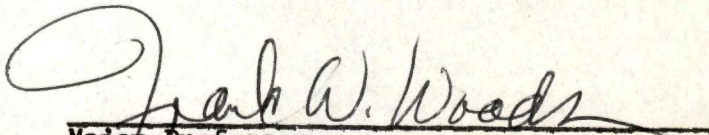


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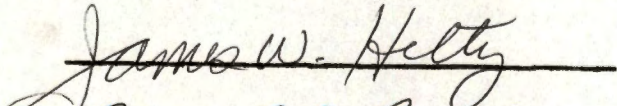
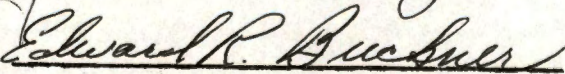
October 1972

To the Graduate Council:

I am submitting herewith a thesis written by Richard H. Martin entitled "The Protective Effect of Mycorrhizae Common in East Tennessee on Loblolly Pine (Pinus taeda L.)." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science with a major in Forestry.


Major Professor

We have read this thesis
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Accepted for the Council:


Vice Chancellor for
Graduate Studies and Research

CRANESHOP REST

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THE PROTECTIVE EFFECT OF MYCORRHIZAE COMMON IN EAST TENNESSEE ON
LOBLOLLY PINE (Pinus taeda L.)

A Thesis
Presented to
the Graduate Council of
The University of Tennessee

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
Richard H. Martin
December 1972

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ABSTRACT

Six loblolly pine stands in East Tennessee were surveyed for fungi possibly mycorrhizal with loblolly pine. Fifty-three species of fungi were collected and identified including Boletus brevipes, Boletus luteus, Cenococcum graniforme, Laccaria laccata, and Pisolithius tinctorius which have previously been shown to be mycorrhizal with loblolly pine. Pure cultures of twelve of the collected species were obtained and tested in synthetic culture with loblolly pine. Agaricus placomyces, Amanita rubescens, Amanita yerna, and Boletus bicolor entered into mycorrhizal relationships with loblolly pine.

Five mycorrhizal fungi, Amanita rubescens, Boletus bicolor, Cenococcum graniforme, Laccaria laccata, and Pisolithius tinctorius, were assayed in plate antagonism tests against thirteen soil-borne pathogens. The presence of mycorrhizal fungi inhibited the growth of the pathogens in 70% of the tests. Boletus bicolor displayed the strongest antagonism inhibiting 90% of the pathogens tested.

Boletus bicolor, Laccaria laccata, and Pisolithius tinctorius were used to test the protection provided live roots by mycorrhizae from pathogens. Sterile loblolly pine seedlings were grown, inoculated with mycorrhizal fungi, and subsequently inoculated with pathogens. Using height growth as an indicator, it was concluded that two of the three mycorrhizal fungi used protected the seedlings from attack by Sclerotium bataticola, Fomes annosus, and Rhizoctonia solani.

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INTRODUCTION

During the past few years, several of the more powerful and widely used pesticides employed in forest management have been either restricted or banned by federal, state, or local governments. It seems probable that others will meet the same fate in the future. In spite of these restrictions, crop producers are faced with the necessity of producing food and fiber for a constantly increasing population.

This situation has recently produced much interest in the use of biological control. Several investigators have demonstrated that many saprophytic soil organisms are antagonistic toward root pathogens (Ark and Hunt, 1941; Allen and Hansler, 1935; Vasudera, 1930). However, attempts to use saprophytic soil organisms for control of root disease have been disappointing. Either there has been difficulty establishing the antagonistic organism or the antagonistic effect was lost in the soil (Garrett, 1956).

Recently interest has been shown in the use of mycorrhizae as protection against root pathogens. Studies by Hacskeylo and Snow (1959), Worley and Hacskeylo (1959), and Hacskeylo (1961) on the relation of mycorrhizae to light, fertililizer, moisture, and pesticides indicate that the number and type of mycorrhizae may be controlled by management practices. Furthermore, it has been theorized that any antagonistic principle produced by mycorrhizal fungi would be in an ideal site to protect roots from pathogens (Marx, 1965).

This investigation is an initial effort to look into the possible use of mycorrhizae as a means of biological control of root disease in East Tennessee. The objectives of this study may be outlined as follows:

- 1) identify the important species of fungi which are mycorrhizal with loblolly pine (Pinus taeda L.) in East Tennessee, 2) determine the potentially important soil-borne pathogens in this same area, and 3) evaluate the antagonistic effects of several of the common mycorrhizal fungi toward soil-borne pathogens.

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CHAPTER I.

REVIEW OF LITERATURE

Since the discovery of the mycorrhizal phenomenon by Frank in 1885, the importance of ectotrophic mycorrhizae has been realized. Reports from the Philippine Islands, Australia, the Dutch East Indies, and Puerto Rico have confirmed the viewpoint that ectotrophic mycorrhizae are essential for the survival of many species of trees (Olicios, 1932; Young, 1936, 1937a; Roelolfs, 1930; Mikola, 1969). In all of these cases attempts at aforestation failed until plantings were inoculated with mycorrhizal fungi. Experiments in nursery soils (Mitchell et al., 1937) and with prairie soils prompted Hatch (1937) to conclude that, "... in all but fertile agricultural soils, pines and other trees which form ectotrophic mycorrhizae are incapable of existing without mycorrhizae."

The literature contains abundant evidence of the universality of mycorrhizae. McArdle (1932), Hatch and Doak (1933), Pessin (1928), and Lohman (1927) have all reported that mycorrhizae are of widespread and habitual occurrence in forest trees. After an extensive survey of the above and other literature on the subject, Hatch (1937) concluded that, "there can no longer be any doubt that mycotrophy is widespread and... represents the normal density of all short roots of trees grown in forest and many other soils." This conclusion is supported by other authors, including Harley (1959) and Trappe (1969).

The role ectotrophic mycorrhizae play in plant growth has been

studied extensively over the past few decades. It has generally been concluded that ectotrophic mycorrhizae increase the absorbing area of the root system (Harley, 1959). Experiments such as those by McComb (1938, 1943), Hatch and Doak (1933), and Mitchell et al. (1937) have shown that mycorrhizal seedlings have more extensive root systems than those of non-mycorrhizal seedlings. The surface area of the absorbing root is also increased by mycorrhizal infection (Harley, 1959). Hatch (1937) attributed this increase to a greater degree of branching, addition of the fungal sheath, and to the larger diameter of mycorrhizal roots.

Increased ion absorption due to mycorrhizal infection has been shown by several authors. Hatch (1937), using pine seedlings in prairie soil, found higher percentages of nitrogen, potassium, and phosphorous in mycorrhizal than in non-mycorrhizal roots. Kramer and Wilber (1949) reported a greater accumulation of P-32 in mycorrhizal roots than in non-infected roots. Cohen (1954) noted greater rates of accumulation of Ca-45, St-90, and P-32 in mycorrhizal roots of loblolly pine (Pinus taeda L.), while Wilson (1957) found that beech (Fagus sylvatica L.) mycorrhizae more readily absorb alkali metals than non-mycorrhizal roots.

It has also been reported that mycorrhizae make unavailable minerals, especially those containing phosphorous, more readily available to plants (Routien and Dawson, 1943; Levisohn, 1956; Rosendahl, 1942).

It has been suggested that mycorrhizae play an important role in nutrient cycling. Harley (1959) has indicated that mycorrhizae may be important in "trapping" very soluble nutrients leached from litter.

Stark (1969) has gone one step further and viewed trees, mycorrhizae, and litter as a closed system in the tropics.

That mycorrhizae may play an important role in biological resistance to disease was probably first suggested by Davis et al. (1942). A hypothesis developed by Zak (1964) proposed several ways by which ectotrophic mycorrhizae may prevent infection: first, by utilizing excess root carbohydrates and other root exudates; second, by supporting a rhizosphere association antagonistic to pathogens; third, by providing a mechanical barrier via the fungal mantle and Hartig net; and fourth, through the production of antibiotics.

Marx (1965) has done considerable work investigating the extent and mode of protection provided by ectotrophic mycorrhizae. He has shown inhibition of a number of pathogens by mycorrhizal fungi in culture, and has shown conclusively that mycorrhizal roots are resistant to infection by Phytophthora cinnamomi Rand. Marx concluded that this resistance was due to antibiotic production and to the physical barriers provided by the fungal mantle and the Hartig net.

I. MYCORRHIZAE AND ROOT DISEASE

Over the past few years there have been several reports that mycorrhizae protect roots from disease. Davis et al. (1942) in a study of forest tree nursery stock, suggested that mycorrhizae may be beneficial by preventing infection by weak pathogens attacking the short roots. Levishon (1954), in a study of Scotch pine (Pinus sylvestris L.) and a Rhizoctonia-like fungus, reported that infection by this fungus prevented

normal mycorrhizal development. In addition, she felt that pathogenicity was dependent on the mycorrhizal status of the seedling. Seedlings with abundant mycorrhizae appeared to be immune to infection. Marks (1965) similarly reported that an unidentified pathogen causing late damping-off of sugar pine (Pinus lambertiana Dougl.) was less virulent when mycorrhizae were well developed.

Redmond (1955), in a study of birch die-back, indicated that the lack of mycorrhizae was a significant factor in the disease. He indicated that an increase in temperature may inhibit the formation of mycorrhizae, thus leaving the roots susceptible to infection. Zak (1964) suggested that a great many "decline" diseases may be explained by the indirect effect of physical factors, such as moisture and temperature, on mycorrhizae formation thereby leaving the roots susceptible to infection.

Saesk (1967) has studied the protection afforded young pine seedlings by mycorrhizal fungi. He found that, under laboratory conditions, a number of mycorrhizal fungi prevented infection by damping off fungi such as Rhizoctonia solani Kühn., Pythium debaryanum Hesse., and Fusarium oxysporum Schl. emend. Syd. & Hans.

The theory that mycorrhizae protect roots from pathogens has been best developed by Zak (1964). He noted several ways through which mycorrhizae may afford protection to roots: first, by the production of antibiotics; second, by favoring protective rhizosphere organisms and utilizing surplus root exudates; and third, by providing a physical barrier via the fungal mantle and Hartig net. To this, Marx (1965) added the possibility that infection by mycorrhizal fungi may induce

the formation of antibodies in the host plant.

II. PRODUCTION OF ANTIBIOTICS BY MYCORRHIZAE

Since the discovery by Duchesne (1897) that some strains of Pennicillium are capable of repressing the growth of certain species of bacteria, a great number of fungi have been found to possess antibiotic properties. Brian (1951) acknowledged 775 species of fungi in which antibiotics have been found to be active.

