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Pinus resinosa Ectomycorrhizae: Seven Host-Fungus Combinations Synthesized in Pure Culture

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Abstract

Seven of ten woodland fungi tested (five gasteromycetes, two hymenomycetes) formed mycorrhizae *in vitro* with red pine (*Pinus resinosa* Ait.). We believe these to be first reports of pure culture syntheses for each of these host-fungus combinations: *Astraeus hygrometricus* (Pers.) Morgan, *Boletus parasiticus* Pers., *Scleroderma bovista* Fr., *S. cepa* Pers., *S. meridionale* Demo. and Malen., *S. polyrhizum* Pers., and *Thelephora terrestris* Fr. Three fungi failed to form mycorrhizae under the conditions provided: *Calvatia gigantea* (Pers.) Lloyd, *Lycoperdon perlatum* Pers., and *Paxillus atrotomentosus* (Batsch:Fr.) Fr. These three fungi produced cellulase and/or phenoloxidase in culture, suggesting saprobic (rather than symbiotic) lifestyles. None of the mycorrhiza-forming fungi gave positive test results for these enzymes.

Keywords: *Astraeus hygrometricus*, *Boletus parasiticus*, *Calvatia gigantea*, cellulase, gasteromycetes, hymenomycetes, *Lycoperdon perlatum*, *Paxillus atrotomentosus*, phenoloxidase, *Scleroderma bovista*, *Scleroderma cepa*, *Scleroderma meridionale*, *Scleroderma polyrhizum*, *Thelephora terrestris*

1. Introduction

Pure culture synthesis of mycorrhizae is an important technique to verify symbiotic relationships between mycorrhizal fungi and host trees (Trappe, 1962, 1967, Harley and Smith, 1983; Molina and Palmer, 1982). Knowledge

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of morphological features of synthesized mycorrhizae aids identification of native mycorrhizae and can also be used to determine effectiveness of seedling inoculations (Trappe, 1977). The purpose of this paper is to report the results of pure culture mycorrhizae synthesis tests made with red pine (*Pinus resinosa* Ait.) and ten known or suspected mycorrhizal fungus isolates native to the northern Great Lakes states. Red pine was used because of its importance to the forest industry.

2. Materials and Methods

Fungus isolates were obtained by tissue isolation from sporocarps onto modified Melin-Norkrans (MMN) (Marx, 1969) agar medium in Petri dishes incubated at room temperature. Table 1 lists the fungus isolates, their source locations, and habitats. Sporocarps from which cultures were obtained were dried as voucher specimens and retained in the authors' laboratory.

The pure culture synthesis method used was the same as that reported by Richter and Bruhn (1986), with only slight modifications. Rooting substrate per jar consisted of 300 ml vermiculite and ground sphagnum peat (10:1 v/v). One hundred fifty ml liquid MMN were added per vessel, followed by autoclaving for 10 min. Upon cooling, the pH of the substrate measured 4.5–5.0, but after several days, when germinated seeds were planted, the pH stabilized at 5.0–5.5.

Surface-sterilized red pine seeds (Michigan source) were plated on potato dextrose agar (PDA) in Petri dishes. The use of PDA allowed easier observation of occasional fungus contaminants than did water agar. Three uncontaminated seedlings were planted in each synthesis vessel. Six to 10 replicates of each host-fungus test were established, along with uninoculated seedlings for the study of nonmycorrhizal root development.

Seedlings were illuminated 18 hr/day under 4, 4 ft, 40 watt Westinghouse "Agro-lites"® positioned approximately 20 cm above the vessels. The vessels were tipped at a 45° angle to directly illuminate their clear glass sides instead of the metal lids. Air temperatures surrounding the vessels ranged 15–20°C dark period and 25–30°C light period.

Following inoculation, vessels were sealed with Parafilm® and returned to illumination for the synthesis period (4–5 months). Finally, vessels were opened under sterile conditions and a portion of the substrate plated on MMN agar. Several mycorrhizae or fine root tips (depending on whether mycorrhizae were formed or not) were aseptically separated, thoroughly rinsed in sterile water, and plated on MMN agar. The entire seedling's room system

Table 1. Sources of fungus basidiocarp isolates used in mycorrhizae synthesis tests with red pine

Fungus	Isolate number	Source	Date isolated	Host and habitat
Astraeus hygrometricus (Pers.) Morgan	DR-123	Richland County, WI	21 Sept. 1985	*Pinus banksiana* and *P. resinosa*; xeric sand dunes
Boletus parasiticus Pers.	DR-8	Schoolcraft County, MI	26 Sept. 1983	*Pinus resinosa* plantation; xeric upland
Calvatia gigantea (Pers.) Lloyd	DR-105	Houghton County, MI	11 Aug. 1985	Mixed conifer-hardwood forest; mesic upland
Lycoperdon perlatum Pers.	DR-84	Houghton County, MI	12 June 1985	*Quercus* forest; mesic upland
Pazillus atrotomentosus (Batsch:Fr.) Fr.	DR-6	Schoolcraft County, MI	26 Sept. 1983	*Pinus resinosa* plantation; xeric upland
Scleroderma bovista Fr.	DR-130	Sauk County, WI	20 Sept. 1985	*Pinus resinosa* plantation; mesic upland
Scleroderma cepa Pers.	DR-121	Houghton County, MI	17 Sept. 1985	*Quercus* forest; mesic upland
Scleroderma meridionale Demo. & Malen.	DR-108	Houghton County, MI	24 Aug. 1985	*Betula/Populus*; xeric mine spoils
Scleroderma polyrhizum Pers.	DR-154	Richland County, WI	20 Sept. 1985	*Pinus resinosa*/ mixed hardwoods; xeric sand dunes
Thelephora terrestris Fr.	DR-24	Gogebic County, MI	27 July 1983	*Pinus resinosa* nursery bed

was washed free of substrate and examined macro- and microscopically. Sizes of structures described are means of 5–10 measurements. Color nomenclature is that of Ridgeway (1912).

