Host specificity, mycorrhizal compatibility and genetic variability of *Pisolithus tinctorius*

Hemavathi Bobbu

Department of Biology, IIIT RK Valley, RGUKT-AP, India

Abstract— *The reaction between the various hosts with Pisolithus tinctorius shows the broad host range of this fungal species showing different degrees of host compatibility. There is wide variation in both rate and extent of ECM formation by different isolates of Pisolithus tinctorius of different geographical regions within a species. Thus Pisolithus tinctorius displays much intraspecific heterogeneity of host specificity and interspecific compatibility. There are variable degrees of plant-fungal isolate compatibility, implying specificity, and this is an important factor influencing successful ectomycorrhiza formation and development. The molecular data also suggested that the Pisolithus tinctorius isolates analyzed from different geographical regions belong to distinct groups. Further studies are therefore warranted to elucidate the molecular, biochemical and physiological differences between the Pisolithus tinctorius isolates at the fungus-root interface of different plant species.*

Keywords— Ectomycorrhiza, Pisolithus tinctorius, Host specificity, mycorrhizal compatibility, genetic variability.

Abbreviations— ECM/EM: Ectomycorrhiza; Pt: Pisolithus tinctorius; AM: Arbuscular Mycorrhizae; VAM: Vesicular Arbuscular Mycorrhizae.

I. INTRODUCTION

Ectomycorrhizal symbiosis, a mutualistic plant–fungus association, formed between fine roots of higher plants, mostly trees, and a wide range of soil ascomycetes and basidiomycetes plays a fundamental role in the biology and ecology of forest trees, affecting growth, water and nutrient absorption, and providing protection from root diseases. It is commonly accepted that there is mutual benefit to the partners, due to the exchange of plant-derived carbohydrates for amino acids and nutrients supplied by the fungus (Smith & Read 1997). Bi-directional movement of nutrients characterizes these symbioses where carbon flows to the fungus and inorganic nutrients move to the plant, thereby providing a critical linkage between the plant root and soil. In infertile soils, nutrients taken up by the mycorrhizal fungi can lead to improved plant growth and reproduction. As a result, mycorrhizal plants are often more competitive and better able to tolerate environmental stresses than are non-mycorrhizal plants. Mycorrhizal associations vary widely in form and function.

Tree roots are exposed to several hundred different species of ectomycorrhizal fungi (Dahlberg *et al.,* 1997). These fungi rely upon basidiospores, mycelial fragmentation, mitotic sporulation and, occasionally, sclerotia as their major, means of dispersal and reproduction (Dahlberg & Stenlid 1995). Each species exists, as a population of many genetic individuals, so-called genets, between which there is almost invariably some phenotypic variation. Each genet arises in a unique mating event and then vegetatively expands as a host root-connected mycelium throughout the humus layer of forest soils (Debaud *et al.,* 1999). The fact that trees are exposed to genetically diverse mycobionts is an important consideration in forest ecology. Genets vary in their ability to colonize different genotypes of host plant, their ability to promote plant growth and adaptation to abiotic factors, such as organic/inorganic nitrogen concentration and soil pH. Each of these factors might affect the course of a beneficial symbiosis and the dissemination of a fungal genet in a forest ecosystem.

The symbiosis between ectomycorrhizal fungi and trees is an essential part of forest ecology and depends entirely on the communication between the two partners for establishing and maintaining the relationship. The identification and characterization of differentially expressed genes is a step to identifying such signals and to understanding the regulation of this process. Symbiotic interactions are highly dependent on communication between the partners for initiation and maintenance. One ecologically important symbiosis is the ectomycorrhiza formed between basidiomycete fungi and their host trees (Buscot *et al*., 2000). By implication, such an understanding can help in the formulation of programmes where economically important tree species (e.g. eucalyptus, oaks, firs, pines, spruces) are inoculated using selected beneficial ectomycorrhizal fungi (Selosse *et al.,* 1999).

Ectomycorrhizal basidiomycetes constitute an important component of forest fungal communities, but little is known about their population structure, genetic variability and mycorrhizal compatibility. Some trees form associations with many different fungi, but some fungi interact with only one host plant. A single fungal species can enter into ectomycorrhizal association with numerous tree species on the same site. A fungus can also develop numerous biotypes or clones in a very limited area of a pure stand (Fries, 1987). Some fungi are apparently host specific, while others have broad host range and form ectomycorrhizae with members of numerous tree genera in diverse families (Marx and Cordell, 1978).

Ectomycorrhizal fungi are mostly basidiomycetes that grow between root cortical cells of many tree species, forming a Hartig net. ECM usually forms between fine roots and dikaryotic mycelia of different Basidiomycete genera. Some Ascomycetes are also involved, e.g. members of Tuberales, and two members from Zygomycetes (Isaac 1992). Both the ECM plants and fungi are generally able to form symbiosis with different species. Most Agaricales (Basidiomycetes) have a heterothallic lifecycle. In this life cycle, fusion between monokaryons, which develop after spore germination, is an essential step. If fusion occurs and a dikaryon develops, both monokaryons are considered to be sexually compatible (hereafter referred to as compatibility in this study). For most species, the cells of the dikaryon have two separate nuclei. The dikaryons can form basidiocarps (mushrooms), the fruit bodies. In the basidiocarp, nuclear fusion and meiosis take place followed by spore formation. Cell fusion and nuclear fusion are thus separated in time. Sexual compatibility between monokaryons has been used to delimit biological species (intercompatibility groups, ICGs) within Basidiomycetes (Peterson, 1995). ECMs are particularly important for tree growth in regions with low nutrient or water status.

