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T. C. Lumley · M. L. Farquhar · R. L. Peterson

Ectomycorrhiza formation between *Pseudotsuga menziesii* seedling roots and monokaryotic and dikaryotic isolates of *Laccaria bicolor*

Abstract Seedling roots of *Pseudotsuga menziesii* were colonized with three monokaryotic isolates and one dikaryotic isolate of *Laccaria bicolor* to assess the effect of fungal genotype on ectomycorrhiza formation. Ectomycorrhizas resulting from colonization by the dikaryotic isolate had a multilayered mantle and a cortical Hartig net. One monokaryotic isolate (ss7) formed ectomycorrhizas comparable in anatomy to those induced by the dikaryotic isolate. Two other monokaryotic isolates (ss5, ss1) failed to form mantles or Hartig nets. Roots colonized by these isolates developed characteristics indicating an incompatible reaction.

Key words *Pseudotsuga* · Douglas fir · *Laccaria* Monokaryons · Genotypes · Ectomycorrhizas Anatomy

Introduction

Pseudotsuga menziesii (Mirb.) Franco (Douglas fir) is an important reforestation species because it is fast growing and capable of adapting to diverse site conditions (Villeneuve et al. 1991). The successful development of *P. menziesii* is dependent, however, upon root colonization by compatible ectomycorrhizal fungi (Trappe and Strand 1969). This requires the proper selection of fungal isolates, i.e. those which are both compatible with roots of *P. menziesii* and the typically cool, wet and acidic growing environment of this species.

The major events that occur during ectomycorrhiza formation have been discussed in detail by many authors (e.g. Harley and Smith 1983; Kottke and Oberwinkler 1986). Following contact with the root, hyphae

become incorporated into the mucilage and, if compatible, penetrate intercellularly into the root epidermis or cortex (Nylund 1980), forming a Hartig net. There is a general paucity of information, however, on the factors affecting compatibility of plants and ectomycorrhizal fungi (Molina et al. 1992). Although plant genotype can influence the susceptibility to colonization by ectomycorrhizal fungi (Marx and Bryan 1971; Dixon et al. 1987; Tonkin et al. 1989), intraspecific variation in fungal species can also have a marked effect on ectomycorrhiza development (Wong and Fortin 1990).

Laccaria bicolor (Maire) Orton (Tricholomataceae, Agaricales) is a widespread fungal species with promise for use as mycorrhizal inoculum in the forestry industry (Kropp et al. 1987; Kropp and Fortin 1988; Wong et al. 1989). It is often found naturally in disturbed areas (Gardes et al. 1991) and has been used as inoculum in poor soils, such as eroded areas, mine spoils, and deforested and prairie areas (Cordell et al. 1987). Wong et al. (1989; 1990a, b) revealed, however, considerable diversity in the ability of monokaryotic and dikaryotic isolates of *L. bicolor* (Maire) Orton to form mycorrhizas with *Pinus banksiana* Lamb. From these observations, they proposed that fungal and plant cell modifications, interface characteristics, degree of interhyphal fusion during mantle formation, and intimacy of hyphal attachment to the root surface are colonization parameters that vary with each fungal isolate, assuming the same phytobiont species is involved.

In ectomycorrhizal associations with *P. menziesii*, *L. laccata* has been correlated with enhanced seedling growth and greater resistance to root pathogens (Sampagni et al. 1986; Sinclair et al. 1975, 1982; Sylvia and Sinclair 1983).

Monokaryons isolated from spores of the same sporocarp provide material that is closely related genetically and allow evaluation of differential gene expression of mycorrhiza-forming ability (Debaud et al. 1988; Lamhamedi et al. 1990). In addition to the practical implications of seedling growth and survival, the manifestation of these genetic differences as anatomical fea-

T. C. Lumley[1] · M. L. Farquhar · R. L. Peterson (✉)
Department of Botany, University of Guelph, Guelph,
Ontario N1G 2W1, Canada
Fax: (519) 767-1991; e-mail: lpeterso@uoguelph.ca

Present address:
[1] Department of Biological Sciences, University of Alberta,
Edmonton, Alberta, Canada

tures could provide insight into the critical steps in ectomycorrhizal establishment.