Each fungus isolate was screened in triplicate for extracellular cellulase activity, using the cellulose-azure (CA) method (Smith, 1977), and for extracellular phenoloxidase activity, using gallic acid (GA) and tannic acid (TA) media (Davidson et al., 1938).

3. Results

All fungus isolates were recovered from the rooting substrate or mycorrhizae at the end of the synthesis period. Mycorrhizae were formed on seedlings inoculated with *Scleroderma meridionale* (Fig. 1), *Astraeus hygrometricus* (Fig. 2), *Boletus parasiticus* (Fig. 3), *S. bovista* (Fig. 4), *S. cepa* (Fig. 5), *S. polyrhizum* (Fig. 6), and *Thelephora terrestris* (Fig. 7). None of these isolates produced a positive reaction on the enzyme test media employed.

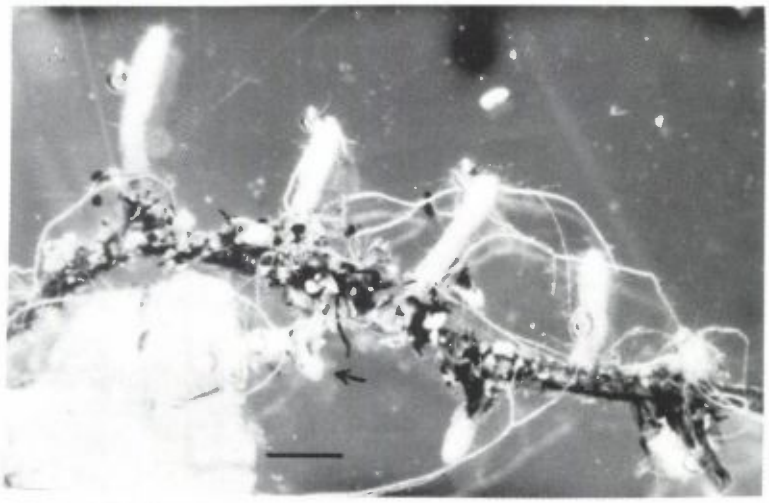


Figure 1. Red pine X *Scleroderma meridionale* mycorrhizae. White simple and bipodal (arrow) mycorrhizae (size range $1 - 5 \times 0.5 - 1$ mm; mantle thickness 10–40 μm). Note mycelial strands. Bar = 2 mm.

Figure 2. Red pine X *Astraeus hygrometricus* mycorrhizae. (A) Mottled pale smoke gray to cinnamon drab, simple and bipodal mycorrhizae (size range $1 - 3 \times 0.5 - 1$ mm). Note mycelial strands and mycelium covering structural root. Bar = 1 mm. (B) Nodulose mycelial strand composed of parallel hyphae. Note clamp connection (arrow). Bar = 20 μm. (C) Cross section of mycorrhiza showing mantle (m) (10–40 μm thick), elongate hyphae of Hartig net (h), and root cortical cells (c). Bar = 20 μm.