1.1. GLOBAL PERSPECTIVE

Mycorrhizal associations vary widely in structure and function. Ericaceous plants, which dominate the acidic, high-organic heath land soils of sub arctic and sub alpine regions, are colonized by a group of ascomycetous fungi, giving rise to the ericoid type of mycorrhiza. This mycorrhizal type is characterized by extensive growth within (i.e., intracellular) cortical cells, but little extension into the soil. The fungi produce extra cellular enzymes that break down organic matter, enabling the plant to assimilate nutrients mineralized from organic compounds present in the colloidal material surrounding roots. Moving along the environmental gradient, coniferous trees replace ericaceous shrubs as the dominant vegetation. These trees are colonized by a wide range of mostly basidiomycetous fungi that grow between (i.e., intercellular) root cortical cells forming the ectomycorrhizal type of mycorrhiza.

Ectomycorrhizal fungi may produce large quantities of hyphae on the root and in soil. These hyphae function in the absorption and translocation of inorganic nutrients and water, but also release nutrients from litter layers by production of enzymes involved in mineralization of organic matter. At the warmer and drier end of the environmental gradient, grasslands often form the dominant vegetation. In these ecosystems, nutrient use is high and phosphorus is frequently a limiting element for growth. The diversity of these root-fungal associations provides plants with a range of strategies for efficient functioning in an array of plant-soil systems. The objective of this paper is to provide an overview of this diversity and to evaluate the role of the mycorrhizal symbiosis in native and managed ecosystems.

1.2. Ectomycorrhizas and their symbiotic associations:

In boreal and temperate forests, ectomycorrhizas (ECMs) are common symbiotic associations in trees and shrubs. Plant hosts include members of Pinaceae, Fagaceae, Betulaceae, Myrtaceae, and include some monocotyledons and ferns (Wilcox 1996). Over 4,000 fungal species, belonging primarily to the Basidiomycotina, and fewer to the Ascomycotina, are known to form ectomycorrhizae. Many of these fungi produce mushrooms and puffballs on the forest floor. Some fungi have a narrow host range, such as *Boletus betulicola* on *Betula* spp., while others have very broad host range, such as *Pisolithus arhizus* (also called *P. tinctorius*) which forms ectomycorrhiza with more than 46 tree species belonging to at least eight genera). *Alnus* (alders), *Salix* (willows), *Populus* (poplars), and *Eucalyuptus* can have both AM and EM associations on the same plant. Some ericoid plants have occasional EM and AM colonization.

The diagnostic feature of ectomycorrhizae (EM) is the presence of hyphae between root cortical cells producing a net like structure called the Hartig net**,** after Robert Hartig who is considered the father of forest biology. Many EM also have a sheath, or mantle, of fungal tissue that may completely cover the absorbing root (usually the fine feeder roots). The mantle can vary widely in thickness, color, and texture depending on the particular plant-fungus combination. The mantle increases the surface area of absorbing roots and often affects fine-root morphology, resulting in root bifurcation and clustering. Contiguous with the mantle are hyphal strands that extend into the soil. Often the hyphal strands will aggregate to form rhizomorphs that may be visible to the unaided eye. The internal portion of rhizomorphs can differentiate into tube like structures specialized for long-distance transport of nutrients and water.

Fungal mantle with varying depth covers them, containing aggregated, branched, and swollen hyphal cells. The outer mantle hyphae are connected to extrametrical mycelium that takes care of the mineral nutrition and water uptake of the symbiotic tissues. The inner mantle hyphae form a network of finger-like branches, which extend between the epidermal (angiosperms) and cortical (gymnosperms) cells of the host plant (Barker *et al.,* 1998). This fungus-plant interface is called the Hartig net and it represents the site of fungal nutrient transfer in exchange for plant photosynthates. In contrast to other mycorrhizal types, the ectomycorrhizal hyphae do not form intracellular structures (Peterson and Farquhar, 1994).

Ectomycorrhizal associations consist of a soil mycelium system, linking mycorrhizal roots and storage or reproductive structures. Trees with ECM association are dominant in coniferous forests, in cold boreal or alpine

regions, and many of the broad-leaved forests in temperate or Mediterranean regions, but they also occur in some tropical or subtropical savanna or rain forests habitats. Ectomycorrhizal associations are formed predominantly on the fine root tips of the host, which are unevenly distributed throughout the soil profile, being more abundant in topsoil layers containing humus than in underlying layers of mineral soil (Brundrett, 1991).

Several functional differences occur between fungal species and strains able to form ECM, which include differences in symbiotic capabilities (Bonfante *et al*., 1998), ability to proliferate (Rousseau *et al*., 1994), and to tolerate drought and heavy metals (Guehl *et al*., 1992). Substantial differences in nutrient uptake capacity between both ECM fungal species and strains have also been revealed (Finlay *et al*., 1992). In general, the ECM fungal species have very different physiologies and morphologies and the total benefit of symbiosis for the host plant depends on the infection pattern of its total root system and the extent of infection by individual varieties of ECM fungal species. ECM fungi are sensitive to variation in soil nutrient status. Additions of abundant fertilizer amounts have significant effects on the ECM fungal diversity and some genotypes may eventually be lost. The reduced mycorrhiza formation of the roots makes them probably more vulnerable to environmental stress and pathogens (Hampp *et al*., 1999).

During the symbiotic interaction between ectomycorrhizal fungi and their hosts the straight, tubular hyphae swell and branch on the surface of plant roots to produce finger-like hyphae. The transition in growth pattern is necessary for the penetration into the intercellular space of the plant root and for the formation of the Hartig net. The Hartig net formation is a good indicator of host-fungus compatibility and is correlated with host growth responses (Smith and Read, 1997; Barker *et al*., 1998). The signal transduction pathways which lead to these fundamental changes in growth pattern are yet unknown. Specific plant flavonoids (Martin *et al*., 1999) have been suggested to trigger symbiotic growth. Treatment with protein kinase inhibitors and drugs that disrupt actin cytoskeleton (Niini, 1998) can mimic the hyphal morphogenesis that takes place in ECM. These observations suggest that the morphogenetic signaling pathways may involve ligand-receptor-interactions and signaling via protein kinase cascades, which lead to reorganization of actin cytoskeleton (Niini 1998; Martin *et al*., 1999).

