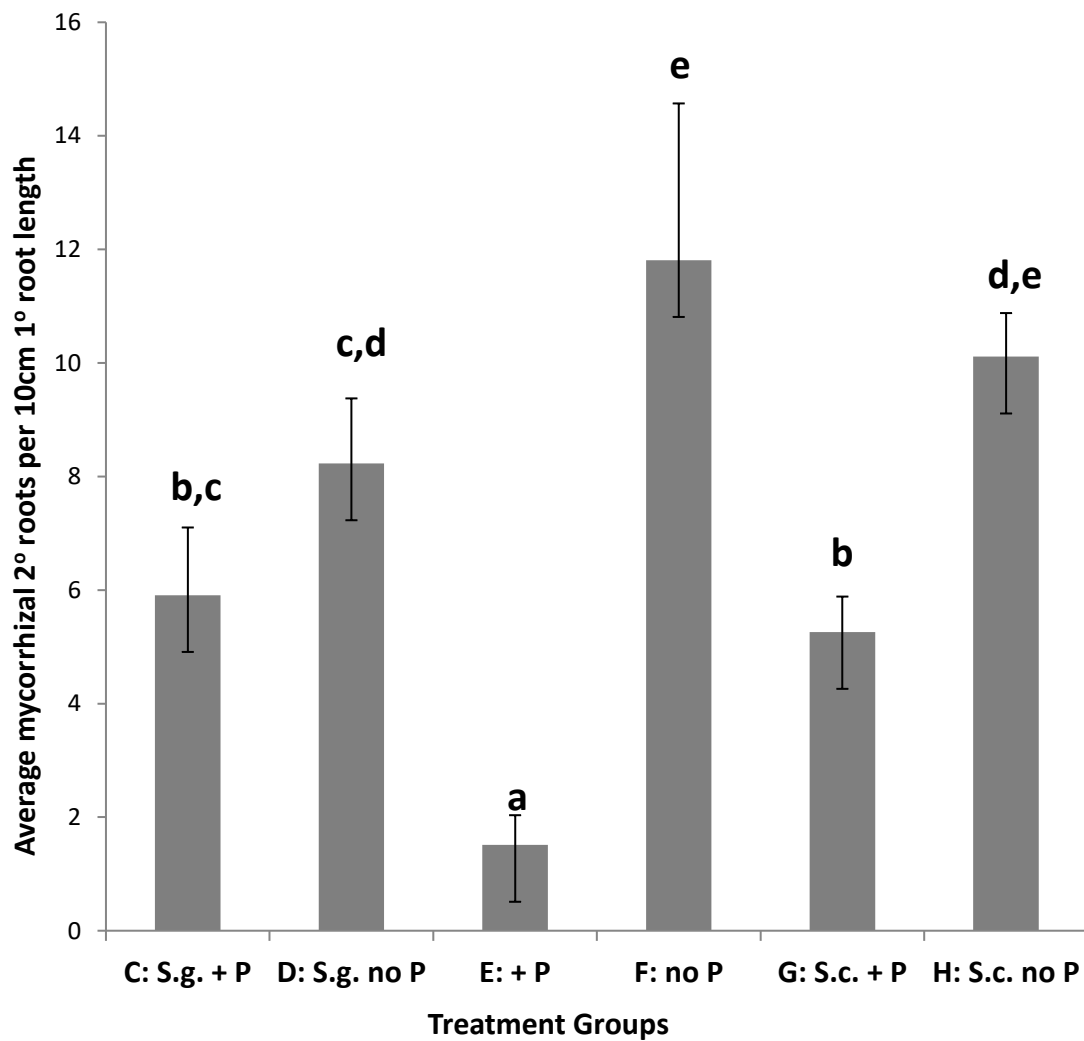


Figure 4. Average number of mycorrhizal 2° lateral roots per 10cm of 1° lateral root length with the frequency that each morphotype was observed.

Bars indicate \pm Standard Error. For each morphotype, columns having the same letter indicate no significant difference. For each experimental group, the asterisk means a significant morphotype difference exists ($P \leq 0.05$). Experimental treatments: C: S.g. + P = *Scleroderma geaster* plus phosphite; D: S.g. no P = *S. geaster* without phosphite; E: + P = no fungus inoculum plus phosphite; F: no P = no fungus inoculum and no phosphite; G: S.c. + P = *Scleroderma citrinum* plus phosphite; H: S.c. no P = *S. citrinum* without phosphite.