Numerous fungi have been found to exert antagonistic effects on other fungi. This phenomenon has been of particular importance in connection with the study of plant diseases. A number of workers have reported antagonism of saprophitic soil fungi toward damping off and root rot fungi (Ark and Hunt, 1941; Allen and Hansler, 1935; Vasudeva, 1930; Weinding, 1932, 1934). Included among the pathogens shown to be inhibited are species of Fusarium, Armillaria, Phytophthora, and Pythium. A reduction in the severity of several diseases has been correlated with the presence of saprophytes in the soil (Waksman, 1945; Garrett, 1956).

Basidiomycetes have been found to be a fruitful source of antibiotics. Wilkins and Harris (1944) surveyed extracts from the sporophores of 700 species for the presence of antibiotic activity. Of those surveyed, 70 species were found to be strongly active and 100 weakly so. Brian (1951) reviewed 1665 species of basidiomycetes which had been tested in culture antagonism tests. Of these, 570 were listed as strongly antagonistic.

Among the genera in which antibiotic activity has been found

are Boletus, Amanita, Lactarius, Inocybe, Russula, and Lycoperdon. These genera include many species of mycorrhizal fungi (Trappe, 1964a).

Few surveys have been carried out on mycorrhizal species. These, however, have shown considerable antibiotic activity. Santoro and Casida (1962) assayed Amanita rubescens Fr., Amanita muscaria Fr., Amanita caesarea Fr., Boletus bicolor Peck., and Boletus luteus L. using acetone and chloroform extracts of pure cultures. Some antibiotic activity was noted for all of these fungi when assayed against bacteria. Paxillus involutus Fr., listed by Trappe (1964a) as a mycorrhizal fungus, has been shown to produce an antibacterial agent in agar plate tests (Robbins et al., 1945). Rypacek (1960) recently reported antagonism of Boletus variegatus Swartz ex Fr. toward Fomes annosus (Fr.) Cke.

Bryan (1960) tested several species mycorrhizal on pine for antagonism toward Phytophthora cinnamomi. Some cultures were not antagonistic while others, particularly species of Boletus, strongly suppressed P. cinnamomi. Krywolap et al. (1964) in a study of an antibiotic produced by Cenococcum graniforme (Sow.) Fer. & Winge, found antagonism against gram positive bacteria. Vaartaja and Salisbury (1965) reported antagonism of Boletus granulatus L. towards a number of virulent pathogens including species of Pythium, Phytophthora, and Fusarium.

Marx has done an extensive survey of antagonism of mycorrhizal fungi towards pathogens. In agar plate tests, Laccaria laccata (Scop. ex Fr.) Berk. & Br., Lactarius deliciosus Fr., Leucopaxillus cerealis var picienus (Peck) Singer & Smith, Pisolithius tinctorius (Pers) Coker & Couch, and Boletus luteus inhibited nearly half of the 48 fungal root

pathogens tested. L. cerealis was found to inhibit 92% of the pathogens tested. Marx identified the inhibiting compound from L. cerealis as a diatrene nitrile. In additional studies, Marx concluded that antibiotic production was an important factor in the protection of shortleaf pine (Pinus echinata Mill.) mycorrhizae from infection by Phytophthora cinnamomi (Marx, 1965).

Saesk and Musilek (1968) demonstrated some antagonism of mycorrhizal fungi toward pathogens when in culture with living pine seedlings. These investigators assayed 17 species of mycorrhizal fungi grown on porous plates in conjunction with Scotch pine seedlings against 12 pathogenic fungi. The mycorrhizal fungi inhibited the growth of the pathogens in 12% of the cultures.

The theory that mycorrhizal antibiotics may be transferred to other portions of the root system has been investigated by Krywolap et al. (1964) and by Grand and Ward (1969). An antibiotic produced by Cenococcum graniforme was found to be present in the roots and foliage of Eastern white pine (Pinus strobus L.), red pine (Pinus resinosa Ait.), and Norway spruce (Picea abies (L.) Karst.) bearing mycorrhizae of the type formed by this fungus (Krywolap et al., 1965). Grand and Ward (1969) confirmed these results and found that extracts of the antibiotic from the foliage were effective in inhibiting Bacillus cereus Cohn. However, they were unable to correlate the strength of antagonism with abundance of Cenococcum mycorrhizae found on root samples, and because trees without Cenococcum mycorrhizae were not available, the presence of antibiotic could not definitely be attributed to Cenococcum graniforme.

II. MYCORRHIZAE, RHIZOSPHERE, AND ROOT DISEASE

Since the introduction of the term by Hiltner in 1904, the rhizosphere of plants has been studied intensively, primarily because it harbors a population of organisms vastly different from the rest of the soil (Kratznelson, 1960). In addition the rhizosphere has been found to be intimately involved in root pathogen relationships (Rovira, 1953).

Garrett (1956) has described the rhizosphere as the plants outermost defensive barrier against invasion by pathogenic fungi. A number of papers have reported diseases which are significantly ameliorated by an antagonistic rhizosphere (Garrett, 1956; Kratznelson, 1965; Walker, 1965; Scroth and Hildebrand, 1964).

Few authors have investigated the effect of mycorrhizae on the rhizosphere. Tribunskayo (1955), investigating the rhizosphere of pine seedlings, reported that the rhizosphere of mycorrhizal roots harbored ten times as many fungal species as non mycorrhizal roots. Neal et al. (1964), working with three morphologically distinct mycorrhizae on douglas fir, found that different mycorrhizae harbored a different microflora and that all differed from the microflora of non-mycorrhizal roots. Kratznelson et al. (1962) reported that mycorrhizal roots of yellow birch stimulated a number of groups of soil bacteria and actinomycetes in the rhizosphere while suppressing total fungal numbers. It is interesting to note that fungal genera such as Pythium and Fusarium predominated in non-mycorrhizal rhizospheres while mycorrhizal roots harbored largely saprophytic genera.

While there is no conclusive evidence that mycorrhizae increase the

antagonistic effect of the rhizosphere, Zak (1964) and Marx (1965) have suggested that the rhizosphere of mycorrhizal roots may be a more effective barrier to attack by pathogens than that of non-mycorrhizal roots.

Roots are known to exude many substances, including a number of nutrients and simple carbohydrates, into the rhizosphere. A number of plant pathogens are chemotactically attracted by these exudates and many must exist saprophytically in the rhizosphere before invading roots (Scroth and Hildebrand, 1964). Mycorrhizae may reduce the virulence of pathogens by utilizing these root exudates. Marx (1965), by inoculating isolated rootlets of shortleaf pine, found that the zoospores of Phytophthora cinnamomi were much less frequent on the surface of mycorrhizal roots than on non-mycorrhizal roots. In other studies, Marx found that hyphae of P. cinnamomi were less frequent and more loosely attached to mycorrhizal roots than non-mycorrhizal roots.

Saesk (1967) found that several mycorrhizal fungi which showed no antibiotic activity in culture tests prevented infection of living seedlings by damping off fungi. He presented the possibility that these mycorrhizal fungi may successfully compete for essential nutrients in the rhizosphere thus preventing infection.

III. MYCORRHIZAE AND ROOT PRODUCED ANTIBIOTICS

Roots of a number of orchid species have been found to produce antibiotics in response to invasion by symbiotic fungi. Bernard (1911), in a study of symbiosis between orchids and Rhizoctonia repens Bernard, felt that an antibacterial agent was formed in the orchid tubers in

response to the fungal symbiont. Gaumann, in further studies, reported that orchids of several species produce orchinol, coumarin, and an unidentified phenolic compound in response to infection by R. repens (Gauman et al., 1960). In addition to inhibiting the symbiont, these antibiotics have been found to inhibit a number of pathogenic fungi as well as bacteria. These antibiotics may provide protection from pathogens through out the life of the plant (Harley, 1965).

Similar antibiotics have not been reported in plants forming ectotrophic mycorrhizae. A number of authors, however, have proposed that the host plant responds to symbiotic infection by producing an antibiotic which plays an important role in maintaining the mycorrhizal fungus in a symbiotic state (Melin, 1963; McDougal and Dufenroy, 1944). Inhibitory compounds which limit mycorrhizal fungi to the cortex of the root may also be active in preventing pathogenic infection (Marx, 1965).

IV. MYCORRHIZAE AS MECHANICAL BARRIERS

Under natural conditions an ectotrophic mycorrhizae includes a tightly woven mantle which covers the root tip and the cortex of the short root. The thickness of the mantle varies but it is normally 20-40 microns thick (Harley, 1959). In addition, the cortical cells are completely surrounded by hyphae.

Zak (1964), discussing mycorrhizae and root disease, noted that most root diseases enter root systems through young unuberized roots. A number of decline diseases apparently involve only these portions of the root system. Mycorrhizal fungi, by quickly infecting young roots, may form

a mechanical barrier which protects them from pathogens.

There is very little proof that the fungal mantle and the Hartig net are formidable barriers; but, to establish infection, a potential pathogen must be capable of penetrating fungal cells as well as the root cortex. Few fungi have the dual ability to digest both the chitin of fungal cells and the cellulose of plant cells. Boosalis (1964), in a review of hyperparasitism, mentioned only two species which are myco-parasites as well as being root pathogens; these being Rhizoctonia solani and a species of Pythium. Furthermore, few parasites have been observed attacking mycorrhizae except for parasitic nematodes (Marx, 1965).

Marx (1965) has been the only author to consider the role of the mechanical mycorrhizal barrier in root pathogen relationships. He attributed at least part of the protection of shortleaf pine roots from infection by Phytophthora cinnamomi to the barriers provided by the fungal mantle and the Hartig net.

V. ROOT DISEASES OF LOBLOLLY PINE

During the last decade seedling production in the Southeast alone has increased to over 500 million seedlings per year. More than 95% of these are loblolly and slash (Pinus elliotii Engelm.) pine (Hodges and Ruehle, 1969). This increased production, often accomplished by increased seedling density, has brought about an increased awareness of root disease problems (Hodges, 1962a).

A great deal of study has been devoted to seedling root diseases, especially in the western yellow pines. Rhizoctonia solani, Fusarium

oxysporum, Fusarium moniliforme Sheld., Fusarium solani (Mart.) Appel. & Wr. emend. Snyder & Hansen, Phytophthora cinnamomi, Phytophthora cactorum (Lebert & Cohn.) Schroeter, Pythium debaryanum, Pythium irregulare Buism, Pythium alphani-dermatum (Edson.) Fitzp., Pythium vexans de By., Pythium ultimum Trow., Pythium spinosum Sawada., Pythium splendens Braun, Botrytis cinera Pers., and Sclerotium bataticola Taub. are among the most commonly mentioned pathogens associated with root rot of yellow pine seedlings (Tint, 1945; Setliff and Hocking, 1966; Cierczak, 1967; Teakle, 1960; Gravett, 1931; Vaartaja and Bumberis, 1964, 1967; Vaartaja, 1967; Vaartaja et al., 1961; Rathburn, 1922, 1923; Davis et al. 1942; Hartly, 1921; Crandall and Hartley, 1938; Spaulding, 1914; Culderone, 1967). Garrett (1970) and Hartley (1921) consider these pathogens to be unspecialized parasites with a wide host range. A number of these pathogens have been isolated from southern forest soils (Table 1) and they are considered as potential parasites on all southern pines (Hodges, 1962b; Campbell and Hendrix, 1967; Hendrix and Campbell, 1968; Campbell, 1951, Miller et al., 1951).

