Chapter 1

The use of specific ectomycorrhizas to improve artificial forestation practices

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Introduction

Microorganisms are present in great numbers on and near the feeder roots of trees, and they play vital roles in numerous physiological processes. These dynamic processes are mediated by associations of microorganisms participating in saprotrophic, pathogenic and symbiotic root activities. The major symbiotic associations on tree roots are mycorrhizas. The word mycorrhiza is used to describe a structure that results from a mutually beneficial association between the fine feeder roots of plants and species of highly specialized, root-inhabiting fungi. Mycorrhizal fungi derive most if not all of their organic nutrition from their symbiotic niche in the primary cortical tissues of roots. The mycorrhizal habit probably evolved as a survival mechanism for both partners in the association, allowing each to survive in environments of low soil fertility, drought, disease, and temperature extremes where alone they could not.

Endomycorrhizas are the most widespread and comprise three groups, ericaceous, orchidaceous, and vesicular-arbuscular mycorrhizas (VAM). The VAM are found on more plant species than all other types of mycorrhizas combined; they have been observed in roots of over 1000 genera of plants representing some 200 families. Over 90% of the 300,000 species of vascular plants in the world form VAM. The commercial use of VAM is considered in Chapter 3.

Ectomycorrhizas occur on about 10% of the world flora. Trees belonging to the Pinaceae, Fagaceae, Betulaceae, Salicaceae, Juglandaceae, Myrtaceae, Ericaceae, and a few others form ectomycorrhizas. Numerous fungi have been identified as forming ectomycorrhizas. In North America alone, it has been estimated that more than 2100 species of fungi form ectomycorrhizas with forest trees. Worldwide, there are over 5000 species of fungi that can form ectomycorrhizas on some 2000 species of woody plants.

Importance of ectomycorrhizas to trees

Ectomycorrhizal fungi aid the growth and development of trees. For some trees, such as *Pinus* sp., they are indispensable for growth under natural conditions. The obligate requirement of pine for ectomycorrhizas in a natural environment has been clearly shown by numerous workers in tree regeneration trials in former treeless areas and in countries without native ectomycorrhizal trees (Marx, 1980a). In trees with abundant ectomycorrhizas the combination of fungus and root provides a much larger, and more physiologically active, area for nutrient and water absorption than is present in trees with few or no ectomycorrhizas. This increase in surface area comes both from the multi-branching habit of most ectomycorrhizas and from the extensive vegetative growth of hyphae of the fungal symbionts from the ectomycorrhizas into the soil. These extramatrical hyphae function as additional nutrient and water-absorbing entities and assure maximum nutrient capture from the soil by the host. Ectomycorrhizas are able to absorb and accumulate nitrogen, phosphorus, potassium, and calcium in the fungus mantles more rapidly and for longer periods of time than nonmycorrhizal feeder roots. Ectomycorrhizas also appear to increase the tolerance of trees to drought, high soil temperatures, soil toxins (organic and inorganic), and very low soil acidity. Ectomycorrhizas also deter infection of feeder roots by root pathogens, such as species of *Pythium* or *Phytophthora* (Marx, 1973; Marx & Krupa, 1978) and hormone relationships induced by fungal symbionts cause ectomycorrhizal roots to have greater lengths of physiological activity than nonmycorrhizal roots (Ek, Ljungquist & Stenstrom, 1983). Not all species of fungi form ectomycorrhizas that have equal benefit to their hosts under specific conditions. Some are more effective than others.

Ecology of ectomycorrhizal fungi

Many species of fungi are normally involved in the ectomycorrhizal associations of a forest stand, on a single tree species, on an individual tree, or even on a small segment of lateral root. As many as three species of fungi have been isolated from an individual ectomycorrhiza (Zak & Marx, 1964). A single fungus species can enter into ectomycorrhizal association with numerous tree species on the same site. A fungus can also develop numerous biotypes or clones in a very limited area of a pure stand (Fries, 1987). Some fungi are apparently host-specific; others have broad host ranges and form ectomycorrhizas with members of numerous tree genera in diverse families.

