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Application of Soil Microorganisms in Planting Stock Production

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Cover photo: abundant ectomycorrhizae on roots of *Hopea odorata* stecklings at ACFTSC.

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Contents

Contents

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Overview

Somyos Kijkar

Importance of vigorous seedlings

Success and failure in reforestation programs depend on various factors: site quality and preparation, climatic conditions, seedling vigor, socioeconomic variables, etc. Of these, site quality and preparation and seedling vigor emerge as the most important.

Soil degradation in tropical regions is spreading quickly and now affects vast areas that need reforestation. Deforestation greatly affects soil fertility, in part, because the loss of trees means that there are few soil microorganisms present. Without soil microorganisms, soils compact and bake, and plant roots cannot penetrate to absorb whatever nutrients and moisture may be available.

However, it is seedling vigor that should be accorded priority. Though the planting site may be fertile and ready for reforestation, poor or weak seedlings can greatly affect plantation establishment. Before plantation establishment, vigorous, healthy seedlings of required species have to be prepared and made suitable for outplanting at the beginning of the rainy season.

Planting stock production is usually a problem in tropical countries because of poor technology, lack of experience, and poor knowledge of contributing factors. The role of microorganisms is one of those factors that has been overlooked and yet is most important.

In addressing the importance of soil microorganisms in planting stock production, it is necessary to deal with more than mycorrhizae. The first papers of these Proceedings describe briefly the role of rhizobia, the nodule bacteria associated with the roots of most leguminous trees. Particular attention is paid to *Frankia*, as this species associates also with many nonleguminous species. Finally, soil microorganisms active in the decomposition of organic matter and necessary in soil improvement, are dealt with.

One of the most important and perhaps least understood zones of biological interaction is the rhizosphere—the area immediately surrounding tree roots—where numerous microorganisms affect the uptake, storage, and processing of nutrients.

ASEAN region

In ASEAN-member countries nursery operations face many difficulties. Brunei-Darussalam set up the Forest Research Centre at Sungai Liang, where large-scale nurseries are operated. Potting media consist of a mixture of swampy peat, horse

dung, and subsoil; it is a pity that there is so little subsoil available. Swampy peat and horse dung raise the temperature in the pots during decomposition and damage valuable microorganisms, resulting in high seedling losses. Wildings collected from natural stands could be potted and tended under the donors using soil from the site itself as an alternative.

Indonesia has been successful in using soil microorganisms. The Faculty of Forestry at the Bogor Agricultural University has considerable knowledge and experience in this area. Researchers have developed strains of bacteria that are very useful in bio-fertilizer decomposition or breaking down biological material in potting mixtures. The Tropenbos Foundation, in cooperation with the Indonesian Forestry Research and Development Agency, has adopted the use of soil microorganisms in hedge orchards and vegetative propagation of dipterocarps. Many other nongovernmental organizations are also working on the application of soil microorganisms in reforestation, especially in Kalimantan and Sumatra.

Researchers in Peninsular Malaysia have done solid research on the role of soil microorganisms, at the Universiti Pertanian Malaysia and the Forest Research Institute of Malaysia. However, the application of research and development findings is limited. In Malaysia, soil microorganisms are generally available from top soil in natural stands.

The situation is similar in Sabah and Sarawak. Though the importance of soil microorganisms is sometimes overlooked, planting material production using top soil from natural stands yields satisfactory results.

In the Philippines, soil microorganism technology is much advanced at a commercial level. Capsules and tablets of mycorrhizae and/or rhizobia are generally available. Work by Prof. Reynaldo de la Cruz and Prof. Mercides U. Garcia at the University of the Philippines at Los Baños, has regional recognition. However, the application of soil microorganisms in planting stock production is also limited, principally because of economic considerations, and naturally occurring microorganisms on planting sites.

In Thailand, the role of soil microorganisms in forestation has been acknowledged for decades. Pine plantations in the north obtain mycorrhizae directly from soil under old plantations or natural pine stands. Recently, fruiting bodies of *Pisolithus tinctoris* (Pt) were collected, dried, and used to inoculate potting media for producing pine seedlings. Mycorrhizal application in dipterocarp planting material using top soil from natural stands of the species is also employed. Research results at the Royal Forest Department on inoculation of potting media are widely distributed. Seedlings of leguminous species, though, do not seem to benefit from inoculation. This greatly affects their growth and yields; more research on this is required.

Singapore is not involved in reforestation along road sides; it is principally interested in trees for shade and enjoyment. Nursery operations in Singapore are advanced and there is no difficulty obtaining soil microorganisms.

As already mentioned, the role of soil microorganisms in planting stock production in ASEAN region needs to be addressed. The ASEAN-Canada Forest Tree Seed Centre Project, through a recommendation of the Working Group on Nursery Technology and Stock Production, organized the training course on which these Proceedings are based to broaden views, concepts, and ideas among researchers and foresters.

Biological nitrogen fixation in forestry

Nantakorn Boonkerd

Introduction

In the tropics, nitrogen is the most common limiting nutrient in crop production. The high cost and limited availability of nitrogen fertilizer preclude its use by many small-scale farmers. The largest reservoir of nitrogen is the atmosphere, which is approximately 80% nitrogen gas. Higher plants and animals, though, cannot incorporate this form of nitrogen. However, certain procaryotic microorganisms, which have a nitrogenase enzyme system, can convert nitrogen gas from the atmosphere to nitrogen compounds usable by plants. This process is known as biological nitrogen fixation (BNF).

There are two sources of nitrogen for plants: 1) nitrogenous fertilizers, and 2) biological nitrogen fixation. Nitrogen is nitrogen, neither source is better; plants cannot distinguish between the two sources. Biological nitrogen fixation is attractive to farmers because nitrogenous fertilizers are too expensive, especially in developing countries.

Nitrogen-fixing microorganisms vary, from freely fixed nitrogen to physiologically and genetically integrated symbioses with host plants. Between these extremes, nitrogen-fixing organisms are found in almost all ecosystems—in the seas, swamps, lakes, and uplands. Nitrogen-fixing microorganisms comprising bacteria, cyanobacteria, and actinomycetes generally fall into two groups: 1) those that fix nitrogen in free-living stage, and 2) those that fix nitrogen in association with higher plants. Examples of free-living nitrogen-fixing microorganisms are bacteria in the genera *Azotobacter, Klebsiella, Azospirillum,* and many genera of cyanobacteria such as *Anabaena, Nostoc,* and *Scytonema.* The amount of nitrogen that can be fixed by free-living organisms is generally small (Table 1). The amount of nitrogen fixed in symbiotic associations with plants is considerably greater than that fixed by free-living organisms (Table 2). Examples of symbiotic nitrogen-fixing systems are: cyanobacteria *Anabaena* and aquatic fern *Azolla;* actinomycetes *Frankia* and actinorrhizal plants such as *Alnus, Casuarina,* and others; and bacteria *Rhizobium* or *Bradyrhizobium,* and leguminous plants. Most nitrogen-fixing trees are associated with *Frankia* and *Rhizobium,* or *Bradyrhizobium.*

Legumes and rhizobium symbiosis

Rhizobia are soil bacteria. They are aerobic, gram negative, rod-shaped, and have the unique ability of infecting leguminous roots to form nodules. Rhizobia in the nodules are transformed into a specific form called "bacteroides." Bacteroides in a nodule can fix a substantial amount of nitrogen, some of which is subsequently translocated to the plant in the form of an amino acid. The plant contributes to the

Table 1 *Biological nitrogen fixation in land*

Land use	Amount of nitrogen fixed (million metric tons/yr)
Agricultural	
Leguminous crops	35
Non-leguminous crops	9
Permanent meadows, grasslands	45
Forest and woodland	40
Unused	10
Total	**139**

Source: *Burns and Hardy 1975*

Table 2 *Biological nitrogen fixing by microorganisms*

Habitat of microorganisms	Example of species	kg N fixed/ha/yr (approx.)
Free-living in soil or water	*Azotobacter, Klebsiella*	1 - 5
Free-living photosynthetic in water or on moist soil	*Anabaena, Nostoc*	1 - 50
Associated with roots (rhizosphere)	*Azospirillum*	2 - 20
Symbiotic, forming nodules on plants	*Rhizobium, Frankia*	10 - 500
Symbiotic with aquatic water fern	*Anabaena Azolla*	5 - 100
Symbiotic with fungi (lichens)	*Nostoc, Calothrix*	1 - 10

Source: *Beringer, J.E. EEC BNF Reports. [Unpublished] .*

symbiosis by providing the bacteria with a suitable environment for nitrogen fixation and photosynthates. This type of symbiosis is considered mutually beneficial.

The effectiveness of the association between rhizobia and leguminous plants can also be determined by nodule color. Effective nodules are normally large and have a deep reddish color inside. The red pigment is leghaemoglobin; it is not a part of nitrogenase—the nitrogen fixing enzyme—it provides the oxygen necessary for bacteroides.

Table 3 *Old and new classifications of rhizobia*

Bacteria	Examples of host genera
Old	
Rhizobium meliloti	*Medicago, Melilotus, Trigonalla*
Rhizobium trifolii	*Trifolium* spp.
Rhizobium leguminosarum	*Pisum, Vicia, Lathyrus, Lens,*
Rhizobium phascoli	*Phaseolus vulgaris, P. multiforis*
Rhizobium lupini	*Lupinus, Ornithopus*
Rhizobium japonicum	*Glycine max*
Cowpea	*Vigna,* many genera and species
New	
Fast growers	
Rhizobium meliloti	*Medicago, Melilotus, Trigonolla*
Rhizobium leguminosarum	
biovar. - *trifolii*	*Trifolium* spp.
biovar. - *phaseoli*	*Phaseolus vulgaris, P. multiforis*
biovar. - *viceae*	*Pisum, Lathyrus, Lens, Vicia*
Rhizobium loti	*Lupinus, Lotus, Anthyllis,*
Ornithopus	
Rhizobium fredii	*Glycine max*
Slow growers	
Bradyrhizobium japonicum	*Glycine max*
Bradyrhizobium spp.	
sp. (*Vigna*)	*Vigna,* and many other genera
sp. (*Lupinus*)	*Lotus pedunculatus, Lupinus* sp.

Source: *Burton, J.C. 1984*

Early classification of rhizobia was based on a specific characteristic of nodulation between leguminous species and a particular type of rhizobia. This classification is known as "cross inoculation." Rhizobia capable of nodulating plants in one of these groups were considered a species regardless of whether or not nitrogen fixation occurred (Table 3).

A new system of classifying rhizobial species based on their physiological and genetic properties has been developed; it was published in the ninth edition of Bergey's *Manual of determinative bacteriology* (Jordan 1984). In this system, two genera of rhizobia are classified based on their growth characteristics, the fast grower is *Rhizobium* and the slow grower *Bradyrhizobium* (Table 3).

The effectiveness of rhizobia are cultivar (host) specific. Some of the rhizobial strains that are effective on a cultivar may not form effective nodules on others. Tropical legumes, on the other hand, are found to form symbiotic associations with several or many species or strains of *Bradyrhizobium* and so are regarded as promiscuous in their rhizobial requirements. Since nitrogen-fixing trees (NFTs) are mostly associated with *Bradyrhizobium* spp., which are found in most tropical soils, it was thought that NFTs were not rhizobia specific. In fact, NFTs are rhizobia specific. Based on our research with *Pterocarpus macrocarpus* and *Leucaena leucocephala*, we found that only 10% of strains tested were effective. To fully benefit from biological nitrogen fixation (BNF), NFTs should be inoculated with proper strains of rhizobia.

Frankia and actinorrhizal plants

Frankia is a genus of nitrogen-fixing actinomycetes. It has the ability of infecting and forming nodules on roots of actinorhizal plants similar to rhizobia and legumes. Root nodules of actinorhizal plants induced by *Frankia* are morphologically and anatomically distinct from legume nodules. These nodules result from a localized stimulus at the infection site of multiple, modified lateral roots whose condensed and repeated branching produces a more or less spherical mass, sometimes up to several centimeters in diameter. Two general types of nodules occur: 1) the *Alnus*-type, with knobby, corolloid structures, and 2) the *Myrica*-type, in which each terminal lobe of the nodule produces a determinate nodule root, which grows more or less vertically (Torrey 1978).

Actinorhizal plants are economically important in forestry programs such as land reclamation and reforestation due to their ability to form symbiotic associations with nitrogen-fixing actinomycetes. *Frankia* are symbiotically associated with a diverse number of angiosperms belonging to 20 genera of eight plant families, as is shown in Table 4 (Baker and Seling 1984).

Ecological features common to all actinorhizal plants are that they are predominantly temperate rather than tropical, perennial, and generally pioneer species, i.e., they are the first plants to colonize a novel or disturbed ecosystem (Baker and Seling, 1984).

Table 4 *Actinorhizal plant genera: families and genera of plants reported to be nodulated by* Frankia (from Baker and Seling 1984).

Family	Genera	Isolated endophytes
Betulaceae	*Alnus*	+
Casuarinaceae	*Casuarina*	+ (?)
Coriariaceae	*Coriaria*	—
Datiscaceae	*Datisca*	—
Elaeagnaceae	*Elaeagnus*	+
	Hippophae	+
	Shepherdia	—
Myricaceae	*Comptonia*	+
	Myrica	+
Rhamnaceae	*Ceanothus*	+ (?)
	Colletia	—
	Discaria	—
	Kentrothamnus	—
	Trevoa	—
Rosaceae	*Cerococarpus*	—
	Chaemabatia	—
	Cowania	—
	Dryas	—
	Purshia	+
	Rubus	—

Note *The right-hand column indicates genera from which endophytes have been isolated and cultured in vitro. Question marks denote Frankia strains unable to reinfect their source host genera.*

Casuarina is probably the only truly tropical actinorhizal genus.

Similar to the situation with legume symbioses, *Frankia* strains are host specific (Baker 1987). In general, the strains of one compatible group are unable to infect the hosts of another strain.

Application of BNF to NFTs

Nitrogen-fixing trees are similar to most leguminous crops in terms of nutrition

requirement and management, especially at the earlier stage of growth. This means that the application of BNF to NFTs should be done in the same manner as leguminous crops.

The application of BNF-organisms (Rhizobia) to leguminous crops normally consists of adding inoculant (bacteria in a carrier material) to seeds before planting. An alternative method consists of applying the inoculant directly to the soil by mixing inoculant with filler such as moist soil or sand or making a suspension of inoculant with water and spraying directly onto seeds in the seed bed. As for the NFTs, the simplest way is to inoculate the nursery stock by inoculating the seeds prior to planting, using the method described above. For bare-rooted stock soil, inoculation by spraying or dropping inoculant suspension directly onto the roots is recommended. A few seedlings should be checked for the presence of nodules. Nodules should appear within 3-5 weeks after planting. If no nodules or few nodules are observed and plants are not healthy after the application of nitrogenous fertilizer, it means that the inoculant is of poor quality or the rhizobial strains are not suitable for this particular legume. In this case, consult with inoculant producers for a possible remedy.

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The importance of mychorrhizae in planting stock production

Aniwat Chalermpongse

Introduction

About 100 years ago, several biologist noticed that certain plant roots, though extensively invaded by fungi, were not diseased. The name "mycorrhiza" (fungus root) was coined by Frank (1885), who provided one of the best early descriptions of the phenomenon. The structure of mycorrhizae has been examined intermittently ever since the first observations made by Unger (1840). Mycorrhizae have attracted the attention of mycologists, tree physiologists, biochemists, biotechnologists, foresters, and nursery specialists. Considerable information exists on their physiology and function. We now know, for instance, that mycorrhizal plants grow better than nonmycorrhizal plants, especially in poor soils..

Mycorrhizae are formed when fungi grow among the outer cells of feeder roots (Marks and Kozlowski 1973). Trees with abundant mycorrhizae have a much larger, physiologically active fungus-root area for nutrient and water absorption than trees with few mycorrhizae (Bowen 1973, Reid 1979).

Successful inoculation of tree seedlings with superior mycorrhizal fungi has resulted in significant improvements in the survival, growth, and quality of forest plantations worldwide (Bowen 1980). Container-grown, and barerooted seedlings with specific and superior mycorrhizal fungi perform significantly better than seedlings with naturally occurring mycorrhizae (Marx et al. 1982). Increases in seedling biomass as a result of mycorrhizal inoculation range from 25 to 450% in field experiments (Marx et al. 1977). The presence of mycorrhizae is known to increase tolerance of trees to extremes of soil moisture (Dixon et al. 1983), temperature (Dixon et al. 1981), toxins (Schramm 1966), and root diseases (Marx 1973). In return, tree hosts provide mycorrhizal fungi with metabolic products such as surplus carbohydrates and vitamins (Hacskaylo 1973).

Deforestation

Tropical forests cover 2970 million hectares (20% of the earth's land surface) and contain half the world's species of flora and fauna. Over the course of the past century, however, tropical forests have been rapidly depleted because of logging to meet, in part, the expanding need for agricultural land. According to one estimate, at least 225 million more hectares will be cleared or degraded by the end of this century. Another major cause of deforestation is shifting agriculture. It is estimated that shifting agriculture now accounts for 70% of deforestation in Africa, 50% in Asia, and 35% in the Americas. At present rates, nine developing countries

will have exhausted their broadleaved forests within 25 years, and a further 13 countries within 50 years (FAO/UNEP 1981).

New plantations can take the pressure off natural forests and help meet requirements of wood, food, fuel, fiber, medicinal plants, and many other badly needed materials.

When establishing new forest plantations, it is important to rear container-grown seedlings that are large and robust within a single growing season. This contrasts with the 2-3-year cycle needed to produce plantable seedlings in barerooted nurseries in the western and northern United States. Most current criteria of seedling quality are limited to the condition and size of seedling stem and foliage. Less attention is paid to the quality of roots in nursery seedlings, though we are well aware of the paramount importance of roots in providing structural support and nutrient and water uptake. To make a complete evaluation of seedling health and accurately predict survival potential, we must increase our awareness of the importance of root quality. In this respect, the presence and abundance of mycorrhizae must be a major factor to consider (Landis et al. 1990).

This paper briefly discusses the role of mycorrhizae in nurseries and forest establishment, including the use of soil, spore, sporophores, vegetative pure mycelial inocula, and mycorrhizal mother-tree seedlings. Recent advances in the use of spore and mycelial inocula of selected fungi and methods used in the production of inoculum are also reviewed.

What are mycorrhizae?

The word "mycorrhizae" literally means "fungus roots." It defines the intimate association between plant roots and specialized soil fungi—the mycorrhizal fungi. Nearly all of the world's land plants form some type of mycorrhizae, and with few exceptions, all major forest tree species form mycorrhizae.

Classes of mycorrhizae

There are three classes of mycorrhizae and they are identified by the arrangement of the hyphae of the fungi within root cortical tissues: 1) ectomycorrhizae, 2) endomycorrhizae (vesicular-arbuscular mycorrhizae), and 3) ectendomycorrhizae.