While there is a great mass of literature on the root diseases of the southern pines, most of it is descriptive and on control measures. In general, unidentified species of Fusarium, Pythium, and Rhizoctonia are associated with root rot in southern pine nurseries. Rhizoctonia solani is the only species mentioned with regularity (Hodges, 1962; Boyce, 1961; Hodges and Ruehle, 1969).

A complex of Pythiaceous fungi, which apparently forms a continuous series, has been indicated as a cause of damping off and root

Table 1. Pathogens isolated from Southern forest soils.

Species isolated	Reference
<u>Pythium irregulare</u> Buism.	Campbell and Hendrix (1967)
<u>Pythium debaryanum</u> Hesse.	"
<u>Pythium spinosum</u> Sawada	"
<u>Pythium helicoides</u> Drechs.	"
<u>Pythium alphani-dermatum</u> (Edson) Fitzp.	Hendrix and Campbell (1968)
<u>Pythium vexans</u> de By.	"
<u>Pythium sylvaticum</u> Campbell & Hendrix	"
<u>Pythium splendens</u> Braun.	"
<u>Phytophthora cinnamomi</u> Rand.	Campbell (1951)
<u>Sclerotium bataticola</u> Taub.	Miller et al. (1957)
<u>Fusarium moniliforme</u> Sheld.	"
<u>Fusarium oxysporum</u> Schl. emend. Syd. & Hansen	"
<u>Pythium ultimum</u> Trow.	Hendrix and Campbell (1968)

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rot of loblolly pine. This series includes Pythium spinosum, Pythium irregulare, Pythium debaryanum, Pythium ultimum, and Pythium pareocandum Drechs, and intermediate forms have been observed by Biesbrock and Hendrix (1967). Pythium ultimum, Pythium irregulare, and Pythium spinosum have been tested with loblolly pine (Hendrix and Campbell, 1968; Campbell and Hendrix, 1967). P. ultimum and P. irregulare caused severe damping off of loblolly pine. P. spinosum was parasitic but the root rot developing from inoculation was less severe.

A number of other Pythium species including Pythium alphanidermatum, Pythium sylvaticum Campbell and Hendrix, Pythium helicoides Drechs., and Pythium splendens, have been found to cause root rot of loblolly pine seedlings in sterile culture (Hendrix and Campbell, 1968).

Species of Fusarium have not been studied in conjunction with loblolly pine even though Hodges (1962a) has stated that Fusarium species appear to be most commonly associated with damping off of Southern pines. Studies with other hard pines indicate that Fusarium moniliforme, Fusarium solani, Fusarium oxysporum, and Fusarium ventricosum Appel & Wollenw. are among the most virulent species and are probably pathogenic on all yellow pines (Hartely, 1921; Tint, 1945).

Numerous species of Phytophthora have been found associated with root disease of Southern pines. Phytophthora cinnamomi, Phytophthora cactorum, and Phytophthora parasitica Dastur. are the most common (Crandell and Hartley, 1938; Campbell and Hendrix, 1967). P. cinnamomi has been found to be a destructive root parasite on jack (Pinus banksiana Lanb.) Scotch, and red (Pinus resinosa Ait.) pine seedlings (Vaartaja

and Cram, 1956) and has been reported to cause damping off of loblolly pine (Robertson, 1969). Phytophthora cactorum and Phytophthora parasitica have caused severe root disease on a number of conifers including jack and red pine, red spruce (Picea rubens Sarg.) and a number of Western yellow pines (Vaartaja and Cram, 1956).

Rhizoctonia solani has been associated with damping off of pines throughout the Southeast (Hodges, 1962) but, sterile culture tests with loblolly pine have not been made. Tests with a large number of conifers, including Western and Central yellow pines, indicate that R. solani is a virulent pathogen with an extremely wide host range (Tint, 1945; Vaartaja et al., 1961; Hartley, 1921).

Sclerotium bataticola has been found to be involved in several serious seedling diseases of loblolly as well as other Southern pines. In sterile culture tests S. bataticola has been shown to be a cause of damping off (Rowen, 1960). In conjunction with several species of Fusarium, S. bataticola produces black root rot reported by Hodges (1962a) as one of the most destructive root rots which occur in Southern nurseries. Loblolly, along with 11 yellow pines, has been rated as very susceptible to black root rot (Rowen, 1960; Henery, 1953). S. bataticola was also concluded to be involved in a similar disease, charcoal root rot, by Seymour (1969).

Due to the difficulty of studying mature root systems, relatively little is known about root disease in mature pines. A number of authors feel that the pathogens which cause root problems in the seedling stage are not a problem in mature trees. (Boyce, 1961; Smith, 1970). Other

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authors, however, feel that these pathogens may be the cause of "decline" diseases (Zak, 1964; Garrett, 1970).

Little-leaf disease is one of these "decline" diseases. Adverse physical factors, especially high soil moisture, in conjunction with Phytophthora cinnamomi progressively kill the succulent roots and result in a general decline in the vigor of the tree. Little-leaf is most important on shortleaf pine, but it is a problem on loblolly pine throughout the Southeast (Zak, 1957).

Fomes annosus (Fr.) Cke. is one of the few root pathogens of mature Southern pines which has received much attention. Fome annosus attacks all Southern pines and has caused considerable damage in plantations throughout the Southeast (Hodges, 1968; Hodges, 1970). Most of the work with F. annosus indicates that the fungus enters through freshly cut stumps and spreads by means of root grafts and root-root contact (Powers and Hodges, 1970; Ross, 1968). Recent work, however, indicates that soil borne inoculum may be important in this disease. Kuhlman (1969) has found that spores of F. annosus percolate into the soil and feels that the spores may germinate on the root surface and cause infection.

Armillaria mellea Vahl. ex Fr. is a common pathogen on many forest trees although it has not been reported as causing severe damage (Lephart, 1963). Oak is the most commonly reported host of A. mellea but it has been reported on a number of Southern pines (Rankin, 1927).

CHAPTER II.

IDENTIFICATION OF MYCORRHIZAL FUNGI COMMON IN EAST TENNESSEE ON LOBLOLLY PINE

A variety of approaches have been used to identify the fungi which form mycorrhizae. The association of sporophores with tree species has been used extensively by Trappe (1957) and Grand (1968a) in the Pacific Northwest to indicate specific host-fungus relationships. This type of evidence is often supported with the observation of mycorrhizae in intimate association with sporophores. However, Melin's (1936) technique of synthesizing mycorrhizae in sterile culture provides the most indisputable means for confirming a mycorrhizal association (Trappe, 1964a).

In the study reported here, six loblolly pine plantations were surveyed for the occurrence of sporophores of possibly mycorrhizal fungi. Species known to be mycorrhizal with loblolly pine were noted. In addition, pure cultures of as many of the collected species as possible were obtained and tested with loblolly pine using Melin's (1936) synthetic culture technique.

I. METHODS

Survey of pine stands

Six Loblolly pine plantations in East Tennessee were surveyed at intervals for the occurrence of sporophores during the period from October, 1970 through December, 1971.

Locations of these stands, agencies responsible for their establishment, and their approximate ages appear in Table 2. All stands except the one at Rose Island were established on abandoned farmland. The stand at Rose Island was a part of the Rose Island Nursery run by the Hiwassee Land Company.

Sporophores were collected periodically from October, 1970 through December, 1971. Most visits were made in the fall and two to three days after heavy rains. Sporophores were collected from the interior of the stand and only from the soil surface.

Immediately after collections were made, spore prints were attempted and morphological descriptions made. A portion of the sporophores were saved for identification and culture; those remaining were dried and preserved as voucher specimens.

Identifications were confirmed by Professor Emeritus Dr. L. R. Hesler from observation of fresh specimens. Morphological descriptions were also compared with descriptions in the literature (Kaufmann, 1918; Coker and Beers, 1943; Hesler, 1960; Grahm, 1944; McIlvaine and Macadam, 1900).

Synthetic culture of mycorrhizae

Attempts were made to isolate pure cultures from all collected species using the procedure described by Hacskeylo (1951). Sections of fresh tissue were taken from the inside of the pileus or stipe, placed on full nutrient sucrose agar, and incubated at room temperature. Plates in which hyphae protruded directly from the sporophore tissue were

Table 2. Locations, agencies responsible for establishment, and approximate ages of loblolly pine stands surveyed for sporophores.

Location	Established by	Age
Andersonville	Tenn. Valley Auth.	21 yrs.
Lafollette	Tenn. Valley Auth.	21 yrs.
Norris	Tenn. Valley Auth.	15 yrs.
Oak Ridge (1)	Tenn. Valley Auth.	20 yrs.
Oak Ridge (2)	Private owners	14 yrs.
Rose Island	Hiwassee Land Co.	14 yrs.

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retained and subcultures established. Pure cultures of Agaricus placomyces Pk., Amanita citrina (Schaeef.) Roq., Collybia maculate Alb. & Schw., Leucopaxillus laterarius (Peck.) Singer & Smith, Lactarius piperatus Fr., and Russula fragilis Fr. were obtained in this manner. Spores of several species were plated on agar by suspending a portion of the hymenium above it with petroleum jelly. The spores of Amanita citrina and Boletus granulatus germinated, but the resulting cultures failed to grow. Additional cultures were obtained by writing to mycologists and other workers engaged in mycorrhizal research (Table 3).

Cultures were maintained on modified Melin-Norkrans agar solution as described by Marx (1965), (Table 4). Cultures were transferred each month.

The technique recommended by HacsKaylo (1953) and modified by Marx and Zak (1965) was used for synthetic culture of mycorrhizae. Half-gallon mason jars were used as culture chambers. A substrate of 600 ml. of vermiculite mixed with 120 ml. of loosely packed peat moss was added to the jars and saturated with 450 ml. of the nutrient solution prescribed for synthetic culture by Melin (1936) and Norkrans (1949)(Table 5). The solution was adjusted to a pH of 4 prior to autoclaving (HacsKaylo, 1953). The jars were plugged with cotton balls reinforced with cheesecloth and immediately autoclaved for 30 minutes at 250 degrees and 15 lbs. pressure.