1.3. Plant benefits from ECM symbiosis

Ectomycorrhizal trees often show better growth and improved resistance in unfavorable conditions than non-mycorrhizal trees. The extrametrical mycelium has potential for water transport and may improve the water relations of the host plant (Guehl *et al*., 1992). The soil surrounding ECM roots, mycorrhizosphere, has a rich microbe flora. The microbial diversity depends on the plant and fungal partners of the ECM association. Many types and species of microbes inhabit the area around the roots in soil, containing organisms that are useful, neutral and harmful for the symbiotic partners (Fitter & Garbaye 1994). The ECM associated bacteria affect mycorrhizal functioning in several ways including the regulation of fungal growth, host root-symbiotic fungus recognition events, nutrient mineralization, and protection against pathogens (Fitter and Garbaye1994).

Ectomycorrhizal fungi pass nitrogenous compounds to plants. They improve N mobilization to plants both by facilitating access to organic N sources and by increasing the uptake of N via extrametrical hyphae (Perez-Moreno and Read 2000). The hyphae mobilize organic N by secreted enzymes such as proteases and take it up by high affinity amino acid transporters (Wallenda and Read 1999). The fungal PEP-carboxylase cycle is important integrator of C and N metabolism, since it supplies C for amino acid skeletons (Martin *et al.,* 1999). The predominant forms of N transferred to the host are glutamine, glutamate, and asparagine (Smith and Read 1997). Phosphorus (P) is often present in the soil as insoluble inorganic or organic forms. The plants can only absorb soluble forms of P and their uptake rapidly causes a P depletion zone around the roots. ECM formation increases plant phosphorus content and the ability of the plant to gain phosphorus (Perez-Moreno and Read 2000). ECM fungal hyphae form polyphosphates from part of the imported P. Polyphosphates are an important P storage compound and together with orthophosphate short chain polyphosphates are the predominant form for P transported towards the host plant (Smith and Read 1997).

1.4. Development of ectomycorrhiza: Initiation and mantle formation

Ectomycorrhiza formation involves changes in the growth pattern of both partners. The development of this "symbiotic organ" facilitates efficient exchange of nutrients in the Hartig net region. In a mature root system newly formed roots are colonized by the Hartig net hyphae of the mother root (Wilcox 1968b). At germination or in planted seedlings, fungal growth towards host roots may involve chemical signaling which induces growth of hyphae in the direction of plant root (Horan and Chilvers 1990), but the specificity of signaling or the nature of substances involved are not known. Fungal cell wall proteins and cell surface polysaccharides have been identified as important molecules in the establishment of symbiosis. The adhesion on the root tips or distal to the root apical meristem (Smith and Read 1997) may involve hydrophobic interactions (Martin and Tagu 1995) and interactions between the plant and fungal polysaccharides and glycoproteins (Martin *et al.,* 1999). After adhesion at root surface, the hyphae make a firm contact on the host cell wall. After the contact with the root surface the fungal cell wall structure loosens (Bonfante *et al.,* 1998).

The hyphae start to swell and branch (Brunner and Scheidegger 1992). This fungal morphogenesis is associated with changes in cytoskeletal organization and regulation of fungal cell wall proteins, and it can be induced by plant flavonoids (Martin *et al.,* 1999). A network of branched hyphae, the hyphal mantle, forms on top of the root surface. The mantle varies in thickness but it usually consists of layers with differing structure and density of hyphae (Brunner and Scheidegger 1992). The region of the mantle closest to root epidermis is called pseudoparenchyma due to appearance of branched and fused hyphae that store lipids, trehalose and polysaccharides (Brunner and Scheidegger 1992). The pseudo parenchymatous hyphae are glued tightly together with extra cellular material that contains polysaccharides and glycoproteins. The mantle separates the host root from soil, and the host plant may depend in large part on the supply of water and nutrients from its symbiotic fungus. Lateral root growth is slowed down by the fungal colonization, and due to the hyphal production of anauxin-betaine, hyphaphorine, root hair formation is prevented (Peterson, 1991, Beguiristan and Lapeyrie, 1997).

1.5. Fungal penetration and Hartig net formation

Penetration between root apical cells is mostly mechanical in nature but also involves production of ECM fungal lysing enzymes for digestion of host cell walls (Dahm *et al.,* 1987). The host cell walls are separated by fungal intrusion and they become swollen and less compact (Brunner and Scheidegger 1992). The damage to plant cell walls causes a transient production of plant defense substances and proteins. According to studies in cell cultures, this defense-response is partly halted by plant enzymes (Salzer *et al.,* 1997). *In plant*a, the eliciting activity of the fungus depends on the extent of compatibility between the symbiotic partners (Burgess *et al.,* 1995; Bonfante *et al.,* 1998). The development of the symbiotic interface is very similar in different host-mycobiont interactions. The finger-like hyphae mostly penetrate the cortex to form a complex, highly ordered web of tightly packed hyphae between the epidermal and cortical cells, the Hartig net. Fungal and plant cell walls merge and form a novel type of interface which contains a complex matrix of constituents from both fungal and plant origin (Bonfante *et al.,* 1998; Niini1998). The formation of a functional Hartig net concludes the development of a structure, which can be referred to as a symbiotic organ.

1.6. Extramatrical hyphae and rhizomorphs

Hyphae extend from the mantle to facilitate nutrient solubilization and transport. Part of the transport takes place in the symplast of living hyphae. Transport in the symplast occurs by motile tubular vacuoles that can move material across long intracellular distances (Shepherd *et al.,* 1993). Most of the basidiomycete ECM fungi, like *Suillus bovinus,* can also form rhizomorphs; linear aggregates of fungal hyphae containing large central ”vessel” hyphae that may represent significant extensions to the root system (Rousseau *et al.,* 1994). At the onset of rhizomorph formation the leading hyphae grow in parallel approaching each other, they form linear aggregates, and allow the formation of branches and intercellular bridges (Cairney 1992). After the tight tubular aggregate of hyphae is formed, cellular contents of the central hyphae disappear and septal cross-walls break down, leading to vessel hypha formation (Agerer, 1992). The vessel hyphae have been implicated for acropetal C transport and the living cortical hyphae for symplastic transport of P and other nutrients (Cairney 1992), but this has not yet been proven. The age of the infected roots varies considerably, but mostly ECM fungal infection prolongs the age of fine roots (Wilcox 1968b). In aged ECMs Hartig net host cell walls disintegrate and cannot be distinguished from the plant-fungal cell wall matrix, which probably leads to a decrease in nutrient transport. Some ECM fungi may survive the death of the cortical cells and live parasitically between the root cells. Others die simultaneously with the collapse of the Hartig net (Wilcox 1996).