1) Ectomycorrhizae

With the use of a microscope, hyphae of ectomycorrhizal fungi can be observed growing internally only around the cortical cells of the roots forming the Hartig net, hence the prefix "ecto." The Hartig net hyphal arrangement appears to replace the middle lamella, which is normally composed of pectins and cemented to the cortical cells. Hyphae of the fungal symbionts, which are present around the outside of the feeder root, are usually tightly interwoven and referred to as the "fungal mantle." The mantle of ectomycorrhizae may be only one of two hyphal

mantles in thickness, or several dozen hyphals in thickness. Ectomycorrhizae can be white, brown, yellow, black, blue, or blends of these colors. The color of ectomycorrhizae is determined apparently by the hyphal color of the specific mycorrhizal fungus encompassing the root.

The need for ectomycorrhizae was first noted when attempts were made to establish new plantations of exotic pines and domestic tree species in various parts of the world. A deficiency of fungal partners on the roots of planting trees resulted in establishment failure. The need of pine, oak, dipterocarp, and *Afzelia* seedlings for ectomycorrhizae has also been demonstrated in the afforestation of treeless areas such as grasslands (Mikola 1973). Even on clear cut pine lands (Ruehle 1982) or amended adverse sites (Ruehle 1980), nonmycorrhizal pine seedlings do not survive or grow well until indigenous fungi form ectomycorrhizae on their roots.

Ectomycorrhizae are formed by fungi belonging to the higher Basidiomycetes, Ascomycetes, *Fungi Imperfecti,* and Zygosporic Phycomycetes of the Endogonaceae (Gerdemann and Trappe 1974, Trappe 1962, 1971). The common ectomycorrhizal-forming fungi are known as Hymenomycetes, and include: *Amanita, Boletus, Boletellus, Cantharellus, Laccaria, Clitopilus, Clitocybe, Cortinarius, Lactarius, Leccinum, Russula, Suillus, Tricholoma, Xerocomus, Corticium, Thelephora, Clavulina, Marasmius, Hebeloma, Paxillus, Hygrophorus, Tylopilus.* Among the Gasteromycetes, are: *Astraeus, Calvatia, Geastrum, Lycoperdon, Scleroderma, Pisolithus, Rhizopogon, Elaphomyces, Tuber, Genea, Hydnotrya, Geopora, Balsamia* and *Sphaerosporella.* Among the Ascomycetes, are: *Cenococcum geophilum* Fr. (Syn. *C. graniforme* (Sow.) Ferde. and Winge), which is the imperfect stage of Elaphomyces (Miller 1982). *Endogone lactiflua* Bk. and Br. and *E. flammicorona* (Trappe and Gerdemann) are among the Endogonaceae of Zygosporic Phycomycetes. All other species of Endogonaceae are endomycorrhizae and belong to other genera (Gerdemann and Trappe 1974).

The host plants of ectomycorrhizal fungi are predominantly trees belonging to the Pinaceae (pine, fir, larch, spruce, hemlock), Fagaceae (oak, chestnut, beech), Betulaceae (Betula, alder, birch), Salicaceae (poplar, willow), Juglandaceae (hickory, pecan), Myrtaceae (eucalyptus), Ericaceae (arbutus), Dipterocarpaceae (*Dipterocarpus, Shorea, Hopea*), Caesalpinaceae (*Afzelia, Bauhinia, Cassia*), and other families.

Ectomycorrhizal fungi occur in soils in the form of spores, free living mycelia, hyphal strands, and sclerotia. Identification can be made by sporophores or fruit bodies that usually develop in association with mycorrhizal roots. Most ectomycorrhizal fungi are difficult to isolate from roots and maintain in artificial cultures in which their growth is slow. But some are very easy to isolate from fruit bodies or sporophores.

2) Endomycorrhizae (vesicular-arbuscular mycorrhizae)

Vesicular-arbuscular (VA) mycorrhizae are strikingly different from ectomycorrhizae. They do not modify root morphology and the fungal component is invisible to the unaided eye. Roots must be differentially stained and observed

under the microscope to discern satisfactorily fungal structures and the degree of root colonization. As implied in the name, two structures characterize the VA mycorrhizae—vesicles and arbuscules. Vesicles are balloon-shaped structures, usually filled with lipids (oil droplets), that serve both as energy storage organs and as reproductive structures. Arbuscules are finely branched, intracellular, short-lived structures that serve as nutrient exchange sites between fungus and host. VA mycorrhizae also have abundant fungal mycelium that ramifies through or inside the root cortex and extends out into the soil.

Zygomycetous fungi (Phycomycetes) in the family Endogonaceae form VA mycorrhizae and there are a few hundred species, about 120 of which have been identified in the genera: *Acaulospora, Entrophospora, Gigaspora, Glomus, Sclerocystis,* and *Scutellospora* (Schenck and Perez 1987).

Because of the difficulty of cultivating endomycorrhizae in pure culture, they are studied in the soil where they occur as free spores (chlamydospores, zygospores, azygospores), sporocarps, or in the extramatrical and intramatrical phases of the host. VA mycorrhizal spores are isolated from the soil by wet sieving and decanting (Gerdemann and Nicolson 1963) or by floatation-adhesion (Sutton and Barron 1972). The fungi are maintained in pure pot culture on the root of host plants in sterilized soil. VAM spores are not wind disseminated, thus their movement is primarily by soil movement, small animals, and insects.

VAM mycorrhizae are distributed in most plant families in the tropics, whereas ectomycorrhizae are distributed widely in temperate timber trees (Sander et al. 1975, Chalermpongse 1987, Jülich 1988).

3) Ectendomycorrhizae

This class is apparently intermediate between the other two classes. These fungi grow into the cortical cells of the root and their appearance is quite different from the arbuscular formation of endomycorrhizae. They grow around the cortical cells in a Hartig net arrangement and may or may not develop a fungal mantle over the surfaces of feeder roots. Ectendomycorrhizae have been observed on roots of certain species of tree seedlings in nurseries, particularly pines and spruces.

The fungi of ectendomycorrhizae are designated as *E.* strains and are nonsporing (Mikola 1965). They are predominant in old forest nurseries raised on agricultural sites and weakly competitive in forest soils. Morphologically, they look like ectomycorrhizae but usually lack the thick, often colorful mantle and visibly abundant external hyphae common to ectomycorrhizae. Although we know little of their ecology or effects on seedling nutrition, growth, and survival, ectendomycorrhizae have been shown to be beneficial in certain instances (LoBuglio and Wilcox 1987, Wilcox and Ganmore-Neumann 1974). The fungi are *Ascomycotina* and mostly lacking mushroom-like fruiting structures, although some form small cup-shaped fruiting bodies on the surface of growing media. The representative

genera are *Phialophora, Chloridium* and *E.* strain. Very few have been researched in tropical countries.

Mycorrhizae and nursery management

Previous land use is important in selecting the nursery site. Growth tends to be poor in nurseries established on agricultural land which has been cropped for many years. Nurseries can be established after clear felling the forest cover; this will ensure the presence of mycorrhizae that occur naturally in the site. The nursery soil should be either acidic or neutral. Conifers are known to grow best in soils with a pH of 4.5-5.5, eucalyptus 5.5-6.5, dipterocarps 6.0-6.8, and leguminous species 6.0-8.0. The development of mycorrhizae is best on arable sites where the soil pH is 5.0-6.5. With a high soil pH, conifer seedlings are known to have difficulty absorbing inorganic fertilizers from the soil.

Seedlings can be raised directly in the nursery on beds consisting of soil mixed with fertilizers or composts to permit adequate mycorrhizal development. The seedlings can remain in the beds until they are ready for outplanting or pricked 2-3 weeks after germination to another nursery bed for more spacing or in polytene tubes or bags.

Need for mycorrhizal inoculation

Tree species in the Pinaceae, Fagaceae, Dipterocarpaceae, and Caesalpiniaceae families require ectomycorrhizae. As already mentioned, this has been convincingly demonstrated in attempts at afforestation in treeless areas such as grasslands, amended adverse sites, old agricultural lands, clear-cut forest areas, and post-wildfire areas.

The following benefits of mycorrhizae in tree growth are based almost exclusively on ectomycorrhizae:

- Tremendous increase in the surface area of feeder roots for absorption of water and nutrients including mycorrhizal hyphae growing from mycorrhizal root systems (Marx and Bryan 1975).

- More selective absorption and accumulation of nutrients, especially phosphorus and other elements.

- Solubilization of minerals and their constituents, which are otherwise unavailable for plant growth.

- Increased longevity of feeder root function; mycorrhizal roots persist longer on root systems than nonmycorrhizal roots.

- Resistance to feeder root infection caused by pathogens such as *Phytophthora* and *Pythium* spp., which are present in many forest and nursery soils.

- Increased tolerance of tree to soil toxins (inorganic and organic), extremes of soil acidity and temperature, etc.

Source of inoculum and inoculation techniques

Soil, spores, sporocarps, vegetative mycelium and mycorrhizal mother-tree seedlings are primary sources of ectomycorrhizal and VA mycorrhizal inoculum for containerized seedlings in nurseries. Each has advantages and disadvantages in relation to the objectives and economics of the inoculation program.

1) Soil inoculum

Historically, soil inocula taken from under ectomycorrhizal host trees have been used extensively, especially in developing countries. In nursery practice, 10-20% (by volume) of soil inoculum is incorporated into the nursery soil (top 10 cm of beds) before sowing. Soil or humus is usually collected from established pine plantations or a successful stand of the same species from the top 10-25 cm of soil, where mycorrhizal trees were closely allied. The soil inoculum should contain, in addition to soil, active mycorrhizal short roots. The material is collected after top debris and litter have been removed. The inoculum should be used 10-15 days after collection to ensure the viability of the fungi. The material should not be exposed to direct sunlight, which can be harmful. After raking or mixing with local soil in the nursery, seed are broadcast in the bed; germinated seedlings will eventually develop mycorrhizae. Mycorrhizal soil inoculation of the nursery bed may be unnecessary if the seedlings are pricked 2-3 weeks after germination into another nursery bed or in polytene tubes, where the mycorrhizae can be introduced. In case of tube seedlings, 2 tablespoonfuls of soil inoculum can be added to every tube prior to sowing or pricking. In some cases, the local soil and the mycorrhizal soil inoculum are mixed in a heap and raked and placed in polytene tubes. This may result in uneven distribution of the inoculum and erratic seedling development.

One of the most serious disadvantages of soil inoculum is that weed seeds, rhizomes, and potential pathogens can invade the nursery. Another disadvantage is the inconsistency of inoculum quality due to varying times and sources of soil collection. For these reasons, this method is used only if other forms of inoculum are not available.

2) Spore inoculum

Spores or macerated fruiting bodies of some ectomycorrhizal mushrooms, puffballs, or truffles (and false truffles) provide good inoculum. Gasteromycetes, such as the puffball-producing genera *Rhizopogon, Scleroderma,* and *Pisolithus,* produce numerous basidiospores that are easier to collect in large quantities than those of other mushroom-producing ectomycorrhizal fungi. Truffles (Ascomycotina) and false truffles (Basidiomycotina) are uniquely suited for this. Their fruiting body tissue consists mostly of spore-bearing tissue and the fruiting bodies can be quite large and edible. If inoculated with oak seedlings and raised in forest plantations, they are a good source of income.

Various authors (Donald 1975, Lamb and Richards 1974, Marx 1982) have demonstrated the value of basidiospores as inoculum. Using a variety of techniques, many scientists have successfully used basidiospore inoculum of *Pisolithus tinctorius* to form specific ectomycorrhizae on pine, oak, and other species of seedlings (Marx 1982). Spores of *P. tinctorius* are collected by crushing sporophores with ruptured peridia over a 25-30 mesh screen. Screened basidiospores are air-dried for several days at low humidity, then stored at 5°C. Spores stored for several years have been used to form *Pisolithus* ectomycorrhizae on pine. Since basidiospores of *P. tinctorius* and many other fungi will not germinate in the laboratory, spore viability can only be determined with ectomycorrhizal synthesis tests.

A simple and effective inoculation procedure involves dusting dry spores of *P. tinctorius* onto soil around young seedlings and leaching them into the root zone using irrigation water. This inoculation has been successful on barerooted and container-grown pine seedlings in the United States. In most tests, 1-2 mg of spores per seedling have been applied. There are about 1.1×10^6 basidiospores of *P. tinctorius* per mg.

Inoculum composed of spores mixed with a moistened carrier such as vermiculite, kaolin, or sand can be broadcast onto soil and mixed into the nursery soil or directly into the growing medium in containers to form *P. tinctorius* ectomycorrhizal pine seedlings.

Basidiospores of *P. tinctorius* have also been mixed with clay to produce tablets or pellets; one tablet is applied to each seedling pot or polytene bag. Only 1 or 2 mg of basidiospores per tablet is sufficient to machine produce tablets.

There are many ways to prepare spore inoculum, and there are advantages and disadvantages to using spores of ectomycorrhizal fungi for inoculation. The formation of ectomycorrhizal basidiospores usually takes 3-4 weeks more than the formation of vegetative inoculum of the same fungus. This can be a disadvantage because during this period pathogenic fungi and other ectomycorrhizal fungi often colonize the root and reduce the effectiveness of the introduced spore inoculum.

One of the major disadvantages of spore inoculum is the lack of standard laboratory tests to determine spore viability. Several workers have tried a variety of physical, chemical, and biological stimuli to germinate basidiospores of P. *tinctorius* without success.

The biggest problem in using spore inoculum is genetic improvement. Basidiospores of *P. tinctorius* collected from different sporophores and locations may have different genetic traits. Genetic variation would be greater if basidiospores from sporophores collected from many geographical areas and different tree hosts were combined into a single inoculum (Marx 1982).

The advantage of using spore inoculum is that it is very light. One gram of basidiospores of *Rhizopogon luteolus* or *Pisolithus tinctorius* contains more than 1

billion spores. More than 450 kg of mature, dry basidiospores of *P. tinctorius* were collected from under pine trees around coal mines near Birmingham, Alabama, U.S.A., in approximately 75 person-days. This is enough basidiospores for more than 225 million pine seedlings, assuming 1 mg of spores/seedling—about 20% of the total number of seedlings grown in nurseries each year in the United States. Thailand produces 50-100 million seedlings each year. It would be nearly impossible to collect as much fungi belonging to the Agaricales and Aphyllophoralles.

3) Mycelial inoculum

Pure mycelial or vegetative inoculum of ectomycorrhizal fungi has often been recommended (Bowen 1965, Marx 1980, Mikola 1973, Trappe 1977) as the most biologically sound method of inoculation. Unfortunately, ectomycorrhizal fungi are difficult to grow in pure culture. Many species have never been isolated and grown in the laboratory. Some species grow slowly, others often die after a few months in pure culture. Most of these fungi require specific growth substances such as thiamine and biotin, in addition to simple carbohydrates. Most are sensitive to growth-inhibiting substances.

The first and most important step in mycorrhizal inoculation of tree seedlings is selecting/breeding superior species or strains of fungi. The genetic and physiological differences are great among ectomycorrhizal fungi. Host specificity is one physiological trait that is important to consider in the selection process. Some species of ectomycorrhizal fungi have broad host ranges, whereas others have a very narrow host range.

Over the past few years, there has been much research on the production and use of pure culture inoculum of selected ectomycorrhizal fungi (Molina and Palmer 1982, Marx and Kenney 1982). A pure culture of a particular fungus is isolated from fungal material (spore germination or vegetative tissue explant) onto special media.

Modified Melin-Norkrans (MMN) agar medium: 0.05 gm $CaCl_2$, 0.025 gm NaCl, 0.5 gm KH_2PO_4, 0.15 gm $MgSO_4$ 7 H_2O, 1.2 mL of 1% $FeCl_3$, 100 mg Thiamine HCl, 3 gm malt extract, and 10 gm glucose in distilled water to equal 1 liter. Fifteen grams of agar/liter are added for forming agar.

After autoclaving, the pH of both the liquid and agar is 5.5-5.7. This medium has proven to be as good or better than most other media for growing many ectomycorrhizal fungi.

A ratio of 28:1 vermiculite and peat moss substrate moistened with MMN equal to approximately half the volume of the dry substrate has proven to be the best substrate. An example is 1400 mL of vermiculite and 50 mL of peat moss mixed thoroughly and then moistened with 750 mL of MMN (Marx and Bryan 1975). Horticultural grade No. 4 vermiculite should have all fine particles removed by screening it through a fine mesh screen. Peat moss should be screened in the same manner. After autoclaving, the pH of the mixed substrate should be 4.5-5.5.

The size of the culture vessel is not critical. One-liter Erlenmeyer flasks can be used as well as 50-L carboys. Mycelial discs from agar plate culture of mildly blended mycelium from liquid culture work well. Four to six evenly spaced agar mycelial discs/L of substrate are recommended. The length of incubation after inoculation with mycelial discs depends on the growth rate of the fungi. Typically, fast growing fungi such as *Thelephora terrestris* and *Pisolithus tinctorius* incubated at room temperature will last 2-4 months in 2-L containers. Slow-growing fungi like *Cenococcum geophilum* will take 8-10 months to colonize the same substrate. Blended mycelial starter inoculum mixed thoroughly in the substrate will reduce incubation time by half.

When vermiculite-peat moss nutrient inoculum is ready to be used in the nursery, take out the inoculum from containers and leach before inoculation into fumigated nursery soil. Leaching is done simply by wrapping 4-6 L of inoculum in several layer of cheese cloth and irrigating it for 2-3 minutes under cool tap water. Excess water is then removed by squeezing the inoculum in the cheesecloth by hand. Drying at room temperature reduces the moisture content to 20-65%. The inoculum is kept in appropriate containers such as plastic bags and placed in cold storage (5°C). About 8-10% inoculum mixed with nursery soil is normally used for inoculation (Marx et al. 1982).

Mycorr Tech Inc. (440 William Pitt Way, Pittsburgh, PA, U.S.A. 15238) is presently producing vegetative inoculum for commercial purposes. Their product comes in 7- to 10-L bags, is effective, and has a reasonable shelf life. Currently, *Pisolithus tinctorius, Hebeloma crustuliniforme,* and *Laccaria laccata* are readily available; other ectomycorrhizal fungi may be produced as demand warrants. In 1988, their product cost approximately US$1.00-2.00 per thousand seedlings, not including the cost of application. Tests have shown their product to be reliable, reproducible, available relatively quickly, and uncontaminated.

4) Mycorrhizal mother-tree seedlings

Mycorrhizal seedlings collected from mother-tree seedlings in nurseries or natural stands containing appropriate mycorrhizae are transplanted in new seed beds at regular spacing (about 0.3 m x 0.3 m) and the beds are subsequently sown. It has been suggested that this is the only successful method for introducing mycorrhizae in *Pinus merkusii* in Indonesia, where traditional methods of soil inoculation are unsuccessful (Marks and Kozlowski 1973).