Sterile pine seedlings were obtained using seed provided by the Hiwassee Land Company. The seed were surface sterilized with 30% hydrogen peroxide as recommended by Trappe (1961). The seed were soaked in hydrogen peroxide for one hour and plated on full nutrient sucrose agar in petri

Table 3. Source of pure cultures of suspected mycorrhizal fungi.

Fungus	Source
<u>Amanita verna</u> Fr. <u>Amanita parcivolvata</u>	Dr. Larry Grand North Carolina State Univ. Raleigh, North Carolina
<u>Amanita rubescens</u> Fr. <u>Boletus bicolor</u> Peck <u>Cenococcum graniforme</u> (Sow.) Fer & Winge	Dr. Edward Hacskeylo Forest Phys. Laboratory Beltsville, Maryland
<u>Clitocybe gibba</u> Fr.	Dr. Howard Bigelow Univ. of Massachusetts Amherst, Massachusetts
<u>Laccaria laccata</u> (Scop. ex Fr.) Berk & Br.	Dr. Donald Marx Southeast. Forest exp. Sta. Athens, Georgia
<u>Pisolithius tinctorius</u> (Pers.) Coker & Couch.	Dr. Bratislav Zak Forest Service Laboratory Corvallis, Oregon
<u>Paxillus atrotomentosus</u> Fr.	Dr. Shoemaker Plant Research Institute Ottawa, Ontario

Table 4. Contents of modified Melin-Norkrans agar medium.

Ingredient	Quantity
CaCl_2	0.05 gms.
NaCl	0.025 gms.
KH_2PO_4	0.50 gms.
$(\text{NH}_4)_2\text{HPO}_4$	0.25 gms.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.15 gms.
FeCl_3 (1% solution)	1.20 ml.
Thiamine HCl	.003 gms.
Malt extract (syrup)	3.00 gms.
Sucrose	10.5 gms.
Agar powder	15.0 ml.
H_2O (distilled)	1000 ml.

Table 5. Ingredients of Melin-Norkrans solution used in synthetic culture of mycorrhizae.

Ingredient	Quantity
KH_2PO_4	0.05 gms.
CaCl_2	0.05 gms.
NaCl	0.025 gms.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.15 gms.
$(\text{NH}_4)_2\text{HPO}_4$	0.25 gms.
Glucose	2.50 gms.
Thiamine HCl	.001 gms.
H_2O (distilled)	1000 ml.

plates and allowed to germinate. Plates which become contaminated were discarded. Germinated seed with radicles 1-2 mm. long were planted in the center of culture jars, one per jar. At the same time the jars were inoculated with blocks of agar cultured with suspected mycorrhizal fungi. Five seedlings were used to test each suspected fungus. Jars were incubated either on light tables or in a greenhouse waterbath (Figure 1).

Amanita citrina, Amanita parcivolvata Pk., Amanita rubescens Fr., Amanita verna Fr., Agaricus placomyces, Boletus bicolor Pk. Clitocybe gibba Fr., Collybia masulata, Lactarius piperatus, Leucopaxillus laterarius, Paxillus atrotomentosus Fr., and Russula fragilis were tested in this manner to determine their ability to form mycorrhizae with loblolly pine. Five seedlings were left uninoculated for use as controls. In addition, three known mycorrhizal fungi, Laccaria laccata, Cenococcum graniforme, and Pisolithius tinctorius, were tested as a check on technique and to determine the extent of mycorrhizae formation under the test conditions.

Two to four months following inoculation all seedlings were removed and examined for the presence of mycorrhizae. At this time, the substrate in each jar was cultured to confirm the presence of the inoculated fungi and the absence of contamination.

II. RESULTS AND DISCUSSION

Survey of pine stands

Fifty-two species of basidiomycetes were collected and identified from the six loblolly pine plantations surveyed (Table 6).

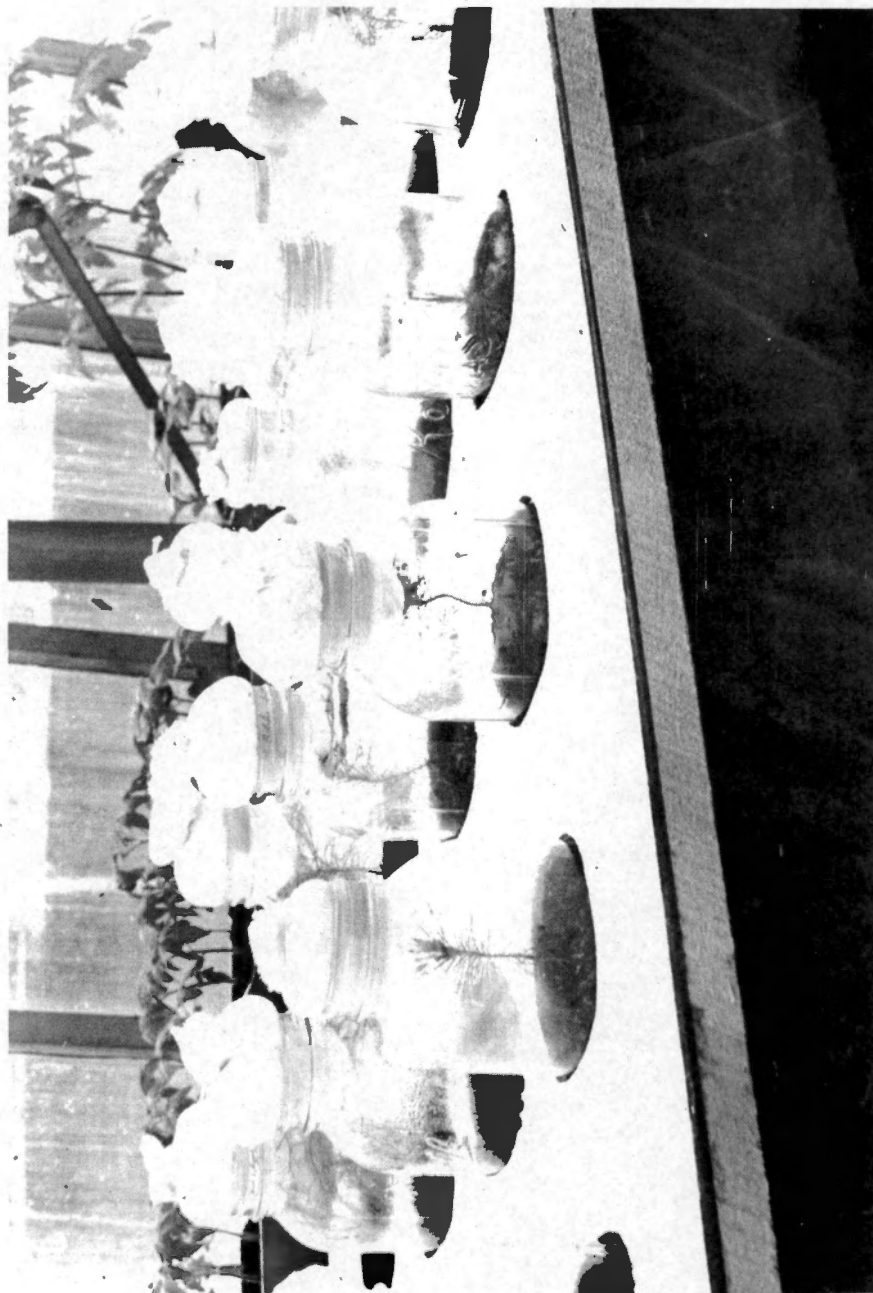


Figure 1. Culture jars incubating in greenhouse waterbath.

Table 6. Species of basidiomycetes collected under
loblolly pine in East Tennessee.

Species collected	Areas of Collection					
	Lafollette	Andersonville	Norris	Oak Ridge (1)	Oak Ridge (2)	Rose Island
<u>Amanita citrina</u> (Schaeef.) Roq.	X	X	X	X	X	X
<u>Amanita flavoconia</u> Atk.	X					
<u>Amanita parcivolvata</u> Pk.						
<u>Amanita rubescens</u> Fr.	X		X			
<u>Amanita strobiliformis</u> Fr.	X					
<u>Amanita verna</u> Fr.	X	X				
<u>Agaricus placomyces</u> Pk.	X	X	X	X	X	
<u>Boletus brevipes</u> Pk.				X	X	
<u>Boletus bicolor</u> Pk.	X					
<u>Boletus cyanescens</u> Bull.	X					
<u>Boletus felleus</u> Bull.	X					
<u>Boletus granulatus</u> L.	X	X	X	X	X	X
<u>Boletus luteus</u> L.					X	
<u>Boletus strobilaceus</u> Scop.					X	
<u>Boletus subaureus</u> Pk.	X					
<u>Clitocybe infundibuliformis</u> Fr.	X					
<u>Clitocybe gibba</u> Fr.	X					
<u>Clitocybe odora</u> (Bull.) Sacc.	X					
<u>Clitocybe subditopoda</u> Pk.	X					
<u>Collybia exculpta</u> Gill.	X					
<u>Collybia maculata</u> Alb. & Schw.	X	X	X	X	X	X
<u>Collybia maculata</u> var <u>immaculata</u> Mass.				X		
<u>Collybia maculata</u> var <u>scorzonerea</u> Gill.				X		
<u>Cortinarius cinnabarinus</u> Fr.	X					
<u>Cortinarius distans</u> Pk.	X					
<u>Cortinarius flaviofolius</u> Pk.	X					
<u>Cortinarius torvus</u> Fr.	X					
<u>Craterellus cantherellus</u> (Schw.) Fr.					X	
<u>Gomphidius vinicolor</u> Pk.			X		X	
<u>Hygrophorus conicus</u> Fr.	X					
<u>Hygrophorus puniceus</u> Fr.	X					
<u>Laccaria laccata</u> (Scop. ex Fr.) Berk. & Br.		X	X	X		
<u>Laccaria ochropurpurea</u> (Berk.) Pk.	X	X				
<u>Lactarius appalachianeses</u> (sp. nov.)				X	X	

(Table 6. Continued)

Species collected	Areas of collection					
	Lafollette	Andersonville	Norris	Oak Ridge (1)	Oak Ridge (2)	Rose Island
<u>Lactarius cinereus</u> Pk.	X					
<u>Lactarius glaucescens</u> Crossl.	X					
<u>Lactarius indigo</u> Schw.	X					
<u>Lactarius lactorubescens</u> sp. nov.	X					
<u>Lactarius picinus</u> Fr.	X					
<u>Lactarius vellereus</u> (Fr.) Fr.	X					X
<u>Lactarius scrobiculatus</u> Fr.	X					
<u>Lactarius piperatus</u> Fr.	X					
<u>Lactarius volemus</u> Fr.				X	X	
<u>Lepiota americana</u> Pk.	X			X		
<u>Paxillus atrotomentosus</u> Fr.	X					
<u>Leucopaxillus laterarius</u> (Peck.) Singer & Smith	X					
<u>Mycena pura</u> Fr.			X	X	X	
<u>Pisolithius tinctorius</u> (Pers.) Coker & Couch.						X
<u>Pleurotius spathulatus</u> Fr.-Pk.	X					
<u>Polyporus cristata</u> (Pers.) Gill.	X					
<u>Russula albidula</u> Pk.	X	X	X	X	X	
<u>Russula densifolia</u> Secr.				X	X	
<u>Russula fragilis</u> Fr.	X	X	X	X	X	X
<u>Russula variata</u> Bann. & Pk.				X		X

In addition, mycorrhizae of the type formed by Cenococcum graniforme were observed on roots in two of the stands.