1.7. Signalling in symbiotic and pathogenic hyphae

During the symbiotic interaction between ectomycorrhizal fungi and their hosts the straight, tubular hyphae swell and branch on the surface of plant roots to produce finger-like hyphae. The transition in growth pattern is necessary for the penetration into the intercellular space of the plant root and for the formation of the Hartig net (Smith and Read 1997; Barker *et al.,* 1998). The signal transduction pathways which lead to these fundamental changes in growth pattern are yet unknown. Specific plant flavonoids (Martin *et al.,* 1999) have been suggested to trigger symbiotic growth. Treatment with protein kinase inhibitors and drugs that disrupt actin cytoskeleton (Niini, 1998) can mimic the hyphal morphogenesis that takes place in ECM. These observations suggest that the morphogenetic signaling pathways may involve ligand-receptor-interactions and signaling via protein kinase cascades, which lead to reorganization of actin cytoskeleton (Niini, 1998; Martin *et al.,* 1999). The growth pattern of pathogenic fungi also changes in compatible interactions with the plant host. Fungal pathogens perceive and respond to molecules from the plant and on the plant cell wall, which trigger pathogenic development. The haploid yeast-like form of the basidiomycete corn smut fungus *Ustilago maydis* is non-pathogenic and the infection process is associated with sexual development.

The formation of dikaryon is necessary for filamentous growth and only the filamentous dikaryon is infectous (Kahmann *et al*., 1999). Both pheromone-receptor interaction and cAMP signalling are needed for pathogenic development in *U. maydis,* and for a successful infection, the fungus also needs an intimate contact with the host plant

(Dürrenberger *et al.,* 1998; Basse *et al.,* 2000). Cross talk between the pheromone-receptor and cAMP signalling pathways is probably mediated by a G-protein subunit Gpa3, which is presumed to activate the enzyme adenylate cyclase that catalyses cAMP production (Kahmann *et al.,* 1999). Specialized infection structures, appressoria, are formed for the penetration of plant cells by many plant pathogenic fungi (Hamer and Talbot 1998). Appressoria are dome-shaped cells with specific, strong cell walls, which facilitate the turgor-driven penetration into the host plant (Thines *et al.,* 2000).

Their formation has been recently studied in the ascomycete pathogen of rice, *Magnaporthe grise*a, where cAMP-linked signaling cascades regulate the formation of appressorium (Adachi and Hamer 1998). In the ascomycete *Cryphonectria parasitic*a, which causes chestnut blight, the best-characterized signaling component for fungal virulence is a G-protein with subunit Cpg1 (Choi *et al.,* 1995). Signalling networks that are conserved in pathogenic development (Kahmann *et al*., 1999) may also regulate hyphal adhesion on plant surface, morphogenesis, and penetration of host tissues during symbiosis. To isolate possible regulators of symbiotic growth, homology-based PCR approach has led to the identification of one putative cDNA and two ras cDNAs from *Suillus bovinus* cDNA library (Raudaskoski *et al.,* 2000). All of these genes are expressed in symbiotic hyphae.

II. INTERACTION OF *PISOLITHUS TINCTORIUS*

To explore further the plant-fungus interaction of ectomycorrhizal basidiomycetes, the present study describes the mycorrhizal compatibility of *Pisolithus tinctorius* with different hosts. *Pisolithus tinctorius* (Pers.) Coker and couch [Syn. = P. archizus (Scop.: Pers.) Rasuschert] (*Pisolithus tinctorius*) is a widespread ectomycorrhizal (ECM) basidiomycete forming mycorrhizas with a variety of hosts (Coker and Couch, 1928, Marx, 1977). In pure culture all isolates to date, irrespective of their original host association, have initiated mycorrhizas on a range of tree genera, confirming the broad host range of this species (Grenville *et al*., 1985). This fungus has a proven host range of over 50 tree species and under field conditions, has been associated with an additional 25 tree species, it has been reported from over 33 countries of the world and 38 states in the United States (Marx, 1977).

Pisolithus tinctorius has been recorded in a range of habitats including forest, urban and orchard sites, as well as eroded and mine-site soils. Usually, seedlings with *P.tinctorius* ectomycorrhizae were the most vigorous seedlings found on adverse sites. These sites are normally characterized by high soil temperature during summer, extreme acidity, droughtiness, low fertility or high levels of toxic metals (Marx, 1977 and Malloch and Kuja, 1979), but carpophores are more usually found in relatively dry sites with little humus or along roadside areas (Castellano and Trappe, 1991). Information from western Australian reforestation sites indicates that the fungus is an early colonizer (Gardner and Malajczuk, 1988) and it is generally regarded as poorly competitive with other ECM fungi (McAfee and Fortin, 1986). It is perhaps for these reasons that *Pisolithus tinctorius* persists best in forestry inoculation programmes in sites subject to edaphic stresses. *Pisolithus tinctorius* isolates produce an extensive extrametrical mycelian phase, which in many cases differentiates into linear organs (Lamhamedi and Fortin, 1991). Based on descriptions linear mycelian organs of *Pisolithus tinctorius* can be described as 'apically diffuse, simple rhizomorphs' (Cairney *et al*, 1991). The formation of rhizomorphs may be important in channeling nutrients and water to and from the host and in protecting extrametrical mycelium against adverse environmental conditions (Cairney, 1992).