5) Vesicular-arbuscular inoculum

Two major features of VA mycorrhizal fungi greatly influence both natural and artificial means of seedling inoculation. First, VA mycorrhizal fungal spores are not wind dispersed, as is the case with many ectomycorrhizal fungal spores. Therefore, VA mycorrhizal fungal spores will not blow in from outside the nursery, or from within the nursery, to naturally inoculate seedlings. Thus, VA mycorrhizal host plants grown in artificial growing media or sterilized soil will not form

mycorrhizae. Second, since VA mycorrhizal fungi can not be grown in pure culture (i.e., without a host), bulk vegetative (mycelial) inoculum is not available. Nonetheless, other techniques for producing inoculum are available for VA mycorrhizal fungi and in many ways parallel those used for producing ectomycorrhizal fungi.

Taking soil from beneath VA mycorrhizal hosts in nature and incorporating it into the container substrate is a simple inoculation method. We discourage this technique, however, because of the potential for introducing unwanted pests and diseases into the nursery as well as the large quantity of soil needed.

Although we cannot yet produce vegetative cultures of VA mycorrhizal fungi, we can still mass procure fungal inoculum by allowing a known VA fungus to grow in association with a host and then using the soil and roots as inoculum. This procedure is called "pot culturing." Generally, spores of a particular VA mycorrhizal fungus are first retrieved from natural soil by various separation techniques (Ferguson and Woodhead 1982). They are identified, surface sterilized, and added to a sterile growing medium in which there is a host such as sorghum, clover, corn, or leguminous seedlings. As the plant grows, it forms VA mycorrhizae with the desired fungus. The fungus then spreads through the growing medium and produces abundant spores. These spores can then be retrieved from the growing medium for use as inoculum or, more commonly, the entire growing medium with the mycelium, spores, and roots (chopped) can be used as inoculum.

VA mycorrhizal fungus pot-cultured inoculum is usually added to growing media in one of two ways (Menge and Timmer 1982):

1) The inoculum is mixed evenly throughout the growing medium prior to filling the cavities.

2) The inoculum is banded 3-5 cm below the surface of the growing medium. Although the banding method may be labor intensive, it assures rapid contact between the roots and fungus as the roots grow down through the inoculum band. Information is variable as to how much inoculum is needed to ensure successful inoculation. From our experience, inoculating with 200-500 spores per seedling is a good beginning for testing inoculum effectiveness in the nursery. For example, Kough et al. (1985) used 20 mL of pot-cultured inoculum (spores + soil + chopped roots) to inoculate successfully cedar and redwood seedlings growing in 160-m^3 containers; the 20 mL of inoculum contained 400-770 spores and 30-68% of root pieces were colonized. VA mycorrhizal fungi are sensitive to high levels of fertilizer, as are many ectomycorrhizal fungi. Careful monitoring of mycorrhizal development under various management practices is needed to develop compatible regimes.

Pot-cultured inoculum provides the best source of VA mycorrhizal fungi. If the pot-cultured inoculum is grown properly, there is little risk of introducing unwanted pests or pathogens. The inoculum is usually reliable, effective, and easily introduced into growing media. Most importantly, pot culturing allows the use of

selected highly beneficial fungal strains for maximum enhancement of seedling growth and survival. Considerable research has been conducted, and continues, on selecting beneficial VA mycorrhizal fungi for plant inoculation. Although most of this research has been conducted on agricultural crops, information is also available on VA mycorrhizal forest tree species.

A commercial source of VA mycorrhizal fungi is now available; others continue to be developed. One promising source of inoculum is being developed and marketed by NPI (417 Wakara Way, Salt Lake City, Utah 84108, U.S.A.). The company can produce inoculum of several VA mycorrhizal fungi and is developing a method for bulk production of hygienically grown inoculum free of pathogens. In 1988, their product cost US$2.00-5.00 per thousand seedlings, depending on inoculation procedure. It should be noted that costs have fallen steadily during the last 2 years. NPI is also involved in site reclamation, which means that their experience incorporating microbial inoculants into plant-rearing programs will be available to nurseries wanting to begin VA mycorrhizal inoculation programs.

As with implementing an ectomycorrhizal inoculation program, nursery managers should have clear objectives for VA mycorrhizal inoculations. VA mycorrhizal inoculation can improve growth in the nursery and reduce fertilizer costs. Inoculated stock can also perform better than noninoculated stock, especially when planted in environmentally stressful habitats or where native VA mycorrhizal fungi are lacking. Whatever the objectives, working with knowledgeable specialists to aid in the selection of VA mycorrhizal fungi, techniques of inoculation, and evaluation of inoculation success is strongly recommended.

Conclusion and recommendations

We cannot overemphasize the importance of mycorrhizae in reforestation and afforestation worldwide. Mychorrhizae must be included in any assessment of root development and seedling quality in forest establishment programs. Trees have evolved with and become dependent on mycorrhizae for survival and healthy growth in all forestry settings. Foresters and nursery managers are well aware of the critical stress that seedlings experience when transplanted. It is of the utmost priority that nurseries grow and send to reforestation sites seedlings with abundant mycorrhizae on their root systems. Seedlings without mycorrhizae will have to form them before they can begin to take up actively water and nutrients from the soil. Seedlings with mycorrhizae are better prepared to begin immediately exploring the soil and stand a better chance of survival and early growth than nonmycorrhizal seedlings.

There is now considerable research in progress on mycorrhizal applications in forestry. A primary focus continues to be the selection of fungi for nursery inoculation based on specific ecological benefits, for example, providing drought-tolerance. Another research direction concentrates on how much natural fungal inoculum is left on disturbed reforestation sites. This is a very important research area because it will help foresters predict which sites are deficient in natural mycorrhizal fungus

and need inoculated nursery stocks. In the future, both research directions will provide nursery and forest management tools to enhance tree regeneration programs worldwide.

The following recommendations are offered to help nursery managers incorporate mycorrhizal management practices in their overall seedling production programs:

- Learn about the basic biology of mycorrhizae.
- Understand why mycorrhizae are important.
- Learn to recognize mycorrhizae, identify different types, and quantify the amount of mycorrhizae on a seedling root system.
- Understand, to avoid negative results, that nursery practices, especially watering, fertilization, and pesticide application affect development of mycorrhizae..
- Examine regularly and keep careful records of feeder root and mycorrhizal development of different stock throughout the nursery; correlate this information with records of other nursery practices to become familiar with how one influences the other.
- Explore the various options for inoculation which are available when the need for an inoculation program develops, and seek the advice of a mycorrhizal specialist for actual implementation.
- Experiment wisely with inoculation, beginning on a small scale and with well-designed studies that include controls.
- Keep abreast of current progress in mycorrhizal technology through reading, attending workshops, or consulting periodically with a mycorrhizal specialist.
- Obtain the following reference texts: M*ethods and Principles of Mycorrhiza Research* (1982), N.C. Schenck, American Phytopathological Society; *Tropical Mycorrhiza Research* (1980), P. Mikola, ed., Oxford University Press; and *Mycorrhiza Research Techniques* (1982), IFS Provisional Report No. 12, Stockholm, Sweden.
- Include measures of mycorrhizal development, when assessing the overall quality of seedlings.
- Inform customers about your inoculation program and its benefits; good mycorrhizal development is an additional selling point in the commercial market.

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Appendix A

Tree host genera of phanerogams (woody plants) with ectomycorrhizae in temperate and tropical native forests (adapted from Mikola 1982, Chalermpongse 1987, Jülich 1988).

Aceraceae
Acer

Angiospermae
Araliaceae
Didymopanax

Betulaceae
Alnus
Betula
Carpinus
Corylus
Ostrya
Ostryopsis

Caesalpiniaceae
Afzelia
Anthonotha
Bauhinia
Brachystegia
Cassia
Erythrophleum
Gilberttiodendron
Julbernardia
Monopetalanthus
Paramacrolobium

Cupressaceae
Cupressus
Juniperus

Dipterocarpaceae
Anisoptera
Balanocarpus
Cotylelobium
Dipterocarpus
Dryobalanops
Hopea
Parashorea
Shorea
Vatica

Ericaceae
Arbutus

Euphorbiaceae
Aldinia
Uapaca
(or Uapacaceae)

Fagaceae
Castanopsis
Fagus
Lithocarpus
Nothofagus
Pasania
Quercus
Trigonobalanus

Gnetaceae
Gnetum

Gymnospermae
Pinaceae
Abies
Cathaya
Cedrus
Keteleeria
Larix
Picea
Pinus
Pseudolarix
Pseudotsuga
Tsuga

Juglandaceae
Carya
Juglans

Letospermaceae
Leptospermum

Myrtaceae
Compomanesia
Eucalyptus
Eugenia
Melaleuca

Nyctaginaceae
Neea
Pisonia

Palmae
Euterpe

Papilionaceae
Ormosia

Polygonaceae
Coccoloba
Rhamnaceae
Rhamnus

Rosaceae
Crataegus
Malus
Pyrus
Sorbus

Salicaceae
Populus
Salix

Sapindaceae
Allophylus
Nephelium

Tiliaceae
Tilia

Ulmaceae
Ulmus

Urticaceae
Cecropin

Appendix B

Distribution of some of the fungi forming the three types of mycorrhizae and some of forest trees involved (adapted from Landis et al. 1990)

Type of mycorrhiza	Fungi		Common forest tree associates
	Class	Representative	
Ectomycorrhiza	Basidiomycotina	*Boletus, Suillus Leccinum, Boletellus*	dipterocarps, pine, eucalyptus
		Clitocybe, Cortinarius	oak, beech, birch
		Tricholoma, Boletinus, Entoloma, Russula, Rhizopogon, Hebeloma,	hemlock, larch, spruce, willow, true fir, poplar
		Lactarius, Scleroderma, Tylopilus, Hymenogaster	Douglas fir, Caesalpiniaceae
		Gautieria, Hymenogaster, Paxillus, Martellia, Cantharellus, Astraeus, Geastrum, Inocybe, Hygrophorus, Pisolithus, Lepiota	hazel
	Ascomycotina	*Tuber, Genea, Hydnotrya Elaphomyces (Cenococcum), Geopora, Balsamia, Sphaerosporell*	beech, birch, Douglas fir, eucalyptus, hazel, hemlock, larch, oak, pine, poplar, spruce, true fir, willow
	Zygomycotina	*Endogone*	Douglas fir, Monterey pine
Ectendomycorrhizae	Ascomycotina	*Phialophora, Chloridium* "E-strain"	birch, pine, spruce

Type of mycorrhiza	Fungi		Common forest tree associates
	Class	Representative	
Endomycorrhizae (Vesicular-arbuscular) (Endogonales)	Zygomycotina	*Acaulospora, Endogone, Entrophospora, Gigaspora, Glomus, Sclerocystis, Scutellospora, Complexipes, Glaziella, Modicella*	ash, cypress, bald cypress, basswood, cedar, eucalyptus, giant sequoia maple, redwood, sweetgum, sycamore, yellow poplar, and most other species of the plant kingdom

Appendix C

Tropical forest trees, shrubs, and herbs in association with endomycorrhizae (vesicular-arbuscular) (adapted from Jülich 1988, Chalermpongse 1987, Sanders et al. 1975, de Alwis and Abeynayake 1980).

Bryophyta
Hepaticae

Spermatophyta
Gymnospermae
Cycadales

Araucariaceae

Cupressaceae

Podocarpaceae

Altingiaceae
Liquidambar

Anarcardiaceae
Buchanania
Campnosperma
Lannea
Mangifera
Semecarpus
Spongias

Annonaceae
Cananga
Cyathocalyx
Goniothalamus
Melodorum
Uvaria
Polyalthia
Xylopia

Apocynaceae
Alstonia
Holasshenia
Wrightia

Aquifoliaceae
Ilex

Barringtoniaceae
Careya

Bignoniaceae
Fernandoa
Oroxylum
Spathodea
Stereospermum
Tabebuia

Bombacaceae
Bombax

Burseraceae
Canarium
Garuga
Protium

Celastraceae
Kurrimia
Siphonodon

Combretaceae
Anogeissus
Terminalia

Compositae
Eupatorium
Guizotia
Tagetes

Cornaceae
Mastixia

Dilleniaceae
Dillenia
Schumacheria
Wormia

Eberaceae
Diospyros

Elaeocarpaceae
Elaeocarpus

Ehretiaceae
Cordia

Euphorbiaceae
Agrostistachys
Aporosa
Baccaurea
Bridelia
Chaetocarpus
Cleistanthus
Croton
Hevea
Ostodes
Phyllanthus
Sapium

Flacourtiaceae
Hydnocarpus

Gramineae
Agrostis
Arandinaria
Bambusa
Cynodon
Dendrocalamus
Digitaria
Imperata
Oryza
Panicum
Paspalum
Sorghum
Zea

Guttiferae
Calophyllum
Cratoxylon
Garcinia
Mesua

Icacinaceae
Gonocaryum
Urandra

Ixonanthaceae
Cinnamomum
Cryptocarya
Irvingia
Litsea
Lauraceae
Neolitsea
Nothophoebe

Leguminosae
Acacia
Adenanthera
Albizia
Arachis
Calopogonium
Cassia
Centrosema
Dalbergia
Desmodium
Glycine
Inga
Leucaena
Milletia
Parkia
Phaseolus
Pithecellobium
Psophocarpus
Pterocarpus
Pueraria
Sindora
Stylosanthes
Trifolium
Xylia

Lythraceae
Lagerstroemia

Mangoliaceae
Liriodendron

Malvaceae
Hibiscus

Melastomataceae
Memecylon

Meliaceae
Aglaia
Amoora
Aphanamixis
Azadirachta
Cedrela
Chukrasia
Khaya
Melia
Swietenia
Toona
Walsura

Moraceae
Antiaria
Artocarpus
Ficus
Streblus

Myristicaceae
Horsfieldia
Myristica

Myrtaceae
Eugenia
Eucalyptus
Syzygium

Ochnaceae
Ochna

Olacaceae
Anacolosa

Oleaceae
Linociera

Palmae
Areca
Caryota
Bactris
Cocos
Elaeis
Roystonea

Rhizophoraceae
Anisophyllea
Carallia
Rhizophora

Rosaceae
Parinari

Rubiaceae
Byrsophyllum
Canthium
Gardenia
Hymenodictyon
Ixora
Mitragyna
Morinda
Randia
Sickinggia
Timonius
Tricalysia

Rutaceae
Aegle
Glycosmis
Micromelum

Sapindaceae
Lepisanthes
Nephelium
Paranephelium
Schleichera
Xerospermum
Zollingeria

Sapotaceae
Madhuca
Palaquium

Simaroubaceae
Ailanthus

Sonneratiaceae
Duabanga

Sterculiaceae
Firmiana
Mansonia
Pterocymbium
Pterospermum
Sterculia
Theobroma

Stilaginaceae
Antidesma

Symplocaceae
Symplocos

Tetramelaceae
Tetrameles

Thymelacaceae
Aquilaria
Gyrinops

Tiliaceae
Grewia

Ulmaceae
Holoptelea

Verbenaceae
Gmelina
Tectona
Vitex

Appendix D

Taxonomic distribution of ectomycorrhizal fungi (from Miller 1982)

Division	Subdivision	Order	Family	Number of confirmed genera
Dikaryomycota	Basidiomycotina	Agaricales	Amanitaceae	2
			Boletaceae	13
			Cortinariaceae	5
			Entolomataceae	1
			Gomphidiaceae	5
			Hygrophoraceae	1
			Paxillaceae	2
			Strobilomycetaceae	3
			Tricholomataceae	6
		Russulales	Elasmomycetaceae	4
			Russulaceae	5
		Gautieriales	Gautieriaceae	1
		Hymenogastrales	Hydnangiaceae	1
			Hymenogastraceae	1
			Octavianinaceae	4
			Rhizopogonaceae	2
		Phallales	Hysterangiaceae	1
		Lycoperdales	Mesophelliaceae	1
		Melanogastrales	Leucogastraceae	2
			Melanogastraceae	2
		Sclerodermatales	Astraceae	1
			Sclerodermataceae	2
		Aphyllophorales	Cantharellaceae	3
			Clavariaceae	7
			Corticiaceae	3
			Hydnaceae	7
			Thelephoraceae	2

Division	Subdivision	Order	Family	Number of confirmed genera
	Ascomycotina	Pezizales	Balsamiaceae	3
			Geneaceae	1
			Helvellaceae	1
			Pezizaceae	1
			Pyronemataceae	3
			Terfeziaceae	4
			Tuberaceae	2
		Elaphomycetales	Elaphomycetaceae	1
	Zygomycotina (Phycomycetes)	Endogonales	Endogonaceae *Endogone flammicorona* Trappe ex Gerd. *E. lactiflua* Bk. ex Br.	2

Appendix E

Occurrence and distribution of mycorrhizal plants with nitrogen-fixing nodules

Family	Genus	Type of mycorrhiza
Betulaceae	*Alnus*	EM, VAM
Casuarinaceae	*Casuarina*	VAM
Coriariaceae	*Coriaria*	EM, VAM
Cycadaceae	*Cycas*	VAM
Dipterocarpaceae	*Dipterocarpus*	EM
Elaeagnaceae	*Elaeagnus*	EM, VAM
	Hippophae	VAM
	Shephardia	VAM
Myricaceae	*Comptoni*	VAM
	Myrica	EM, VAM
Leguminosae	Herbaceous	VAM
	Woody	EM, VAM
Mimosaceae	*Xylia*	VAM
Papilionaceae	*Dalbergia*	VAM
Rhamnaceae	*Calletia*	VAM
	Ceanothus	VAM
	Discardia	VAM
	Pterocarpus	VAM
Rosaceae	*Cercocarpus*	EM, VAM
	Dryas	EM, VAM
	Pursina	VAM
	Rubus	VAM
Ulmaceae	*Parasponia*	VAM

Note: EM = ectomycorrhizae
VAM = vesicular-arbuscular mycorrhizae

Ectomycorrhizae

Uthaiwan Sangwanit

Introduction

The roots of most higher plants are greatly modified by mycorrhizae. These structures are formed as a result of the invasion of young, living roots by hyphae of certain fungi. The association between root cells and fungi is truly symbiotic, that is, both organisms benefit from the association. The plant supplies carbohydrates and other metabolites that are beneficial to the fungus; in return, the fungus benefits the plant by increasing the availability of nitrogen, phosphorus, and other nutrients (Harley 1969).

Mycorrhizae are often divided into three types:

1) ectomycorrhizae: the fungal hyphae grow over the feeder root surfaces, forming an external mantle or sheath; they occur in the intercellular spaces of the root cortical cells, forming a network of hyphae around the cells called the "Hartig net."

2) endomycorrhizae: hyphae grow intracellularly in the root cortex, forming specialized absorbing structures called "arbuscules" and "vesicles"; they do not form a fungal mantle on the root surfaces.