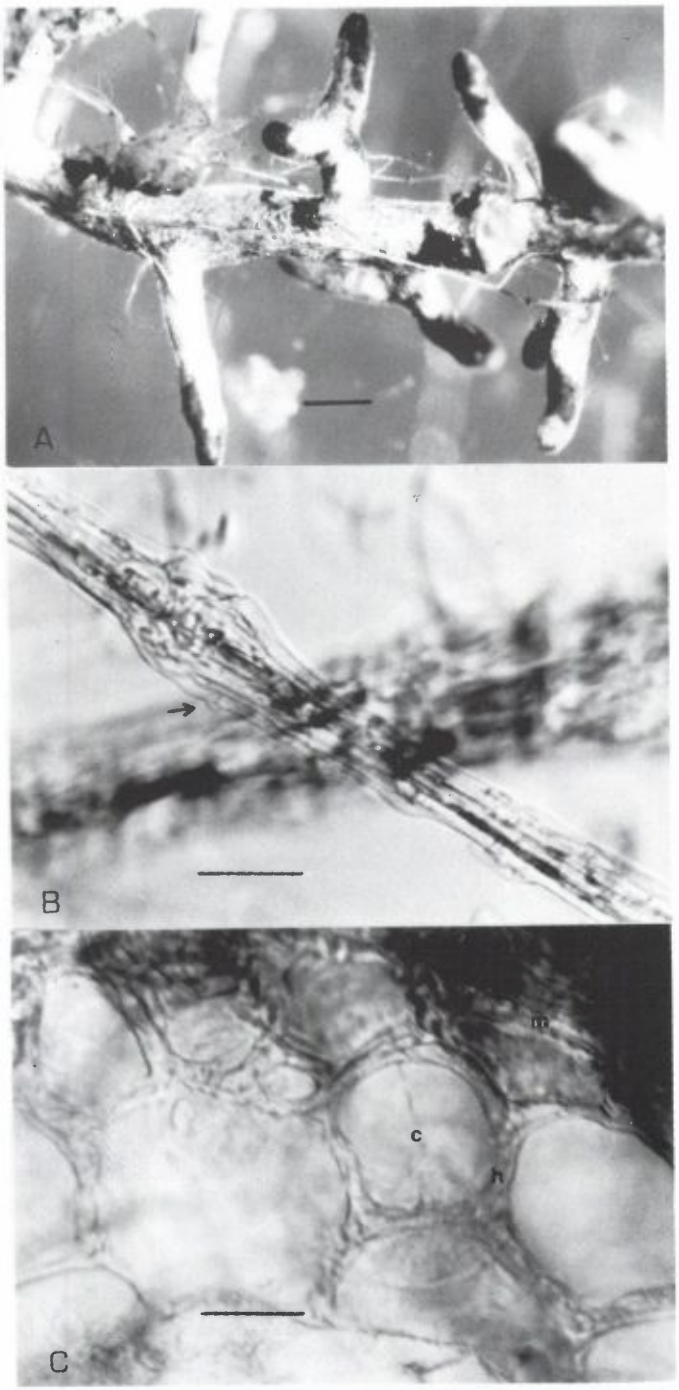


Figure 2.

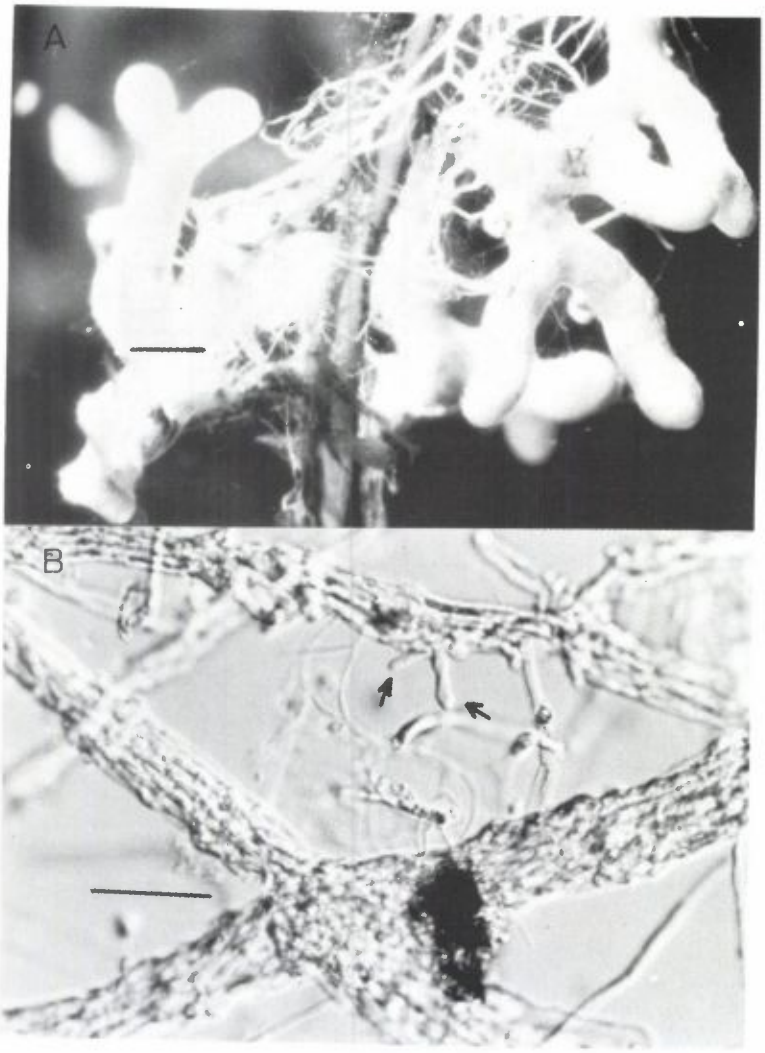
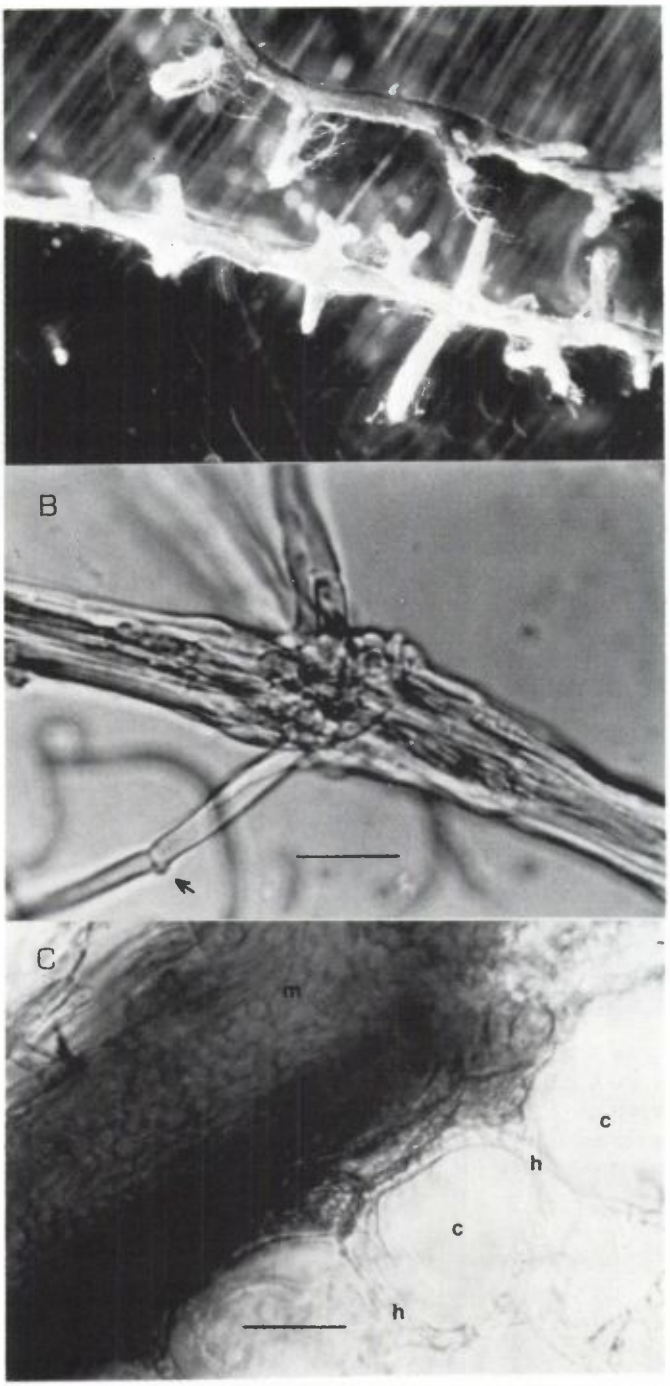