Several species occurred with such regularity as to be worthy of special mention. Amanita citrina, Boletus granulatus, and Russula fragilis were the most common species observed and were abundant in all stands visited. Collybia maculata also occurred in all stands surveyed, but at least four morphologically distinct varieties were collected. Only two of these, C. maculata var immaculata Mass. and C. maculata var scorzonerea Gill., could be identified. Agaricus placomyces and Russula albidula Pk. were also quite common and occurred in all stands except the one at Rose Island.

During collection the sporophores of several species were found with mycelial connections with mycorrhizae (Figure 2). Mycorrhizae were found connected to the base of sporophores of Boletus grantlatus, Laccaria laccata, Russula albidula, Russula densifolia Secr. and a new species tentatively named Lactarius appalachianesis (sp. nov.) by Hesler.

Use of the association of sporophores with a species of tree to indicate a mycorrhizal relationship has been criticized by Trappe (1957) and others (Grand, 1968b; Bryan and Zak, 1961). There is no way to tell from the occurrence of a sporophore whether the fungus which formed it is mycorrhizal, saprophitic, or pathogenic. At present, however, the use of sporophores is the only practical way to identify basidiomycetes and Trappe (1960) has suggested that their use can be quite valuable if the genera which are commonly involved in mycorrhizal relationships are kept in mind.

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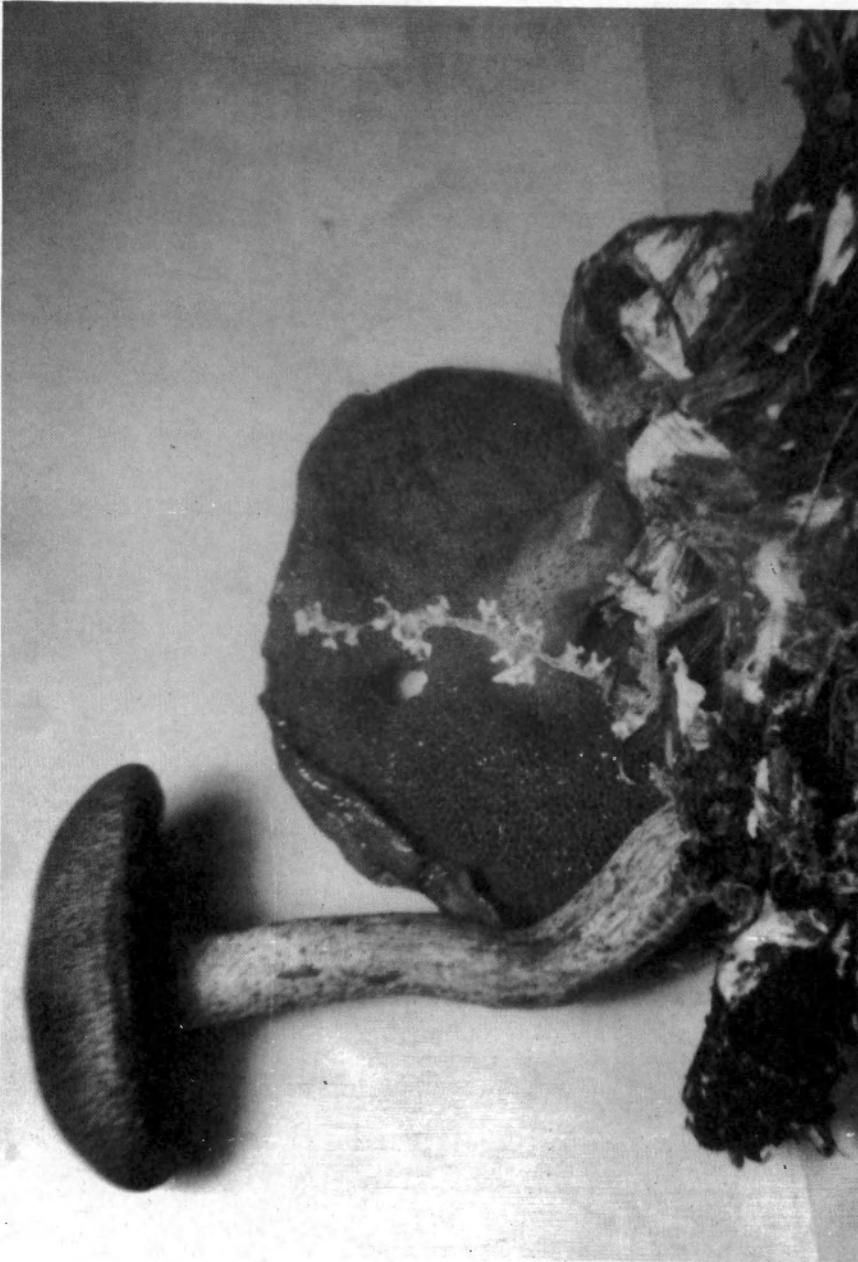


Figure 2. The association of mycorrhizae with the sporophores of Boletus granulatus.

Synthetic Culture of Mycorrhizae

Only 19 species of fungi have been shown to be mycorrhizal with loblolly pine in synthetic culture (Table 7). Five of these, Boletus granulatus, Boletus brevipes Pk., Boletus luteus L., Laccaria laccata, and Pisolithius tinctorius, were collected in the six plantations of loblolly pine surveyed in East Tennessee.

Of the twelve species tested in sterile culture, Agaricus placomyces, Amanita rubescens, Amanita verna, and Boletus bicolor entered into mycorrhizal relationships with loblolly pine (Figure 3). Boletus bicolor produced mycorrhizae over the entire root system, whereas Agaricus placomyces, Amanita rubescens, and Amanita verna produced mycorrhizae only where roots came in contact with areas of heavy fungal growth in the substrate.

Amanita citrina, Amanita parcivolvata, Paxillus atrotomentosus, and Russula fragilis caused the production of mycorrhizae-like structures (Figure 4). The structures formed by these species were morphologically different from each other, but all were branched dichotomously and hypertrophied, while root hairs were suppressed. However, these mycorrhizae-like structures lacked the fungal sheath characteristic of fully developed mycorrhizae.

Clitocybe gibba, Collybia maculata, Lactarius piperatus, and Leucopaxillus laterarius did not form mycorrhizae with loblolly pine. The short roots of seedlings inoculated with these species were not branched, were not swollen and all had root hairs.

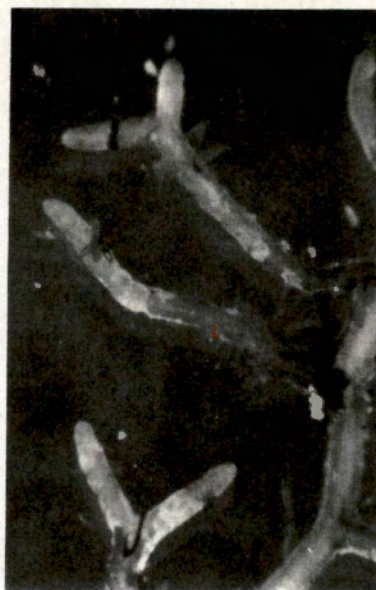
The three known mycorrhizal fungi, Cenococcum graniforme, Laccaria

Table 7. Species shown to be mycorrhizal with
loblolly pine by synthetic culture.

Species	Reference
<u>Amanita muscaria</u> Fr.	Bryan and Zak (1961)
<u>Boletus betula</u> Schw.	Bryan and Zak (1962)
<u>Boletus brevipes</u> Pk.	Doak (1934)
<u>Boletus chromapes</u> Frost.	Doak (1934)
<u>Boletus chysenteron</u> Bull.	Trappe (1964a)
<u>Boletus communis</u> Bull.	Bryan and Zak (1961)
<u>Boletus eximus</u> Bull. ex Fr.	Trappe (1964a)
<u>Boletus granulatus</u> L.	Young (1940)
<u>Boletus luteus</u> L.	Bryan and Zak (1962)
<u>Boletus miniato-olivaceus</u> Frost.	Doak (1955)
<u>Boletus pictus</u> Pk.	Doak (1934)
<u>Cantharellus cibarius</u> Fr.	Doak (1934)
<u>Cenococcum graniforme</u> (Sow.) Ferd & Winge	Trappe (1964b)
<u>Clitocybe laccata</u> Fr.	Bryan and Zak (1961)
<u>Laccaria laccata</u> (Scop. ex Fr.) Berk & Br.	Trappe (1964a)
<u>Pisolithius tinctorius</u> (Pers.) Coker & Couch	Reid (1967)
<u>Rhizopogon roseolus</u> (Corda.) Hollos.	Young (1940)
<u>Rhizopogon luteolus</u> Fr. & Nordh.	Young (1937b)
<u>Russula lepida</u> Fr.	Doak (1934)



A



B

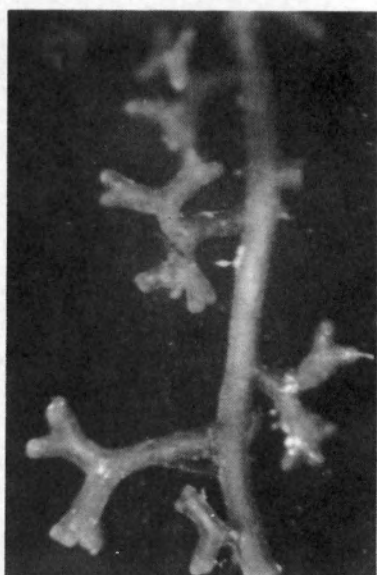


C

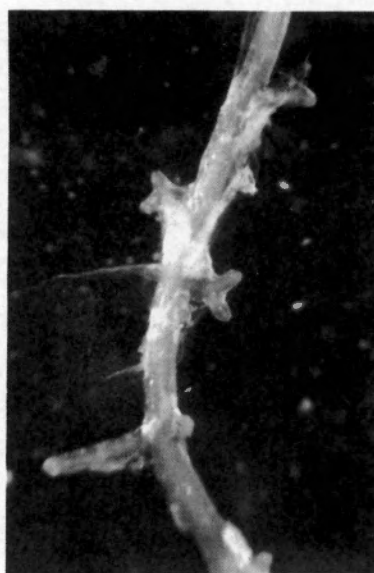


D

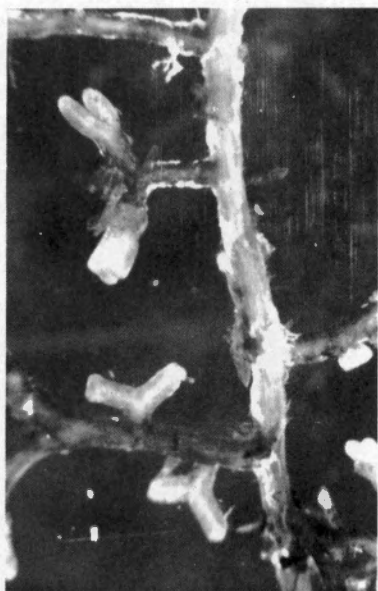
Figure 3. Mycorrhizae produced in sterile culture by A) Amanita verna, B) Amanita rubescens, C) Agaricus placomyces, D) Boletus bicolor.



