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Review

Root Colonization and Intraspecific Mycobiont Variation in Ectomycorrhiza

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Abstract

Ectomycorrhizae are common symbiotic fungus-root associations which are recognized by the structural features known as the mantle and the Hartig net. The development of these structures is probably preceded by interactions involving remote factors and hypha-root contact. Studies on root colonization by different fungi and others on pure cultures of different fungi have provided hypotheses concerning the mechanisms determining ectomycorrhizal structures, mechanisms which remain to be demonstrated. There is a need to use model partnerships to comprehensively study all the developmental stages. Models which consider intraspecific variations could be used in comparative studies which focus on the colonization process and test hypothetical mechanisms. Intraspecific variations have already been reported in fungi with respect to ectomycorrhizal structures and to certain physiological aspects with hypothetical roles in fungus-root interactions. Future work relating these two types of variations is an approach which may demonstrate critical interactions determining ectomycorrhizal development.

Keywords: development, ectomycorrhiza, fungi, interactions, intraspecific variation, root colonization

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1. Introduction

The uptake of soil nutrients by land plants is performed partly by mycorrhizae, organs resulting from symbiotic association between roots and fungi (Marks and Kozlowski, 1973; Harley and Smith, 1983). In the simplest terms, the plant in this association benefits from the absorption capability of the fungus while the fungus benefits from the carbon fixed by the plant. However, both symbionts can also benefit in several other ways, some interrelated and others yet to be clearly identified. There is presently no convenient method for quantifying the benefits, i.e. mycorrhizal functions.

Mycorrhizae have been mainly categorized according to their structure (Harley and Harley, 1987). Ectomycorrhizae are distinguished by the absence of fungal penetration of root cells prior to the onset of senescence. Their characteristic structures include the mantle and the Hartig net (Fig. 1). The mantle is a compact fungal tissue which encloses root segments and/or apices. Hyphae extending outwards from the mantle increase the biological interface with the substrate. The Hartig net is a fungal network which penetrates the root intercellularly, extending at most to the endodermis. Although mantles and Hartig nets are also recognized in arbutoid, ectendo- and monotropoid mycorrhizae, these classes of mycorrhiza are characterized by hyphal penetration of root cells. This review considers only the structural development of mantles and Hartig nets in ectomycorrhizae. Other aspects of ectomycorrhizal structure, such as cell expansion and dichotomous branching, have been discussed previously by Slankis (1973), Rupp and Mudge (1985), Gay (1988) and Nylund (1988).

Several hypotheses have been advanced concerning the causal determinants of ectomycorrhizal structures but none have yet been supported by rigorous physiological, biochemical or molecular evidence. An approach which may be useful for exploring these hypotheses is the examination of intraspecific variation, natural or induced, in both the fungus and the plant. This approach exploits the smaller degree of genetic and physiological difference in intraspecific comparisons relative to interspecific comparisons. Similar approachs have been used in the analysis of plant pathogens (Hammond and Lewis, 1986; Durrands and Cooper, 1988; Roelfs, 1988; Sneh et al., 1989) and an ericoid mycorrhizal fungus (Bonfonte-Fasolo et al., 1987). Intraspecific variations in ectomycorrhizal aggressivity have been reported in several fungi (Table 1) as have intraspecific variations in ectomycorrhizal structures (Marx et al., 1970; Tonkin et al., 1989; Wong et al., 1989). It may be possible to link these ectomycorrhizal variations to physiological and biochemical variations among the

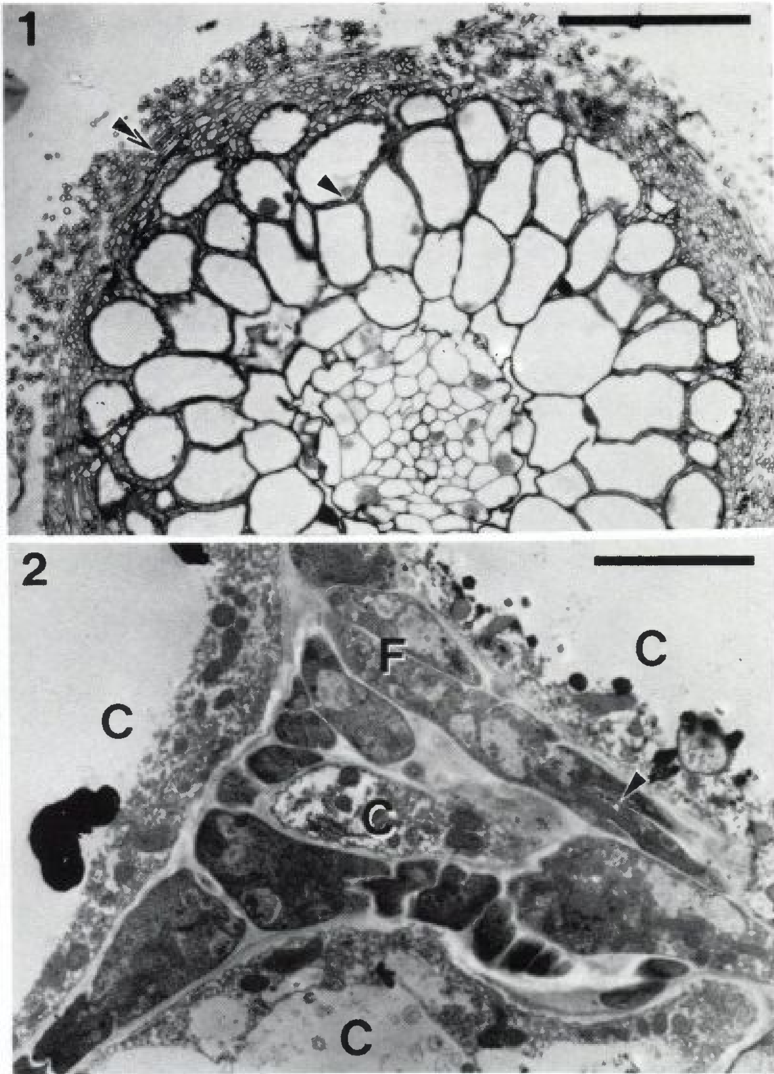

Figure 1. Transverse section of a *Laccaria bicolor-Pinus banksiana* ectomycorrhiza which was photographed using light microscopy and shows the mantle (hyphal cover of the rootlet; double arrowhead) and the Hartig net (fungal penetration between cell walls of the rootlet; arrowhead). Bar = 100 μm.