There are distinct early- and late-stage fungi in ectomycorrhizal fungus successions in forests. In aseptic culture (i.e. without competition and other stresses) early- and late-stage fungi form ectomycorrhizas on seedlings equally well. However, only the early-stage fungi are able to rapidly

colonize seedlings in natural, nonsterile soil that harbours competitors and other environmental stresses. Early-stage fungi may not totally disappear from mature stands, but they are supplanted by more dominant species (Dighton, Poskitt & Howard, 1986) or are suppressed in reproduction because of changes in canopy and root/soil characteristics.

Increases in fungal species diversity are associated with ectomycorrhizal fungus succession as forest stands age and numbers of host species increase (Last *et al*., 1984). Fruit body production by these fungi, which is how fungal succession is measured, is also strongly influenced by seasonal changes, rainfall amount and frequency, organic content of soil, root density, and degree of ectomycorrhizal development (Wilkins & Harris, 1946).

Use of specific ectomycorrhizal fungi on seedlings

Ectomycorrhizal fungi have been introduced into deficient soils in various inocula to provide seedlings with adequate ectomycorrhizas to create man-made forests. Most research on inoculation with ectomycorrhizal fungi has been based on two premises. First, any ectomycorrhizal association on roots of tree seedlings is far better than none. Success in correcting deficiencies has contributed greatly to our understanding of the importance of ectomycorrhizas to trees. Second, some species of ectomycorrhizal fungi on certain sites are more beneficial to trees than other fungal species that naturally occur on such sites. Much work in recent years with a few fungal species has been aimed at selecting, propagating, manipulating, and managing the more desirable fungal species to improve tree survival and growth and this has recently been reviewed (Marx & Ruehle, in press). Consequently, the purpose of the present paper is to discuss the research and development that led to present-day ectomycorrhizal technology in the United States.

Selection of candidate fungi

The first and most important step in any inoculation program of tree seedlings is the selection of fungi. Physiological and ecological differences among ectomycorrhizal fungi are great and these differences can be used as criteria for selection. Host specificity is a physiological trait important to consider in the selection process. The consistent association of certain fungi with only a few specific tree hosts is well documented in the literature. Many other fungi are associated with a great number of different tree hosts; these are probably early-stage fungi. Any candidate fungus should exhibit the physiological capacity to form ectomycorrhizas on the desired hosts and the more hosts the better. Isolate variability within any candidate symbiont is another criterion to consider. Several isolates from different tree hosts and geographic regions should be used, at least initially, to determine the amount of variation that exists between isolates.

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This point has been stressed by Moser (1958a) and demonstrated with iso-
lates of various fungi. For example, isolates of *Pisolithus tinctorius* from
various pines form abundant ectomycorrhizas on pine and oak, but iso-
lates from various oak species form few ectomycorrhizas on pine. Some
oak isolates formed no ectomycorrhizas on either host, nor did other pine
isolates from Australia and Brazil (Marx, 1979; 1981). Another criterion
is the ability of the selected fungus to grow in pure culture and withstand
manipulation. A variety of culture media and methods of isolation can be
used to obtain pure cultures of the selected fungus (Schenck, 1982; see
also chapter 2). Ideally, the fungus should be able to grow rapidly. Once
the growth characteristics of a fungus have been confirmed, it is import-
ant to determine its capacity to withstand physical, chemical, and
biological manipulations. Producing large quantities of vegetative inocu-
lum of a fungus is of little value if the fungus cannot survive the rigours of
various manipulations, such as physical processing (blending, leaching, or
drying), shipment, and soil incorporation. The fungus inoculum must also
be able to survive a minimum of 4 to 6 weeks between nursery soil inocu-
lation and seed sowing, germination, and the production of short roots by
the seedlings. During this period, it must survive fluctuations of soil mois-
ture, temperature and microbial competition. Ideally, the fungus should
also be able to survive several weeks of storage between inoculum pro-
duction and use.

Another criterion is the ecological adaptation of the selected fungus to
the major type of site on which the seedlings are to be planted. Of equal
importance is the ability of the fungus to survive and grow under cultural
conditions used in nurseries. In other words, the candidate fungus must
be an early-stage fungus in normal succession if it is to be effective on
seedlings in the nursery and in the early successional stage of stand estab-
lishment. According to Trappe (1977), the ecological adaptability of an
ectomycorrhizal fungus hinges on the metabolic pathways it has evolved
to contend with environmental variation. Extremes of soil and climate, an-
tagonism from other soil organisms including other ectomycorrhizal fungi,
pesticide application, physical disruption of mycelium from nursery cul-
tural practices (undercutting and root pruning), and the abrupt
physiological adjustment from a well fertilized and irrigated nursery soil
to an uncultivated, low-fertility planting site with all its normal stresses
are only a few of the environmental variations to which the selected fun-
gus must adapt.