3) ectendomycorrhizae: have some of the features of both ectomycorrhizae and endomycorrhizae.

The fungal hyphae of these three types of mycorrhizae occur only in the root cortical cells of the host; they never extend into the endodermis and stele (Pritchett 1979).

Tree and fungal symbionts of ectomycorrhizae

Ectomycorrhizae are restricted almost entirely to trees and found in association with most forest tree species (Marks and Foster 1973). They are characteristic of the families Pinaceae, Fagaceae, and Betulaceae—the principal families of trees found in cold and temperate forests. Eucalyptus and some tropical hardwood species are generally ectomycorrhizal, whereas some angiosperm families such as Salicaceae, Juglandaceae, Tilliaceae, and Myrtaceae are either ectomycorrhizal or endomycorrhizal, depending on soil conditions (Prichett 1979).

The gross morphology of ectomycorrhizal roots differs from uninfected roots of similar age. They are thicker, more brittle, more branched, usually colored differently, and sometimes have hyphae and rhizomorphs emanating from the

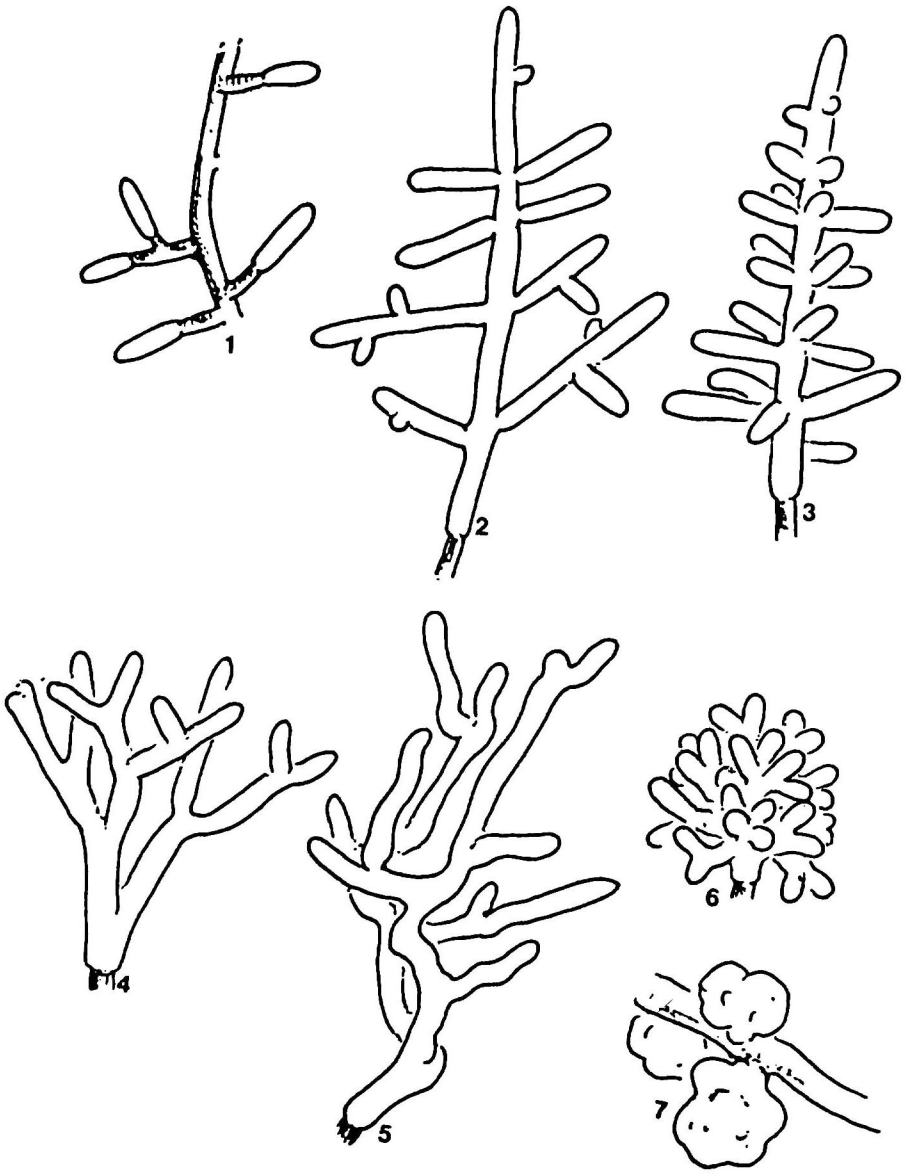

Figure 1 *Typical shapes of ectomycorrhizae: 1) simple or unramified; 2) monopidial-pinnate; 3) monopodial-pyramidal; 4) dichotomous; 5) irregularly pinnate, dichotomous-like; 6) coralloid; 7) tubercle-like (from Agerer 1987).*

Table 1 *Synopsis of orders and families of fungi known to form ectomycorrhizae (from Miller 1984).*

Subdivision	Order	Family
Basidiomycotina	Agaricales	Amanitaceae
		Boletaceae
		Cortinariaceae
		Entolomataceae
		Gomphidiaceae
		Hygrophoraceae
		Paxillaceae
		Strobilomycetaceae
		Tricholomataceae
	Aphyllophorales	Cantharellaceae
		Clavariaceae
		Corticiaceae
		Thelephoraceae
	Gautieriales	Gautieriaceae
	Hymenogastrales	Hydnangiaceae
		Hymenogastraceae
		Octavianinaceae
		Rhizopogonaceae
	Lycoperdales	Mesophelliaceae
	Melanogastrales	Leucogastraceae
		Melanogastraceae
	Phallales	Hysterangiaceae
	Russulales	Elasmomycetaceae
		Russulaceae
	Sclerodermatales	Astraceae
		Sclerodermataceae
Ascomycotina	Eurotiales	Elaphomycetaceae
	Pezizales	Humariaceae
	Tuberales	Eutuberaceae
		Geneaceae
		Hydnotryaceae
		Pseudotuberaceae
		Terfeziaceae
Zygomycotina	Mucorales	Endogonaceae

mycorrhizal system. Seven typical shapes of ectomycorrhizae are shown in Fig. 1 (Agerer 1987).

Worldwide, more than 5000 species of fungi are known to form ectomycorrhizae on some 2000 species of trees (Marx et al. 1992). These fungi fall into three subdivisions within the division Eumycota: 1) Basidiomycotina, 2) Ascomycotina, and 3) Zygomycotina. The largest number of ectomycorrhizal fungi are in the Basidiomycotina, there are a few genera in the Ascomycotina, and only two species (*Endogone*) in the Zygomycotina. Orders and families containing ectomycorrhizal fungi are listed in Table 1. It should be noted that, in some families, the entire family has evolved as mycorrhizal symbionts of higher plants, but in other families, only certain genera have been reported as mycorrhizal fungi.

Identification of the fungal partners of natural ectomycorrhizae is possible using the pure culture ectomycorrhiza synthesis technique described in detail by Molina and Palmer (1984).

In this technique, aseptically germinated seedlings are grown together with the test fungus in a large test tube in vermiculite mixed with peat moss and moistened with nutrient solution. After several months, mycorrhizae form in the test tube. Although this method is extremely useful in many studies, by itself it is not suitable for identifying fungal symbionts of natural mycorrhizae. Since the technique is carried out in a wholly artificial environment, the synthesized mycorrhiza may not have the same morphology as its natural counterpart (Zak 1973). The failure of a fungus to form ectomycorrhizae with a host using this technique does not prove that this would be the case in a natural setting (Trappe 1962). The technique is limited by the difficulty of isolating and growing many species of fungi in pure culture.

Benefits of ectomycorrhizae to trees

Ectomycorrhizae can enhance growth and development of forest trees (Pritchett 1979) by:

1) Increasing nutrient and water absorption; the formation of ramified short roots and mycelia permeating the soil around short roots greatly increases surface absorption area.

2) Increasing nutrient mobilization through biological weathering; the fungi of ectomycorrhizae can break down complex minerals and organic substances in the soil and change them to forms that are available to host plants.

3) Increasing feeder root longevity by providing a biological deterrent to root infection by soil pathogens; the fungal mantle around the root surfaces and Hartig net in the root cortex serve as a physical barrier to infection. In addition to the physical barrier, there is an antibiotic substance in mycorrhizal roots that can also be translocated to adjacent nonmycorrhizal roots, making them resist-

ant to attack by pathogenic fungi. The symbiotic fungus can also provide the host plant with growth hormones, including auxins, cytokinins, gibberellins, and growth-regulating B vitamins.

Factors affecting ectomycorrhizal development

Many environmental factors can influence ectomycorrhizal development by affecting either the tree roots or the fungal symbionts. In the successful development of ectomycorrhizae, there must be:

1) a susceptible feeder root produced by the host plant

2) viable inoculum of an ectomycorrhizal fungus in the rhizosphere

3) favorable chemical, physical, and biological soil conditions (Marx et al. 1992).

The main factors influencing the susceptibility of roots to mycorrhizal infection appear to be high light intensity and low-to-moderate soil fertility (Pritchett 1979). Under these conditions, large amounts of soluble carbohydrates or photosynthates are synthesized and low quantities of available nitrogen and phosphorus are absorbed from soils and translocated upward to the shoot. Consequently, the rate of assimilation during formation of new protoplasm and cell walls is slow. A lot of soluble carbohydrate remains and it is translocated to the root, where it accumulates, making it more susceptible to symbiotic colonization.

Many factors in the soil regulate the survival of fungal symbionts or their growth on roots. For example, extreme soil temperatures (above 35°C and below 5°C), alkaline pH, low moisture and oxygen contents, and antagonistic soil microorganisms can reduce the survival of symbionts and influence their inoculum potential in the soil (Marx et al. 1984). Under certain conditions, fungicides used in plant-disease control can inhibit mycorrhizal fungi, or they may stimulate mycorrhizal development by reducing microbial competition (Pritchett 1979).

Nursery soils fumigated with certain fungicides do not suffer from the lack of ectomycorrhizal fungi after treatment because the fungi produce wind-borne spores that soon recolonize the soil.

Application of ectomycorrhizae in silviculture

The failure experienced with planting exotic pines in various parts of the world stimulated researchers, especially foresters, to look into the significance of mycorrhizal associations of forest trees. Moreover, foresters always have difficulty establishing new plantations on adverse sites or areas that have long been planted with agricultural crops. They speculated that low growth and survival rates of trees could be due to the lack of ectomycorrhizal fungi in the soil. As a consequence, much research was undertaken on inoculating forest tree seedlings in nurseries with ectomycorrhizal fungi to obtain vigorous seedlings for outplanting.

Selection of ectomycorrhizal fungi

The first and most important step in any inoculation program for tree seedlings is selecting ectomycorrhizal fungi (Trappe 1977). The selection criteria are:

1) Fungus should exhibit the physiological capacity to form abundant ectomycorrhizae on seedlings of the desired hosts and on the largest possible number of hosts.

2) Selected fungus must be able to grow rapidly in pure culture and withstand physical, chemical, and biological manipulation.

3) Selected fungus should be able to survive several weeks of storage between inoculum production and use.

4) Selected fungus should have high ecological adaptation to the type of site on which most of the seedlings are to be planted.

Methods of inoculation

Four inoculation techniques have proven successful for mycorrhizal inoculation in nurseries (Pritchett 1979, Marx 1992).

1) Soil from natural forests or nurseries

Soils have been the most widely used inocula because of the simplicity of the operation. But, the method has major disadvantages: soil is expensive to transport, species composition in the inoculum is not controlled, and the inoculum may contain pathogenic organisms and weed seeds.

2) Mycorrhizal seedlings

Vigorous mycorrhizal seedlings are planted in seed beds at 1- to 2-m intervals before sowing the seeds. These mycorrhizal seedlings will serve as a source of infection for adjacent new seedlings. This method has been used for *Pinus merkusii* in Indonesia, but it is also suitable for small-scale nursery operations.

3) Pure cultures of mycorrhizal fungi

The use of pure cultures of ectomycorrhizal fungi has been recommended for providing the most biologically sound material. Unfortunately, this technique has been used rarely in large-scale nursery operations because of difficulties in mass-producing quality inoculum.

4) Spores of various mycorrhizal fungi

Basidiospores are produced by ectomycorrhizal fruiting bodies or mushrooms.

When we collect the fruiting bodies from forest floors, we obtain the basidiospores. These spores can be effective in various forms: mixed with a sand, clay or vermiculite carrier; suspended in water and drenched or irrigated; dusted or sprayed; pelleted and broadcast; encapsulated or coated onto seeds; and incorporated into hydrocolloid chips.

Spore inoculum usually remains viable in storage from one season to the next. But, spore inoculum has several disadvantages: the viability of basidiospores of many ectomycorrhizal fungi is difficult to determine in the laboratory; basidiospores usually take longer to form ectomycorrhizae than vegetative inoculum of the same fungus; and genetic diversity in basidiospore inoculum is high, if they are collected from different environments.

Summary

- Mycorrhizae are the symbiotic associations between fungi and young, living roots of higher plants. There are three types of mycorrhizae: 1) ectomycorrhizae, 2) endomycorrhizae, 3) ectendomycorrhizae.

- Mycorrhizae differ according to the presence of fungal hyphae surrounding root surfaces and the location of hyphae in the root cortex of hosts. Most forest trees are associated with ectomycorrhizae. Fungi reported to form ectomycorrhizae are classified into three subdivisions in the division Eumycota: Basidiomycotina, Ascomycotina, and Zygomycotina. However, the majority of ectomycorrhizal fungi are Basidiomycotina.

- Ectomycorrhizae can enhance growth and survival rates of trees by increasing nutrient and water absorption of roots, increasing nutrient mobilization through biological weathering, and increasing feeder root longevity from attack by soil pathogens.

- Many environmental factors influence the development of ectomycorrhizae. High light intensity and low to moderate soil fertility favor development. Extreme soil temperatures, low moisture content, alkaline pH, low oxygen tension, and antagonistic soil microorganisms reduce development.

- Inoculation of nursery soils with ectomycorrhizal fungi to produce vigorous seedlings suitable for outplanting on adverse sites can be done by using three types of inoculum: soil from natural forests or nurseries, mycorrhizal seedlings, pure culture of ectomycorrhizal fungi, and basidiospores of the fungi.

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Application of VA Mycorrhizal Fungi

Omsub Nopamonrbodi

Introduction

Until about 20 years ago, vesicular-arbuscular mycorrhizal (VAM) fungi were largely ignored by soil and plant scientists. This has now changed because it has been demonstrated that under controlled greenhouse conditions VAM fungi can

- increase the uptake of phosphorus (P)
- play a role in the uptake of other plant nutrients
- be important in the biological nitrogen fixation of Rhiz*obium*
- control root pathogens
- increase plant resistance to drought.

As a result, these microorganisms attract the interest of scientists in several agricultural disciplines (Powell and Bagyaraj 1984). It has also been shown that, under greenhouse conditions, these soil-borne fungi benefit plant growth, especially in the tropics where soils are P-deficient (Nopamornbodi et al. 1987). In addition, VAM fungi result in more efficient use of applied P fertilizers, either in soluble or nonsoluble forms (Mikhall 1976).

Definition of mycorrhiza

There are two main types of mycorrhizae: 1) ectomycorrhizae and 2) endomycorrhizae. In the former, the fungus grows intercellularly in the cortex of the plant's roots (Hartig net) (Table 1). In the latter, the fungus grows inter and intracellularly, forming specific fungal structures within the cortical cells. Ectomycorrhizae (of which there are 5000 species) are a major characteristic of temperate to boreal forest trees. They are mainly different Basidiomycetes, especially *Boletus* spp., *Cortinarius*, *Russula*, and *Tricholoma* ectomycorrhizae are very important in forest nutrient recycling. The vast majority of tropical tree species are endomycorrhizal of the VAM type. Ectomycorrhizae are essential for the growth of pines and eucalypts, both widely used in reforestation in the tropics. When such mycorrhizal trees are used, they must be inoculated artificially in nurseries.

By far the most important and widely distributed (geographically as well as within the plant kingdom) type of mycorrhiza is the VAM type. It is found under natural conditions in almost all tropical and subtropical agronomic crops

Table 1 *Types of mycorrhizae (after Harley and Smith 1983)*

Main type	Subtype	Characteristic structures	Fungal partners	Host plants (general)
Ectomycorrhiza		hyphal mantle around root; Hartig net: hyphae between cortical cells	Basidiomycetes Ascomycetes Phycomycetes	trees; shrubs of Gymnospermae and Angiospermae
Ectendomycorrhiza		hyphal mantle not necessarily present; Hartig net: hyphal coils in root cells	Basidiomycetes Ascomycetes	trees; shrubs of Gymnospermae and Angiospermae
	Arbutoid	hyphal mantle; Hartig net: hyphal coils in cells	Basidiomycetes	Ericales
	Monotropiod	hyphal mantle; Hartig net: unbranched baustoria in cells, colorless mycelium	Basidiomycetes	Monotropeae
	Ericoid	no hyphal mantle; Hartig net: unbranched baustaria in cells; colorless mycelium	Ascomycetes Ericales (Basidiomycetes)	
	Orchid	no hyphal mantle; Hartig net: hyphal coils in cells; unbranched baustaria possible, colorless mycelium	Basidiomycetes	Orchidaceae
	Vesicular-arbuscular	no hyphal mantle; Hartig net: hyphal coils in cells (arbuscules); vesicle formation in or between cells posible	Endogonaceae	trees; graminoid and herbaceous plants of Gymnospermae and Angiospermae; lower plants (algae and ferns) of Bryophyta and Pteriodphyta

Biology of VAM

A basic requirement for the manipulation and management of VAM fungi is a knowledge of their biology and the development of the infection in plant roots and their identification and occurrence in the plant kingdom.

Morphology of VAM infection

In contrast to ectomycorrhizal infection, VAM infection does not change the morphology of the root. To determine VAM infection and eventually quantify the infection, microscopic examination of roots is necessary. Washed roots can be examined for fungal structures under a compound or fluorescence microscope at 100-400 X.

VAM infection is characterized by the formation of unseptate hyphae outside the root and inter and/or intracellular hyphae in the cortical cell layers of the root. Arbuscules are formed in the cells and vesicles may be formed inter and/or intracellularly.