Figure 2. Transverse section of a *Laccaria bicolor-Pinus banksiana* ectomycorrhiza which was photographed using transmission electron microscopy and shows labyrinthic growth (arrowhead) of the fungus (F) in the Hartig net between root cortical cells (C). Bar = 5 μm.

Table 1. Reports of intraspecific variation in ectomycorrhizal agressivity in fungi

fungal species	reference	No. of strains	No. of nonmycorrhizal strains	plant species
Amanita muscaria (L.: Fr.) Pers.	Mason, 1975	5	1	*Betula verrucosa* Ehrh.
	Gibson and Deacon, 1990	2	0	*B. pubescens* Ehrh.
Boletus subtomentosus Fr.[a]	Lundeberg, 1970	5	4	*Pinus mugo* Turra, *P. sylvestris* L., *P. virginiana* Mill.
Cenococcum geophilum Fr.	Godbout and Fortin, 1983	2	0[e]	*Alnus crispa* (Ait.) Pursh., *A. rugosa* var. *americana* (Regel) Fern.
Hebeloma crustuliniforme (Bull.) Quél.,	Giltrap, 1982b	2	0	*Betula pendula* Roth, *B. pubescens*
	Gibson and Deacon, 1990	2	0	*B. pubescens*
H. sinapizans (Paul: Fr.) Gill., *H. truncatum* (Schaeff.: Fr.) Karst	Giltrap, 1982b	2	0	*B. pendula*, *B. pubescens*
Laccaria bicolor (Maire) Orton	Kropp et al., 1987	21[b]	1	*Pinus banksiana* Lamb.
	Kropp and Fortin, 1988	28[b]	–	*P. banksiana*
	Wong et al., 1989	10[b]	1	*P. banksiana*
	Doudrick et al., 1990	6	1	*Picea mariana* (Mill.) B.S.P.
	Kropp, 1990a	–[b,c,d]	–	*Pinus strobus* L.
L. laccata (Scop.: Fr.) Berk. & Br.	Sylvia and Sinclair, 1983a	2	1	*Pinus resinosa* Ait., *Pseudotsuga menziesii* (Mirb.) Franco
L. laccata var. *moelleri* Singer	Doudrick et al., 1990	3	1	*P. mariana*
L. proxima (Boud.) Pat.	Gibson and Deacon, 1990	2	0	*B. pubescens*
Lactarius pubescens (Schrad.) Fr.	Gibson and Deacon, 1990	2	0	*B. pubescens*
Paxillus involutus (Batsch.: Fr.) Fr.	Laiho, 1970	18	6	*Pinus* sp.

Pisolithus tinctorius (Pers.) Coker & Couch	Marx et al., 1970	2	0	*Pinus taeda* L.
	Molina, 1979	6	0	*Pinus contorta* Dougl., *P. menziesii*
	Marx, 1981	21	6	*P. taeda*
	Grenville et al., 1986	5	0	*Eucalyptus pilularis* Smith
	Dixon et al., 1987	3	0	*P. taeda*
	Tonkin et al., 1989	2	0	*Eucalyptus marginata* Donn.: Sm.
	Lamhamedi et al., 1990	106[b]	15	*P. pinaster* (Ait.) Sol.
		78[b]	8	*P. banksiana*
	Malajczuk et al., 1990	2	0	*Eucalyptus urophylla* S.T. Blake
Sclerotina citrinum Pers.: Pers.	Godbout and Fortin, 1983	3	2	*A. crispa*, *A. rugosa* var. *americana*
	Godbout and Fortin, 1985	2	0	*Populus tremuloides* Michx.
Suillus bovinus (L.: Fr.) Kuntze	Sen, 1990b	11	1	*P. sylvestris*
S. variegatus (Schwartz: Fr.) Kuntze	Sen, 1990b	11	2	*P. sylvestris*
Thelephora terrestris Ehrl.: Fr.	Marx et al., 1970	2	0	*P. taeda*
Tricholoma fulvum (DC.: Fr.) Sacc.	Gibson and Deacon, 1990	2	0	*P. pubescens*

[a] possible cases of misidentification

[b] includes sib-monokaryons and dikaryons made from their crosses

[c] includes monokaryons regenerated from protoplasts of the same dikaryon

[d] – = not applicable

[e] both strains were weak ectomycorrhiza formers, one was nonmycorrhizal on *A. crispa* and the other on *A. rugosa*

fungal strains, thus identifying some of the critical processes in ectomycorrhizal development.

2. Development of Ectomycorrhizae

An individual root system is a heterogeneous substrate for ectomycorrhizal fungi. Not only do root segments differ in anatomy and morphology, they differ in the degree of root cell activity, root exudation and cell wall differentiation. Differentiated segments of roots are already known to be unreceptive to colonization by ectomycorrhizal fungi (Chilvers and Gust, 1982; Melville et al., 1987). Furthermore, lateral roots of different orders may have Hartig nets of different structures, apparently due in part to differential root anatomy (Wong et al., 1989). The heterogeneity within root systems therefore adds an extra dimension to ectomycorrhizal colonization. Any single root system may also have segments colonized by different ectomycorrhizal fungi (Zak and Marx, 1964; McAfee and Fortin, 1986, 1987; Gibson and Deacon, 1988) or conspecific strains (Gardes et al., 1990d; Malajczuk et al., 1990) and segments at various stages of colonization.

Primary colonization by ectomycorrhizal fungi may be theoretically divided into the following stages:

1. remote stimuli (diffusible factors acting before fungus-root contact)
2. fungus-root attachment
3. development of the mantle and the Hartig net
4. steady state
5. senescence

Factors at each stage may contribute to determining the overall compatibility of the interacting partners. In the following sections, each stage will be discussed with respect to possible determinants of symbiotic compatibility and ectomycorrhizal development. The discussion will concentrate on the ectomycorrhizal symbionts but it must be kept in mind that the rhizosphere and soil environment do modify ectomycorrhizal interactions and may thus determine ecological specificity in some partnerships. Modifying factors are known to be produced by many rhizospheric microorganisms (Barea and Azcon-Aguilar, 1982; De Oliveira and Garbaye, 1989; Garbaye and Bowen, 1989). In considering symbiotic compatibility, it must also be kept in mind that the mechanisms may act by promoting symbiotic partners, by inhibiting nonsymbionts, by not inhibiting symbiotic partners and/or by not promoting nonsymbionts. Any

combination of these mechanisms may explain the general lack of strick specificity in most ectomycorrhizal partnerships (Harley, 1984; Duddridge, 1986c, 1987; Anderson, 1988).