The effect of temperature on different species and ecotypes of ectomy-
corrhizal fungi is perhaps the most widely researched environmental
factor. Upper and lower temperature limits of the candidate fungi should
be determined. Moser (1958a) studied the ability of fungi to survive long
periods (up to 4 months) of freezing at -12°C and to grow at 0 to 5°C. He

found that high elevation ecotypes of *Suillus variegatus* survived freezing for 2 months, but valley ecotypes were killed after freezing for only 5 days. However, the ability of certain fungi to survive freezing was not correlated with their ability to grow at low temperature. Generally, mountain eco-types and species had much lower temperature optima than lowland ones. *Pisolithus tinctorius* can grow at temperatures as high as 40 to 42°C (Hung & Chien, 1978) and has a hyphal thermal death point of 45°C (Lamb & Richards, 1971). It not only survives and grows well at high temperatures but it also grows at 7°C (Marx, Bryan & Davey, 1970; Marx & Bryan, 1971) and withstands frozen soil (Marx & Bryan 1975).

Reaction of candidate fungi to soil moisture, organic matter, and pH are also important traits to consider. *Cenococcum geophilum* is not only drought tolerant but forms ectomycorrhizas in natural soils ranging in pH from $3 \cdot 4$ to $7 \cdot 5$ (Trappe, 1977). Unfortunately, the drought tolerant char-acteristics of *C. geophilum* also make it difficult to establish on pine seedlings in irrigated nurseries where it can be rapidly supplanted by natu-rally-occurring *Thelephora terrestris* (Marx, Morris & Mexal, 1978). *Pisolithus tinctorius* ectomycorrhizas on pine and oak have been observed in drought-prone coal spoils as acid as pH $2 \cdot 6$. *Suillus bovinus* (Levisohn, 1956) and *Paxillus involutus* (Laiho, 1970) form abundant ectomycorrhi-zas on seedlings in nurseries with high organic matter, but these ectomycorrhizas are supplanted by others after seedlings are planted out on sites having low organic matter.

The production of hyphal strands and sclerotia are also important traits in candidate fungi. Uptake of nutrients, especially phosphorus (Bowen, 1973), and translocation of carbon compounds (Reid & Woods, 1969) take place through hyphal strands. In Australia, one of the initial criteria for selection of fungi for inoculation programmes is its ability to produce hyphal strands. Although research data are lacking, abundant hyphal strand production by *P. tinctorius* apparently enhances water and nutrient absorption and increases its survival potential under adverse conditions. Yellow-gold hyphal strands of this fungus, easily visible to the naked eye, have been traced through coal spoils as far as 4 m from young seedling roots to fruit bodies. The production of sclerotia by *P. tinctorius* (Marx *et al.*, 1982) and *C. geophilum* (Trappe, 1969) in soil or container rooting media should enhance the abilities of these fungi to survive harsh soil con-ditions. Therefore, sclerotia production is also a favourable trait in candidate fungi.

All the criteria mentioned are meaningless unless the candidate fungus is aggressive and can form abundant ectomycorrhizas on seedlings as soon as short roots are produced. This is another characteristic common among early-stage fungi. The fungus should be able to maintain superiority over

naturally occurring fungi on seedling roots in the nursery. Even though the effect of a fungus on seedlings may only be temporary until it is supplanted by other fungi in the field, this brief advantage may make the difference between survival or death of newly planted seedlings. For tree seedlings to obtain any measurable benefit from a specific ectomycorrhizal association, there is a threshold amount of the specific ectomycorrhizas that must be present on seedling roots.

Types of inocula

The majority of reports on inoculation techniques with ectomycorrhizal fungi involve basidiomycetes on pines, oaks, and eucalypts. The techniques were developed out of necessity to grow tree species requiring ectomycorrhizas in areas of the tropics where ectomycorrhizal fungi were absent. Several types of natural and laboratory-produced inocula and several methods of application have been used through the years. Many of the techniques have proven successful, others have not.