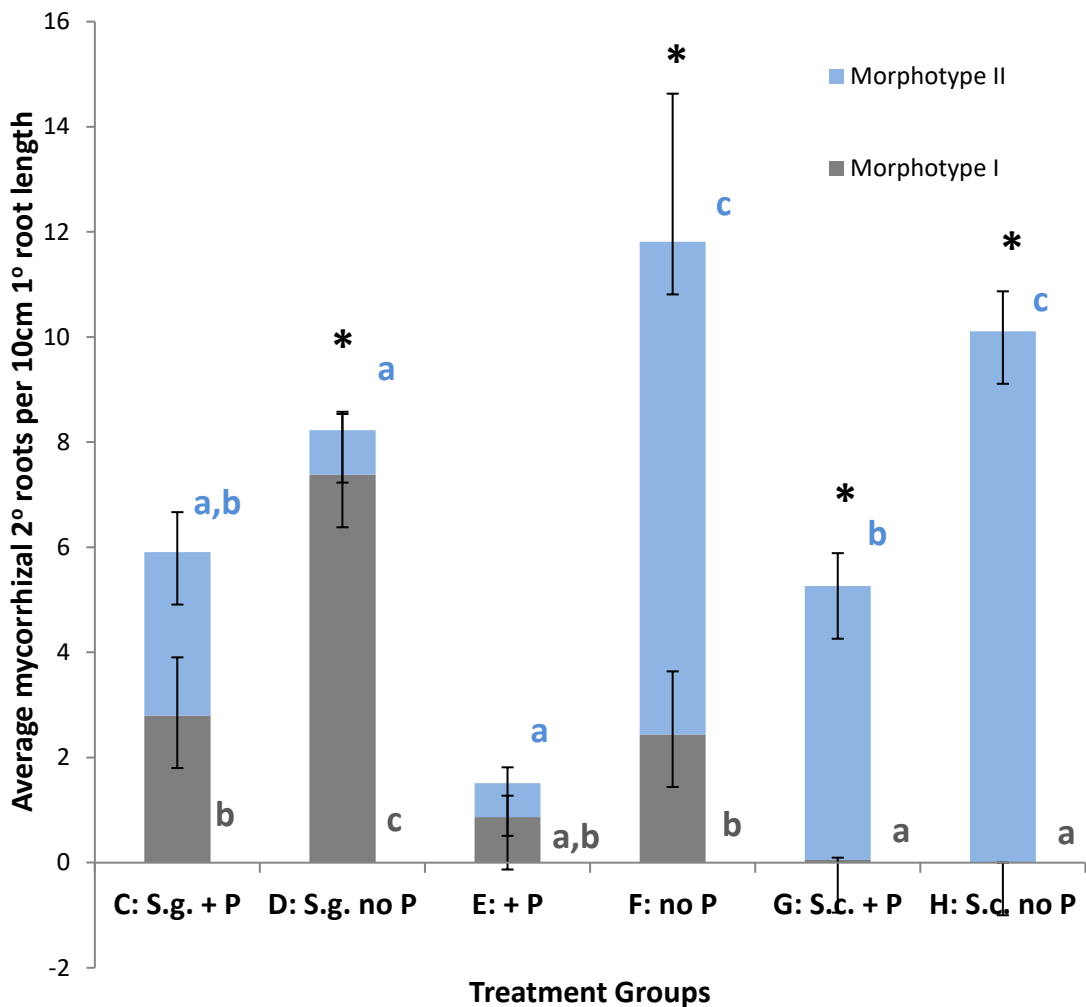
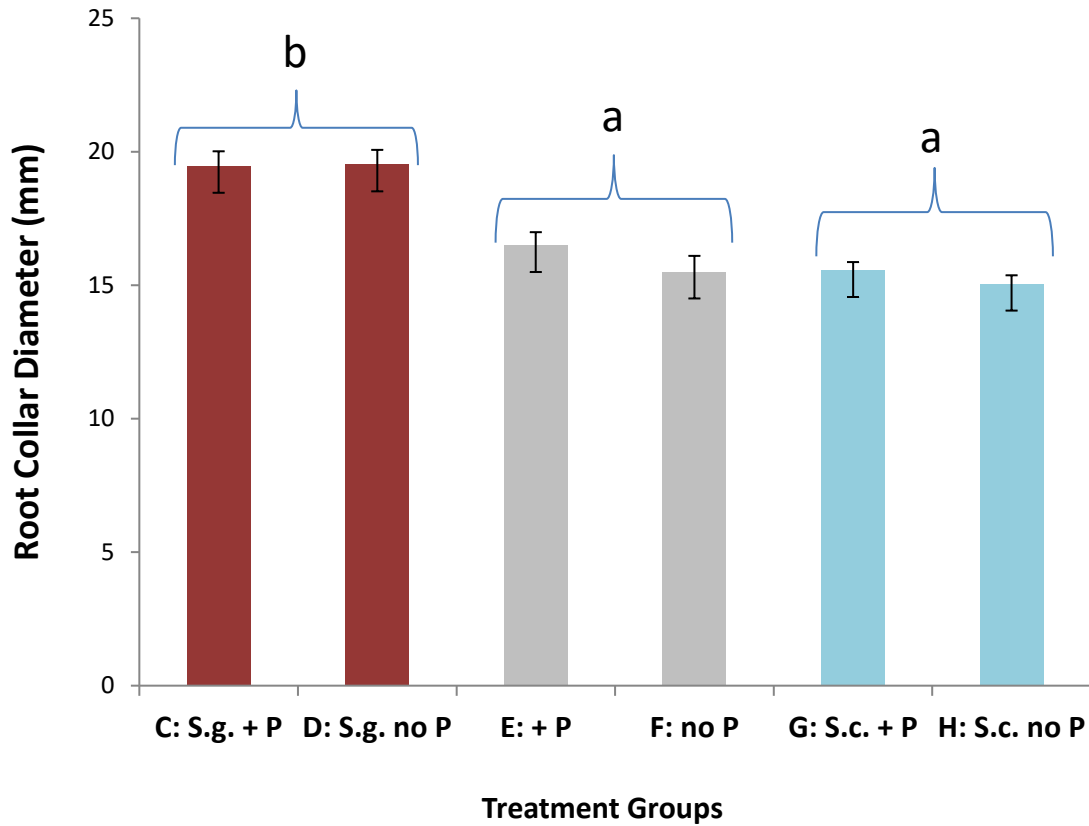


Figure 5. Average root collar diameter (mm). Experimental treatments: C: S.g. + P = *Scleroderma geaster* plus phosphite; D: S.g. no P = *S. geaster* without phosphite; E: + P = no fungus inoculum plus phosphite; F: no P = no fungus inoculum and no phosphite; G: S.c. + P = *Scleroderma citrinum* plus phosphite; H: S.c. no P = *S. citrinum* without phosphite. Colors correspond to different fungus inocula. Fungus inoculum types with the same letter indicate no significant difference.



VI. References

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