Remote stimuli

Root exudates include a variety of carbohydrates, amino acids and organic acids which are released by leaching from living and dead cells as well as by active secretion from living cells (Barea, 1986). They are utilized by microorganisms, including ectomycorrhizal fungi, in the rhizosphere. Ectomycorrhizal fungi in turn secrete a variety of plant growth regulators (for list of references, see Wong, 1989), anti-microbial agents (Duchesne et al., 1989; Kope and Fortin, 1989) and likely other substances, all potentially able to influence the state of plants as well as their rhizosphere prior to ectomycorrhiza formation. The metabolic state of plants determines their exudates and may thus feedback to the fungi. Remote stimuli in both directions should therefore contribute to facilitating fungus-root contact. These diffusible stimuli may continue to be important after ectomycorrhiza establishment. The following sections consider root and fungal stimuli which may act as signals rather than as nutritional sources for their respective partners.

Root stimuli

The existence of a M-factor had been hypothesized because living roots stimulate *in vitro* growth of certain ectomycorrhizal fungi on an apparently complete nutrient medium containing sugar, salts, B-vitamins and amino acids (Melin and Das, 1954; Melin, 1962). The analysis of M-factor production by roots had been complicated by the occurrence of both stimulatory and inhibitory substances (Melin, 1963). There were also observed differences in response among fungal species. For *Lactarius rufus* (Scop.: Fr.) Fr., *L. mitissimus* (Fr.) Fr. (Melin, 1962), *Rhizopogon roseolus* (Corda) Hollos (Melin, 1963) and *Suillus variegatus* (Schwartz: Fr.) Kuntze (Melin and Das, 1954), roots increased the initial growth rate but not the final yield. In contrast, little growth was observed in *Lactarius helvus* (Fr.) Fr., *Russula aeruginea* Lindblad, *R. fragilis* (Pers.: Fr.) Fr. (Melin, 1962), *R. sardonia* Fr. (Melin, 1963) and *R. xerampelina* (Schaeff.) Fr. (Melin and Das, 1954) unless roots were present. M-factor activity may therefore act on fungi in different ways or involve more than one root factor. Furthermore, since M-factor activity apparently occurred in nonhost plants such as alfalfa, garden cress, pea, tomato and wheat (Melin and Das, 1954; Benedict et al., 1965; Straatsma et al., 1986), it may be provided by common metabolites. The active component has been

proposed to be carbon dioxide (Straatsma et al., 1986), cytokinin (Gogala, 1970 (cited by Oort, 1974)) or nicotinamide adenine dinucleotide (H. Nilsson, unpublished (cited by Melin, 1963)). The involvement of a gaseous factor such as carbon dioxide may explain the numerous unreported failures to isolate and characterize M-factor.

M-factor activity may also be the result of root removal of fungal growth inhibitors. For example, Fortin (1967) had proposed that the indole-3-acetic acid produced by ectomycorrhizal fungi has a self-regulatory function which is modified by root uptake because this auxin could inhibit the growth of *Amanita muscaria* (L.: Fr.) Pers., *A. rubescens* (Pers.: Fr.) Pers., *Suillus granulatus* (L.: Fr.) Roussel and *S. variegatus*. This hypothesis was subsequently corroborated by the alleviation of the observed growth inhibition in the two *Suillus* spp. by the addition of excised roots of *Pinus resinosa* Ait. or *P. sylvestris* L. (Fortin, 1970).

Besides stimulating hyphal growth, roots have also been reported to stimulate *in vitro* spore germination in many ectomycorrhizal fungi. The generally poor germinability of spores of ectomycorrhizal fungi suggests that root stimulation contributes to compatibility determination in ectomycorrhizal partnerships (Fries, 1984). This hypothesis is supported by observations that certain ectomycorrhizal fungi appeared to be stimulated in general by tree species but not by herb species (Melin, 1962; Birraux and Fries, 1981; Fries, 1981; Fries and Swedjemark, 1986; Ali and Jackson, 1988). There are however cases where spore germination is stimulated by nonhost roots such as carrot (Fries and Swedjemark, 1986), clover (Ali and Jackson, 1988), lupin (Birraux and Fries, 1981) and tomato (Melin, 1962; Straatsma et al., 1985). Moreover, certain spores can germinate without roots (Melin, 1962; Fries, 1977, 1978, 1979, 1981, 1983a, 1983c; Fries and Birraux, 1980; Birraux and Fries, 1981; Straatsma et al., 1985; Ali and Jackson, 1988) and others do not germinate even when tree roots are present (Fries, 1981, 1983a; Ali and Jackson, 1988). It would appear that root stimulation of spore germination is not an absolute nor specific prerequisite for ectomycorrhizal development in all instances. This is further illustrated by the identification of abietic acid as a factor released by *P. sylvestris* which could stimulate spore germination in *S. granulatus, S. grevillei* (Kl.: Fr.) Sing., *S. luteus* (L.: Fr.) Roussel and *S. variegatus* but not in *Hebeloma mesophaeum* (Pers.) Quél., *Paxillus involutus* (Batsch.: Fr.) Fr. nor *Thelephora terrestris* Ehrl.: Fr. (Fries et al., 1987).

Other observations indicate that spore germination in ectomycorrhizal fungi could also be stimulated by conspecific mycelium (Fries, 1978, 1979, 1983a, 1983c, 1984), by mycelium of other ectomycorrhizal fungi (Birraux and Fries, 1981) and by other microorganisms (Oort, 1974; Fries, 1977, 1981, 1984, 1987;

Ali and Jackson, 1989). The conspecific mycelial factor which stimulated spores of *P. involutus* was apparently volatile but not that of *Leccinum scabrum* Bull.:Fr.) S.F. Gray (Fries, 1978). Furthermore, species of *Leccinum* could be divided into two groups such that mycelia within each group stimulated only spore germination within the group; one group consisted of *L. holopus* (Rostk.) Watl. and *L. scabrum* and the other *L. aurantiacum* (Bull.) S.F. Gray, *L. insigne* A.H. Smith, *L. quercinum* (Pil.) Pil., *L. variicolor* Watl., *L. versipelle* (Fr. & Hök) Snell and *L. vulpinum* Watl. (Fries, 1979, 1983b). It appears that many different factors can affect spore germination. They must be considered in any evaluation of root stimuli along with the possible loss in spore germinability (Bulmer and Beneke, 1964; Fries, 1983c; Theodorou and Bowen, 1987) and presence of inhibitors (Fries, 1978, 1984; Bjurman, 1984).

Although roots can clearly have a positive stimulus on ectomycorrhizal fungi, there is yet little understanding concerning the active compounds, their functions and their importance. Presently, there is no evidence that plants release stimulants specifically active on compatible ectomycorrhizal fungi. A lack of specificity does not reduce the importance of such stimulants but it does indicate that compatibility determinations are made by alternative methods.