Pisolithus tinctorius also produces sclerotia as part of its extrametrical mycelian phase (Dennis, 1980, Fortin *et al*., 1983). While environmental conditions may influence the shape and structural detail of sclerotia, in all cases they comprise an external melanised ring surrounding a cortex and medulla (Grenville *et al*., 1985). In common with other fungal sclerotia, histochemical staining indicates that the cortex and medulla are rich in protein, lipids and carbohydrate, implying a storage function and the ability to allow *Pisolithus tinctorius* to withstand edaphic stresses in a vegetative state (Grenville *et al*., 1985). The production of sclerotia, along with aggregation of extrametrical mycelium into rhizomorphs, may thus be important in the reported success of *Pisolithus tinctorius* in stressful soil conditions. Genets of *Pisolithus tinctorius* are able to spread vegetatively through soil in excess of 30 m (Baar *et al.,* 1994; Anderson *et al.,* 1998).

2.1. Host-fungus specificity

Pisolithus forms ECM with a wide range of hosts (Table 1). *Pisolithus tinctorius* forms its association with most of *Eucalyptus spp.* and is a broad-host-ranging (Table 2). But some broad-host-ranging fungi did not form ectomycorrhizas with eucalypts: *Lactarius deliciosus*, *Boletus edulis* and *Thelephora terrestris* (Molina and Trappe, 1982b). Even isolates from different conifer species are not necessarily intercompatible (Marx, 1981). The *Pisolithus tinctorius* isolated from carpophores collected in association with Pinus spp. are poor colonizers of Eucalyptus spp. (Burgess *et al*., 1994). An isolate of *Pisolithus tinctorius*, originally isolated from under pine in the USA (isolate 270; D.H.Marx, 1982) has been used extensively in mycorrhizal inoculation programmes world wide with variable results.

The Eucalyptus spp. and P. radiata became mychorrhizal with several broad-host-ranging fungi, yet were unable to form mycorrhizas with fungi host specific to each other, and indicates that a variety of recognition phenomena may be

determining compatibility or incompatibility between these symbionts. Such recognition phenomena has been demonstrated for legume-Rhizobium symbioses (Schmidt, 1979), but we are unaware of such information for ectomycorrhizal hosts and fungi, because different isolates of *Pt* vary in mycelial growth and in ability to form ectomycorrhizae with different species (Molina, 1979 and Marx, 1981).

Table.1: Host genera with which Pisolithus tinctorius forms ectomycorrhizas

Genus	Source
Abies	Marx, 1977
Acacia	Ba *et al*., 1994
Aflezia	Ba and Thoen, 1990
Allocasuarina	Theodorou and Redell, 1991
Alnus	Godbout and Fortin, 1983
Arbutus	Zak, 1976
Arctostaphylos	Molina and Trappe, 1982a
Betula	Marx, 1977
Carya	Marx, 1977
Castanea	Martins *et al*., 1996
Castanopsis	Tam and Griffiths, 1994
Casuarina	Theodorou and Reddell, 1991
Eucalyptus	Marx, 1977
Hopea	Yazid *et al*., 1994
Larix	Molina and Trappe, 1982b
Pinus	Marx, 1977
Populus	Godbout and Fortin, 1985
Pseudotsuga	Marx, 1977
Quercus	Marx, 1977
Tsuga	Marx, 1977

The response of various species of eucalypts to inoculation with different species of ectomycorrhizal fungi has now been established in both glasshouse and field experiments and suggests that there is potential for increasing productivity from eucalypts grown as short rotation crops in plantations. Growth responses with wide range of ectomycorrhizal fungi in Eucalypts showed having either a broad or narrow host range (Bougher and Malajczuk, 1990 and Bougher *et al*., 1990). However, for each host, there were a range of responses to inoculation by the fungi, which implies that differences between species isolates affect growth promotion. Differences in physiological and growth characteristics between a number of species and isolates of ectomycorrhical fungi have been reported (Bougher and Malajczuk, 1990). Further, some *Pisolithus tinctorius* isolates show ECM compatibility with clones derived from mature eucalypt trees, but is poorly compatible with clonal host plants generated from young seedlings, suggesting that the developmental maturity of host material can also influence compatibility (Tonkin *et al*., 1989). *Pisolithus tinctorius* displays much intraspecific heterogeneity of host specificity, physiology and the benefits the fungus can impart upon the host plant. It is not clear at present how far such heterogeneity reflects systematic segregation within *Pisolithus tinctorius* (Cairney and Chambers, 1997).

A recent, detailed study of interactions between 20 *Pisolithus tinctorius* isolates from different geographical regions and *Eucalyptus grandis* indicates wide variation in both rate and extent of ECM formation by different isolates showing intraspecific variation in intercompatibility with *Pisolithus tinctorius* isolates (Burgess *et al*., 1994). The extent of ECM formation varied from a fully developed sheath and Hartig net in compatible isolates through isolates that formed only a superficial sheath, to isolates that formed no identifiable roots can also be expressed in polyphenol accumulation in host tissue and thickening of host cell walls abutting the incompatible isolate (Lei *et al*., 1990b). For example Acacia species often have associations with putative ectomycorrhizal fungi, which have a mantle with little or no Hartig net, but are still considered to have ECM (Brundrett *et al*., 1995). Further Fortin *et al*., (1980) described synthesis of ectomycorrhiza on *Alnus strobus* seedlings within five days after inoculation with *Pt*.

Field observations of *Pisolithus tinctorius* fruiting in Australia suggest that the isolates associated with eucalypts do not invade plantation stands of exotic pines. Furthermore, eucalypts planted as exotics outside Australia do not become invaded by isolates of pine- *Pisolithus tinctorius* (Malajczuk *et al*., 1990). Field inoculation studied by Garbaye *et al* (1988) showed that eucalypt hybrids in the Congo responded to inoculation with an introduced isolate of *Pisolithus tinctorius* collected from under pine (isolate 270). However, this fungus was replaced by an isolate of scleroderma within 2 years after out planting of seedlings in the field. This scleroderma is probably an Australian species, which has successfully colonized soils at the plantation site (Malajczuk *et al*., 1990). These observations imply that the question of host species fungal isolate specificity as applied to fungi in the broad host range group such as *Pisolithus tinctorius* may need to be considered in the selection of appropriate fungi for inoculation of tree seedlings.