The most widely used natural inoculum, especially in developing countries, is soil or humus collected from established pine plantations (Mikola, 1973; Marx 1980a). In most instances, the original soil inocula came from mature pine plantations on other continents. A major drawback with soil or humus inoculum is the lack of control of species of ectomycorrhizal fungi in the inoculum. There is no assurance that soil inoculum contains the most desirable fungi for the tree species to be produced or for the site on which the seedlings are to be planted out. Soil inoculum from mature pine forests would likely contain more late-stage than early-stage fungi. Also, soil inoculum may contain harmful microorganisms and noxious weeds. Some of these microorganisms may be potentially harmful not only to the tree seedling crop, but also to nearby agricultural crops. Exotic pathogens introduced to the areas in soil inoculum create potential hazards for epiphytotics. The use of natural inoculum, however, does satisfy the premise that any ectomycorrhizas on seedlings used in forest regeneration are better than none.

Spores of various fungi have been used as inoculum to form specific ectomycorrhizas on tree seedlings. The major advantages are that spores require no extended growth phase under aseptic conditions, and spore inoculum is very light. One gram of basidiospores of *Rhizopogon luteolus* or *Pisolithus tinctorius* contains about 1×10^9 potentially infective basidiospores. Another advantage of spores, at least those of certain fungi, is their ability to maintain viability in storage from one season to the next. Such survival is important because spores collected in the summer or early autumn must be stored until the following spring if they are to be used to inoculate spring-sown nursery beds. However, one of the major disadvantages of spore inoculum of most ectomycorrhizal fungi is the lack of

appropriate laboratory tests to determine spore viability. Currently, time-consuming ectomycorrhizal synthesis tests are the only reliable means of determining spore viability of many of these fungi. Another disadvantage is that sufficient sporophores of many fungi may not be available every year. One would need ideal storage conditions to maintain a large inventory of spores in order to ensure a constant supply of spore inoculum from year to year.

Formation of ectomycorrhizas by basidiospores usually takes 3 to 4 weeks longer than vegetative inoculum of the same fungus (Theodorou & Bowen, 1970; Marx, Bryan & Cordell, 1976). This delay can be a disadvantage because, during this period, pathogenic fungi or other ectomycorrhizal fungi can colonize the roots and reduce the effectiveness of the introduced spore inoculum. Also, seedlings experiencing a delay in ectomycorrhizal formation lose the physiological advantage that ectomycorrhizas furnish during this period. It should be pointed out, however, that in parts of the world where the natural occurrence of ectomycorrhizal fungi is erratic or deficient, this delay may not have a significant effect on the final results of inoculation. Another significant problem in using spore inoculum is the lack of genetic definition. Genetic diversity in basidiospores can be enormous, particularly if spores are collected from many geographic areas and from different tree hosts and combined into a single inoculum. Basidiospore inoculum of *Pisolithus tinctorius* has been used on an experimental scale, and more recently on an operational scale, in the United States and elsewhere. Recent studies have proved that these spores are effective in various inoculum forms such as: (1) mixed with a physical carrier before soil inoculation, (2) suspended in water and drenched onto soil, (3) dusted or sprayed onto soil, (4) pelleted and broadcast onto soil, (5) encapsulated or coated onto pine seeds, and (6) in hydrocolloid chips (Marx & Ruehle, in press).

Pure mycelial or vegetative inoculum of ectomycorrhizal fungi has been repeatedly recommended as the most biologically sound material for inoculation. Unfortunately, ectomycorrhizal fungi as a group are difficult to grow in the laboratory. Many species have never been isolated and grown in pure culture. Some species grow slowly, others often die after a few months in culture. Most of these fungi require specific growth substances, such as thiamine and biotin, in addition to simple carbohydrates (Schenck, 1982). Several researchers in various parts of the world have developed cultural procedures for producing vegetative inoculum of a variety of fungi for research purposes. Unfortunately, large-scale nursery applications of pure mycelial cultures, even those involving only a few thousand tree seedlings, have been severely hampered by the lack of sufficient quantities of viable inoculum. It is relatively simple to produce a sufficient volume of inoculum, i.e., 30 to 40 litres, for research studies carried out in small con-

tainers, pots, microplots, or even small nursery plots, but it is a completely different matter to produce sufficient quantities of commercial vegetative inoculum for large-scale nursery inoculation in a practical programme (Marx, 1985). Tens of thousands of litres of vegetative inoculum would be needed to inoculate just one of the nurseries in the southern US, where over 1·5 billion pine seedlings are produced annually on some 625 hectares of nursery soil (see also chapter 2).