Fungal stimuli

Not only may plant growth regulators produced by fungi alter the level of root exudation, they could also influence root morphogenesis and lateral root proliferation (Gay, 1988). The effects of exogenously added auxins (natural or their synthetic analogs) on root initiation and proliferation have been well documented in tree species (Selby and Seaby, 1982; Ross et al., 1983; Simpson, 1986; Baser et al., 1987) and certain reports have suggested similar roles for ethylene (Graham and Linderman, 1981; Rupp and Mudge, 1985; Stein and Fortin, 1990) and kinetin (Barnes and Naylor, 1959). Root proliferation would increase the quantity of available colonization sites whereas increased root exudation could stimulate fungal growth. Another role suggested for fungally produced auxins is the regulation of chitinase activity in roots which may determine defensive reactions or facilitate metabolite exchange across fungal cell walls (Sauter and Hager, 1989). However, none of these types of fungus-root interactions can completely determine ectomycorrhizal compatibility because many other microorganisms produce plant growth regulators (Strzelczyk and Pokojska-Burdziej, 1984).

Results have not suggested that plant growth regulators are limiting factors in ectomycorrhiza formation. Graham and Linderman (1981) have found that the ethylene releasing compound ethephon did not affect the number of

lateral roots and ectomycorrhizae on *Pseudotsuga menziesii* (Mirb.) Franco inoculated with *Hebeloma crustuliniforme* (Bull.) Quél. Similar results were obtained also when *Pinus mugo* was inoculated with *Laccaria laccata* (Scop.: Fr.) Berk. & Br. or *Pisolithus tinctorius* (Pers.) Coker & Couch (=*P. arhizus* (Pers.) Rauschert) (Rupp et al., 1989). Moreover, indolebutyric acid did not increase ectomycorrhiza formation on *Quercus velutina* Lam. by *P. tinctorius* even though there was an increase in lateral root development (Baser et al., 1987).

Fungus-root attachment

The initial contact between symbionts provides a new opportunity for compatibility determination. Relatively few studies on ectomycorrhizae have considered fungus-root attachment even though adhesion processes have been studied in many host-pathogen and symbiotic interactions (Barak et al., 1986; Bonfonte-Fasolo et al., 1987; Diaz et al., 1989; Finlay and Falkow, 1989; Kamoun et al., 1989). Piché et al. (1983a, 1983b) reported the presence of amorphous carbohydrate material on the surfaces of *P. tintorius* hyphae and *Pinus strobus* L. roots prior to contact and suggested that these materials serve in recognition processes as well as in attachment. These amorphous materials may be similar to the glycoprotein fibrils reported at hypha-root interfaces during initial contact between an isolate of *P. tinctorius* and *Eucalyptus urophylla* S.T. Blake (Lei et al., 1990b). That amorphous materials are implicated in fungus-root attachment is not surprising when considering that plant cells themselves are attached by an amorphous middle lamella consisting mainly of pectin. Whether they play a role in specificity determination remains a question which may be examined by a comparative study of fungal colonizers with host preferences. Lei et al. (1990a) reported that one *P. tinctorius* isolate is associated with more extracellular fibrils when colonizing its preferred host *P. caribaea* (Mor.) than when colonizing *Eucalyptus* spp.

Development of the mantle and the Hartig net

Hyphae of ectomycorrhizal fungi tend to proliferate on root surfaces. Nylund and Unestam (1982) suggested that a sparse hyphal envelope develops prior to the initiation of the Hartig net. Since hyphal colonization of root surfaces has been observed with apparently incompatible partners (Theodorou and Bowen, 1971; Molina, 1981; Malajczuk et al., 1984; Duddridge, 1986a), hyphal envelopes may simply be the result of growth on root exudates and in fact not require fungus-root attachment. Mantles, in contrast, are compact tissues specific to ectomycorrhizal development.

There is a current controversy concerning the sequence of events during ectomycorrhizal development. While Hartig nets have been reported to precede mantles in ectomycorrhizae found on pine (Laiho and Mikola, 1964) and *P. sylvestris* (Robertson, 1954; Duddridge and Read, 1984b) and in those of *Piloderma bicolor* (Peck) Jülich-*Picea abies* (L.) Karst (Nylund and Unestam, 1982), mantles apparently precede Hartig nets in ectomycorhizae found on *Fagus sylvatica* L. (Clowes, 1951) and in those of *Hebeloma cylindrosporum* Romagnési-*Dryas integrifolia* Vahl. (Melville et al., 1987), *P. involutus-Betula pendula* Roth (Grellier et al., 1984), *P. tinctorius-Eucalyptus globulus* (Kirkp.) (Horan et al., 1988), *P. tinctorius-E. marginata* Donn.: Sm. (Tonkin et al., 1989) and *Tuber melanosporum* Vitt.-*Cistus incanus* L. (Fusconi, 1983). However, in ectomycorrhizae of *Laccaria bicolor* (Maire) Orton-*Pinus banksiana* Lamb. which take 2 days for completion, there is an apparently simultaneous appearance and development of mantles and Hartig nets (Wong et al., 1990b). These contradictory results may be due in part to inconsistent distinction of mantles and hyphal envelopes and in part to differences among partnerships.

For angiosperms, mantles may precede Hartig nets because their roots are relatively resistant to Hartig net penetration (Godbout and Fortin, 1983). Furthermore, hyphal extension on root surfaces may be generally more rapid than that within roots, as observed in *Pinus resinosa* Ait. by Wilcox (1968). Rather than whether one structure develops more rapidly than the other, there is an interest to assess whether one is a prerequisite for the other. Such a dependence is not suggested by the contradictory results among different partnerships nor by the occurrence of incomplete ectomycorrhizal colonization where one of the structures is partially or entirely absent (Kope and Warcup, 1986; Tonkin et al., 1989; Wong et al., 1989; Brunner et al., 1990).

The mantle

Mature mantles have a compact characteristic reminiscent of tissues in sporocarps (Marks and Foster, 1973) and may also have distinct layers (Harley and Smith, 1983; Ashford et al., 1988; Moore et al., 1989). The lamellar characteristics may be the result of differential cytoplasmic features, fungal aging, hyphal sizes and/or extracellular materials. The extracelllar matrix found in mantles on *Pisonia grandis* R. Br. differentiated during mantle maturation (probably as a result of both cell debris and excretion), reduced interhyphal spaces, showed a variation across the mantle and became resistant to apoplastic transport (Ashford et al., 1988). The structural integrity of mantles probably requires cementing materials, the adhesive component of which may be the

same throughout the mantle and in fact identical to that involved in fungus-root attachment.