The in vitro synthesis of ectomycorrhizas with *E.urophylla* using the isolates of *Pisolithus tinctorius* from eucalypt and pine, showed far more growth and colonization with eucalypt isolate than pine isolate. Further, in competition studies using dual inoculation, the rapid growth of the eucalypt isolate (H445) in association with the eucalypt root contributed to the replacement of the pine isolate (270) as the dominant fungus forming ectomycorrhizal roots (Malajczuk *et al*., 1990). The diversity in host response implies, however, that there is need for a rigorous assessment of isolates showing growth stimulation characteristics prior to their use in any field inoculation program. In past studies of ectomycorrhizal formation by *Pisolithus tinctorius* in pure culture it has been generally found that all isolates were effective in forming mycorrhizas on a wide range of tree host genera, giving the fungus a broad range host status (Malajczuk *et al*., 1982).

However there were major differences in the rate of ectomycorrhizal development on eucalypt roots when they are inoculated with different isolates of *Pisolithus tinctorius* in aseptic conditions. Even the *Pt* isolated from *E.camaldulensis* when cross-inoculated to *E.teriticornis*, the ectomycorrhizas developed showing the compatibility of fungus-host belonging to the same species (Vijaya and Srivasuki, 2001).

Table.2: Eucalyptus species that showed association to Pt

Eucalyptus Sps.	Source
E.bridgesiana Bak	Chilvers, 1973
E. Camuldulensis Dehnh.	Neumann, 1959
E.dalrympleana Maid	Chilvers, 1973
E.grandi Hill in Maid	Marx, 1977
E.gummifera(Gaertn.) Hochr	Mullette, 1976
E.leucoxylon Muell	Chilvers, 1973
E.mandata Hook	Marx, 1977
E.microcarys Muell	Chilvers, 1973
E.polyanthemos Schauer	Chilvers, 1973
E.radiata Sieb. Ex DC.	Chilvers, 1973
E.robusta Smith	Marx, 1977
E.sieberi Johns	Chilvers, 1973

Ectomycorrhizal fungi show specificity at the host genus level rather than at the host species level (Chilvers, 1973). Molina (1979, 1981) earlier concluded that some fungi can form ectomycorrhizas with a wider range of hosts, at least in pure culture, than predicted from their sporocarp association with specific hosts in the field. These observations may help in understanding of specificity of ectomycorrhizal fungi for tree hosts and allow, in the long term, development of laboratory methods for the selection of the most appropriate fungi for target trees in commercial inoculation programmes.

Several separate *Pisolithus* species, *including P. kisslingi E. Fisch, P. pusillum Pat*. and *P. aurantioscabrosus Walting et al.,* have, however, been described in tropical south East Asia, based on distinctive carpophore and basidiospore morphology (Walting *et al*., 1995), because individuals within *P.tinctorius* (*Pt*) display considerable variation in carpophore and basidiospore morphology, several species have been proposed within the group currently described as *Pisolithus tinctorius* (Bronchart *et al*., 1975).

2.2. Physiology of compatibility:

Genera with dual ECM/VAM associations include *Alnus*, *Acacia*, *Casuarina*, *Eucalyptus*, *Populus*, *Salix* and *Uapaca* (Moyersoen and Fitter, 1998). The VAM in plants with dual associations may be relictual (due to an inability to fully exclude them), functional (providing greater or wider access to nutrients), or a backup mechanism for situations when inoculums of ECM fungi is limited. Evidence for the last option is provided by plants with dual associations that only have substantial amounts of VAM when growing in disturbed habitats, flooded soils, or as young seedlings (Moyersoen and Fitter, 1998).

Within a host plant, the degree of branching in ECM short roots varies with different mycorrhizal fungi (Godbout and Fortin, 1985; Newton, 1991). It is thought that plant growth regulators supplied by the ECM fungus influence root swelling, extension and branching, as these chemicals can induce similar changes in the absence of fungi (Kaska *et al*., 1999). Associations of angiosperms like *Eucalyptus*, *Betula*, *Populus*, *Fagus* and *Shorea* have a Hartig net confined to epidermal cells, while the Hartig net of gymnosperms like *Pinus* extends into the cortex (Massicotte *et al*., 1987). Angiosperms with a cortical Hartig net are rare, but *Dryas* is an exception (Melville *et al*., 1987). Hosts with an epidermal Hartig net, such as *Quercus* and *Betula* species, usually have a relatively narrow cortex with cells that can be massively lignified perhaps as an adaptation to withstand hydraulic pressure (Brundrett *et al*., 1996).

The success of eucalypts in establishment of exotic plantations and as invaders of indigenous communities (Lamb, 1979) can be attributed in part to their compatibility with broad host-ranging fungi. Successful, long-term development of exotic plantations of these hosts however, is generally accompanied by appearance of host-specific fungi. In native stands of eucalypts a natural succession of mycorrhizal fungi occurs as stands mature, and that this succession tends over time from broad-host-ranging fungi towards dominance by host-specific fungi. In plantations of ectomycorrhizal exotics, this succession is qualitatively and perhaps quantitatively restricted by the depauperate mycoflora of host specific fungi originally introduced with planting stock. If this hypothesis is correct, its implications to problems in exotic plantations, e.g. second rotation decline, urgently need to be explored (Malajczuk *et al*., 1982). Field observations of fungal fruiting however suggest that successful, long-term development of exotic stands of these hosts is largely related to appearance of host-specific ectomycorrhizal fungi rather than broad-host-ranging fungi.