Development of VAM infection

a) Preinfection: resting spores of the fungus, fungal hyphae in the soil, or root fragments with fungal structures are sources of infective propagules where fungal development can start.

b) Primary infection (root penetration): since VAM fungi can infect a large number of different host plants, it appears that the fungus does not recognize specific taxa. On the other hand, some plant families (Chenopodiaceae, Brassicaceae, and Caryophyllaceae) or species of certain families (e.g., *Lupinus*) are never infected in normal circumstances; a phenomenon that has not yet been explained. Normally, the fungus penetrates the root between the epidermal cells and often forms an appressorium in the first cell layers. After this stage, autotrophic growth terminates.

c) Formation of arbuscules and vesicles: after root penetration, hyphae grow inter and intracellularly. Fungal growth is restricted to the epidermis, endodermis, xylem, phloem, and meristematic tissue. Above-ground shoot tissue and chlorophyllous plant parts are not colonized. Arbuscules are often formed within cells shortly after penetration (2-5 days). Arbuscules result from strong branching of hyphae after having penetrated the cell wall. The finely branched hyphae are closely surrounded by the plasma lemma of the cell. Because of the large surface contact, arbuscules are the most intensive connection between the fungus and the plant. Arbuscule formation increases the metabolic activity of the host cell, which is mainly due to the bidirectional transfer of metabolites and nutrients to and from the fungus.

   Arbuscules live for only 4-15 days as they degenerate and are digested by the host cell. After arbuscule decline, plant cell function returns to normal. Arbuscule

formation and degeneration occurs simultaneously in the root. At the time of arbuscule formation or shortly after, some VAM fungi form inter and/or intracellular vesicles.Vescicles are apical or intercalary swellings of hyphae that contain lipids and act as reserve organs for the fungus. Under stress (e.g., low supplies of metabolites from the host plant), these reserves are used by the fungus and the vesicles degenerate. Fungal species belonging to the genera *Gigaspora* and *Scutellospora* never form vesicles; other VAM fungal species do so, but rarely. The two genera mentioned above produce auxiliary cells in the root-external mycelium.

d) Extension of fungus into roots and rhizosphere: the extension of the infection in the root is divided into three phases: 1) initial (lag) phase, when primary infection takes place; 2) exponential phase, when the fungus spreads in the root faster than root growth itself; 3) plateau phase, when root and fungal growth are at the same rate.

e) Spread of fungus in the soil: after the primary infection and during the first phase of infection in the root, hyphae grow out of the root and rhizosphere into the soil. This part of the fungal structure (i.e., the root-external mycelium) is the most important one as concerns the uptake of elemental nutrients and transport of nutrients to the root.

f) Reproductive structures of VAM fungi: VAM fungi form resting spores on the external mycelium. The diameter of the spores depends on the species of fungus and can range from 15 m to more than 800 m. Some species of the VAM-forming genera *Acaulospora* and *Glomus*, as well as all *Sclerocystis* spp., are sporocarpic. The process of spore formation is known for several fungal species of different genera. With certain species, spore formation can start 3-4 weeks after roots are infected, whereas with other species of VAM up to 6 months are required before spore formation begins. The species of fungus and host plant, and soil and environmental conditions all affect the time and extent of sporulation. The fungal mycelium inside and outside the root is another reproductive structure of VAM fungi. The fungal mycelium can germinate and infect new roots. However, whereas spores can survive for up to several years in the soil, the infectibility of fungal mycelium (separated from the host plant or death of the host) lasts only 2-4 weeks.

Function of VA mycorrhiza

Under field conditions almost all crop plants are infected by VAM fungi. It is difficult , however, to demonstrate the function of VAM fungi in the field. Most of the research findings are from experiments conducted under artificial conditions. Although it is known that the physiology of a plant is completely changed after its association with VAM fungi (Dehne 1986), comparing mycorrhizae is often the only way to investigate their function.

Carbohydrate demand

It is estimated that VAM fungi require 1-17% of the carbohydrates that the plant submits for root biomass production. The maximum hypothetical photosynthate requirement could be as high as 40-60% (Stribley et al. 1980), to the extent that the carbohydrate demand of the fungus limits the plant's biomass.

Increase of the rhizosphere by VAM

VAM form a mycelium around the roots at the same time that they are formed in those roots. Internal and external fungal hyphae are in contact with up to 10 entry points per cm of root surface (Ocampo et al. 1980).

The external mycelium considerably increases the contact of the root with the medium in which it grows. Without mycorrhiza, 1 cm of root can explore about 1-2 cm^3 of soil using root hairs. Assuming radial growth of VAM hyphae around the root, VAM mycelium can increase this from 5 to 200 times. A rhizospheric soil volume of 200 cm^3 may be the exception, but 12-15 cm^3 per infected root is common.

VAM fungal mycelium appears more resistant than the root itself to abiotic stresses such as drought, toxic elements, and soil acidity (Ahmadsad 1985). A plant with mycorrhiza remains in close contact with the soil for a longer period of time than a plant without mycorrhiza. The life span of external mycelium appears to decline rapidly 3-4 weeks after the first infection of the plant by the fungus (Schubert et al. 1987).

Plant nutrient uptake: the principal function of mycorrhiza is to increase the soil volume explored for nutrient uptake and enhance the efficiency of nutrient absorption.

Soil aggregation: fertile soils have a high percentage of stable aggregates. VAM fungi can bind and aggregate soil particles through the intensively growing mycelium. Additionally, binding of soil by VAM fungi is a potential control mechanism in soil erosion. The function of VAM fungi in soil aggregation has often been underestimated. Today, it is known that VAM mycelium not only aggregates soil particles but also that the hyphae are bound to the particles through amorphous polysaccharides.

Climatic stress: VAM plants recuperate faster after a short period of water stress than plants without VAM.

Control of pathogenic microorganisms: VAM fungi can increase, decrease, or have no effect on pathogens. VAM fungi increase the resistance of the plant to root pathogens if they colonize the root before it is attacked. VAM fungi increase some leaf and stem diseases caused by viruses and pathogenic fungi. However, VAM

plants may suffer less from the disease than plants without mycorrhizae. The interaction between VAM fungi and pathogenic bacteria has not been investigated in depth. The incidence of nematodes is reduced and their damage is mitigated.

Inoculum production

It is well known that the technology of VAM inoculation is limited principally by lack of production and commercial distribution of VAM inocula. Worldwide, there are only two commercial sources of VAM inocula: Native Plants Inc., Salt Lake City, Utah, U.S.A., and Jira-Agroindustrias, Tulua, Colombia. It is not clear why there are not more commercial sources. Several techniques for producing VAM inoculum are now available and some have been recently patented.

Sources of VAM inocula

VAM fungi are obligate biotrops and, unlike other symbiotic microorganisms such as *Rhizobium*, cannot be multiplied on artificial growth media without a host. VAM fungi depend for reproduction on photosynthates of the plant or on root components when the root is manipulated to grow meristematically on artificial growth media under axenic conditions.

Sources are defined by the biology of VAM fungi. All infective structures of the fungi can be used. These are:

- fungal spores
- mycelium produced inside or outside the host root
- arbuscules and auxiliary mycelium produced inside or outside the host root
- arbuscules and auxiliary cells in infected roots and substrates (carriers that contain infected roots and/or VAM mycelium and spores) are common sources of VAM inocula.

Application of VAM in planting stock production

Techniques of VAM inoculation in the field are intrinsically related to crop production systems. The methods of application depend on facilities available for fitting the inoculation technology into the technical process of crop establishment. The selection of the source, the amount of inoculum, and the application technique depend on the system of planting and the source of planting material. For fruit trees or forest plants, inoculation VAM are applied to the seed bed by incorporating inoculum with the soil or medium where seedlings are grown. To inoculate seedlings or cuttings that will grow in the field, a small hoe is dug with a suitable tool, inoculum is applied through the hole and the cuttings, stalks, or seedlings are planted vertically on top of the inoculum. The plant material is then covered with soil.

Benefits of VAM inoculation

- Higher plant yields
- Greater biomass
- Higher plant survival rates
- Faster relative growth rates
- Higher quality plants
- Savings on agronomic inputs (fertilizer, irrigation, and pesticides).
- Protection (e.g. erosion control).

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Microbial organic fertilizer production in Thailand

Pitayakon Limtong and Vanlada Sunantapongsuk

Introduction

Soil fertility plays a crucial role in agricultural production. In Thailand, data gathered by the Soil Survey and Classificiation Division of the Land Development Department indicate that there are 36 million hectares of land (about 70% of the country's total land area) considered unfertile with a low content of plant nutrient. Of this area, 30.5 million ha (60%) are thought seriously degraded.

Many of the factors responsible for poor soil fertility are due to tropical conditions: monsoon rain patterns, slope of the land, shallow soil surface, and poor parent material. Inappropriate cultivation methods and lack of fallow periods are also responsible for poor soil fertility.

Soil organic matter

Soil organic matter tends to be poor in Thailand. This is particularly so in the east and northeast, where sandy soils predominate. In the central plains, where clayish soils predominate, rice has been cultivated for a long period of time and this has led to loss of fertility and compaction. The sandy loam found in the north of the country has been degraded by deforestation and shifting cultivation. In the south, some areas have very poor soil fertility because of undulating topography, a shallow soil surface, a high rate of leaching and erosion because of heavy precipitation (Limtong 1988).

To increase agricultural production in areas where soils are degraded the role of organic matter should be emphasized, in addition to the use of organic and chemical fertilizers. Many experiments conducted in eastern and northeastern Thailand show that the application of organic and chemical fertilizers increases growth and yields of plants. Moreover, organic fertilizers slowly release nutrients in the second and third years of cultivation. Since farmers can make their own organic fertilizer, the application of such is appropriate for them.

Microbial aspects of organic fertilizer production

Compost is produced from various solid wastes through microbial processes during decomposition. Compost making is appropriate for many farmers because of low production costs and the easy availability of materials such as rice straw, corn stalk, and other agricultural wastes. Some industrial by-products can also be used

for making composts, e.g., bagasse, rice husk, and saw dust. Factors that limit the use of industrial wastes are, for instance, the difficulty of transporting bulky material and toxic material. Other sources of raw material are municipal waste and aquatic plants such as water hyacinth. Table 1 presents data on major types and quantities of organic solid wastes in Thailand, and Table 2 details the chemical properties of these wastes.

In 1981, the Land Development Department received financial support from the Farmer's Aid Fund for an extension program in compost production and use in 26

Table 1 *Type and quantity of various organic solid wastes in Thailand*

Type	Quantity (Tons/yr)
Crop wastes	
rice straw	43 000 000
corn residue	1 000 000
soy bean and mung bean residue	500 000
water hyacinth	5 800 000
sugar cane residue	2 000 000
tree leaves in residential areas	1 000 000
Industrial wastes	
bagasses	6 000 000
saw dust	30 000
coconut dust	30 000
rice hull	5 000 000
others	30 000
Municipal wastes	
Bangkok and other main cities	3 000 000
Animal wastes	
cattle manure	16 700 000
pig manure	3 100 000
poultry manure	5 000 000
other animal manure	500 000
Night soil	14 000 000

Source: *Technical report on organic matter for soil improvement. Land Development Department, 1992*

Table 2 *Chemical properties of various organic solid wastes in Thailand*

Type of waste	N (%)	P_2O_5 (%)	K_2 (%)	C:N	pH
Farm wastes					
rice straw	0.55	0.09	2.39	89	8.2
cassava peel	1.82	0.48	1.99	27	8.5
corn residue	0.53	0.15	2.21	62	8.2
soy bean residue	3.34	1.04	2.54	16	8.1
Industrial wastes					
bagasses	0.40	0.15	0.44	146	6.0
saw dust	0.32	0.16	2.45	196	5.4
coconut husk	0.36	0.05	2.94	167	6.1
rice hull	0.36	0.09	1.08	152	6.1
Municipal waste	1.20	1.56	10.34	39	5.9
Others					
water hyacinth	1.27	0.71	4.84	34	7.8
peat soil	1.10	0.04	0.26	41	3.5
azolla	2.98	0.61	2.05	14	6.8

Source: *Technical report on organic matter for soil improvement. Land Development Department, 1992.*

provinces. The target was 50 000 tons. During the first year of the program, production exceeded the target.

In 1982, a 5-year project called "Organic Recycling for Soil Improvement" was set up. It was part of the Fifth National Economic and Social Development Plan (1982-1986) and included 12 500 villages in 38 provinces. In the following Plan (1987-1991), the project included 50 000 villages in 72 provinces.

The compost-production component of the project included modern means of production that included the use of microbial inoculum to accelerate decomposition.

Research

A microbial research laboratoy was established to collect basic data on microbial activity during decompostion and other phases. There were two objectives: 1) determining the quality of microbial inoculum from private-sector sources, 2) se-

lecting microorganisms from various natural sources for producing inoculum. The research program is also concerned with the use of specific microorganisms for improving compost quality, nitrogen fixation, and phosphate solubilization.

Emphasis was placed on cooperative research with other interested institutes to maximize the use of resources and available expertise in microbiology, agronomy, biotechnology, and bioengineering. In 1985, project staff collaborated with compost researchers at Kasetsart University to present a preliminary report on the status on the use of microbial inoculum in compost production in Thailand. This collaboration continued in 1986.

Implementation

Initially, implementation was at the laboratory level; this limited possible industrial-scale application. Production was estimated at 50 000 packages for use during the next fiscal year. In the next Plan (1992-996), during which the Project will also have a 5-year implementation phase, 100 000 packages of inoculum will be produced with a targer compost production figure of 100 000 - 300 000 tons annually. This requires a more generous budget for equipment and labor. The Project now calls for private-sector involvement under the general guidelines of the Land Development Department. A committee will be established consisting of representatives of several institutes.

Production process

Several mesophylic and thermophylic microorganisms were isolated from various sources in Thailand—soil samples, decomposted plant residues, composts, and animal manures. The technique used was that described by Murao et al (1979)

Selection of cellulolytic microorganisms comprises two stages. The first consists of determining cellulose production based on the band of clear zone in the agar tube by using Walseth (Rautella and Cowling 1966) in the primary screening. Determining cellulose activity in broth and solid state cultivation during the secondary screening uses the technique proposed by Mandel and Sternberg (1976). The second stage consists of estimating decomposition efficiency of rice straw in an incubator at 45-50°C as well as under field conditions (Stutzenberger et al 1970).

The population of selected strains of microbes was increased in an appropriate medium and cultivation conditions depended on each strain. The time required for this should be short and the method economical. Fungi and Actinomycetes were suitable for cultivation in solid state. Bacteria were appropriate for cultivation in broth under controlled conditions.

After the microbial population is increased, selected strains were mixed together and then mixed with a suitable carrier. The moisture content of the mixed carrier was reduced in the tray under air-dry conditions. The low moisture content of the

mixed carrier was packed into plastic or foil bags weighing approximately 150 gm each.

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Recovery and quantitative estimation of vesicular-arbuscular mycorrhizal fungi from soil

Poonpilai Suwannarit

Introduction

Propagules of vesicular-arbuscular mycorrhizal (VAM) fungi consist of chlamydospores, azygospores, soil-borne vesicles, mycelium, and infected root pieces. These propagules must be collected from field soils or pot cultures in which VAM fungi are increased in the greenhouse on living hosts. Because, so far, VAM fungi cannot be cultured in any artificial media, there are several methods by which propagules in soil can be recovered and measured.

Recovery from soil

Wet sieving and decanting (Gerdemann and Nicolson 1963)

1) Prepare 250 cc of soil and 1000 mL of water.

2) Mix and let stand for a few seconds.

3) Pour mixture through a 500 µ-sieve > collect debris

4) 250 µ-sieve > collect debris

5) 125 µ-sieve > collect debris

6) 45 µ-sieve > collect debris

7) Transfer debris from each sieve to a petri dish and examine under a dissecting microscope.

Density gradient centrifugation (Ohms 1957)

1) Pour 10 mL of a 50% sucrose solution into a clear 50-mL centrifuge tube.

2) Using a hypodermic syringe with a curved glass tube extension to direct the liquid against the side of the tube, add a 10-mL layer of a 25%-sucrose solution to the 50% sucrose solution layer, then add a layer of water above that.

3) Add a suspension of sievings and centrifuge 5 min at 3100 rpm (approx. 1100 X g).

4) Remove debris that collects in the middle layer, place on a fine sieve and wash with water.

Sucrose centrifugation (Jenkins 1964)

1) Place a 100-500-cc soil sample on a 20-mesh screen and wash with water to remove large debris. Collect sieved soil and water in a container.

2) Mix the sieved soil by stirring and allow mixture to stand for 30 sec. Decant through a 270- or 325-mesh screen and collect the residue from the sieve in a beaker. Add water to the sieved soil and repeat decantation. Discard remaining soil.

3) Transfer the collected residue into two 50-mL centrifuge tubes and centrifuge for 4-5 min at 1750 rpm (approx. 325 X g) in a horizontal rotor.

4) Decant the supernanant liquid carefully and add resuspend pellet to a sucrose solution (454 gm cane sugar/L). Centrifuge again for 0.5-1 min.

5) Pour the supernanant (with spores) onto a 325-mesh screen and rinse with water to remove the sugar.

Quantitative estimation

Eelworn counting slide

This slide is similar to a hemacytometer but has a 1-mL capacity. The slide is etched into rectangles or can be specially ordered with a rectangle divided into 30 parallel lines. The number of spores per mL is calculated by counting the spores in one section of the slide and multiplying accordingly.

Most probable number (Porter 1979, Powell 1980)

1) Using the test soil, make a 10-fold series of soil dilutions to 10^{-3}, 10^{-4}, or 10^{-5} using steamed, autoclaved, or fumigated soil as the diluent.

2) Place soil in small vials (30-100 cc) using five replicate vials per dilution.

3) Sow seeds or pregerminated seedlings of a test plant into each vial.

4) Grow plants in a greenhouse or growth chamber for 6 weeks. Wash, clear, and stain roots, then examine under a dissecting microscope. Determine whether or not there is infection.

5) Referring to Table 1, calculate the most probable number of VAM propagules.