A



B



C



D

Figure 4. Mycorrhizae-like structures produced in synthetic culture by A) Amanita parcivolvata, B) Russula fragilis, C) Amanita citrina, and D) Paxillus atrotomentosus.

laccata, and Pisolithius tinctorius, produced mycorrhizae under the test conditions. P. tinctorius and L. laccata produced abundant mycorrhizae while C. graniforme produced mycorrhizae only where roots contacted heavy fungal growth.

A number of authors have stressed that the results of synthetic culture are not conclusive (Harley, 1959; Zak and Bryan, 1963; Zak and Marx, 1964). The fact that a fungus enters the mycorrhizal relationship under the artificial conditions of synthetic culture indicates that the fungus has the ability to form mycorrhizae. Whether or not it does so under more natural conditions is open to question (Harley, 1959). Negative results in synthetic culture are especially questionable. Several investigators have shown species to be mycorrhizal with further testing even though they were first reported as non-mycorrhizal (Bryan and Zak, 1961; Bryan and Zak, 1963).

Summary

Fifty-three species of fungi were collected and identified under six loblolly pine stands. Of these species Boletus brevipes, Boletus granulatus, Boletus luteus, Cenococcum graniforme, and Pisolithius tinctorius have previously been shown to be mycorrhizal with loblolly pine. This study adds Amanita rubescens, Amanita verna, Agaricus placomyces, and Boletus bicolor to this list. The results of the survey of pine stands indicate that these species are probably common mycorrhizal symbionts with loblolly pine in East Tennessee.

In addition, Russula albidula, Russula densiflora, Lactarius appalachianesis, Amanita citrina, Amanita strobiliformis Vitt., Lactarius

piperatus, Lactarius vellerius Fr., and Russula fragilis can be cited as probable symbionts with loblolly pine. R. albidula, R. densifolia, and Lactarius appalachianesis were common under loblolly pine and were found attached to mycorrhizal roots. The remaining five were quite common in the stands surveyed and all are known to be mycorrhizal with one or more Southern yellow pines (Trappe, 1964a).



CHAPTER III

EVALUATION OF ANTAGONISM OF MYCORRHIZAL FUNGI TOWARD PATHOGENS

Several approaches have been used to study the effect of mycorrhizae on root pathogen relationships. The most common approach has been the assay of mycorrhizal fungi for antibiotic activity. The assays have been largely plate antagonism studies (Robbins et al., 1945; Bryan, 1960; Marx, 1965) although extracts of pure cultures have also been used (Santoro and Casida, 1962; Marx, 1965).

Marx (1965) studied the protection of live roots by mycorrhizae by isolating individual mycorrhizae in sterile glass vials. The vials were then inoculated with Phytophthora cinnamomi and observed under a binocular scope. Saesk (1968) has studied the protection afforded young pine seedlings by mycorrhizae against several pathogens. Saesk grew mycorrhizal seedlings in sterile culture and subsequently inoculated them with pathogens.

In the study reported here, agar plate antagonism tests were used to assay several fungi which are mycorrhizal with loblolly pine in East Tennessee for antagonism toward a number of pathogens. Six pathogens were chosen and tested under sterile culture for pathogenicity to loblolly pine. On the basis of these studies three pathogens and three mycorrhizal fungi were chosen and tested in sterile culture to measure the protection afforded seedlings against pathogens by mycorrhizae.

I. METHODS

Plate Antagonism Studies

Five fungi mycorrhizal on loblolly pine, Amanita rubescens, Boletus bicolor, Cenococcum graniforme, Laccaria laccata, Pisolithius tinctorius, were assayed for antagonism toward thirteen pathogens (Table 8).

The procedure described by Marx (1965) was used for plate antagonism studies. Standard 9 cm. petre dishes were filled with 40 ml. of modified Melin-Norkrans solution (Table 4, page 24) and randomly arranged in sets of ten. An 8 mm. disk cut from the edge of a mycorrhizal colony was transferred to five of the ten plates. All ten dishes were then incubated on a laboratory bench for two to three weeks until radial growth of the mycorrhizal colonies reached approximately 2 cm. (Table 9). At this time an 8 mm. disk cut from a pathogen colony was transferred to all ten plates. In the five test plates the pathogen disk was placed three cm. from the edge of the symbiont colony. In the five control plates the disk was placed in a comparable position.

Plates were placed in random order and incubated for 3-21 days (Table 10) until radial growth in the control plates reached 3 cm. or until the two test organisms grew within 3 mm. of each other. The above procedure was repeated for each combination tested.

Radial growth of pathogens toward symbiont colonies was measured and compared with radial growth in the control plates measured in a comparable direction (Figure 5). Variances of the two sets of radii were tested for homogeneity of variance with an F test (Snedecor and

Table 8. Source of pure cultures of pathogens used in evaluation of antagonism of mycorrhizal fungi toward pathogens.

Fungus	Source
<u>Armillaria mellea</u> Vahl. ex Fr. <u>Fomes annosus</u> (Fr.) Cke. <u>Fusarium solani</u> (Mart.) Appel. & Wend. emend. Snyder and Hansen	Dr. W. J. Stambaugh Duke University Durham, North Carolina
<u>Sclerotium bataticola</u> Taub.	Dr. S. J. Rowan Forest Sciences Laboratory Athens, Georgia
<u>Fusarium moniliforme</u> Scheld. <u>Botrytis cinera</u> Pers. <u>Pythium ultimum</u> Trow.	Dr. Larry F. Grand North Carolina State Univ. Raleigh, North Carolina
<u>Phytophthora cinnamomi</u> Rand. <u>Phytophthora parasitica</u> Dastur. <u>Pythium irregulare</u> Buism. <u>Rhizoctonia crocorum</u> (Pers) D. C. <u>Fusarium oxysporum</u> Schl. emend. Snyder & Hansen	Dr. Donald Marx Forest Sciences Laboratory Carlton Street Athens, Georgia
<u>Rhizoctonia solani</u> Kuhn.	James L. Bricker University of California Riverside, California

Table 9. Incubation time and average radial growth for five mycorrhizal fungi.

Species	Incubation time	Ave. radius
<u>Amanita rubescens</u> Fr.	21 days	1.55 cm.
<u>Boletus bicolor</u> Pk.	21 days	1.03 cm.
<u>Cenococcum graniforme</u> (Sow.) Ferd and Winge	21 days	0.69 cm.
<u>Laccaria laccata</u> (Scop. ex Fr.) Berk & Br.	14 days	1.66 cm.
<u>Pisolithius tinctorius</u> (Pers.) Coker and Couch.	21 days	1.37 cm.

Table 10. Incubation time and average radial growth of thirteen pathogens used in antagonism studies.

Species	Incubation time	Ave. radius
<u>Sclerotium bataticola</u> Taub.	4 days	3.33 cm.
<u>Phytophthora cinnamomi</u> Rand.	5 days	2.83 cm.
<u>Fomes annosus</u> (Fr.) Cke.	6 days	3.03 cm.
<u>Pythium ultimum</u> Trow.	2 days	4.19 cm.
<u>Rhizoctonia solani</u> Kuhn.	5 days	3.01 cm.
<u>Fusarium oxysporum</u> Schl. emend. Snyder. and Hans.	9 days	2.23 cm.
<u>Fusarium moniliforme</u> Sheld.	8 days	2.01 cm.
<u>Pythium irregulare</u> Buism.	2 days	3.06 cm.
<u>Botrytis cinera</u> Pers.	17 days	2.63 cm.
<u>Phytophthora parasitica</u> Dastur.	14 days	2.24 cm.
<u>Armillaria mellea</u> Vahl. ex Fr.	27 days	1.28 cm.
<u>Rhizoctonia crocorum</u> (Pers.) D. C.	33 days	1.22 cm.
<u>Fusarium solani</u> (Mart.) Appel & Wend. emend. Snyder. & Hans.	16 days	1.89 cm.

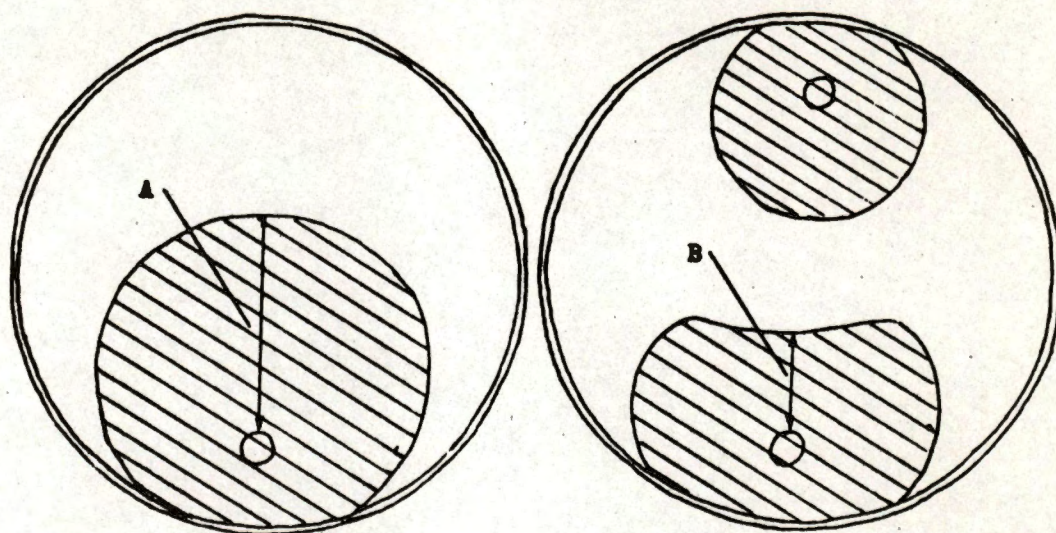


Figure 5. Measurements used for comparisons in plate antagonism tests. A. Radius measured in each control plate. B. Radius measured in each test plate.