At the innermost layer of the mantle, hyphae in contact with the root epidermis generally show the labyrinthic growth pattern which is commonly associated with hyphae of Hartig nets (Fig. 2). This phenomenon has been reported in ectomycorrhizae found on *P. abies* (Blasius et al., 1986) and *P. grandis* (Ashford et al., 1988) and in those of *A. muscaria-P. abies* (Kottke and Oberwinkler, 1986b) and *T. melanosporum-C. incanus* (Fusconi, 1983).

The Hartig net

Whereas Hartig nets may extend to the endodermis in gymnosperm roots, they generally do not penetrate beyond the epidermal layer of angiosperm roots (Godbout and Fortin, 1983). This difference may be determined by differences in cell wall chemistry (Timell, 1967) and root anatomy, many members of the latter taxon having an exodermis (Shishkoff, 1987) which may limit fungal penetration in a manner similar to the endodermis (Godbout and Fortin, 1983). In a survey of 8 gymnosperms and 13 angiosperms, Brundrett et al. (1990) found that all the gymnosperms had cortical Hartig nets and lacked the root exodermis whereas the angiosperms had epidermal Hartig net and 12 of them had an exodermis and/or cortical cell wall thickenings. Cell wall changes during root differentiation may also explain the loss of root susceptibility to ectomycorrhizal colonization.

Increased lignification of cell walls has been suggested to be a root response against incompatible fungi which can limit the extent of Hartig net development (Molina and Trappe, 1982). Although increases in safranin staining (Molina, 1981; Molina and Trappe, 1982) and electron opaqueness (Duddridge, 1986a) of cell walls have been observed in several apparently incompatible partnerships, these changes do not always prevent Hartig net penetration. Lignification therefore may only be an indicator and not a determinant of ectomycorrhizal incompatibility. Partial fungal tolerance to lignification may involve the establishment of the Hartig net prior to the completion of lignification. Alternatively, ligninolytic enzymes may be implicated in certain cases because low levels of ligninolytic activities have been reported in *A. muscaria, Cenococcum geophilum* Fr., *P. involutus, Rhizopogon luteolus* Fr. & Nord, *R. roseolus, Suillus bovinus* (L.: Fr.) Kuntze, *Tricholoma aurantium* (Schaeff.: Fr.) Ricken (Trojanowski et al., 1984; Haselwandter et al., 1990).

Accumulation of polyphenolics in outer root cells, forming the 'tannin layer', also does not totally prevent Hartig net penetration (Marks and Foster, 1973). Such accumulations throughout eucalypt roots were observed with certain

incompatible partners (Malajczuk et al., 1982, 1984; Tonkin et al., 1989). However, since apparent polyphenolics have also been reported in uninoculated control roots (Ling-Lee et al., 1977; Piché et al., 1981; Malajczuk et al., 1984; Duddridge and Read, 1984a, 1984b; Tonkin et al., 1989; Wong et al., 1990a), the antimicrobial activity of polyphenolics or their products may be directed against other rhizospheric microorganisms. Sylvia and Sinclair (1983b) found that pathogen resistance in *P. menziesii* is more dependent on its phenolic production in response to *L. laccata* than on the antibiotic capability of this fungus. The production of phenoloxidase activities by certain ectomycorrhizal fungi (Levisohn, 1959; Laiho, 1970; Lundeberg, 1970; Giltrap, 1982a; Ramstedt and Söderhäll, 1983) may contribute to a degree of fungal tolerance towards plant phenolics. Since the polyphenolics may be the source of materials for both antimicrobial compounds and lignification, these two defense mechanisms in roots may be intimately related.

Warrington et al. (1981) found openings on uncolonized roots of *Pinus taeda* L. which were apparently used as entrance sites by hyphae of *P. tinctorius*. Although these openings may be exploited by ectomycorrhizal fungi, they cannot be the sole entry points because Hartig nets uniformly penetrate root surfaces at all middle lamellae. Blasius et al. (1986) suggested that ectomycorrhizal fungi in fact penetrate in the form of broad lobed fronts.

The means of fungal penetration has been suggested to be mechanical because hyphae appear wedge-shaped in cross-sections of certain Hartig nets and the middle lamella splits without the gross structural disorganization that would be expected after enzymatic hydrolysis (Nylund and Unestam, 1982; Duddridge and Read, 1984b). However, it remains possible that a combination of mechanical and enzymatic means is involved (Marks and Foster, 1973). Since intercellular penetration runs along the pectin-rich middle lamella, several authors have suggested a role for fungal pectinolytic enzymes. Viscometric assays using pure cultures of an isolate of *Boletus subtomentosus* Fr. (Lundeberg, 1970; Lindeberg and Lindeberg, 1977) and one of *S. luteus* (Giltrap and Lewis, 1982) suggested that pectinolytic activities are secreted by these two ectomycorrhizal species. However, similar assays on other isolates and on isolates of other ectomycorrhizal species have detected either no pectinolytic activity or extremely low amounts (Lundeberg, 1970; Lindeberg and Lindeberg, 1977; Ramstedt and Söderhäll, 1983; Dahm et al., 1987). These negative results may be due to the absence of regulatory factors from roots. Low pectinase activity, as well as its control by catabolite repression (Giltrap and Lewis, 1982) or its localization on hyphal surfaces, could explain the lack of apparent root damage during Hartig net formation.

There are also alternative means of promoting plant cell wall separation.

First, plant growth regulators are known to affect cell wall metabolism and structure (Kappler and Kristen, 1986; Fry, 1989; Osborne and Jackson, 1989). Their production by ectomycorrhizal fungi may have a role in altering root cell walls, or in retarding wall completion, in order to accommodate fungal penetration (Harley, 1985). Ectomycorrhizal fungi may therefore be exploiting the enzyme apparatus in plants which is implicated in cell wall synthesis, seed germination and abscission. Second, calcium ions appear to improve the structural integrity of cell walls by cross-linking pectic substances in the middle lamella (Demarty et al., 1984). The removal of these ions, in the form of calcium oxalate, is thought to be responsible for the development of air spaces in leaves of *Typha angustifolia* L. (Kausch and Horner, 1981). Therefore, the oxalate crystals reported on ectomycorrhizae (Lapeyrie et al., 1987) may be indicating fungal production of oxalic acid which serves a role in Hartig net development. However, several other roles have been hypothesized for calcium oxalate accumulation (Snetselaar and Whitney, 1990) and detrimental cytological effects in plants can be caused by oxalic acid (Tu, 1989).