An examination of short roots of the Eucalyptus species inoculated with incompatible ectomycorrhizal fungi (i.e. conifer-specific symbionts) showed intense accumulations of probable phenolic compounds in the epidermal and cortical cells, even without fungal mantling of short roots (Kosuge, 1969). Molina (1981) presented evidence for a response mechanism by Alnus to ineffective ectomycorrhiza formation by *Paxillus involutus*. In this case, epidermal and cortical cells in immediate contact with the mantling mycelium accumulate polyphenolic compounds. Since phenolic compounds are associated with host reactions to pathogen invasion indicating incompatibility.

Ling-Lee *et al* (1977) have described the presence of various phenolic compounds in both mycorrhizal and non-mycorrhizal roots of *Eucalyptus fastigiata* Deane and

Maiden and suggested that accumulation of phenols in the epidermal cells of mycorrhizas is a response to the presence of the fungal symbiont. Again the phenolic production by the host may differ depending on the colonizing fungus, particularly between compatible and incompatible host-plant/fungus relationships represents a form of hypersensitive reaction typical of incompatible host-root/pathogen reactions (Deverall, 1977). Clearly further research is needed on how host phenolics may influence ectomycorrhiza formation, as well as host-fungus specificity and compatibility. Hydrophobins are a class of fungal cell wall proteins involved in making cell-cell or cell-surface contact (wosten *et al*., 1994). They are essential for the growth of hyphae from a hydrophilic medium into the air. In ectomycorrhiza, hydrophobins also have been detected among the proteins corresponding to expressed sequence tags of *Pisolithus tinctorius* (Tagu *et al*., 1998). Hydrophobin HydPt1 from *P. tinctorius*, are important not only in the aerial mycelium but also in the symbiotic tissues of the Hartig's net (Tagu *et al*., 2001).

Based on spores isolated from carpophores originating in North America, South America, Australia and Europe, it appears that tetra poplar incompatibility (four mating types) exists within *Pisolithus tinctorius* monokaryons (Kope and fortin, 1990; Rosado *et al*., 1994b). Crosses between some monokaryons and dikaryons have also been achieved in the laboratory (Kope, 1992). Both monokaryons derived as single spore isolates and reconstituted dikaryons are capable of ECM formation with Pinus spp. (Lamhamedi *et al*., 1990). Individual mono and dikaryons derived from a single carpophore show differential affinities for mycorrhiza formation on Pinus spp. But *Pisolithus tinctorius* monokaryons are generally less efficient in ECM formation than dikaryotic mycelia (Lamhamedi *et al*., 1990). Dikaryosis is thus thought to be required for the full expression of ECM forming abilities. Reconsituted *Pisolithus tinctorius* dikaryons show variability in the growth form of the extramatrical mycelia, particularly in extension rates and the degree to which rhizomorphs are formed. They also show variable abilities to improve host plant growth, drought tolerance and mineral nutrient content (Lamhamedi and fortin, 1991;Lamhamedi *et al*., 1992a),

The interaction between *Pisolithus tinctorius* and a host root begins prior to fungus root contact. Diffusible substances released from the host appear to stimulate a chemotropic growth response of compatible *Pisolithus tinctorius* hyphae towards the host (Horan and Chilvers, 1990). With in 1 day of the introduction of the fungus to a eucalypt root system, and before fungus-root contact, there is evidence of a chemical interaction between fungus and host in the form of a browning reaction in outer root cap cells (Horan *et al*., 1988). Shortly after contact, fibrils (believed to be glycoproteins of fungal origin) can be observed at the fungus-root interface in compatible interactions, but not in incompatible *Pt*-host interactions (Lei *et al*., 1990b). Molecular investigations of the *Pt*-eucalypt interaction indicate an altered polypeptide expression in both partners, including the appearance of mycorrhiza specific polypeptides ('ectomycorrhizins') during ECM formation (Burgess *et al*., 1995a). Thus, it is suggested that apoplastic chitinases in the root cortex destroy elicitors from the ectomycorrhizal fungi without damaging the fungus. By this mechanism, the host plant could attenuate the elicitor signal and adjust its own defense reactions to a level allowing symbiotic interaction (Salzer *et al*., 1997).

2.3. Genetic variability of *Pisolithus tinctorius*:

The symbiosis between ectomycorrhizal fungi and trees is an essential part of forest ecology and depends entirely on the communication between the two partners for establishing and maintaining the relationship. The identification and characterization of differentially expressed genes is a step to identifying such signals and to understanding the regulation of this process. Mankel *et al.,*(2002) determined the role of hydrophobins produced by *Tricholoma terreum* in mycorrhiza formation and hyphal development. The gene is expressed in aerial mycelium and in mycorrhiza and. detected a hydrophobin in the symbiosis between *T. terreum* and its native pine host *Pinus sylvestris*. The hydrophobin was found in aerial mycelium of the hyphal mantle and also in the Hartig net hyphae, which form the interface between both partners. Interestingly, this was not the case in the interaction of *T. terreum* with a host of low compatibility, the spruce *Picea abies*. The differential expression with respect to host compatibility was verified at the transcriptional level by competitive PCR. Genes specific for mycorrhization probably encode proteins involved in nutrient transfer or structural components of fungal/host interaction. In ectomycorrhiza, hydrophobins also have been detected among the proteins corresponding to expressed sequence tags of *Pisolithus tinctorius* (Tagu *et al*., 1996).

To date, the population genetic structure of natural ectomycorrhizal fungi has been studied for six species of basidiomycetes: *Hebeloma cylindrosporum* (Gryta *et al*.1997), *Laccaria bicolor* (Selosse *et al.,* 1999), *Suillus variegatus* (Dahlberg 1997), *S. bovinus* (Dahlberg and Stenlid 1990, 1994), *S. pungens* (Bonello *et al.,* 1998) and *Pisolithus tinctorius* (Anderson *et al*., 1998). Somatic incompatibility groupings in *S. bovinus* (Dahlberg and Stenlid 1990, 1994), and molecular analysis of *S. pungens* populations (Bonello *et al.,* 1998), revealed that genets of ectomycorrhizal basidiomycetes might be both large and old (> 150 years old). Genets of *L. bicolor* and *Pisolithus tinctorius* are also able to spread vegetatively through soil in excess of 30 m (Anderson *et al*., 1998). In contrast, the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum*, found in nutrient-poor and unstable sandy soils of coastal sand dunes, produces a high-density of short life span (< 1 year) genotypes with a limited spatial extension (Gryta *et*

al., 1997). Dahlberg and Stenlid (1990) have argued that the occurrence of numerous small genetic individuals may suggest a recent colonization by basidiospores, whereas fewer, larger genets are indicative of old mycelial structures that have grown from a point source over decades; such old individuals are often found in mature closed forest ecosystems (Dahlberg and Stenlid 1995).