The objective of this study was, therefore, to characterize the ability of three sibling-monokaryons of *L. bicolor* to colonize seedling roots of *P. menziesii*. Although *L. bicolor-P. menziesii* ectomycorrhizas have been observed macroscopically (Coleman et al. 1990), their structure has not been characterized. Colonization of *P. menziesii* roots by a wild-type dikaryotic strain of the fungus, known for its mycorrhizal capabilities, was documented, therefore, for comparison with the monokaryons.

Materials and methods

Fungal cultures

Four isolates of *L. bicolor* (Maire) Orton were obtained from Centre de Recherche en Biologie Forestiere (CRBF) as CRBF 0101, CRBF 0347, CRBF 0348, and CRBF 0498. CRBF 0101 is a wild-type dikaryon that has been in culture at the University of Guelph since 1982 and is designated as "B". The isolates 0347, 0348, and 0498 are monokaryons that were cultured from spores of a single sporocarp collected in 1984 at St.-Etienne de Lauzon, Quebec and have been previously referred to as ss5, ss1, and ss7, respectively (Kropp et al. 1987). All isolates were maintained at CRBF on agar slants at 4° C or in liquid nitrogen (B. Kropp, personal communication). To maintain actively growing cultures, isolates were subcultured every 3–4 weeks onto modified Melin-Norkrans (MMN) nutrient medium in 1.2% (w:v) bacto-agar (Difco) at pH 5.5 (Marx 1969) and stored in the dark at 21–22° C. Voucher specimens were also stored at 5° C in sterile distilled water (after Richter and Bruhn 1989). Cultures were routinely examined for the presence of clamp connections; their absence confirmed the monokaryotic status of the three monokaryotic isolates.

Seedling growth and inoculation

Seeds of *P. menziesii* (Mirb.) Franco var. *menziesii* were obtained from the Petawawa National Forestry Institute (PNFI). They had been collected in 1984 from Cassidy, British Columbia, lat. 49°02′, long. 123°52′, elev. 45 m. Seeds were surface sterilized in approximately 100 ml of 30% hydrogen peroxide for 90 min with the addition of 3–4 drops of Tween 20 for the last 5 min, then rinsed thoroughly, plated on a medium containing 1.0% dextrose and 0.08% agar in distilled water and allowed to germinate in the dark at 24–25° C. After 10–14 days, when the primary roots were 2–3 cm long, the seedlings were transplanted, under sterile conditions, to Seed-pack growth pouches (Vaughan's Seed Co) which had been previously saturated with distilled water and autoclaved for 20 min at 121° C (Fortin et al. 1980). Preinoculation seedlings were maintained in a growth chamber with 16-h light (270 μE m^{-2} s^{-1}) at 25° C and 8-h dark periods (20° C), both at 90% relative humidity. Sterile distilled water was supplied at a rate of 5 ml per week until the primary root reached the bottom of the pouch, at which time only enough water was supplied to keep the paper wick damp. Postinoculation seedlings were supplied with only 2–3 ml of water per week.

Seedlings with first-order lateral roots longer than 4 cm were inoculated in sterile conditions by placing a regenerated fungal plug immediately basipetal to an elongating first-order lateral tip. Approximately 20 seedlings were inoculated with each of the isolates.

Light microscopy

Mycorrhizal short roots were excised from 10 plants for each isolate and fixed for 2 days at 4–5° C in a solution of 3% glutaralde-

hyde in 0.1 M HEPES buffer pH 6.8. Root tissue was then rinsed three times in HEPES buffer and dehydrated at room temperature in a graded ethanol series (30, 50, 70, 90, 95%) completed with three changes of 100% ethanol. Tissue was gradually infiltrated with LR White resin (London Resin Co). Individual roots were then put into fresh resin and polymerized in PVC moulds in a vacuum oven for 1–2 h at 65° C. Sections (1–2 μm) of 10 roots of each treatment were stained with 1% methylene blue and 1% Azure B (1:1) in 1% sodium tetraborate and counterstained with 0.05% aqueous basic fucshin.