Table 1 *Table of most probable numbers for use with 10-fold dilutions and 5 tubes per dilution (Cochran 1950, as reproduced in Alexander 1965)*

P_1	P_2	Most probable number for indicated values of P_3					
		0	1	2	3	4	5
0	0	—	0.018	0.036	0.054	0.072	0.090
0	1	0.018	0.036	0.055	0.073	0.091	0.11
0	2	0.037	0.055	0.074	0.092	0.11	0.13
0	3	0.056	0.074	0.093	0.11	0.13	0.15
0	4	0.075	0.094	0.11	0.13	0.15	0.17
0	5	0.094	0.11	0.13	0.15	0.17	0.19
1	0	0.20	0.040	0.060	0.80	0.10	0.12
1	1	0.040	0.061	0.081	0.10	0.12	0.14
1	2	0.061	0.082	0.10	0.12	0.15	0.17
1	3	0.083	0.13	0.15	0.17	0.19	0.22
1	4	0.11	0.13	0.15	0.17	0.19	0.22
1	5	0.13	0.15	0.17	0.19	0.22	0.24
2	0	0.045	0.068	0.91	0.12	0.14	0.16
2	1	0.68	0.092	0.12	0.14	0.17	0.19
2	2	0.093	0.12	0.14	0.17	0.19	0.22
2	3	0.12	0.14	0.17	0.20	0.22	0.25
2	4	0.15	0.17	0.20	0.23	0.25	0.28
2	5	0.17	0.20	0.23	0.26	0.29	0.32
3	0	0.78	0.11	0.13	0.16	0.20	0.23
3	1	0.11	0.14	0.17	0.20	0.23	0.27
3	2	0.14	0.17	0.20	0.24	0.27	0.31
3	3	0.17	0.21	0.24	0.28	0.31	0.35
3	4	0.21	0.24	0.28	0.32	0.36	0.40
3	5	0.25	0.29	0.32	0.37	0.41	0.45
4	0	0.13	0.17	0.21	0.25	0.30	0.36
4	1	0.17	0.21	0.26	0.31	0.36	0.42
4	2	0.22	0.26	0.32	0.38	0.44	0.50
4	3	0.27	0.33	0.39	0.45	0.52	0.59
4	4	0.34	0.40	0.47	0.54	0.62	0.69
4	5	0.41	0.48	0.56	0.64	0.72	0.81
5	0	0.23	0.31	0.0.43	0.58	0.76	0.95
5	1	0.33	0.46	0.64	0.84	1.1	1.3
5	2	0.49	0.70	0.95	1.2	1.5	1.8
5	3	0.79	1.1	1.4	1.8	2.1	2.5
5	4	1.3	1.7	2.2	2.8	3.5	4.3
5	5	2.4	3.5	5.4	9.2	16.	—

Note: *P_1, P_2, and P_3 are the numbers of tubes showing growth*

Plate method for population studies (Smith and Skipper 1979)

1) Add 1 gm of soil to 9 mL of distilled water and shake vigorously.

2) Pipet immediately 1 mL in parallel lines onto a 9-cm filter paper disc in a petri dish.

3) Count spores (either when wet or after air drying) under a dissecting microscope (7-30 X).

Modified plate method (Suwannarit et al. 1992)

1) Wet sieve and decant 100 gm of soil.

2) Collect remaining debris in a volumetric flask.

3) Add water to 50-or 100-mL mark.

4) Shake spores well and pipette immediately 1 mL in parallel lines onto a filter paper in a petri dish.

5) Count spores under a dissecting microscope.

6) Calculate the number of spores per gm of soil.

Assessment of colonization after clearing and staining

Nonsystematic method

1) Using dissecting needles, spread roots uniformly in a petri dish to eliminate clumping and enhance light transmission.

2) Rotate root sample carefully on the microscope's stage.

3) Count the number of susceptible feeder roots to determine the percentage of roots with mycorrhizae.

Assessment of colonized roots is usually based on the following classification, which is used at the Institute for Mycorrhizal Research and Development at the Forest Service of the United States Department of Agriculture):

Class 1: 0-5%
Class 2: 6-25%
Class 3: 26-50%
Class 4: 51-75%
Class 5: 76-100%

Systematic methods

Grid line intersect method (Giovannetti and Mosse 1980)

1) Ink the grid line to form 1.27-cm squares on a circular piece of acetate and place it on the bottom of the inverted plate top.

2) Spread root sample evenly in the bottom of a petri dish.

3) Place the bottom portion of the plate containing the sample inside the top.

4) Scan vertical and horizontal grid lines.

5) Record presence or absence of colonization at each point where a root intersects a line.

6) Calculate the proportion of the root length that is colonized.

Slide method

1) Select root segments approximately 1 cm in length at random from a stained sample and mount on microscope slides in groups of 10.

2) Use 30-100 root segments from each sample.

3) Using a magnification of 100-250, determine the length of cortical colonization in millimeters for each root segment.

4) Express as a percentage of root length colonized.

The slide method can be simplified by recording only the absence or presence of colonization in each root segment and expressing the result as the percentage of root colonized.

Assessment of noncleared roots

Colorimetric assay (Daft and Nicolson 1972)

1) Assess mycorrhizal colonization of entire root system by measuring the relative intensity of the yellow water-soluble pigment in root (using tomato as sample).

2) Use an ultraviolet mercury vapor lamp to assess visually the intensity of the yellow pigment.

Notes:

- Using this method, the intensity correlates with the intensity of mycorrhizal colonization based on a systematic assay procedure that uses paired root systems.

- Accuracy is good under low-nutrient conditions, when root colonization percentages are high.

- The yellow pigmentation is conspicuous under pot conditions in heavily colonized roots but is not detectable in field-grown plants.

- Mycorrhizal colonization occurs only in certain hosts.

Becker and Gerdemann (1977)

1) Extract yellow pigment from onion root by auclaving root systems for 30 min at 121°C.

2) Remove roots after cooling and read absorbance of the water extract at 400 m against a water blank in a Bausch and Lomb Spectronic-20 spectrometer.

3) Keep extract in the dark, if absorbance cannot be read immediately after cooling, to prevent the pigment from fading.

This method is an alternative for root assessment for controlled short-term experiments, but not for long-term experiments in which phenolic compounds of dying roots could interfere with absorbance of the water extract.

Herrera and Ferrer (1978)

1) Clear and stain roots with trypan blue in lactophenol.

2) Elute absorbed stain from fungal tissue.

3) Assume that the amount of stain eluted correlates with total colonization.

There is a possibility that contamination from other microorganisms can interfere with measurement.

Chemical assay (Hepper 1977)

Perform a chitin assay from endomycorrhizal and nonmycorrhizal roots. Chitin is converted to glucosamine and the absorbance at 650 m is compared with purified glucosamine. The amount of glucosamine is correlated with the total weight of the root samples.

Ultraviolet autofluorescence assay (Ames et al. 1982)

Colonization is assessed from fresh endomycorrhizal root segments with an epifluorescence microscope equipped with epifluorescent condensers, mercury lamps, exciter filters that pass wave lengths of 455-490 nm, and barrier filters that

allow wave lengths of 520-560 nm to pass to the viewer. Arbuscules autofluoresce in the root segments of all plants.

Under ultraviolet light, only arbuscules autofluoresce; vesicles, spores, and hyphae do not.

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Isolating fungi from soil and other organic materials

Leka Manoch and Siangjeaw Piriyaprin

Introduction

Soil fungi play an important role in the degradation of organic compouds present in soil. In addition, they are very important to mankind in providing useful pharmaceutical products such as antibiotics and producing certain organic acids, enzymes, etc., which are valuable in fermentation and other industrial processes. These fungi include several species of *Aspergillus, Mucor, Monascus,* and others (Smith 1968). Several species of soil fungi are very important in biological control of plant pathogen (e.g., *Trichoderma* vs. *Sclerotium*) and insect pests (e.g., *Metarhizium anisopliae* on *Rhinoceros* beetle).

On the other hand, soil microfungi can cause heavy losses in agriculture, forestry, and industry. They can cause many serious plant diseases such as *Phytophothora* spp., *Fusarium* spp., *Pythium* spp., and others. Some soil fungi such as *Aspergiullus fumigatus, Sporothrix schenckii,* and *Histoplasma capsulatum,* may infest birds and various mammals including humans and cause serious diseases such as aspergillosis, sprotrichosis, and histoplasmosis. *Histoplasma farciminosum* is the causal agent of peizootic lymphagitis, a serious disease of horses and mules. *Aspergillus flavus, Beauveria bassiana, Metarhizium anisopliae,* and other soil fungi cause endoparasitism in insects. Soil fungi such as *Aspergillus flavus, Aspergillus ochraceous, Fusarium* spp., *Chaetomium* spp., and others can also produce mycotoxins in certain substrates.

Using various methods, many species of soil fungi can be isolated. Selective techniques and media have been used to isolate a certain group of new, noteworthy soil fungi.

Materials and methods

Soil samples and organic debris are collected from various agricultural and forest sites in plastic bags, taken to the laboratory and placed in a refrigerator at about 10°C. Various methods for isolating fungi are employed, e.g., dilution plate, soil plate, modified Warcup's alcohol treatment, actidione treatment, baiting technique, and filter paper.

Prior to isolation, the soil samples and organic debris are taken out of storage and allowed to come to room temperature. Various procedures for isolation are carried out as follows:

Table 1 *Genera of soil fungi isolated from various locations by using different methods (adapted from Manoch 1992, pp 744-745)*

Fungi	Vegetation and location	Isolation method
Absidia sp.**	pine, Chiang Mai	SP[1]
*Acremonium murorum****	pine, Chiang Mai	SP
Ascodesmis sp.***	forest, Kanchanaburi	alc[3]
*Aspergillus deflectus****	shallot, Chiang Mai	SP
*Aspergillus flavus****	Combretum, Chachoengsao;	SP
	KU, Bangkok	Actidione
*Aspergillus niger****	forest, Chiang Mai	SDP[2]
*Aspergillus ochraceus****	lichee, Kanchanaburi;	SP
	KU, Bangkok	Actidione
*Aspergillus sulphurea****	lichee, Kanchanaburi	SP
*Byssochlamys fulva****	forest, Chiang Mai	alc
Ceratocyystis sp.***	eucalypt, Chon Buri	SP
Chaetomiun spp.***	eucalypt, Chon Buri;	SP
	corn, Chiang Mai;	SP
	forest, Chiang Mai	SDP
*Cunninghamella echinulata***	forest, Chiang Mai	SP
Coniochaeta sp. ***	eucalypt, Chon Buri	SP
*Curvularia geniculatus****	pine, Chiang Mai	SP
*Curvularia lunata****	forest, Chiang Mai	SDP
*Doratomyces stemonitis****	pine, Chiang Mai	SP
*Emericella nidulans****	pear, Chiang Mai;	SP
Eupenicillium sp. ***	stawberry, Chiang Mai	SP
	forest, Kanchanaburi	alc
*Fusarium moniliforme****	pine, Chiang Mai	SP
*Fusarium oxysporum****	pine, Chiang Mai	BP[6]
*Fusarium solani****	pine, Chiang Mai	BP
Gelasinospora sp.****	rice, Ubon Ratchatani	SP
Gilmaniella sp.****	cabbage, Kanchanaburi	SP
*Gliocladium roseum****	pine, Chiang Mai	SP
*Gliocladium viride****	pine, Chiang Mai	SDP
Humicola sp.****	oil palm, Phuket	SP
Micoascus sp.***	KU, Bangkok	Actidione[5]
Mucor sp.**	pine, Chiang Mai	SP
Microsporum sp.****	KU, Bangkok	BH[8]
Neocosmospora sp.***	orange, Bangkok	SP
Neosartorya sp.***	bamboo, Chachoengsao;	heat[4]
	forest, Chiang Mai	alc
*Paecilomyces lilacinus****	forest, Chiang Mai	SDP
	forest, Kanchanaburi	Actidione

*Paecilomyces variotii*****	forest, Kanchanaburi	alc
Penicillium sp.****	forest, Nakhon Ratchasima	SP
*Pythium aphanidermatum**	egg plant, Loei	BP (CMA+BNPRA)
*Pythium spinosum**	pine, Chiang Mai	SP (CMA+BNPRA)
*Pythium vexans**	pine, Chiang Mai;	BP (CMA+BNPRA)
	Japanese apricot, Chiang Mai	SP
*Pithomyces chartarum*****	Ruzzi grass, Nakhon Ratchasima	SP
*Rhizoctonia solani*****	rice, Ubon Ratchatani	BR[6]
Rhizopus sp.**	pine, Chiang Mai	SP
Sordaria sp.***	Japanese apricot, Chiang Mai	BR
Spegazzinia sp. ****	forest, Kanchanaburi	SDP
*Scopulariopsis brevicaulis*****	KU, Bangkok;	Actidione
	peach, Chiang Mai	SDP
Sporothrix sp.****	KU, Bangkok	Actidione
Talaromyces sp.****	bamboo, Bangkok;	alc
	Dipterocarpus, Loei	alc
Thielavia sp.***	corn, Chiang Mai	SP
Trichoderma sp.****	Japanese apricot, Chiang Mai	BR
Westerdykella sp.***	orange, Pathumtani	SP
unidentified species	melon, Chaing Mai	SP (PCNB-Peptone agar)

Notes:

*	= Pythiaceae
**	= Zygomycetes
***	= Ascomycetes
****	= Hyphomycetes
SP[1]	= soil plate
SDP[2]	= soil dilution plate
alc[3]	= alchohol treatment
heat[4]	= heat treatment
actidione[5]	= actidione treatment
BP[6]	= baiting with pine seed
BR[7]	= baiting with rice grain
BH[8]	= baiting with human hair.

Dilution plate method (Barron 1971)

A 10-gm soil sample is added to 100 mL of sterile distilled water (or 0.15% water agar) in a 250-mL Erlenmeyer flask. Suspensions are vigorously shaken until thoroughly mixed. Then, 10 mL of the suspension are mixed with 100 mL of sterile distilled water in a flask. Ten millilitre samples are then transferred through a succession of 100 mL sterile distilled water until the desired dilution is reached.

One-mL aliquots of the selected dilutions (usually 1/100, 1/1000, 1/10 000) are pipetted into each of five petri dishes for each selected dilution and 10-15 mL of warm (45°C), melted glucose-ammonium nitrate agar ("GAN") are poured over the soil and the plates are rotated gently to distribute the particles. GAN includes rose bengal to limit the growth of all developing colonies and streptomycin, which is added to the medium just before plating, to avoid bacterial development. GAN was devised by Gochenaur (1964) and has been used successfully as a medium for isolating soil microfungi by many researchers.

Petri plates are incubated in sterilized boxes at room temperature (26-28°C) for 3-5 days. They are incubated in the dark since rose bengal has been shown to have a fatal photosensitizing effect on many fungi when there is light. Following a 3-5 day incubation (Figure 1B), hyphal tips of the different colonies are transferred to potato dextrose agar (PDA) slants using a transfer needle. After initial isolations are completed, plates are examined at various intervals over a 2-week period to obtain slow-growing forms. The tube slants are incubated in indirect light at room temperature (26-28°C) for 2 weeks. The cultures are then grouped into presumed entities on the basis of the colony's texture, color, sporulation, etc. The total number of isolates of each entity is recorded. Each tube represents a presumed fungal species. All isolates not discarded at this time are kept for further study and stored at 10°C (Figures 1D, 9A, 9B, and Table 1).

Identification is based on morphological features observed in plate cultures on suitable media and observations under the microscope. Imperfect fungi develop best on malt agar and PDA, whereas the ascosporic stages do better on cornmeal agar. Czapek-Dox agar is ideal for *Penicillium* and *Aspergillus*. Fungus genera of special interest are kept for more critical examination.These are regrown on plates of suitable agar media, mainly on PDA. The cultures are incubated under light at room temperature (20-28 °C) for 10-14 days; cultural features are usually recorded and microscopic features studied. Morphological characteristics are described based on direct microscopic examination of undisturbed colony surfaces and followed by study of lactophenol mounted slide preparations or slide cultures (Figure 3C). Measurements of spores and other structures are made on water mounts. In describing colony color, a mycological color chart is used.

Pure cultures of the various fungi isolated in this study are currently being maintained in the Mycology Laboratory, Department of Plant Pathology, Kasetsart University and Organic Matter and Waste Products Subdivision, Land Development Department, where they are available for future reference. The isolates are kept on PDA slants, sterile distilled in water or liquid paraffin. Soil cultures are refrigerated.

Soil plate method (Warcup 1950)

A small amount of soil (0.005-0.015 gm) is placed into a sterilized petri dish. About 10 mL of warm GAN medium with streptomycin (50 mg/mL) is added and the petri dish is gently rotated to disperse the soil particles. This method has proven to

be the most effective and convenient and is used successfully for isolating soil fungi. Soil fungi isolated by this method include Mucorales, Hyphomycetes, Coelomycetes, and Ascomycetes (Figures 1A, 3B, 5B, 5C, 7A, 7B, and Table 1).

After the agar solidifies, all procedures described above are followed.

Alcohol treatment method (Warcup and Baker 1963)

A modification of Warcup's alcohol treatment technique is used. A small amount of soil (0.3 gm) is placed in a dry, sterile, 18-mm test tube.

The tube is filled approximately three-quarters with 65% ethanol, shaken, and allowed to stand for 15 min. The excess alcohol is decanted and 15 equal portions of the treated soil are transferred to 15 dry, sterile petri plates using a small sterile spatula. A thin layer of warm (45°C), melted GAN is poured over the soil and the plates gently rotated to distribute the particles. After the agar solidifies, the same procedures described in the previous method are followed.

Higher percentages of ascomycetes and other fungi that produce reproductive structure such as cleistothecia, pycnidia, and sclerotia, are isolated using the alcohol treatment technique than using the standard dilution plate method (Figures 8A, 8B, 8C, 10A, 10B, and Table 1).

Heat treatment method (Modified Warcup and Baker 1963)

Soil samples (0.3 gm) are placed in a sterile test tube and put in a water bath at 60-80°C for 10-15 min. Excess water is drained off and soil particles placed into a sterilized petri dish. After the agar solidifies, the same procedures described in the previous method are followed. This method is used for isolating heat-resistant ascomycetes fungi from soil and other substrates (Figures 8A, 8B, 8C, and Table 1).

Actidione treatment method (Kuehn and Orr 1962)

Actidione (cycloheximide, Upjohn Co., Kalamazoo, Michigan, U.S.A.) is a chemical compound with fungistatic properties. It has been extensively used for isolating Gymnoascaceae or keratinophilic fungi from soils. Three concentrations of actidione (0.00015, 0.0005, and 0.00075 mg/mL) are added to warm, melted PDA before plating onto a small amount of soil particles in a petri dish. The plates are then incubated at room temperature (26-28°C) for a few days. Above-mentioned methods are followed.

Baiting technique (Manoch 1992)

Various types of sterilized seed, e.g., vegetable, jute, pine, and rice are placed in soil samples and incubated for 24-36 hrs. The baits are then taken off and washed with sterile, distilled water and 20% H_2O_2. They are placed on PDA or corn meal agar. This method has been used extensively for isolating fast-growing fungi, e.g.,

Rhizootonia spp. Selective media such as PDA + BNPRA and PDA + PCNB have been used for isolating *Pythium* spp. and *Fusarium* spp., respectively (Fujisawa and Masago 1975, Mircetich and Kraft 1975, Nash and Snyder 1962).

Pythium and *Fusarium* can be isolated from soil by using soil plate or soil dilution on selective media, as mentioned earlier, or isolated by the baiting technique using vegetable seeds. After a few days' incubation, the infested seeds are transferred onto selective media (Figure 1C). Above-described methods are followed. Pure cultures are observed under microscope (Table 1, and Figures 2A-E).

Some plant pathogenic fungi such as *Phytophthora* can be isolated by using the host plant, e.g., orange, apple, mango, durian, etc., as baits. They are placed in the soil under the diseased plant for a few days. After being removed from the soil, the infected host tissue is cut into small pieces and placed on a petri dish containing selective media (PDA + BNPRH) (Fujisawa and Masago 1975, Tsao and Guy 1972).