Propagation of vegetative inoculum

Moser (1958a, b, c) in Austria was one of the first to make a serious attempt to produce vegetative inoculum of ectomycorrhizal fungi. For production of inoculum, mycelium of *Suillus plorans* was first grown in liquid culture then in sterile peat moss. Takacs (1961, 1964, 1967) in Argentina modified Moser's techniques to produce inoculum for new pine nurseries established in formerly treeless areas lacking native ectomycorrhizal fungi. Inoculum of *Amanita verna*, *Suillus granulatus*, *S. luteus*, *Hebeloma crustuliniforme*, a *Russula* sp., *Scleroderma verrucosum*, and *S. vulgare* were produced with this technique. It is now recognized that the above species of fungi represent both early- and late-stage fungi in normal forest succession. Quantitative data on ectomycorrhizal development after such inoculations are not available, and it is difficult to evaluate the success of this inoculation method. Hacskaylo & Vozzo (1967) initiated a series of inoculation experiments with pure vegetative cultures of various fungi to correct a deficiency of these fungi on the island of Puerto Rico. Following Moser's general technique, they grew *Cenococcum geophilum*, *Corticium bicolor*, *Rhizopogon roseolus*, and *Suillus cothuranatus* in polypropylene cups containing a 2:1 ratio of sterile peat moss and vermiculite moistened with nutrient solution. After root inoculation in the nursery, all fungi except *C. geophilum* formed ectomycorrhizas to varying degrees on *Pinus caribaea* seedlings.

Since 1966, the USDA Forest Service Institute for Mycorrhizal Research and Development (IMRD) has been studying the significance of ectomycorrhizas formed by *Pisolithus tinctorius* and other fungi to survival and growth of tree seedlings on a variety of sites. The interest in *P. tinctorius* was prompted by its relatively wide geographic and tree host distribution, range of environmental tolerance, and ease of pure culture propagation (Schramm, 1966; Marx, 1977, 1980a, 1981). This fungus is now known to form ectomycorrhizas with over 100 species of woody plants. Vermiculite and peat moss moistened with modified Melin-Norkrans medium (MMN) was found to be an excellent substrate for the production of effective vegetative inoculum (Marx, 1969).

Vermiculite-peat moss-MMN inoculum used directly from the container and mixed into fumigated nursery soil is rapidly colonized by

saprophytic microorganisms. Heavy colonization reduces the effectiveness of this inoculum to form ectomycorrhizas on pine. Leaching the inoculum before inoculation to remove most nonassimilated carbohydrates reduces this microbial colonization and increases inoculum effectiveness (Marx, 1980a). Leached inoculum has been used by many workers, but it is difficult to use because it has a very high bulk density (750 g l^{-1}), a high moisture content, and the physical consistency of a sticky paste. These problems were corrected by slowly drying the inoculum to final bulk densities from 320 to 390 g l^{-1} and moisture contents from 10 to 35%. Final pH of inoculum ranged from $4 \cdot 4$ to $5 \cdot 2$. The original volume of inoculum was reduced by nearly 60% after leaching and drying. Dried vegetative inoculum mixed more uniformly in soil than the wetter, non-dried formulations, so it was effective when used at lower rates. Also, dried inoculum stored at 5°C for as long as 9 weeks and at room temperature for 5 weeks was still effective in forming ectomycorrhizas (Marx, 1980a).