Scanning electron microscopy

For scanning electron microscopy, the mycorrhizal short roots and nonmycorrhizal roots with hyphal proliferation from 10 pouches for each isolate were fixed and dehydrated as for light microscopy. Roots were then critical point dried, mounted on aluminum slug mounts using two-sided tape, sputter coated with gold/palladium and viewed using a JEOL JSM 35C scanning microscope. At least 10 roots were examined for each inoculum type.

Results

The dikaryon CRBF 0101 (B) was vigorously mycorrhizal on seedling roots of *P. menziesii*. Hyphae contacted the root surface, generally at some distance from the root apex (Fig. 1), and penetration of the root surface occurred soon after hyphal contact (Figs. 1, 2). Some interaction between hyphae and root hairs also occurred (Fig. 2). Emerging laterals were colonized heavily, resulting in dense mantles covering the apices of both long and short lateral roots (Fig. 3). Long roots had a sparse covering of hyphae (Fig. 3). The mantle on the short laterals consisted of an inner dense layer with extensive interhyphal fusion (Fig. 4) and adhesion to the root surface, and an outer diffuse layer with obvious and frequent clamp connnections. Longitudinal sections of colonized lateral roots showed a small apical meristem, a root cap consisting of a few cell layers and a mantle which covered the root apex (Figs. 5, 6). Hyphae appeared to adhere tightly to root cap cells at the root apex (Fig. 6). The mantle in the pre-Hartig net region of the root consisted of a compact inner region and a more diffuse outer region (Fig. 7); hyphae surrounded root cap cells, some of which had collapsed (Fig. 7). The uniseriate Hartig net was 2–3 cortical cell layers deep and caused complete separation of the vacuolated cortical cells (Fig. 8).
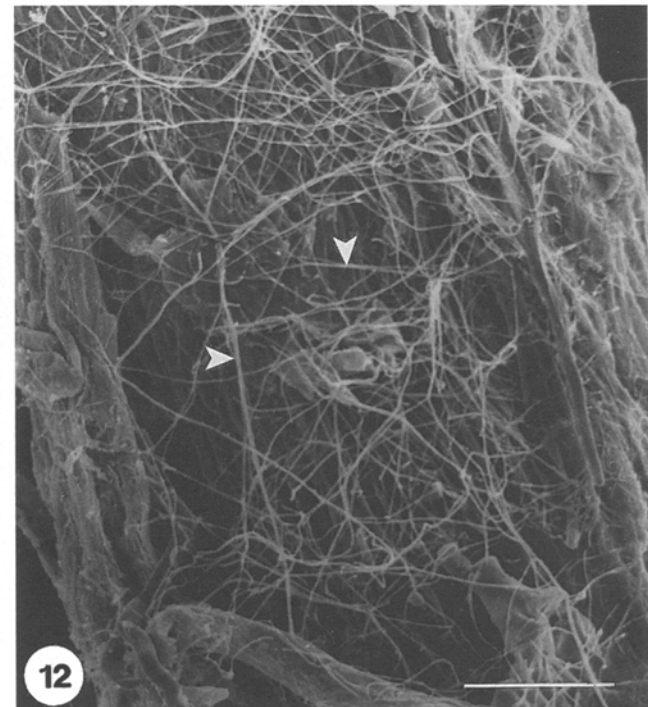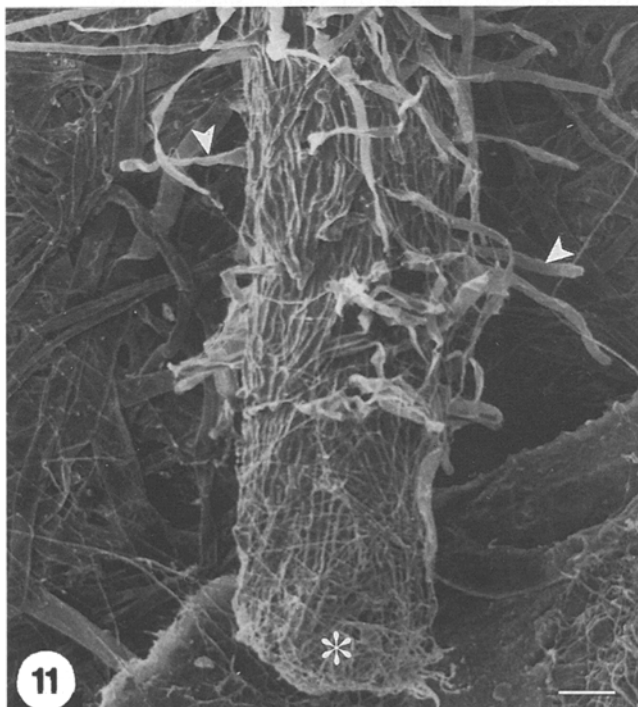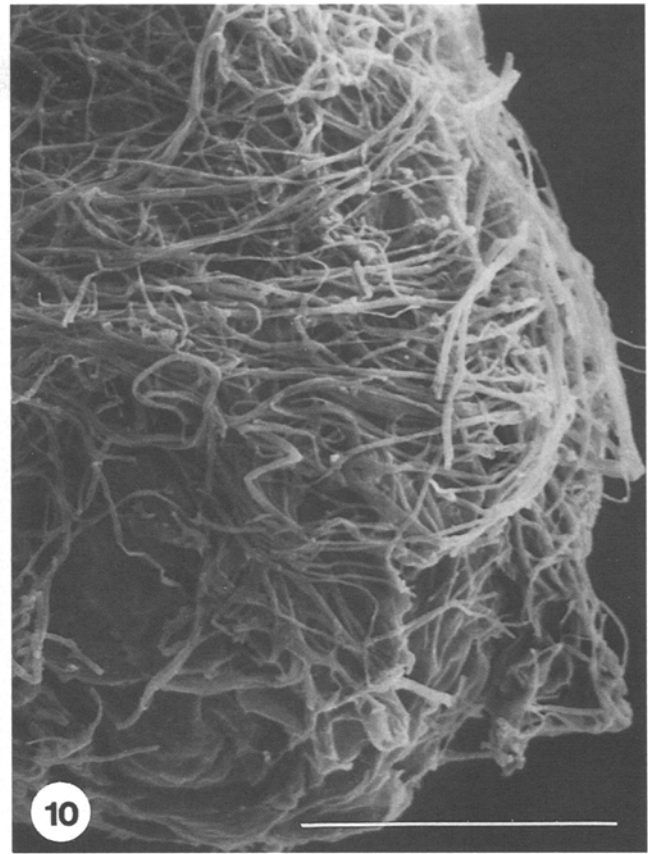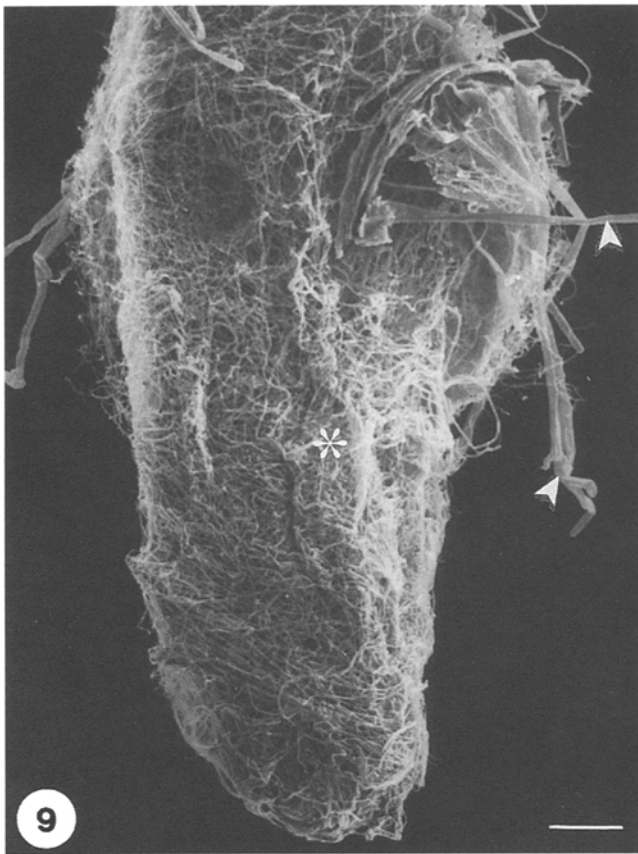
Figs. 1–4 Scanning electron microscopy of ectomycorrhiza development between seedling roots of *Pseudotsuga menziesii* and a dikaryon of *Laccaria bicolor*. **Fig. 1** Early contact of hyphae (*arrowheads*) with root cap cells (***) some distance from the root apex; *bar* 0.1 mm. **Fig. 2** Contact of hyphae with a root hair (*RH*). A branch of a hypha (*arrowhead*) has penetrated between root cap cells; *bar* 0.01 mm. **Fig. 3** Two lateral roots (***) showing mantles that cover the entire root. A few hyphae (*arrowheads*) are found on the long root; *bar* 0.01 mm. **Fig. 4** A higher magnification of a mantle. The inner mantle hyphae (*arrowheads*) are more compactly arranged than hyphae in the outer mantle (*double arrowheads*). Clamp connections (*arrows*) are evident; *bar* 0.01 mm