Another method that can be used consists of cutting the host plants (e.g., leaves of durian or mango) into small (5 x 5 mm) pieces and submerging in infected soil and water in a beaker. After 2 or 3 days of incubation, the infected tissues are transferred onto PDA + BNPRH in a petri dish (Figure 6A). Above-described methods are followed. Pure cultures are observed under microscope (Figure 6B).

Human hair and snake skin (shedded) have been used as baits for isolating keratinophilic fungi, e.g., *Microsporum* spp. from soil (Table 1). They are placed on soil to which a few drops of water and antibiotic are added and incubated at room temperature for 1 week. White mycelium can be found on the baits (Figure 4A) and conidia can be observed under light microscope (Figure 4B).

Method for isolating thermophilic fungi (Cooney and Emerson 1964)

The soil plate and soil dilution plate methods are used to isolate thermophilic fungi from soil and plant debris. They are cultured on selective media, e.g., yeast glucose agar or yeast starch agar to which a few drops of antibiotic are added to eliminate bacteria. They are incubated at 45-55°C for a few days. Hyphal tips are transferred to PDA slant using a transfer needle. They are incubated at 45-55°C for 1 week. Above-described methods are followed.

Filter paper method (Berg et al. 1972, Murao et al. 1979)

Soil samples (1 gm) are placed in sterilized petri dishes and covered with sterile No.1 filter paper (9 cm in diameter). Liquid cellulolytic fungus media are poured on the filter paper discs and incubated at 30-45°C for 3-5 days. Microbial growth (colony) on the filter paper is transferred to slant cellulolytic media. This method is used for isolating soil cellulolytic or decomposing microorganisms.

Culture media for isolating soil microorganisms

Glucose-ammonium nitrate agar for isolating soil fungi in general (Gochennaur 1964)

NH_4NO_3	1.0	gm
Glucose	5.0	gm
Yeast extract	1.0	gm
K_2HPO_4	1.0	gm
$MgSO4.7H_2O$		
Rose bengal	0.03	gm
Agar	18.0	gm
Distilled water	1000	mL
Streptomycin	30	ppm

After medium has been autoclaved, 4 mL of 30 ppm streptomycin are added.

Yeast glucose agar for isolating thermophilic fungi (Cooney and Emerson 1964)

Yeast extract	15.0	gm
Glucose	10.0	gm
Agar	20.0	gm
Distilled water	1000	mL

Yeast starch agar for isolating thermophilic fungi(Cooney and Emerson 1964)

Yeast extract	4.0	gm
$K2HPO_4$	1.0	gm
$MgSO_4.7H_2O$	0.5	gm
Soluble starch	15.0	gm
Agar	20.0	gm
Distilled water	1000	mL

Cellulolytic fungi media (Murao et al. 1979)

Carboxy methyl cellulose	6.0	gm
$(NH4)_2SO_4$	2.0	gm
KH_2PO_4	0.6	gm
K_2HPO_4	0.4	gm
$MgSO_4.7H_2O$	0.5	gm
Ferric citrate	10.0	mg
ZnSO4.7H2O	4.4	mg
$MnSO_4.4H_2O$	5.0	mg
CaCl2	55.0	mg
$CoCl_2.6H_2O$	1.0	mg
Thiamine hydrochloride	100.0	mg
Yeast extract	1.0	gm

Agar	15.0	gm
Distilled water	1000	mL
Streptomycin	30	ppm

After autoclaving the medium, 4 mL of 30 ppm streptomycin are added.

Cellulolytic actinomycetes media (Murao et al. 1979)

Carboxy methyl cellulose	6.0	gm
$(NH4)_2SO_4$	1.0	gm
KH_2PO_4	0.8	gm
K_2HPO_4	0.2175	gm
$Na_2HPO_4.7H_2O$	0.334	gm
$MgSO4.7H_2O$	0.5	gm
$CuSO4.5H_2O$	0.001	gm
$FeSO4.7H_2O$	0.001	gm
$MnSO4.7H_2O$	0.001	gm
$ZnSO4.nH_2O$	0.001	gm
Yeast extract	0.1	gm
Agar	15.0	gm
Distilled water	100	mL
Actidione	30	ppm

After autoclaving the medium, 4 mL of 30 ppm actidione are added.

Cellulolytic bacteria media (Berg et al. 1972)

Carboxymethyl cellulose	5.0	gm
Peptone	5.0	gm
$MgSO4.7H_2O$	0.5	gm
Yeast extract	0.5	gm
Agar	15.0	gm
Distilled water	1000	mL
Actidione	30	ppm

After autoclaving the medium, 4 mL of 30 ppm actidione are added.

BNPRA medium for isolating *Pythium* sp. (Fujisawa and Masago 1975, Mircetich and Kraft 1973)

Rifampicin	10.0	ppm
Nystatin (Mycostatin)	50.0	ppm
Viccillin (Ampicilin)	500.0	ppm
PCNB (Terrachlor)	0.0667	gm
Rifampicin	25.0	ppm
Benlate	10.0	ppm
Distilled water	100.0	mL

For preparing the BNPRA solution, 11.5 mg of Rifampicin (Rifadin 300 mg/capsule); 2.2 mL Nystatin (Mycostatin 100 000 unit/mL); 700 mg Viccillin (Ampicillin 250 mL/capsule); 33 mg PCNB (Brasscicol 75% ai) and 20 mg Benlate (Benomyl 50% ai) are mixed with 100 mL distilled water. Before pouring medium in petri dishes, 1 mL BNPRA solution is added to 10 ml CMA or PDA.

BNPRH medium for isolating *Phytophthora* spp. (Masago et al. 1977, Tsao and Guy 1977)

Rifampicin	10	ppm
Nystatin	50	ppm
Viccillin	500	ppm
PCNB	25	ppm
Benlate	10	ppm
Hymexazol (HMI)	50.0	mg
Distilled water	100	mL

For preparing the solution of BNPRA, 0.5 mg Hymexazol (Tachigarin or 3-hydroxy-5-methylisoxazol) are added. Before pouring medium in petri dishes, 1 mL BNPRH is added to 10 mL CMA or PDA.

PCNB (peptachloronitrobenzene) peptone agar medium for isolating *Fusarium* sp. (Nash and Snyder 1962)

Peptone	7.5	gm
$MgSO_4.7H_2O$	0.025	gm
KH_2PO_4	0.5	gm
Agar	6.0	gm
Distilled water	500	mL
PCNB	0.5	gm

Culture media for growing soil fungi

Potato dextrose agar (PDA) for growing fungi in general

Potato	200.0	gm
Dextrose	20.0	gm
Agar	18.0	gm
Distilled water	1000	mL

Potato carrot agar (PCA) for growing fungi in general

Carrot	100.0	gm
Dextrose	20.0	gm
Agar	18.0	gm
Distilled water	1000	mL

Cornmeal agar (CMA) for growing fungi in general

Difco cornmeal agar	17.0	gm
Distillted water	1000	mL

Czapek-Dox agar for growing Aspergillus *and* Penicillium

$NaNO_3$	3.0	gm
K_2HPO_4	1.0	gm
$MgSO_4.7H_2O$	0.5	gm
KCI	0.5	gm
$FeSO_4.7H_2O$	0.01	gm
Sucrose	30.0	gm
Agar	15.0	gm
Distilled water	1000	mL

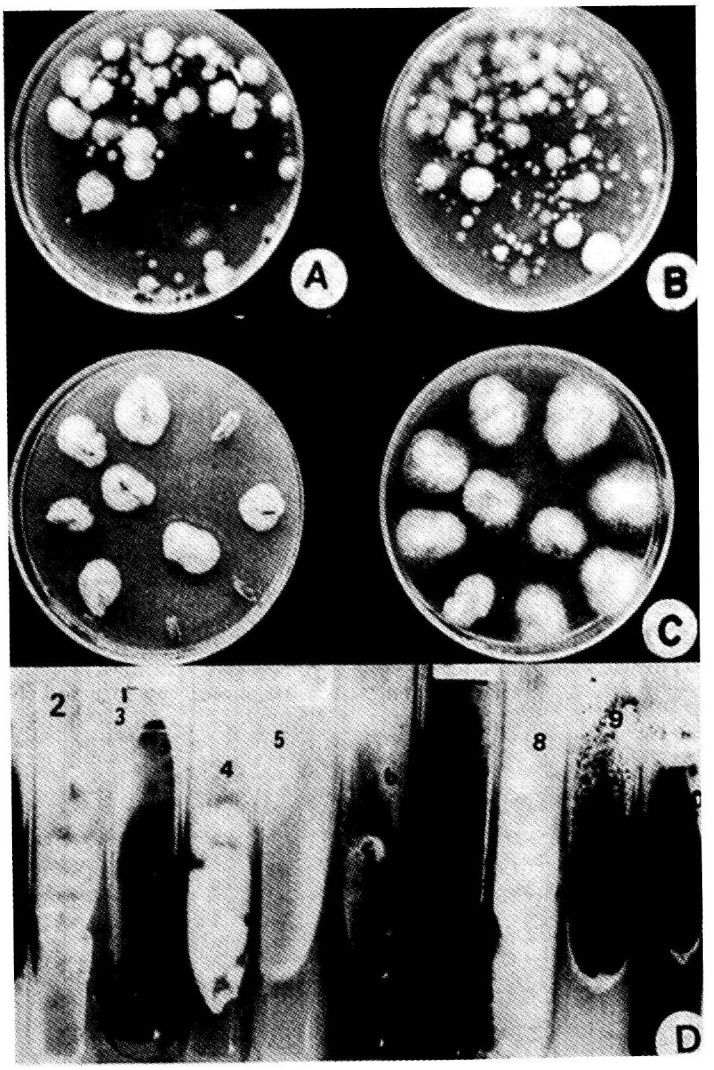

Figure 1 *Colony of soil fungi from various methods: (A) soil plate, (B) soil dilution plate, (C) baiting technique with rice grain, (D) pure cultures of soil fungi on PDA. (From Manoch 1992.)*

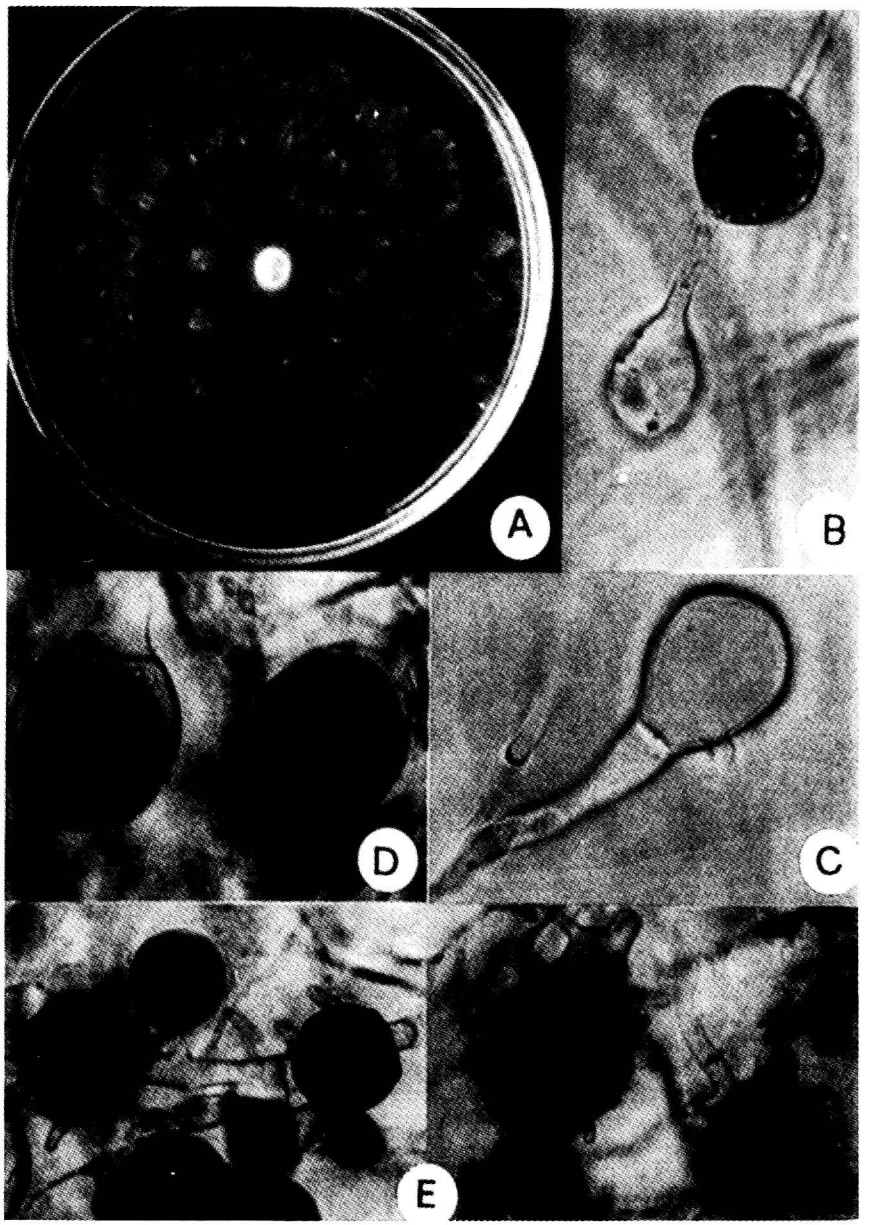

Figure 2 Pythium vexans *and* P. spinosum *from soil: (A) colony of* P. vexans *on PCA, (B) sporangium, discharge tube, and vesicle, (C) empty sporangium and part of the discharge tube, (D) oospore in oogonium, (E) P. spinosum showing oospore. (From Manoch 1992.)*

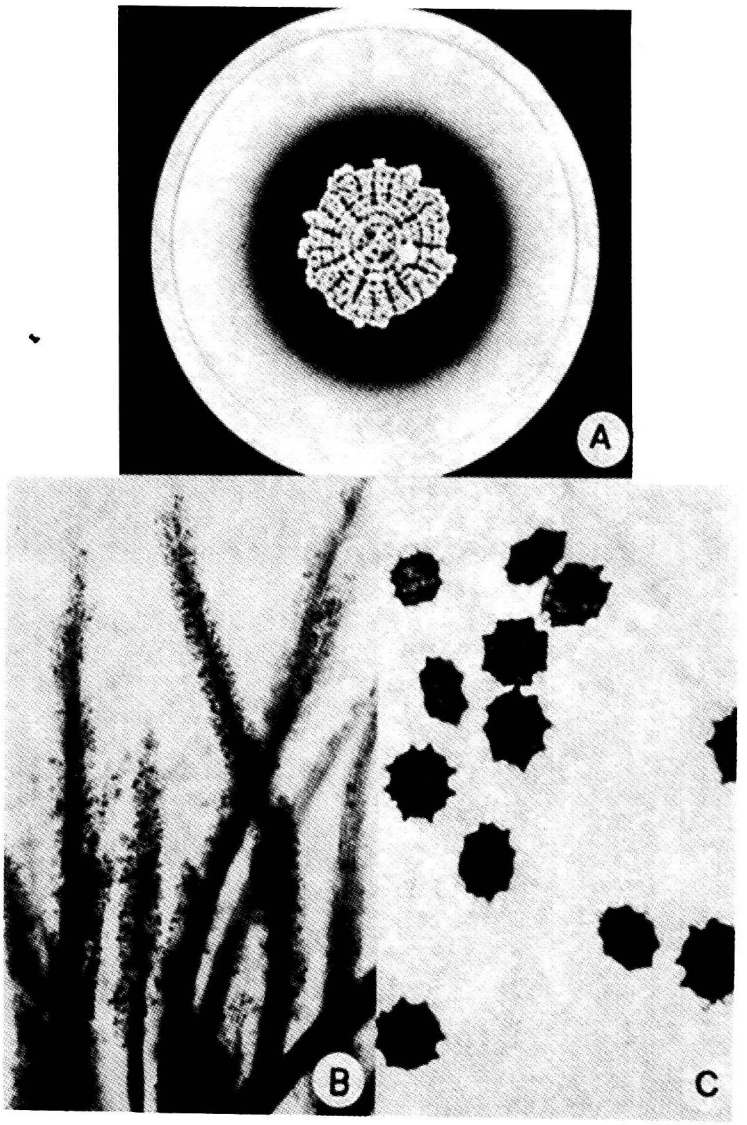

Figure 3 *Hypnomycetes collected from soil: (A) colony of* Penicillium *producing red pigment of PDA, (B) synnemata of Doratocystis stemonitis, (C) conidia of Spegazzinia sp. (From Manoch 1992.)*

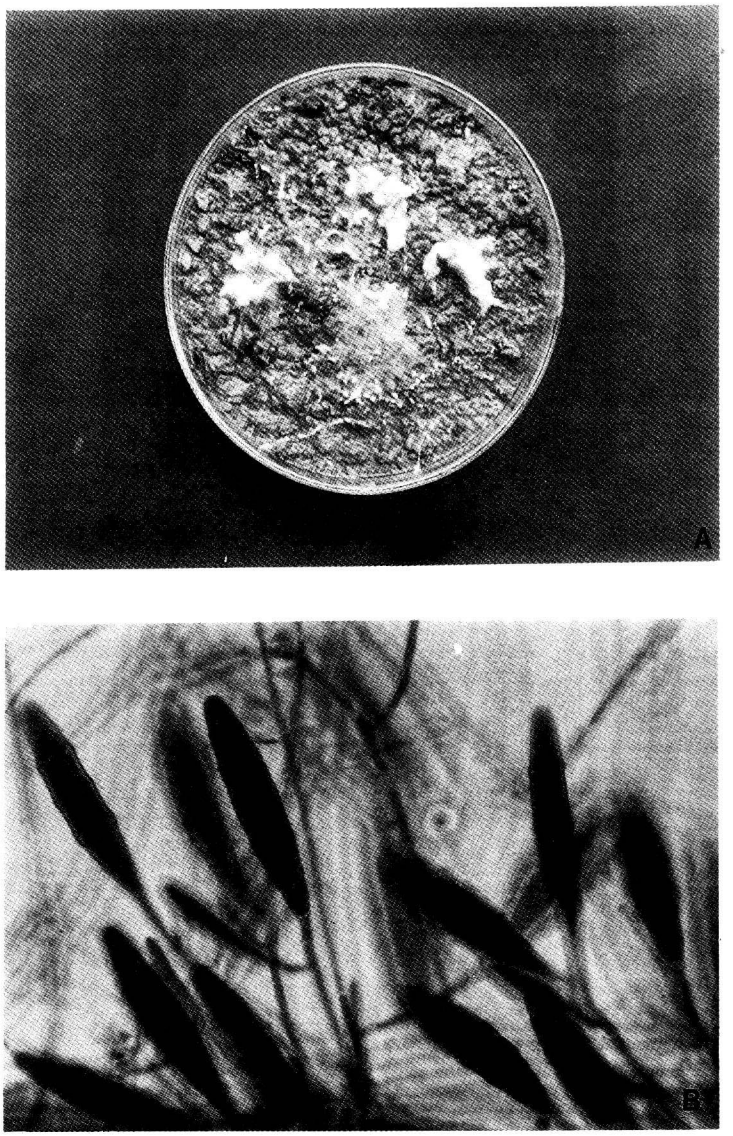

Figure 4 Microsporum *sp.: (A) mycelium and conidium on human hair using baiting technique, (B) hypha and conidia. (From Manoch 1992.)*