Figure 3. Red pine X *Boletus parasiticus* mycorrhizae. (A) Pure white bipodal and multiply-forked mycorrhizae (size range $2-3 \times 0.5-1$ mm; mantle thickness 40–80 μm). Note abundant mycelial strands. Bar = 1 mm. (B) Mycelial strands composed of interwoven hyphae which lack clamp connections. Note short hyphal elements protruding from strands (arrows). Bar = 20 μm.

Figure 4. Red pine X *Scleroderma bovista* mycorrhizae. (A) White simple and bipodal mycorrhizae (size range $0.5-4 \times 0.25-0.75$ mm). Note mycelial strands and mycelium covering portions of structural root (bottom). Bar = 2 mm. (B) Nodulose mycelial strand composed of parallel hyphae. Note clamp connection (arrow). Bar = 20 μm. (C) Cross section of mycorrhiza showing mantle (m) (10–20 μm thick), spherical hyphae of Hartig net (h), and root cortical cells (c). Bar = 20 μm.



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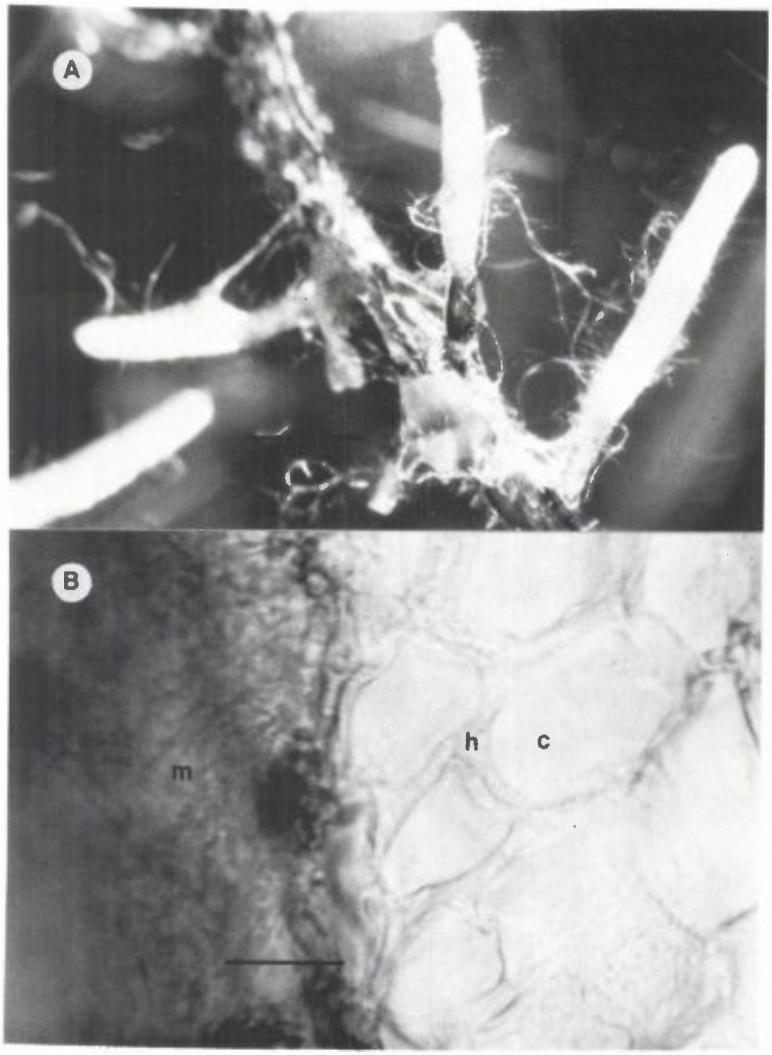