Cochran, 1967). If the F value was consistent with the hypothesis that the variances were equal the two sample means were compared with an unpaired, pooled variance "t" test. If the null hypothesis that the two variances were equal was rejected the two means were compared with an unequal variance "t" test (Snedecor and Cochran, 1967). All tests were run at the 5% level of significance.

Virulency Test of Several Pathogens

A test was made with six pathogens, Botrytis cinera, Fomes annosus, Fusarium moniliforme, Phytophthora cinnamomi, Rhizoctonia solani and Sclerotium bataticola, to determine pathogenicity on loblolly pine seedlings under sterile culture conditions.

The experiment was arranged in a randomized complete block design with four blocks and seven treatments (Table 11). Blocks were arranged across a greenhouse bench in order to remove variation caused by heat and humidity conditions.

Culture chambers were prepared in the same fashion as for synthetic culture of mycorrhizae except that one quart mason jars were used. The quantity of substrate and nutrient solution was correspondingly reduced. Sterile seedlings were obtained as previously described.

Seedlings were incubated on a greenhouse bench for three weeks prior to inoculation. At this time contaminated jars were removed and inoculations made with three 1 cm. by 1 cm. blocks of agar cultured with one of the test pathogens. After three weeks the difference between the height at the time of inoculation and the present seedling height was measured. These data were subjected to an analysis of variance. Treat-

Table 11. Treatments used for virulency test of pathogens

Treatment #	Treatment
1	Control: uninoculated
2	Inoculated with <u>Sclerotium Bataticola</u> Taub.
3	Inoculated with <u>Fusarium moniliforme</u> Sheld.
4	Inoculated with <u>Phytophthora cinnamomi</u> Rand.
5	Inoculated with <u>Fomes Annosus</u> (Fr.) Cke.
6	Inoculated with <u>Rhizoctonia solani</u> Kuhn.
7	Inoculated with <u>Botrytis cinera</u> Pers.

ment effects were studied with individual degree of freedom comparisons.

Protection from Pathogens by Mycorrhizae

To test the effect of mycorrhizae on resistance of living roots to pathogens, sterile seedlings were grown, inoculated with mycorrhizal fungi, and subsequently inoculated with pathogens.

This investigation was arranged in a randomized complete block design with a four by four factorial arrangement of treatments (Table 12). Thus there were four mycorrhizal treatments: 1) non-mycorrhizal, 2) Boletus bicolor, 3) Laccaria laccata, and 4) Pisolithius tinctorius; and four pathogen treatments: 1) no pathogen, 2) Sclerotium bataticola, 3) Fomes annosus, and 4) Rhizoctonia solani. Blocks were arranged across the light tables used for incubation (Figure 6).

The seedlings were grown in culture chambers as described in synthetic culture of mycorrhizae. Sterile seedlings were obtained as previously described. Seedlings were grown for one week after planting. At this time contaminated jars were removed and the remaining jars randomly arranged in four sets of 16 jars each.

After the seedlings were one week old the appropriate jars were inoculated with one of the three mycorrhizal fungi, Boletus bicolor, Laccaria laccata, or Pisolithius tinctorius. These species were chosen because all were known to develop fully formed mycorrhizae under sterile culture conditions. These species also represented a spectrum from strongly antagonistic, Boletus bicolor, to weakly antagonistic, Pisolithius tinctorius, in plate antagonism tests. Seedlings were inoculated with cultured blocks of agar, approximately 1 cm. by 1 cm.,

Table 12. Treatments used to test the effect of mycorrhizae on the susceptibility of roots to pathogens.

Treatment #	Mycorrhizal species	Pathogen species
1	None	None
2	<u>Boletus bicolor</u>	None
3	<u>Laccaria laccata</u>	None
4	<u>Pisolithius tinctorius</u>	None
5	None	<u>Scletotium bataticola</u>
6	<u>Boletus bicolor</u>	<u>Sclerotium bataticola</u>
7	<u>Laccaria laccata</u>	<u>Sclerotium bataticola</u>
8	<u>Pisolithius tinctorius</u>	<u>Sclerotium bataticola</u>
9	None	<u>Rhizoctonia solani</u>
10	<u>Boletus bicolor</u>	<u>Rhizoctonia solani</u>
11	<u>Laccaria laccata</u>	<u>Rhizoctonia solani</u>
12	<u>Pisolithius tinctorius</u>	<u>Rhizoctonia solani</u>
13	None	<u>Fomes annosus</u>
14	<u>Boletus bicolor</u>	<u>Fomes annosus</u>
15	<u>Laccaria laccata</u>	<u>Fomes annosus</u>
16	<u>Pisolithius tinctorius</u>	<u>Fomes annosus</u>

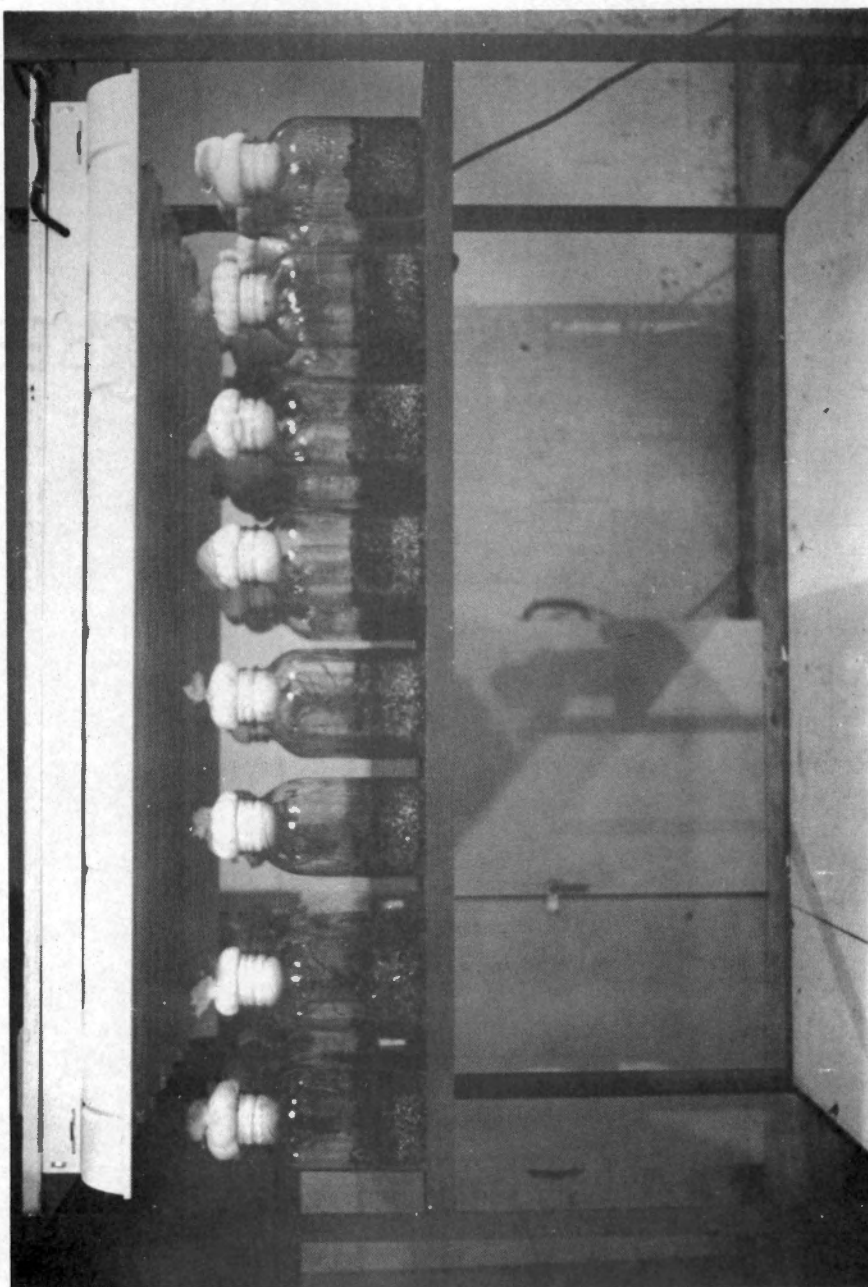


Figure 6. Culture jars incubating on one of the light tables used.

on three sides to assure even mycorrhizal development. All jars were then incubated for six weeks to allow for mycorrhizae formation.

After a seven week growth period the appropriate jars were inoculated with one of the three pathogens. These three pathogens were chosen as the test pathogens because they were the most virulent under sterile culture and all were inhibited to some extent in plate antagonism studies. After inoculation, the jars were incubated for an additional eight weeks.

The data analyzed were the growths of the seedlings during the eight week interval after inoculation with pathogens. This growth was taken as the height at the end of 15 weeks growth less the height of the seedling at the time of inoculation with pathogens. These data were subjected to an analysis of variance with individual degree of freedom comparisons. Individual error variances were tested with a Bartlett's test for homogeneity of variance. All tests of hypothesis were run at the 5% level of significance.

Following completion of the 15 week growth period one complete replication was chosen at random for reisolation to confirm infection by the inoculated pathogens. Each seedling was removed from the substrate and four one cm. segments cut from the roots. Segments were cut around the junction of healthy tissue with any discolored or necrotic areas. The segments were washed for three hours under running tap water to remove any substrate and then cut into 3 mm. sections. These segments were surface sterilized in 10% clorox for 40 seconds, rinsed in sterile distilled water, and plated on water agar. The plates were incubated

at room temperature for one week and examined for the presence of the inoculated pathogens.

II. RESULTS AND DISCUSSION

Plate Antagonism Studies

Sixty antagonism tests were made between mycorrhizal fungi and pathogens. Table 13 presents a summary of the test results. Of the mycorrhizal fungi tested, Boletus bicolor displayed the strongest antagonism inhibiting 90% of the pathogens tested. Cenococcum graniforme, Amanita rubescens, and Laccaria laccata displayed moderate antagonism inhibiting the growth of 69%, 54% and 75% of the pathogens respectively. Pisolithius tinctorius inhibited 64% of the pathogens tested but, in most cases the antagonism was weak.

There are a number of objections to this type of test, most important of which is the fact that nutrient and pH changes in the agar may result in inhibition of the test organism rather than antibiotic production (Marx, 1965; Saesk and Musilek, 1968). Marx (1965) studied the effect of the mycorrhizal fungi he used on chemical changes in the agar. Marx discovered that there were chemical and pH changes in the agar near the symbiont colony. He concluded, however, that they were not unfavorable to the growth of pathogens.