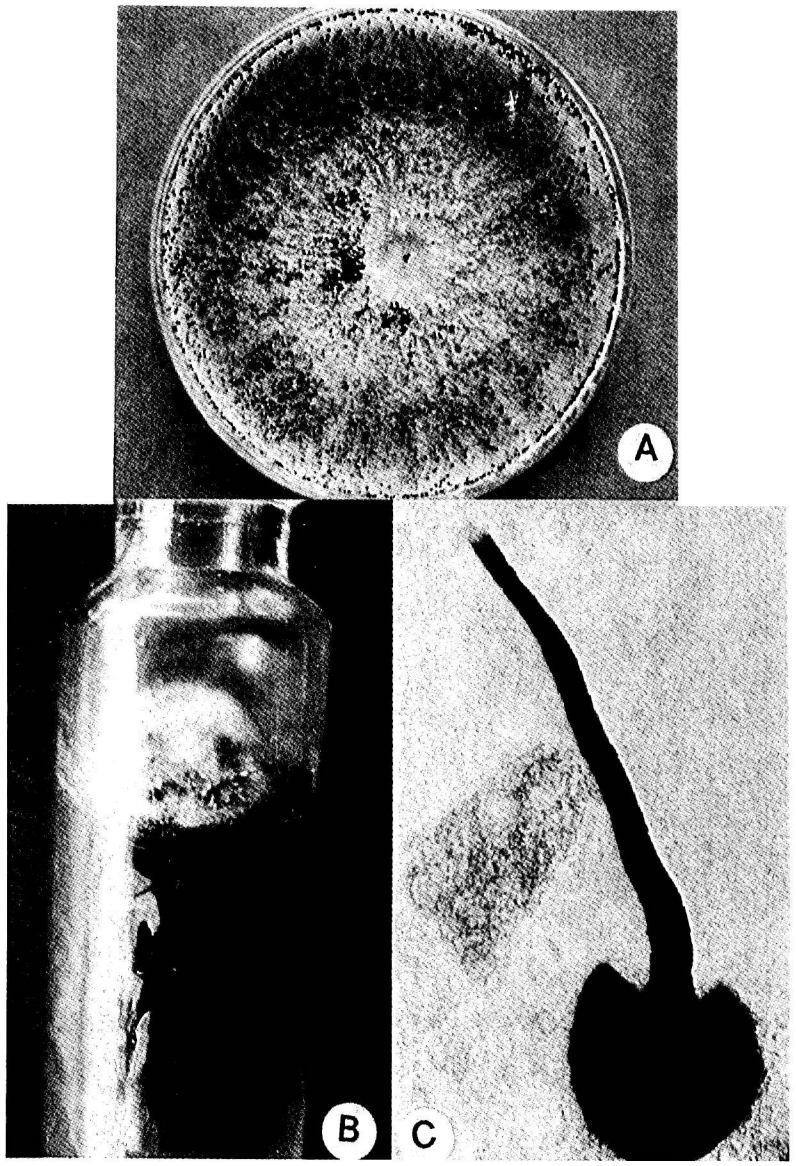

Figure 5 *A: colony of* Sordaria *sp. on PDA with white mycelium and black perithecia; B: submerged mycelium with dichotomous, root-like structure of unidentified fungus; C: Perithecium of* Ceratocystis *sp., with typical long beak. (From Manoch 1992.)*

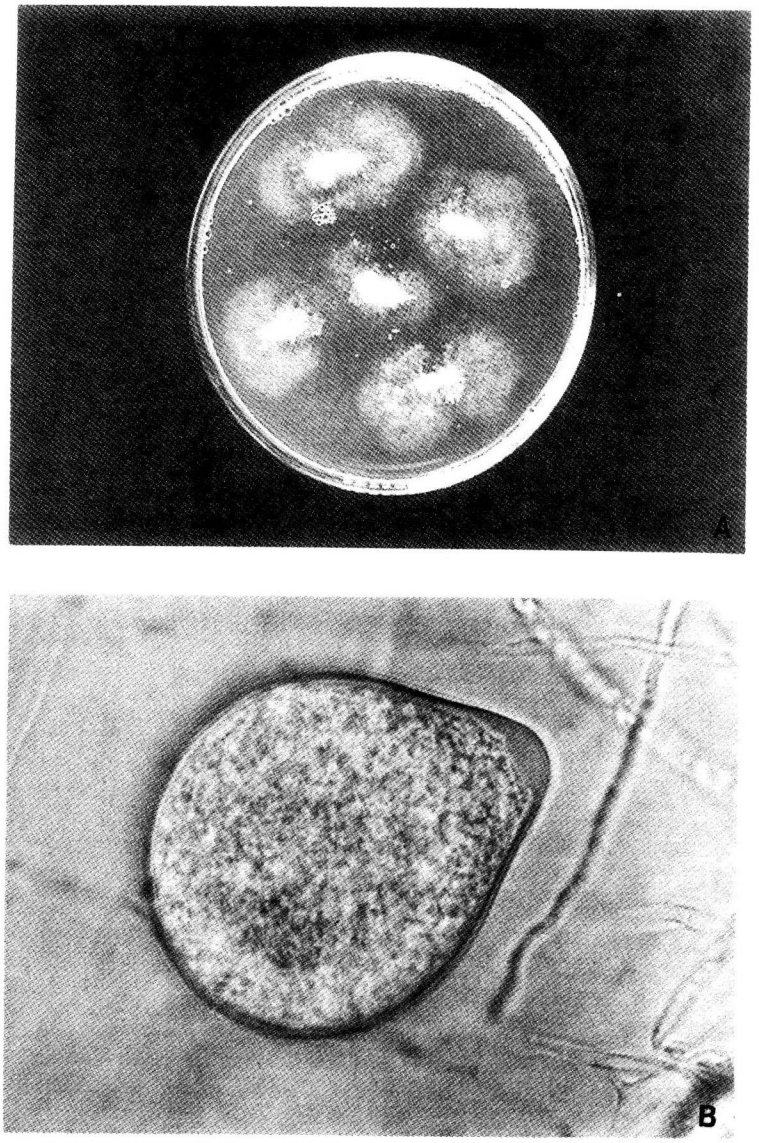

Figure 6 Phytophthora parasitica: *(A) tissue transplanting on PDA+BNPRH, (B) sporangium. (From Manoch 1992).*

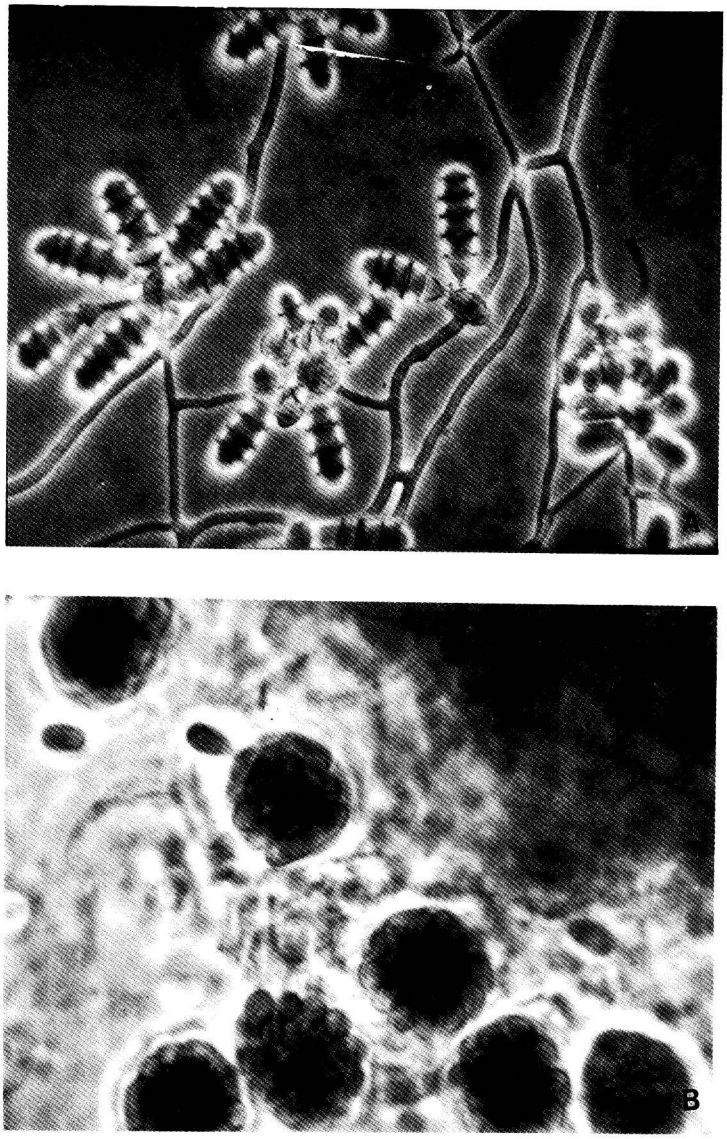

Figure 7 *(A)* Cephaliophora *sp.: conidia and conidiophore, (B)* Westerdykella *sp.: asci and ascospore; isolated using soil plate method. (From Manoch 1992).*

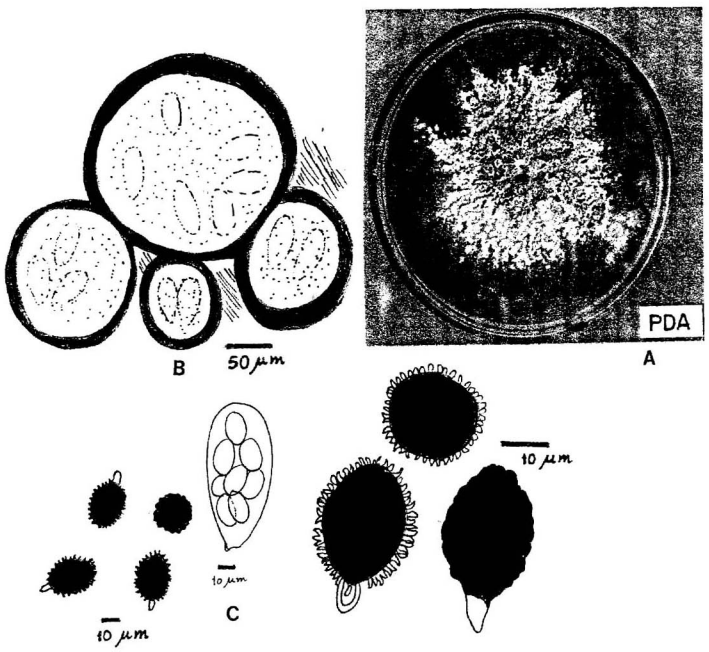

Figure 8 Apiosordaria *sp. (A) colony on PDA, (B) camera lucida, drawing of perithecium with ascus and ascospore, (C) camera lucida drawing of asci and ascospore; isolated using alcohol or actidione treatment method. (From Manoch 1992).*

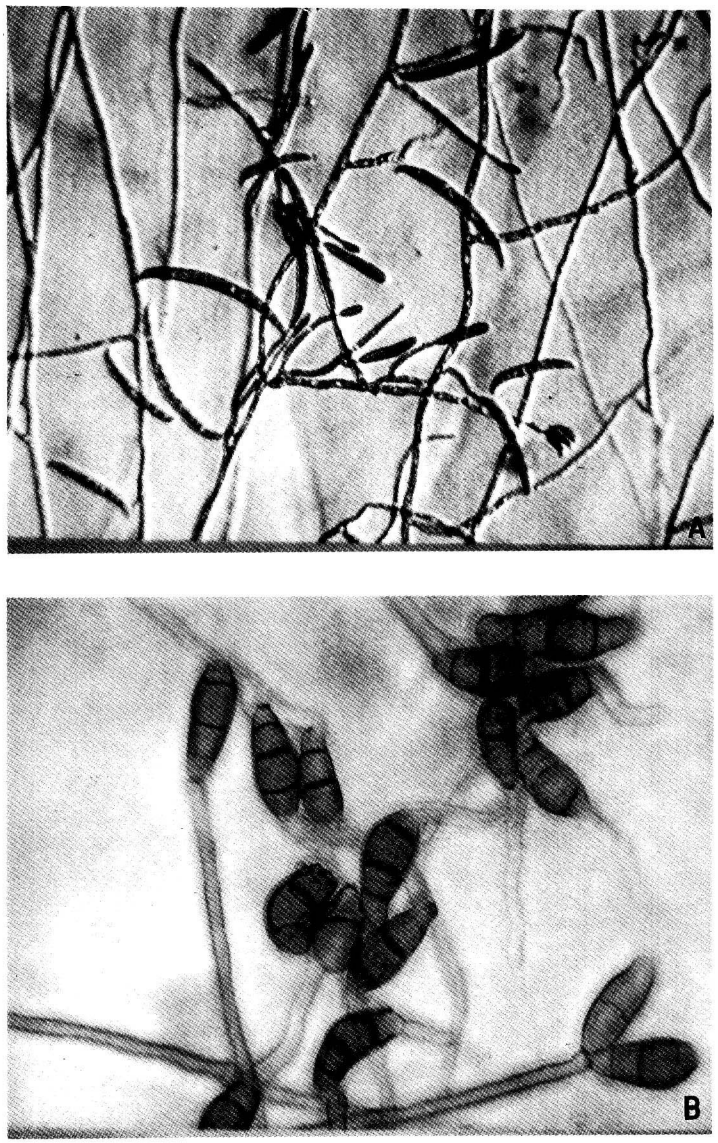

Figure 9 *(A)* Fusarium *sp.: macroconidia and microconidia, (B)* Curvularia *sp.: conidia and conidiophore; isolated using soil or soil dilution plate method. (From Manoch 1992).*

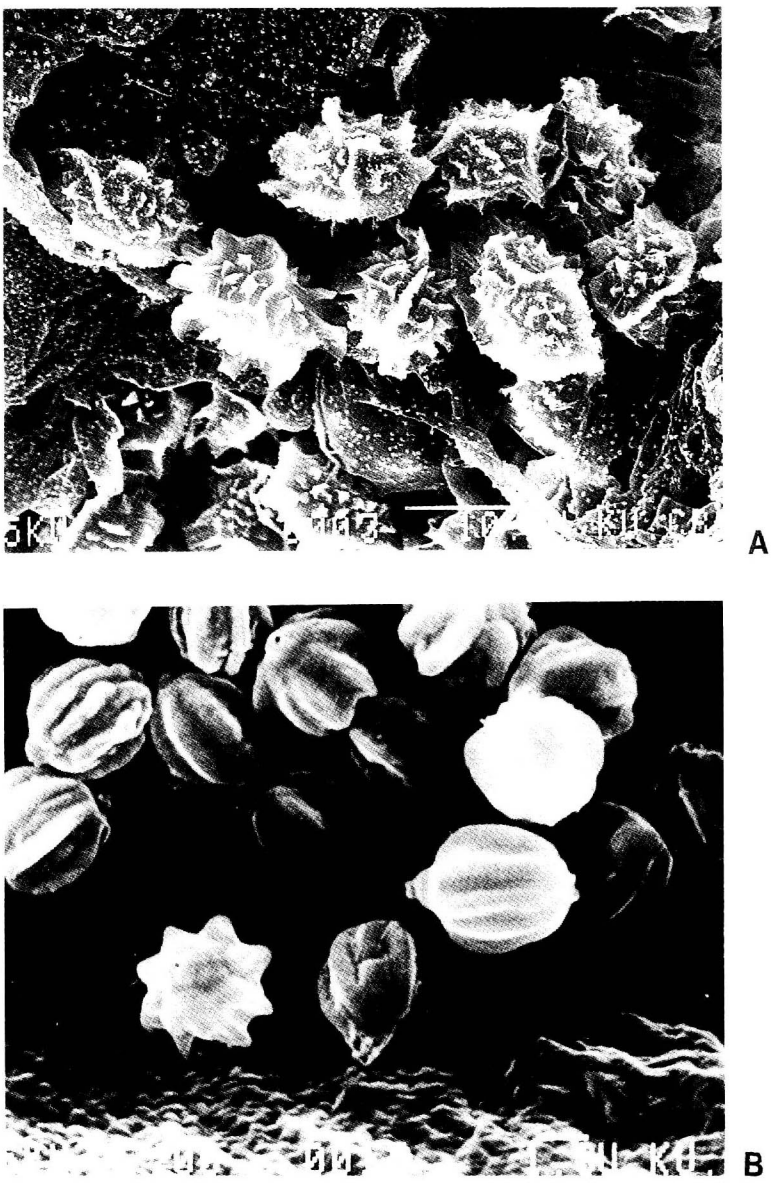

Figure 10 *Scanning electron microscope photographs of (A)* Aescodesmis *sp.: ascopore, (B)* Penicillium *sp. conidia; isolated using alcohol treatment method. (From Manoch 1992).*

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Errata

p. iii, line 5, "*dillligent*" should read "*diligent.*"

p. vii, line 5, "*Nakom*" should read "*Nakon.*"

p. 7, line 12, "*Trigonalla*" should read "*Trigonella.*"

p. 7, line 18, "*Cowpea*" should read "*Cowpea type.*"

p. 7, line 21, "*Trigonolla*" should read "*Trigonella.*"

p. 7, line 27, "*Ornithopus*" should be placed under the second column "*Examples of host genera.*"

p. 11, line 1, "*mychorrizae*" should read "*mycorrhizae.*"

p. 28, line 1, "*Letospermaceae*" should read "*Leptospermaceae.*"

p. 32, line 5, "*Eberaceae*" should read "*Ebenaceae.*"

p. 47, line 2, "*Nopamonrbodi*" should read "*Nopamornbodi.*"

p. 50, line 22, "*15 m . . . 800 m*" should read "*15 μ . . . 800 μ.*"

p. 59, line 4, "*Leaungvutivirog*" should read "*Leaungvutiviroj.*"

p. 59, line 21, "*Junrungreaug*" should read "*Junrungreang.*"

p. 70, line 16, "*Ceratocyystis*" should read "*Ceratocystis.*"

p. 70, line 17, "*Chaetomiun*" should read "*Chaetomium.*"

p .70, line 20, "*Doratomyces*" should read "*Ceratomyces.*"

p. 70, line 36, "*Micoascus*" should read "*Microascus.*"

p. 74, line 1, "*Rhizootonia*" should read "*Rhizoctonia.*"

p. 78, line 8, "*KCI*" should read "*KCL.*"

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