The role of direct surface-to-surface interactions at Hartig nets as well as inner mantles is not known. These interactions may determine labyrinthic growth of the fungus (Nylund and Unestam, 1982; Duddridge and Read, 1984a; Warmbrodt and Eschrich, 1985; Blasius et al., 1986; Massicotte et al., 1986; Kottke and Oberwinkler, 1987) (Fig. 2), plant cell wall ingrowths in the epidermis (Ashford and Allaway, 1982; Massicotte et al., 1986) and cortex (Duddridge and Read, 1984c; Kottke and Oberwinkler, 1986b, 1988) of certain roots and embedding of hyphae into the pectic material of the middle lamella (Duddridge and Read, 1984a,b). The two types of cell wall modifications are thought to increase the active symplastic surface area for nutrient transfer.

Since root cell wall ingrowths were first reported in ectomycorrhizae of *P. grandis* where Hartig nets were restricted to the root epidermis (Ashford and Allaway, 1982), they were thought to be effective functional replacements for Hartig nets which enter the root cortex. However, they may be unessential replacements because they are absent in epidermal Hartig nets of *Alpova diplophloeus* (Zeller & Dodge) Trappe & A.H. Smith-*Alnus rubra* Bong., *L. bicolor-Betula alleghaniensis* Britt., *P. tinctorius-Eucalyptus pilularis* Smith (Massicotte et al., 1987b) and *P. tinctorius-B. alleghaniensis* (Massicotte et al., 1990). Analyses of their composition indicated that those in *P. sylvestris* are not callose deposits (Duddridge and Read, 1984c) and those in *Alnus crispa* (Ait.) Pursh. contain sugar residues (Massicotte et al., 1986) including *N*-acetylglucosaminyl, L-fucosyl and D-mannosyl residues (Massicotte et al., 1987a).

The development of Hartig nets may differ substantially between lateral

roots of different orders. The differences observed in *P. banksiana* seedlings may be the result of large pre-existing intercellular spaces at cell corners in the cortex of first-order root laterals which do not occur in second-order laterals (Wong et al., 1989, 1990a). These intercellular spaces appeared to be readily accessible to hyphal elongation and thus apparently permit Hartig net penetration without labyrinthic growth of the fungus and without separation of cortical cells. Hyphal growth in intercellular spaces has also been reported in the primary root of *P. abies* (Nylund and Unestam, 1982) and *P. menziesii* (Brown and Sinclair, 1981) colonized by *Piloderma croceum* Erikss. & Hjortst. and *L. laccata*, respectively, and in roots of *Pinus nigra* Arnold and *P. sylvestris* colonized by *S. grevillei* (Duddridge, 1986a,b). Brown and Sinclair (1981) suggested that these intercellular spaces are connected directly to root surface and thus permit hyphal entrance in the manner reported by Warrington et al. (1981).

Steady state

After the completion of the Hartig net and the mantle at a root segment, there is generally a stage which shows few structural changes while symbiotic functions proceed in both partners. The maintenance of this mature state may require a degree of communication between the partners. The interactions maintaining this state have not yet been studied nor have the reasons for their eventual failure. The former may involve stimuli exchanged between the partners and the latter may be dependent on vascular differentiation which changes the supply route of photosynthates. Gene expression in the two partners has been reported to differ before and after association (Hilbert and Martin, 1988a,b; Sen, 1990b).

Ectomycorrhizal development is actually a continuing process because the fungus may extend longitudinally on the root, the root may continue to elongate and other root segments may become colonized through secondary colonization. Also important is the mycelial development extending into the soil from the mantle. This extraradical mycelium is responsible for soil nutrient accumulation (Skinner and Bowen, 1974; Finlay and Read, 1986b), metabolite transfer among plants (Finlay and Read, 1986a) and sporocarp development (Godbout and Fortin, 1990). Its structural characterization has however received relatively little attention.

Senescence

Senescent ectomycorrhizae show deterioration of mantle structure and fungal penetration of root epidermal and cortical cells (Harley, 1984). Nylund et al. (1982) reported papillae formation in plant cell walls of senescent ectomycorrhizae of *P. bicolor-P. abies, P. tinctorius-P. abies* and *P. bicolor-P. sylvestris*, structures similar to other wound responses in plants. During senescence, the fungus has apparently taken on pathogenic or saprophytic behavior and bi-directional nutrient transfer has ended. Although the factors controlling senescence have not yet been studied, the observed intracellular penetrations demonstrate that ectomycorrhizal fungi do have the potential to penetrate plant cell walls. This is also supported by observations that certain ectomycorrhizal fungi can enter root cells of certain hosts (Harley, 1984).

3. Intraspecific Variation in Ectomycorrhizal Fungi

Studies on intraspecific variation in ectomycorrhizal fungi have dealt with intersterility or mating-type groupings (Fries, 1983c, 1985; Fries and Mueller, 1984; Kropp et al., 1987; Kropp and Fortin, 1988; Doudrick and Anderson, 1989; Fries and Neumann, 1990; Mueller and Gardes, 1990), with enzyme or isozyme patterns (Ho, 1987a,b, 1989; Ho and Trappe, 1987; Mousain et al., 1988; Wagner et al., 1988, 1989; Zhu et al., 1988; Kropp, 1990b; Sen, 1990a), with restriction fragment length polymorphisms (Armstrong et al., 1989; Gardes et al., 1990a,b,c) and with growth characteristics and nutrient utilization in culture (Ferry and Das, 1968; Laiho, 1970; Lundeberg, 1970; Hung and Trappe, 1983; Samson and Fortin, 1986; Cline et al., 1987; Sen, 1990a). Although the vigor of fungal growth can affect ectomycorrhizal development (Duddridge and Read, 1984b; Duddridge, 1986b), growth characteristics offer little potential in differentiating critical points in ectomycorrhizal development because of the general nature of the effects. Nevertheless, other genetic variations such as those in ectomycorrhizal aggressivity and structures may provide insights concerning ectomycorrhizal development and interactions.

In any assessment of intraspecific variation, one question which arises is the identity of the fungal cultures. Furthermore, taxonomical species recognized using structural criteria are not always sufficient because they may constitute biological species which are intersterile. Such species complexes have already been found in *L. bicolor* (Kropp and Fortin, 1988; Doudrick and Anderson, 1989; Gardes et al., 1990b; Mueller and Gardes, 1990), *L. laccata* (Fries, 1983c; Fries and Mueller, 1984; Gardes et al., 1990a,b), *P. involutus* (Fries,

1985), *P. tinctorius* (Kope and Fortin, 1990) and *S. granulatus* (Fries and Neumann, 1990). Since the relatedness of biological species remains debatable, it is important to confirm the existence of intraspecific variations using sexually compatible cultures. Such a confirmation for ectomycorrhizal phenotypes has been provided for *L. bicolor* and *P. tinctorius* (Table 1).