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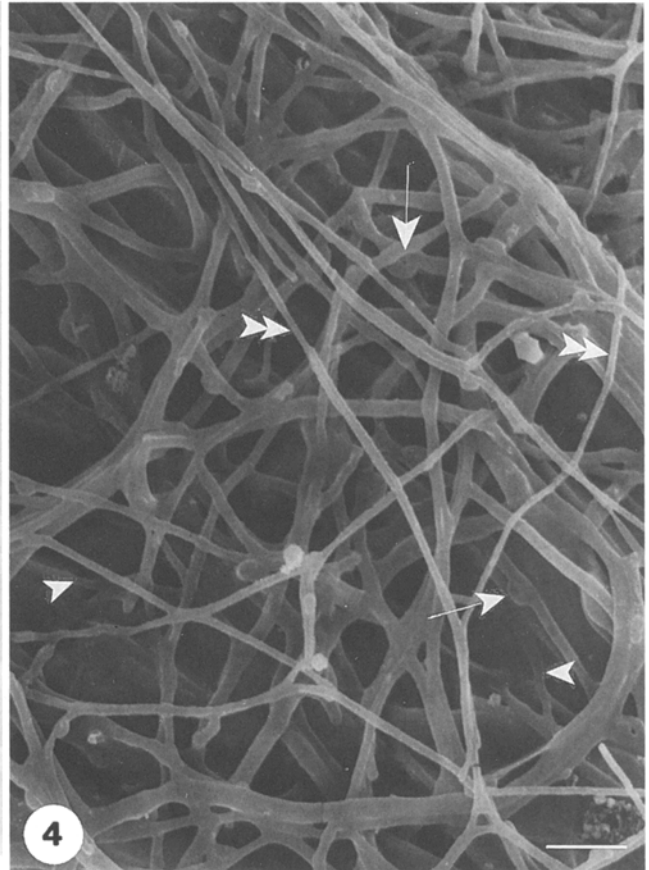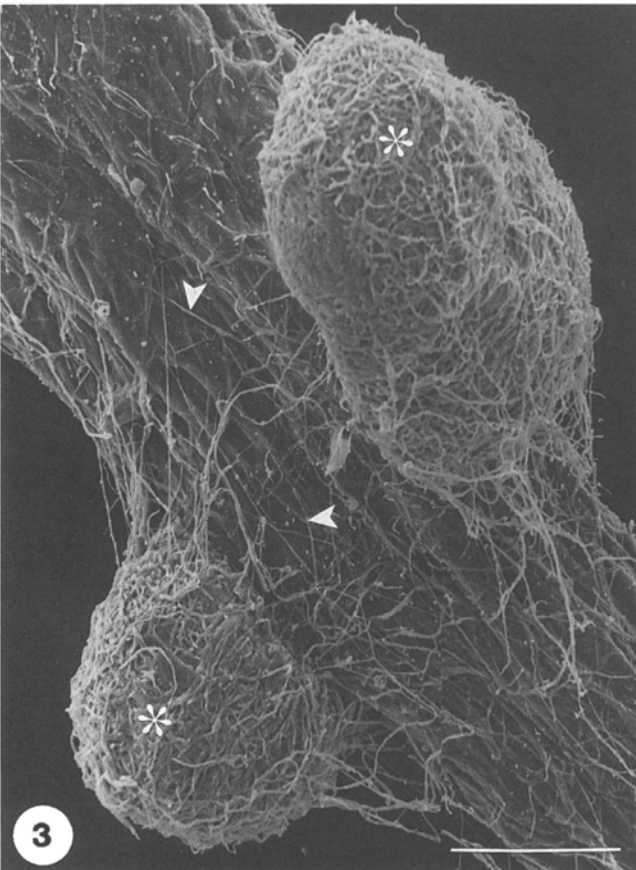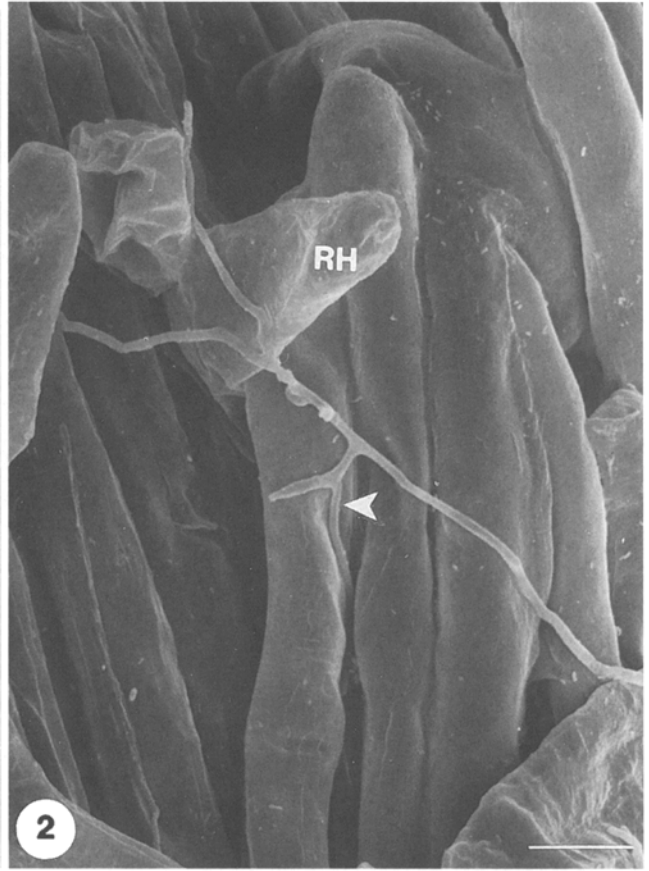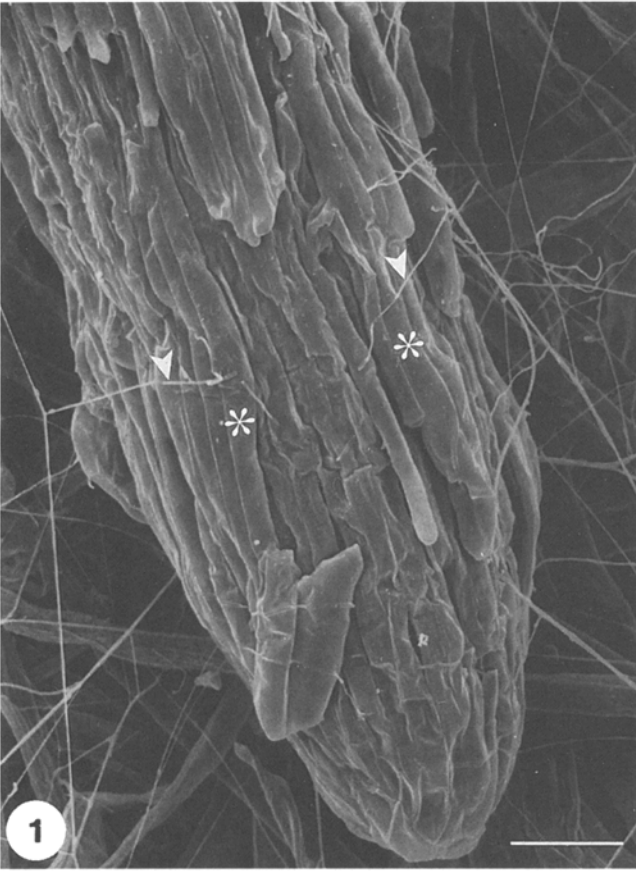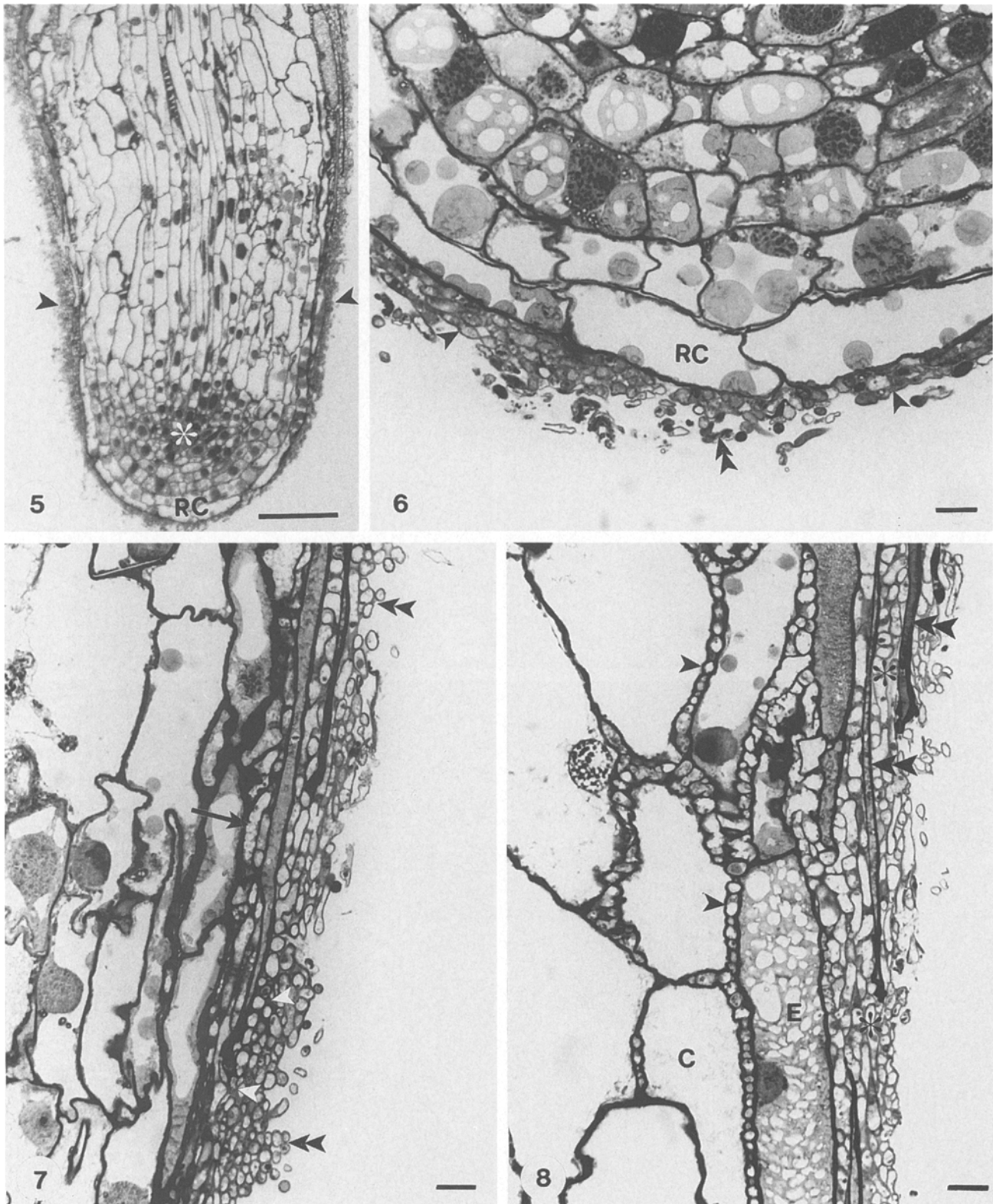
Figs. 5–8 Light microscopy of longitudinal sections of *P. menziesii* ectomycorrhizas formed by a dikaryon of *L. bicolor*. **Fig. 5** Seedling root with an apical meristem (*), a small root cap (*RC*) and a mantle of fungal hyphae (*arrowheads*); *bar* 0.1 mm. **Fig. 6** Portion of root apex showing root cap cells (*RC*) with adhering mantle hyphae (*arrowheads*). The outer mantle hyphae (*double arrowheads*) are loosely organized; *bar* 0.01 mm. **Fig. 7** Portion of root basipetal to root apex showing pre-Hartig net stage. Inner mantle hyphae (*arrowheads*) are compactly organized while outer mantle hyphae (*double arrowheads*) are loosely organized. Hyphae have penetrated between collapsing root cap cells (*arrow*); *bar* 0.01 mm. **Fig. 8** Region of root in which a Hartig net has formed between epidermal (*E*) and cortical (*C*) cells. The Hartig net (*arrowheads*) is mostly uniseriate. Collapsing root cap cells (*double arrowheads*) and a mantle (*) are evident; *bar* 0.01 mm