The strength of antagonism is especially open to question. Marx (1965) was able to relate the strength of antagonism to such factors as stiffness of the agar medium. He also found that strength of observed antagonism varied in different growth media, suggesting that the

Table 13. Mean differences between pathogen colony radii in control and test plates in plate antagonism studies (in centimeters).

Pathogen	Mycorrhizal fungi				
	P. tinctorius	L. laccata	A. rubescens	C. graniforme	B. bicolor
S. bataticola	0.69*	2.15*	1.34*	0.91*	1.36*
P. cinnamomi	0.89*	0.67*	0.81*	1.12*	1.47*
F. annosus	0.50*	1.12*	0.57*	1.97*	1.85*
P. ultimum	0.54*	3.42*	0.20	0.24	2.96*
R. solani	0.05	0.74*	0.67*	0.39	1.81*
F. oxysporum	0.01	0.15*	0.01	0.04	0.76*
F. moniliforme	0.02	0.11	0.12	0.08	0.20
P. irregulare	0.83*	nt	3.21*	2.92*	2.72*
B. cinera	0.48*	0.85*	0.35	0.99*	1.09*
P. parasitic	nt	0.47*	1.40*	1.02*	1.06*
A. mellea	0.15	0.11	0.26*	0.47*	0.56*
R. crocorum	0.60*	0.88*	0.28	1.06*	nt
F. solani	nt	0.33	0.19	0.63*	nt

*Indicates mean difference was concluded to be greater than zero at the five percent level of significance

nt=Not tested

antagonistic principle may be lost under more natural conditions.

Virulency Test of Several Pathogens

Analysis of the height data in the test of six pathogens resulted in the conclusion that three of the six pathogens, Sclerotium bataticola, Fomes annosus, and Rhizoctonia solani, had a significant effect on height growth.

The height differences between seedling heights before and three weeks after inoculation with pathogens appear in Table 14. The analysis of variance and associated tests of hypotheses appear in Table 15.

As a result of this test, three pathogen species, Sclerotium bataticola, Rhizoctonia solani, and Fomes annosus were chosen for further testing.

Protection from Pathogens by Mycorrhizae

The increase in height of the seedlings during the eight week incubation period following inoculation with pathogens appear in Table 16. These data are summarized with treatment means in Table 17. The analysis of variance with associated individual degree of freedom comparisons appears in Table 18.

Statistical analysis. The analysis of variance resulted in the following conclusions: There was no discernable variation in height growth due to mycorrhizae or to pathogen treatments. However, the largest part of the variation among mycorrhizae treatments was within the comparison of non-mycorrhizal seedlings with mycorrhizal seedlings. Likewise, the largest part of the variation among pathogen treatments was within the comparison of uninoculated seedlings with seedlings inoculated with pathogens.

Table 14. Seedling height growth during the three weeks following inoculation with pathogens (in centimeters).

Treatment	Block number				Mean
	I	II	III	IV	
1. Control	0.3	1.7	1.8	3.3	1.8
2. <u>Scletotium bataticola</u> Taub.	0.0	0.0	0.2	1.3	0.4
3. <u>Fusarium moniliforme</u> Sheld.	0.1	1.8	2.2	2.2	1.6
4. <u>Phytophthora cinnamomi</u> Rand.	0.0	1.8	1.4	1.8	1.2
5. <u>Fomes annosus</u> (Fr.) Cke	0.0	0.8	2.0	0.8	0.9
6. <u>Rhizoctonia solani</u> Kuhn.	0.0	0.6	1.5	0.1	0.6
7. <u>Botrytis cinera</u> Pers.	0.0	1.5	1.5	1.1	1.0

Table 15. Analysis of variance of height growth in virulency test of pathogens.

Source of variation	d,f	Est. of var.	F ratio
Among replications	3	3.339	10.337*
Among treatments	6	1.046	3.238*
Treat. 1 vs. 2	1	3.920	12.136*
Treat. 1 vs. 3	1	0.080	0.248
Treat. 1 vs. 4	1	0.551	1.706
Treat. 1 vs. 5	1	1.531	4.740*
Treat. 1 vs. 6	1	3.001	9.291*
Treat. 1 vs. 7	1	1.120	3.467
Treatment x Reps.	18	0.323	0.323

*Indicates that the null hypothesis that the associated variance was equal to zero was rejected at the 5% level of significance.

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Table 16. Seedling growths during the eight week period following inoculation of mycorrhizal and non-mycorrhizal seedlings with pathogens (in centimeters).

Treatment		Replication			
Mycorrh.	Path.	I	II	III	IV
A	1	1.05	2.10	1.25	1.25
A	2	1.75	0.00	0.40	0.50
A	3	0.00	0.45	0.40	0.50
A	4	0.15	0.30	1.55	0.60
B	1	1.55	3.30	0.10	0.70
B	2	3.40	2.15	0.05	0.85
B	3	4.65	0.60	0.60	2.45
B	4	3.10	0.10	0.70	1.20
C	1	2.05	2.65	1.80	2.95
C	2	0.55	1.55	1.05	1.00
C	3	3.05	1.75	0.95	2.50
C	4	1.60	0.30	2.65	0.40
D	1	0.95	1.75	1.45	0.80
D	2	2.40	0.75	0.85	0.90
D	3	1.35	0.00	0.65	1.10
D	4	1.50	1.05	1.35	0.25

Mycorrhizae treatments:

A=uninoculated.

B=inoculated with Pisolithius tinctorius.

C=inoculated with Boletus bicolor.

D=inoculated with Laccaria laccata.

Pathogen treatments:

1=uninoculated.

2=inoculated with Sclerotium bataticola.

3=inoculated with Fomes annosus.

4=inoculated with Rhizoctonia solani.

Table 17. Mean seedling growth by treatment during the eight week period following inoculation of mycorrhizal and non-mycorrhizal seedlings with pathogens (in centimeters).

Mycorrhizae	Pathogens				Mean
	None	<u>Sclerotium bataticola</u>	<u>Fomes annosus</u>	<u>Rhizoctonia solani</u>	
None	1.41	0.66	0.34	0.45	0.76
<u>Pisolithius tinctorius</u>	1.41	1.61	2.08	1.28	1.59
<u>Boletus bicolor</u>	2.41	1.04	2.06	1.26	1.68
<u>Laccaria laccata</u>	1.26	1.22	0.78	1.04	1.07
Mean	1.61	1.13	1.35	1.05	

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Table 18. Analysis of variance of seedling growth during the incubation period following inoculation of mycorrhizal and non-mycorrhizal seedlings with pathogens.

Source of variation	df	SS	MS	F. ratio
Total	63	62.6050		
Among replications	3	6.5882	2.1961	2.6291
Among mycorrhizae	3	9.0176	3.0059	2.3635
A vs BCD	1	5.5522	5.5522	4.3656
Among pathogens	3	2.9045	0.9682	0.4458
1 vs 234	1	2.3298	2.3298	1.0728
Interaction	9	6.5072	0.7230	1.6088
(A 1) vs (A 234)	1	2.2317	2.2317	4.9659*
(ABCD 1) vs (ABCD 234)	1	2.3298	2.3298	5.1842*
(A234) vs (BCD 234)	1	6.0570	6.0570	13.4779*
(A 234) vs (B 234)	1	7.3151	7.3151	16.2775*
(A 234) vs (C 234)	1	4.8150	4.8150	10.7143*
(A 234) vs (D 234)	1	1.2504	1.2604	2.8046
(B 234) vs (CD 234)	1	1.4450	3.3134	3.2154
(C 234) vs (BD 234)	1	0.1013	0.1013	0.2254
(BCD 1) vs (BCD 234)	1	1.6212	1.6212	3.6070
Reps. X Mycorrhizae	9	11.5135	1.2718	
Reps. X Pathogens	9	13.9404	2.1715	
Reps. X Mycor. X Path.	27	12.1334	0.4494	

Mycorrhizae treatments: A=non-mycorrhizal, B=Pisolithius tinctorius,
C=Boletus bicolor, D=Laccaria laccata.

Pathogen treatments: 1=no pathogen, 2=Sclerotium bataticola,
3=Fomes annosus, 4=Rhizoctonia solani.

The analysis resulted in the conclusion that there was no discernable variation due to the interaction of mycorrhizae with pathogens. However, there was variation within several specific interaction comparisons. It was concluded that there was variation within the comparison of uninoculated, non-mycorrhizal seedlings with non-mycorrhizal seedlings inoculated with pathogens. There was variation within the comparison of inoculated, non-mycorrhizal seedlings with inoculated, mycorrhizal seedlings. Similar comparisons made with Pisolithius tinctorius and Boletus bicolor were also concluded to contain variation.

Reisolation of pathogens. Contamination made the results of the reisolation attempt difficult to interpret. However, it was confirmed that the pathogens used could infect loblolly pine seedlings under the culture conditions used. Pathogens were recovered from seedlings inoculated with Pisolithius tinctorius and Fomes annosus, Boletus bicolor and Sclerotium bataticola, and from three of the four seedlings inoculated with Rhizoctonia solani.

Conclusions. It was concluded that two of the three mycorrhizal fungi, Boletus bicolor and Pisolithius tinctorius, protected loblolly pine seedlings from the three pathogens used. Grouped together as well as singly, inoculation with these species resulted in greater height growth when compared with non-mycorrhizal seedlings when pathogens were present. Protection was concluded to be incomplete even though there was no statistical difference between mycorrhizal seedlings inoculated with pathogens and mycorrhizal seedlings left uninoculated. Height growth of mycorrhizal seedlings inoculated with pathogens was lower and these seedlings become chlorotic shortly after inoculation with pathogens.

The fact that there was no statistically significant interaction between mycorrhizae treatments and pathogen treatments throws considerable doubt on these conclusions. Furthermore there is some question as to whether a test under sterile culture conditions is a valid test of the effect of mycorrhizae on pathogens. If the mycorrhizal fungi used are antagonistic toward the pathogens used, their mere presence in the substrate may prevent infection.

Summary

Five fungi mycorrhizal on loblolly pine, Amanita rubescens, Boletus bicolor, Cenococcum graniforme, Laccaria laccata, and Pisolithius tinctorius, were assayed against thirteen pathogens in plate antagonism tests. These mycorrhizal fungi inhibited the growth of the pathogens tested in 70% of the tests. Boletus bicolor displayed the strongest antagonism inhibiting 90% of the pathogens tested.

Sterile loblolly pine seedlings were grown, inoculated with Boletus bicolor, Laccaria laccata, or Pisolithius tinctorius and subsequently inoculated with pathogens. Using height growth as an indicator, it was concluded that two of the three mycorrhizal fungi, Boletus bicolor and Pisolithius tinctorius, protected the seedlings from attack by Sclerotium bataticola, Fomes annosus, and Rhizoctonia solani.

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