Ectomycorrhizal aggressivity

For reasons related to convenient quantitation, ectomycorrhizae are generally considered to be rootlets which have their apex covered by a fungal mantle. This definition has its drawbacks because it does not account for actual symbiotic interfaces and because it disregards ectomycorrhizal colonization which does not cover root apices. Nevertheless, the ectomycorrhizal aggressivity of a fungus may be assessed by the number of ectomycorrhizae formed on inoculated root systems. Such assessments have revealed substantial variation among strains of many fungi (Table 1), different tree species and synthesis conditions having been tested in some cases. Synthesis conditions can affect the comparison of conspecific strains (Marx et al., 1970; Kropp et al., 1987; Kropp and Fortin, 1988; Wong et al., 1989; Gibson and Deacon, 1990) probably because the variants have different sets of optimal conditions. In general, growth and ectomycorrhiza formation rates of ectomycorrhizal fungi are known to depend on temperature, pH, nutrients, moisture, aeration, external carbohydrates and other abiotic factors (Marx et al., 1970; Slankis, 1974; Duddridge, 1986b; Nylund, 1988). Intraspecific variations have also been observed in the number of seedlings successfully colonized (Molina, 1979; Marx, 1981; Wong et al., 1989), possibly the result of differential susceptibility among host genotypes (Marx and Bryan, 1971; Cline and Reid, 1982; Dixon et al., 1987; Tonkin et al., 1989).

The occurrence of nonmycorrhizal strains has been reported in several ectomycorrhizal fungi (Table 1). Lundeberg (1970) suggested that ectomycorrhizal strains are less saprophytic than nonmycorrhizal strains, the former generally having a relatively slow growth rate and low production of extracellular degradative enzymes *in vitro*. This hypothesis is similar to that made by Norkrans (1950) that ectomycorrhizal species are relatively less saprophytic. The nonmycorrhizal strain identified in *L. bicolor* by Wong et al. (1989) was apparently not restricted by diffusible factors since it did not completely inhibit ectomycorrhiza formation by other strains in a remote fashion (i.e. with contact prevented by a membrane) nor was its root colonization stimulated by them (Wong and Fortin, 1988). Furthermore, when roots were co-inoculated

with this nonmycorrhizal strain and another ectomycorrhizal strain, ectomycorrhiza formation occurred (K.K.Y. Wong, unpublished). However, similar co-inoculation experiments suggested that an aggressive isolate of *P. tinctorius* is slightly inhibited by a much less aggressive isolate (Malajczuk et al., 1990).

The genetic mechanisms determining ectomycorrhizal aggressivity remain unknown. Their analysis is complicated by the possibility of gradual and complete loss of aggressivity in certain fungal isolates (Marx and Daniel, 1976; Kropp et al., 1987), aggressivity of certain *P. tinctorius* isolates having been recovered through exposure to a host (Marx, 1981). The picture is further complicated by the inconsistencies observed among dikaryons obtained by crossing the same pairs of monokaryons of *L. bicolor* (Kropp et al., 1987; Wong et al., 1989; Kropp 1990a). Reisolation of the individual nuclei of these dikaryons by protoplast formation suggested that the overall ectomycorrhizal characteristic of each was stable (Kropp, 1990a). Irrespective of the source of intraspecific variation, fungal strains with stable characteristics could be used to study ectomycorrhizal development.

Ectomycorrhizal development

Differences in responses to root stimuli have been observed between two spore collections of *Cantharellus cibarius* Fr. (Fries, 1981; Straatsma et al., 1985) and of *L. helvus* (Melin, 1962; Fries, 1981). However, this variation may be host independent because the germinability of spore collections of the same species is known to be inherently variable (Fries, 1979, 1981, 1983c, 1984; Fries and Birraux, 1980; Doudrick and Anderson, 1989). Nevertheless, there may be competition among conspecific genotypes during pre-infection stages of ectomycorrhiza development. Host mediation of such competition is elsewhere suggested by the variable response of hyphal growth among *C. cibarius* strains to the presence of roots (Straatsma et al., 1986).

Variation in ectomycorrhizal interactions may also be considered at the structural level with respect to fungus-root interfaces and the development of mantles and Hartig nets. An early report was made by Marx et al. (1970) who found a difference in the mantle thickness developed on *P. taeda* by 2 isolates of *P. tinctorius*. Marx (1981) however did not mention any differentiation among 21 isolates although mantle thicknesses ranged from 12–48 μm. Differences in Hartig net development were recently reported in 2 isolates of *P. tinctorius*, one failing to form Hartig nets on certain clones of *E. marginata* (Tonkin et al., 1989). These incomplete associations were apparently related to an accumulation of root polyphenolics. In a study of 2 other *P. tinctorius* isolates, hypha-root interactions during initial colonization of *E. urophylla* were found

to be a distinguishing feature. The more aggressive colonizer was associated with more glycoprotein fibrils at the hypha-root interface during attachment (Lei et al., 1990b) whereas the less compatible isolate induced epidermal cell wall thickening in the host. This latter isolate was more compatible with *P. caribaea* and its hyphae were associated with more extracellular fibrils during initial interactions with this host (Lei et al., 1990a). However, the question as to whether these fibrils are symptoms or determinants of ectomycorrhizal compatibility remains to be answered.

Although Debaud et al. (1988) did not mention any structural differences among ectomycorrhizae formed on *Pinus pinaster* (Ait.) Sol. by 4 sib-monokaryons of *H. cylindrosporum* and their mother dikaryotic culture, Wong et al. (1989) reported substantial differences in the colonization of *P. banksiana* among 10 sib-monokaryotic and dikaryotic strains of *L. bicolor.* Root colonization among *L. bicolor* strains could be differentiated in terms of mantle thickness, Hartig net penetration, width of Hartig net separation of root cortical cells, polyphenolic accumulation in the endodermis of first-order laterals and hyphal penetration of epidermal cells (Wong et al., 1989). Differentiation could also be made with respect to the development rate of these structural features (Wong et al., 1990b) and to the fungal morphology in Hartig nets (Wong et al., 1990a). Although extracellular glycofibrils at hypha-root interfaces were consistently rare during initial interactions (Wong et al., 1990a), an aggressive strain was associated with more Concanavalin A binding sites in these interfaces than a less aggressive strain (Lei et al., 1990c). Since supposedly identical dikaryons (made by crossing the same set of monokaryons) differed substantially, differential gene expression could apparently determine some of the observed differences.