Figure 5. Red pine X *Scleroderma cepa* mycorrhizae. (A) White simple mycorrhizae (size range $1 - 5 \times 0.25 - 1$ mm; few bipodal forms were present). Bar = 2 mm. (B) Cross section of mycorrhiza showing mantle (m) (10–20 μm thick), elongate hyphae of Hartig net (h), and root cortical cells (c). Note interlocking pattern formed by hyphae of the Hartig net (lower right). Bar = 20 μm.

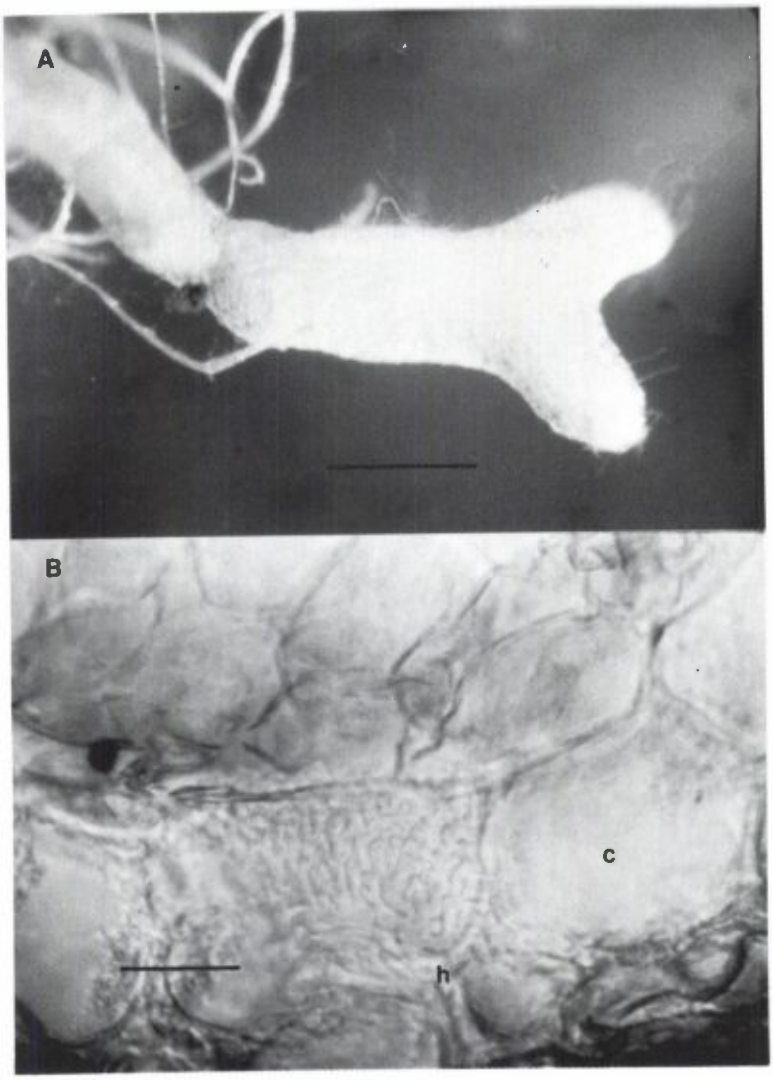

Figure 6. Red pine X *Scleroderma polyrhizum* mycorrhizae. Close-up view of white bipodal mycorrhiza (size range $1 - 3 \times 0.5 - 1$ mm; simple forms predominated). Note sparingly tomentose surface and mycelial strands. Bar = 1 mm. (B) Cross section of mycorrhiza showing hyphae of Hartig net (h) and root cortical cells (c). Note interlocking pattern formed by hyphae of the Hartig net (center). A portion of the mantle (10–30 μm thick) can be seen in the lower right. Bar = 20 μm.