In 1976, the IMRD and State and Private Forestry, USDA Forest Service, and Abbott Laboratories, North Chicago, Illinois, began a cooperative program of research and nursery evaluation to develop commercial methods of producing vegetative inoculum of *Pisolithus tinctorius*. The effectiveness of different vegetative inoculum formulations was tested under diverse cultural regimes on various tree species in bare-root and container nurseries in the United States and Canada. Inoculum from IMRD and Abbott formed ectomycorrhizas on 13 species of trees in tests with several types of containers and cultural practices at six locations in the United States and one in Canada (Marx *et al.*, 1982). Tests of IMRD and Abbott inoculum formulations in over 75 bare-root seedling nursery tests were completed in 1981 (Marx et al., 1984). In 33 different tree nurseries in 25 states on 14 tree species, research showed that inoculum formulations effective on containerized seedlings were also effective on bare-root seedlings (Fig. 1.1A & 1.1B). Results from over 30 nursery tests with loblolly pine in the southern US showed that abundant *P. tinctorius* ectomycorrhizas reduced seedling losses by over 25% and increased fresh weights of seedlings by over 20%. These were unexpected benefits to the nurserymen. The Abbott inoculum, which was trademarked > MycoRhiz®, was also grown in the vermiculite-peat moss-MMN medium. Liquid cultures (MMN) were grown in shake flasks, 14-litre fermenters, or large industrial fermenters and were used as starter inoculum for the vermiculite-peat moss-MMN substrate. Liquid cultures were continuously agitated and highly aerated at temperatures of 28 to 32°C for 7 to 14 days.

Beds of medium were sterilized by injection of high pressure steam in either deep-tank or rotary drum fermenters. The sterile medium was inoculated with the starter inoculum of *Pisolithus tinctorius*. The level of
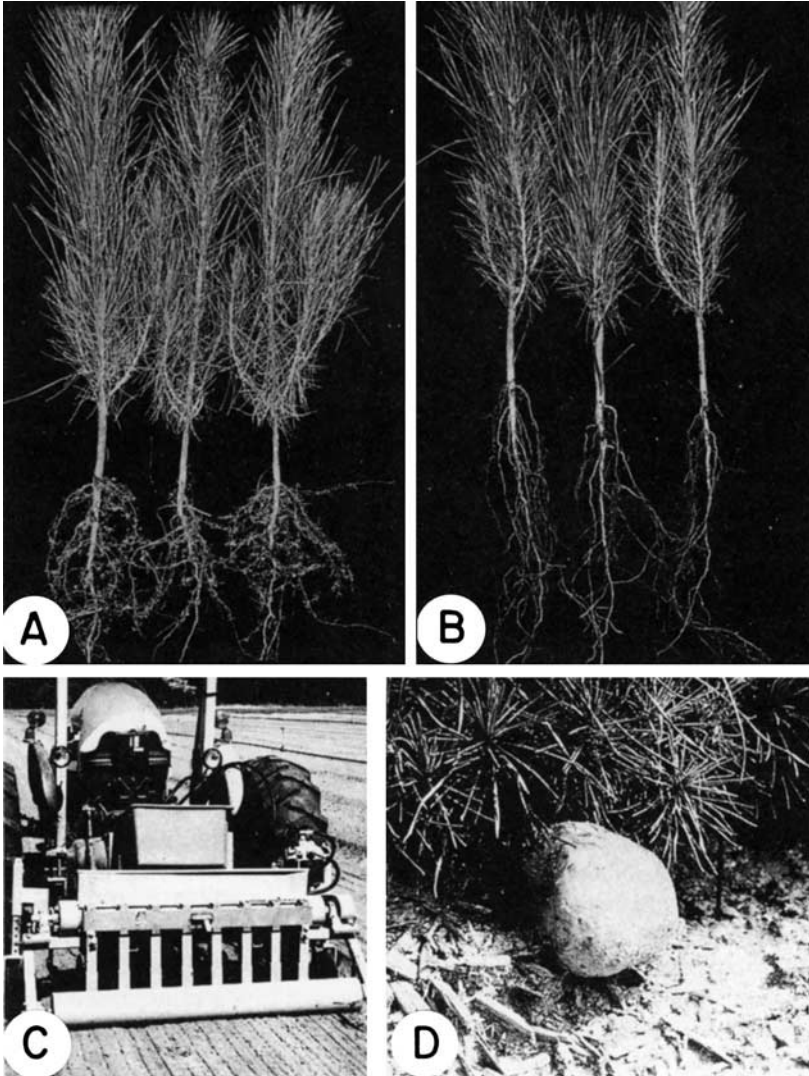
Fig. 1.1. Loblolly pine (*Pinus taeda*) seedlings with abundant *Pisolithus tinctorius* ectomycorrhizas following inoculation with commercial vegetative inoculum (A) and nursery-run seedlings with only naturally-occurring ectomycorrhizas (B). Soil was inoculated by machine (C) and, following successful mycorrhizal development, fruit bodies of *P. tinctorius* (D) are produced in abundance.