The typical size of genets varies greatly between fungal species, from a few mm in bark fungi (*Collybia fusipes*; Marçais *et al.,* 1998) to more than 1 km in tree root pathogens (e.g. *Armillaria bulbosa*; Smith *et al.,* 1992). The size of genets of ectomycorrhizal fungi is thought to vary with the forest age (Dahlberg & Stenlid 1995). Estimated population densities range from 30 to 5000 genets/ha, depending on the species and forest age (Dahlberg & Stenlid1994, 1995). Populations of ectomycorrhizal species (e.g. *S. bovinus*, *S. variegatus*) in old forests mainly consist of discrete, large (up to 27 m) and old (> 150 years) genets, which do not seem to intermingle (Dahlberg & Stenlid 1994, 1995; Dahlberg 1997). Although spatial characteristics of populations of ectomycorrhizal fungi may differ between taxa, it appears that vegetative dissemination dominates over spore colonization in established forests, typified by canopy closure, lack of young trees and organic nitrogen-rich litter (Dahlberg and Stenlid 1995). It may also suggest an irregular process of sporophore fructification within and amongst genets. Interestingly 95% of the genotypes identified in the first sampling of 1994 were not found less than 3 weeks after. This observation confirmed that the fructification phenotype of genets growing on a small area could be highly heterogeneous (Selosse *et al.,* 1999).

Recently analyzed electrophoretic patterns of expressed mycelial proteins from *Pisolithus tinctorius* isolates associated to *P.microcarpus* (Cke. and Mass.) Cunn collected from different geographical regions within Australia indicate much variability in polypeptide pattern within *Pisolithus tinctorius* and a correlation between groupings based on polypeptide pattern and geographical origin. Several distinct groups of polypeptide patterns appear to exist even within the state of Western Australia based on differences in basidiospore type (Burgess *et al*., 1995b). A variety of molecular approaches (RAPD, ITS-RFLP, micro satellite and sequence analysis) indicate considerable polymorphism within *Pisolithus tinctorius* isolates collected from around the Sydney region (NSW, Australia) (Cairney and Chambers, 1997).

A cDNA library of 4-day-old *Eucalyptus globules* - *Pisolithus tinctorius* ectomycorrhiza was constructed and sequenced 850 cDNAs cloned randomly or obtained through suppression subtractive hybridization (SSH). Based on the absence of a database match, 43% of the ectomycorrhiza ESTs are coding for novel genes. At the developmental stage analyzed (fungal sheath formation), the majority of the identified sequences represented 'housekeeping' proteins, i.e. proteins involved in gene/protein expression, cell-wall proteins, metabolic enzymes, and components of signaling systems. Thus screened arrayed cDNAs to identify symbiosis-regulated genes by using differential hybridization. Comparisons of signals from free-living partners and symbiotic tissues revealed significant differences in expression levels (differential expression ratio >2.5) for 17% of the genes analyzed. No ectomycorrhiza-specific gene was detected. The results successfully demonstrate the use of the cDNA array and SSH systems as general approach for dissecting symbiosis development, and provide the first global picture of the cellular functions operating in ectomycorrhiza (Voiblet *et al*., 2001)**.** Development of the ectomycorrhizal symbiosis leads to the aggregation of fungal hyphae to form the mantle. SRAPs may form part of a cell-cell adhesion system needed for aggregation of hyphae in ectomycorrhizas (Laurent *et al*., 1999).

Twenty *Pisolithus tinctorius* isolates from different geographic locations and different hosts were characterized by the random amplified polymorphic DNA technique to calculate genetic distances among the isolates. Cluster analysis based on the amplified fragments grouped the isolates according to their host and geographical origins. Group I contained isolates collected in Brazil and group II those collected in the Northern Hemisphere. In addition to the diversity seen at the molecular level, the isolates also showed host specificity. Greenhouse experiments demonstrated that isolates from the Northern Hemisphere colonized mainly Pinus whereas isolates from Brazil colonized only Eucalyptus. The molecular data suggest that the *Pisolithus tinctorius* isolates analyzed belong to two distinct groups (Junghans *et al*., 1998**)**.

Ectomycorrhiza development alters gene expression in the fungal and plant symbionts. The identification of a large number of genes expressed exclusively or predominantly in the symbiosis will contribute greatly to the understanding of the development of the ectomycorrhizal symbiosis. Gene's specific for mycorrhization probably encode proteins involved in nutrient transfer or structural components of fungal/host interaction. Thus, comparative investigations of single (or even a few) isolates of different species are unlikely to provide reliable information on functional capabilities. Extensive screening of taxonomically well-defined isolates is required. This must take into account spatial and temporal variation in gene expression in populations of ectomycorrhizal fungi growing in axenic culture or in association with a host plant, together with study of the origin and maintenance of their genetic variation, are therefore critical for under-standing how populations of ectomycorrhizal fungi evolve. In this review of the current state of knowledge of interactions between *Pisolithus tinctorius* and its hosts we demonstrate that *Pisolithus tinctorius* displays much intraspecific

heterogeneity of host specificity, dominant interspecific compatibility and the benefits the fungus can impart upon the host plant. Thus it is under such conditions that mycorrhizas with their ability to enhance nutrient uptake and drought tolerance are expected to play an important role in the success of reforestation efforts.

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