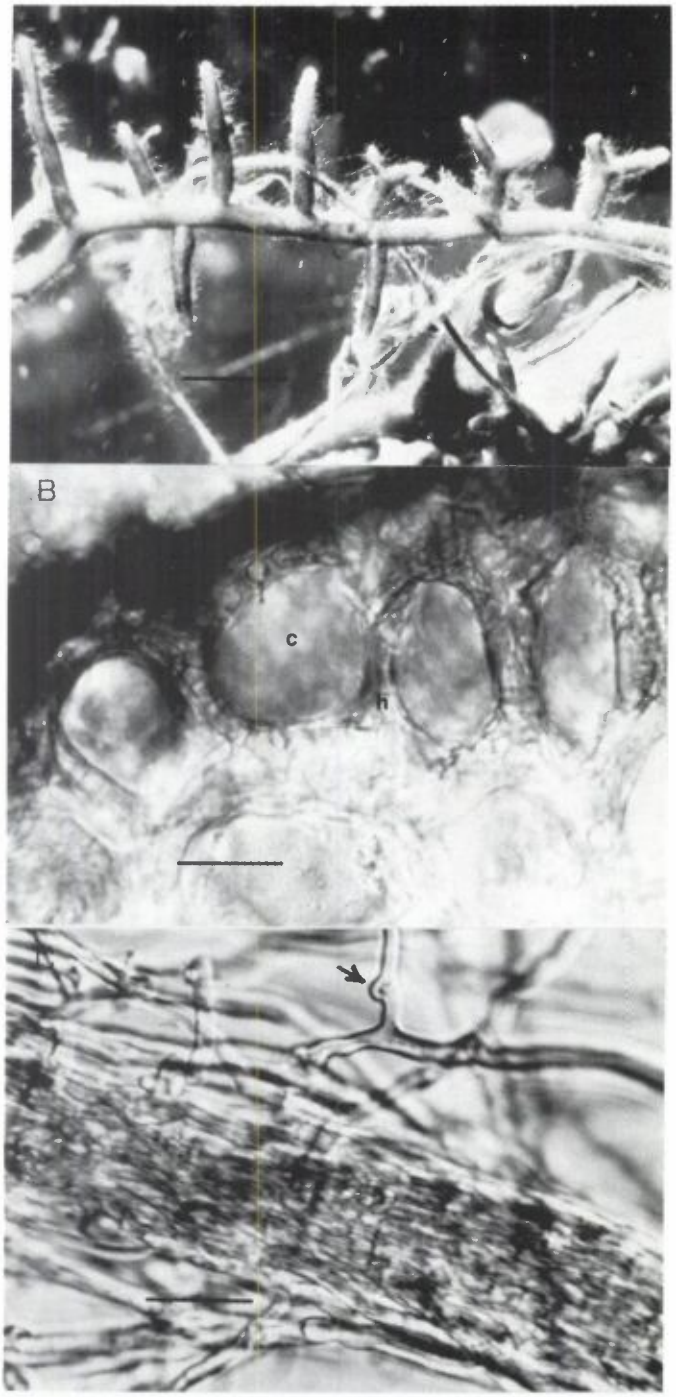

Figure 7.

Figure 7. Red pine X *Thelephora terrestris* mycorrhizae. (A) Sudan brown to Hay's pusset, simple and bipodal mycorrhizae showing lighter tips and tomentose texture (size range $1 - 3 \times 0.25 - 0.5$ mm; multiply-forked forms were also present). Note thick mycelial strands. Bar = 2 mm. (B) Cross section of mycorrhiza showing mantle (m) (20–40 μm thick), spherical hyphae of Hartig net (h), and root cortical cells (c). Note several layers of hyphae between outermost and inner cortical cells. Bar = 20 μm. (C) Mycelial strand composed of parallel hyphae. Note clamp connection (arrow). Bar = 20 μm.

Mycorrhizae were not formed on either uninoculated seedlings or those inoculated with *Calvatia gigantea*, *Lycoperdon perlatum* and *Paxillus atrotomentosus*. *Calvatia gigantea* tested weakly positive for cellulase production. *Lycoperdon perlatum* tested strongly positive for cellulase production and weakly positive (with no growth) on GA medium. *Paxillus atrotomentosus* grew well and produced a moderate reaction on both GA and TA media.

Height of seedlings was quite uniform among the successful syntheses, with means ranging from 7.4 to 11.1 cm. Seedlings main root length was more variable among the successful syntheses, with means ranging from 13.1 to 30.9 cm. Seedlings remained healthy in all successful syntheses, as did those inoculated with *C. gigantea*. Seedlings inoculated with *L. perlatum* generally became chlorotic, while those inoculated with *P. atrotomentosus* varied from healthy to moribund.

All fungi which formed mycorrhizae also formed mycelial strands, though differing in their composition. In addition, all mycorrhizae possessed the interlocking hyphal pattern on the inner mantle surface overlying root cortical cells.

4. Discussion

Three of ten attempted mycorrhize synthesis tests failed. Failure to form mycorrhizae in pure culture is not conclusive proof that a fungus is non-mycorrhizal (Trappe, 1967; Riffle, 1973; Kropp, 1982; Molina and Palmer, 1982). Pure culture syntheses are highly artificial, presenting conditions which may not be conducive to forming the same relationship found in nature. Nevertheless, mycorrhizae formed in pure culture may still bear similarity to those formed in nature (Trappe, 1967; Riffle, 1973; Molina and Palmer, 1982). Morphologically similar mycorrhizae have been formed using the pure culture technique described here and under operational greenhouse conditions. and under operational greenhouse conditons with the fungi *S. citrinum* Pers. (Richter and Bruhn, 1986, 1987, 1989) and *S. meridionale* (Richter and Bruhn, 1987).