4. Analysis of Ectomycorrhizal Development Using Intraspecific Variation

The intimacy of symbiotic associations has always been an obstacle for attempts to dissect the mechanisms determining symbiosis. Ectomycorrhizae constitute numerous fungus-root associations which are recognized by common structural features. The general similarities in ectomycorrhizal structures suggest the existence of common developmental mechanisms among species but it cannot be assumed that a unique set of mechanisms determines each of the structural features.

In recent years, there have been several extensive microscopic studies on ectomycorrhizal structure and development (Nylund and Unestam, 1982; Fusconi, 1983; Duddridge and Read, 1984b; Grellier et al., 1984; Kottke and

Oberwinkler, 1986a; Massicotte et al., 1986, 1987b, c, 1990; Melville et al., 1987; Horan et al., 1988; Moore et al., 1989) and on their variations among species (Marx and Bryan, 1970; Molina and Trappe, 1982; Malajczuk et al., 1982, 1984; Duddridge, 1986a; Kope and Warcup, 1986; Brunner et al., 1990). These studies have led to hypotheses concerning mechanisms determining ectomycorrhizal structures. Although much more work can still be done on structural aspects of ectomycorrhizal colonization, the demonstration of critical determinants of ectomycorrhizal structures will require other experimental approachs. There is also a need for a comprehensive examination of all stages of ectomycorrhizal development using a selected ectomycorrhizal partnership.

One approach to evaluating hypothetical determinants of ectomycorrhizal development is the comparative examination of intraspecific variations. Such analyses have begun for ectomycorrhizal fungi (Lei et al., 1990a,b,c; Tonkin et al., 1989; Wong et al., 1989, 1990a,b) and plants (Tonkin et al., 1989). There are already interesting results which warrant further investigation, including the occurrence of extracellular glycoprotein fibrils in *P. tinctorius*, the accumulation of polyphenolics in *E. marginata* and the great variation in mantle thickness among closely related genotypes of *L. bicolor*. Other observations indicate that conspecific isolates can have different host preferences (Godbout and Fortin, 1983; Lei et al., 1990a; Tonkin et al., 1989; Lamhamedi et al., 1990) and may therefore be useful for studying host specificity.

The observed intraspecific variations suggest that it is possible to develop mutants deficient in certain aspects of ectomycorrhizal development. The genetic analysis of mutant and other variant genotypes would require the completion of the sexual cycle and thus is not convenient using host trees. Among the ectomycorrhizal fungi, *H. cylindrosporum* and *L. bicolor* have shown promising results in terms of fruit-body formation and spore germination under controlled conditions (Debaud and Gay, 1987; Kropp et al., 1987; Debaud et al., 1988; Godbout and Fortin, 1990). Furthermore, monokaryotic haploid strains of these two fungi can form both mantles and Hartig nets (Debaud et al., 1988; Wong et al., 1989). Continued studies on natural variability should clarify our ideas concerning targets for mutations and reveal other potential targets. This line of research should eventually identify genes implicated in ectomycorrhizal development.

The structural characterization should be complemented with biochemical and molecular information. Intraspecific variation has already been reported in the production of pectinolytic activity (Lundeberg, 1970; Lindeberg and Lindeberg, 1977; Giltrap and Lewis, 1982; Dahm et al., 1987), phenoloxidase activity (Levisohn, 1959; Laiho, 1970; Lundeberg, 1970; Giltrap, 1982a) and plant growth regulators (Graham and Linderman, 1980; Ek et al., 1983; Gay

and Debaud, 1987; Ho, 1987a,b; Ho and Trappe, 1987) by several ectomycorrhizal fungi but it has yet to be demonstrated during ectomycorrhizal interactions. Furthermore, the ectomycorrhizal status of many of these strains has not yet been thoroughly characterized. A correlation between the intraspecific variation found in certain biochemical characteristics with that in ectomycorrhizal structures would corroborate their hypothetical roles in ectomycorrhizal development. Co-inoculation experiments using conspecific strains may also be helpful for evaluating pre-infection interactions involving remote stimuli and fungus-root attachment. These studies would require the development of biochemical or molecular markers (Gardes et al., 1990c,d; Sen, 1990a,b) which can be used for *in situ* identification of hyphae by immunocytochemical localization (Herman, 1988) or *in situ* hydridization (Angerer et al., 1985; Lum, 1986), respectively.

A comparative approach could be used to screen ectomycorrhiza-specific gene products in order to identify those apparently responsible for variant ectomycorrhizal structures. This strategy may be particularly fruitful in the analysis of variations among closely related genotypes because the reduced allelic variation would permit a more reliable analysis of gene expression among the variants, using biochemical or molecular probes developed from one fungal strain or plant clone. To date, ectomycorrhiza-specific proteins have been documented in a *P. tinctorius-E. globulus* partnership (Hilbert and Martin, 1988a,b). In the future, recent developments in immunocytochemical localization, *in situ* hydridization, isolation of differentially expressed genes (Sargent, 1987) and cDNA library construction from minute quantities of biological material (Belyavsky et al., 1989) will permit a detailed molecular analysis of ectomycorrhizal development and interactions.

There is clearly much more work required before the mechanisms determining ectomycorrhizal development are deciphered. The analysis of symbiotic associations requires an integrated approach which exploits the techniques available in diverse domains of modern biology. A coherent analysis of selected model systems would appear to be the most profitable approach. At present, apparently suitable model plants include micropropagated clones of the angiosperm *E. marginata* (Tonkin et al., 1989) and the conifer *Pinus radiata* D. Don (Smith, 1986). The amount of work already reported on *H. cylindrosporum, L. bicolor* and *P. tinctorius* makes these species suitable candidates as the model fungus. Model systems may exploit intraspecific variations in order to focus on certain aspects of ectomycorrhizal development and to test hypothetical mechanisms. The information collected from a few partnerships may subsequently be used to make generalizations among species. Similar approachs can be used to study functional characteristics to complete

our understanding of the ectomycorrhiza symbiosis. Growth response in plants is already known to be dependent on the genotype of the mycobiont (Laiho, 1970; Marx et al., 1970; Dixon et al., 1987; Kropp and Fortin, 1988; Gibson and Deacon, 1990; Lamhamedi et al., 1990; Sen, 1990b). Interesting intraspecific variations in *P. tinctorius* have also been observed in the structure of extraradical mycelia and the drought tolerance response of colonized seedings of *P. pinaster* (M.S. Lamhamedi, CRBF, pers. comm.).

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