Laboratory nutritional studies can supplement evidence provided by a mycorrhize synthesis failure. Mycorrhizal fungi are presumed to be fastidious organisms, dependent almost solely on their host for energy sources (Harley and Smith, 1983). Except for a few reports of ectomycorrhizal fungi degrading cellulose and lignin (Giltrap, 1982; Trojanowski et al., 1984), these carbon sources are apparently not available to most ectomycorrhizal fungi, due to their inability to produce appropriate cellulases and/or ligninases (Melin, 1953; Meyer, 1974; Nilson and Ginns, 1979; Harley and Smith, 1983; Martin et al., 1987). The CA method determines the ability of an organism to liberate an azure dye bound to cellulose powder in the absence of other carbon sources (as used in this study). In studies with *Aspergillus niger* cellulase and *Clostridium thermocellum* cultures, dye release has been shown to be a sensitive and quantifiable indicator of extracellular cellulase activity (Hotten et al., 1983). However, the ability of various fungi to produce cellulases can be influenced by the growth medium. For example, *P. atrotomentosus*, like most brown-rot fungi, can not degrade pure, non-associated cellulose (Nilsson and Ginns, 1979). The failure of *P. atrotomentosus* to give a positive reaction on CA medium is apparently due to the non-associated nature of the cellulose in the medium.

Growth rate and degree of agar discolorization on GA or TA media have been used as standard tests of fungus extracellular phenoloxidase activity (Davidson et al., 1938). Nearly all white-rot fungi tested by Davidson et al. (1938) gave a positive reaction on either one or both of these media, whereas approximately 20% of the brown-rot fungi tested gave inconsistent reactions. Inconsistency and much of the variation in strength of reactions among fungi may be attributed to the wide range of phenoloxidases produced by fungi.

Unsuccessful syntheses

In this study the three isolates which failed to form mycorrhizae produced a positive reaction on one or more of the enzyme test media. The combined results of our synthesis trials and enzyme screening suggest that *C. gigantea*, *L. perlatum* and *P. atrotomentosus* may be saprobic rather than mycorrhizal fungi.

Various species of *Calvatia, Lycoperdon*, and *Paxillus* have been suggested to be mycorrhizal fungi based on field observations (Trappe, 1962). Riffle (1983) failed to produce mycorrhizae in pure culture between *Pinus ponderosa* Laws, and either *Calvatia craniiformis* (Schw.) Fr. and *Lycoperdon rimulatum* Pk. Kropp (1982) was unable to synthesize mycorrhizae between

P. atrotomentosus and *Tsuga Leterophylla* (Raf.) Sarg. This fungus commonly fruits on brown-rotted wood, known to contain abundant conifer mycorrhizae in forest ecosystems (Harvey et al., 1976). The mycorrhizal fungus *Scleroderma citrinum* is often found on rotted wood (Smith, 1951). However, other species of *Paxillus* are known either to be mycorrhizal or to cause a brown-rot type of wood-decay (Redhead and Ginns, 1985). *Hygrophoropsis aurantiaca* (Wulf.:Fr.) Maire, also in the Paxillaceae and found on brown-rotted wood (Redhead and Gins, 1985), failed to form mycorrhizae with red pine in pure culture (Richter and Bruhn, 1986).

Successful synthesis

Formation of mycorrhizae by seven of the fungus isolates examined demonstrates that a symbiotic relationship between these fungi and red pine can be formed (Trappe, 1962, 1967; Riffle, 1973; Kropp, 1982; Molina and Palmer, 1982). We believe that this is the first report of pure culture mycorrhizae synthesis for each of these host-fungus combinations, and the first report of any pure culture synthesis for *B. parasiticus*, *S. bovista* and *S. polyrhizum*.

Species of *Boletus* are generally considered mycorrhizal (Trappe, 1962; Miller, 1982; Harley and Smith, 1983). *Boletus parasiticus* is usually found fruiting from the base of *Scleroderma vulgare* Fr. (= *S. citrinum* [Guzman, 1970]) (Smith and Thiers, 1971). The sporocarp from which our isolate was obtained was not directly associated with *S. citrinum*, although the latter occurred in the immediate vicinity.

Thelephora terrestris is common in North America and is considered to be an early and opportunistic colonizer of both coniferous and deciduous tree seedlings (Trappe, 1977; Harley and Smith, 1983). Trappe (1962) provided no records of *T. terrestris* in his extensive review of mycorrhizal associations. Few reports of pure culture mycorrhizae syntheses with this fungus and conifers are available; it has been reported with *Tsuga heterophylla* (Kropp and Trappe, 1982), *Pinus taeda* L. (Marx et al., 1970), and *Picea sitchensis* (Bong.) Carr. (Thomas and Jackson, 1979).

Nearly 100% of available fine roots were colonized by *T. terrestris*, demonstrating that experimental conditions were adequate for mycorrhizae formation by this fungus. Such efficient colonization of a seedling by a fungus may indicate its role as an "early-stage" mycorrhizal fungus (Dighton and Mason, 1985). Abundant mycelial strands were formed by *T. terrestris*, in contrast to *L. laccata* (Scop.:Fr.) Berk. & Br., another early-colonizing mycorrhizal fungus found in nurseries (Trappe, 1977; Harley and Smith, 1983).

Of the gasteromycetes tested, *A. hygrometricus*, *S. bovista*, *S. cepa*, *S. meridionale*, and *S. polyrhizum* formed mycorrhizae with red pine. Mycorrhizae and mycelial strands formed by these isolates were morphologically quite similar. *Astraeus hygrometricus* has a wide distribution across North America, especially in sandy forested areas (Smith, 1951; Molina and Trappe, 1982; Taber and Taber, 1984). The four species of *Scleroderma* have a worldwide distribution, generally in temperate forested regions (Guzman, 1970; Demoulin and Malencon, 1971; Miller, 1983; Garrido, 1986).

Mycorrhizae formed by the *Scleroderma* spp. coincided closely with literature descriptions of mycorrhizae synthesized between other species of this genus and various other hosts (Marx and Bryan, 1969; Molina and Trappe, 1982; Richter and Bruhn, 1986, 1987; Kannan and Naturajan, 1987). In contrast to the nearly complete colonization of fine roots by *T. terrestris*, colonization of root tips was 50% or less by the species of *Scleroderma* tested. We suspect that these *Scleroderma* spp. represent "later-stage" mycorrhizal fungi (Dighton and Mason, 1985). In greenhouse inoculations of red pine, *Laccaria bicolor* (Maire) Orton, probably an "early-stage" mycorrhizal fungus (Dighton and Mason, 1985), far surpassed *S. citrinum* in rate of seedling colonization (Richter and Bruhn, 1989).

No reports of pure culture mycorrhizae synthesis with *A. hygrometricus* are listed in Trappe's early review (1962). Schramm (1966) reported field observations of mycorrhizae formed by *A. hygrometricus* with *P. virginiana* Mill. on coal mine spoils in Pennsylvania. *Astraeus hygrometricus* has formed mycorrhizae in pure culture with *Betula pendula* L. (Gaie and Heinemann, 1980) and *P. banksiana* (Danielson, 1984). Mycorrhizae of *Astraeus pteridis* (Shear) Zeller have been synthesized with a number of coniferous and deciduous hosts (Trappe, 1967; Molina, 1979; Molina and Trappe, 1982).

Reports of *Scleroderma* spp. forming mycorrhizae in pure culture are fairly numerous, with *S. citrinum* (=*S. aurantium* Pers., =*S. vulgare* [Guzman, 1970]) being represented most often. Trappe (1962) lists reports of pure culture syntheses for *S. citrinum* with *Picea abies* L., *Pinus mugo* Turra., *P. strobus* L., *P. sylvestris* L., and *P. virginiana* Mill. He also cites numerous likely coniferous and deciduous hosts based on field observations. Other pure culture syntheses with *S. citrinum* have been reported for *P. patula* Schl. & Cham. (Kannan and Naturajan, 1987) and *P. resinosa* (Richter and Bruhn, 1986). This fungus has also been successfully used to inoculate greenhouse seedlings of *Quercus rubra* L. (Beckjord and McIntosh, 1984). Schramm (1966) reports *S. citrinum* to be an earlier colonizer of *P. virginiana* on coal mine spoils.

Scleroderma bovista has formed mycorrhizae with pecan in greenhouse pots (Marx and Bryan, 1969). Trappe (1962) lists no references of pure culture mycorrhizae synthesis with this species, and lists only *Pinus radiata* D. Don, *Pseudotsuga menziesii* (Mirb.) Franco, and *Eucalyptus* spp. as likely hosts based on field observations. In the northern U.S., we have observed *S. bovista* associated with *Betula, Pinus, Populus* and *Quercus.*

Scleroderma cepa (=*S. flavidum* Ell. & Ever. [Guzman, 1970]) has formed mycorrhizae in pure culture with *Betula papyrifera* Marsh (Jones et al., 1986). Trappe (1962) lists no pure culture syntheses with this species, and cites only an *Eucalyptus* sp. as a possible host. In the northern U.S., *S. cepa* has been found associated with species of *Betula, Pinus, Populus* and *Quercus* (Richter and Bruhn, 1987).

Neither *S. meridionale* (=*S. macrorrhizon* Wall. [Demoulin, 1974]) nor *S. polyrhizum* (=*S. geaster* Fr. [Guzman, 1970]) is included in Trappe's list (1962). Mycorrhizae synthesis has been reported between *S. meridionale* and *P. banksiana* (Danielson, 1984). *Scleroderma polyrhizum* has been implicated in forming mycorrhizae with *Casuarina* sp. in Australia based on field association (Miller, 1983). In the northern U.S., *S. meridionale* and *S. polyrhizum* have been found associated with the same tree genera as *S. cepa*, along with *Picea* for *S. meridionale* (Richter and Bruhn, 1987).

5. Conclusions

The synthesis method employed is suitable for demonstrating the mycorrhizae-forming ability of a variety of fungi symbiotic with *P. resinosa.* Red pine seedlings grew well and were generally in good condition at the end of the 4–5 month synthesis period. Only those seedlings paired with two of the apparently nonmycorrhizal fungi declined in condition.

Three of the ten fungi used in this study failed to form mycorrhizae and are suspected to be nonmycorrhizal. These isolates were also shown to produce extracellular enzymes more characteristic of a saprobic than a symbiotic lifestyle.

The fungus isolate which colonized root systems most thoroughly in our syntheses was *T. terrestris.* This is not surprising, in light of its common occurrence in greenhouses and nurseries of both coniferous and deciduous tree seedlings (Trappe, 1977; Harley and Smith, 1983). Our results support the contention that this is an "early-stage" mycorrhizal fungus, particularly adapted to the physiology of seedlings rather than mature trees (Dighton and Mason, 1985). Less thorough colonization of fine roots by the other

mycorrhizae-forming fungi suggests that they might be better adapted to older seedlings or trees.

This report further extends the mycorrhizal role for species of *Scleroderma*, and even suggests a largely mycorrhizal lifestyle for the genus.

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