Figs. 9–12 Scanning electron microscopy of *P. menziesii* seedling roots colonized by various monokaryons of *L. bicolor*; *bars* 0.1 mm. **Fig. 9** Roots colonized by the monokaryon ss7 showing well-developed mantle (*). Root hairs (*arrowheads*) have persisted. **Fig. 10** Enlargement of mantle showing rather loose arrangement of mantle hyphae. **Fig. 11** Long root colonized by the monokaryon ss5. Hyphae tended to accumulate at the root apex (*). Many root hairs (*arrowheads*) persisted. **Fig. 12** Long root colonized by the monokaryon ss1. Few, loosely organized hyphae (*arrowheads*) were present on the root surface

Hyphae of the monokaryotic isolate CRBF 0498 (ss7) formed a mantle over the root apex and for some distance back but root hairs persisted (Fig. 9). The mantle appeared to be somewhat patchy at the root apex (Fig. 10). The Hartig net hyphae penetrated into the cortex in such a way that the outer cells became separated by one or more layers of hyphae (Figs. 13, 14). The Hartig net showed few signs of labyrinthine growth and Hartig net hyphae were rarely branched (Fig. 14). Cortical cell walls stained intensely in these roots (Figs. 13, 14). Hyphae accumulated on long laterals, but showed no subsequent penetration. Although there was no attempt at quantification, this isolate clearly formed far fewer mycorrhizas per seedling than isolate B.

The monokaryon CRBF 0348 (ss5) showed an affinity for long roots, and hyphae often accumulated at the root apex (Fig. 11); a full mantle was never observed. Hyphae were occasionally observed in contact with short roots, but these tips soon darkened and penetration did not occur. Longitudinal sections of roots showed that hyphae were only loosely associated with roots (Fig. 15). Occasionally, hyphae adhered to degenerating root hairs (Fig. 16).

The monokaryon CRBF 0347 (ss1) showed some hyphal contact with the root, but the ensuing accumulation of hyphae was always diffuse and the hyphae only sporadically adhered to the root surface (Fig. 12). Consequently, most of the hyphae were lost during the processing of tissue for light microscopy (Fig. 17). As with the monokaryon ss5, a few hyphae sometimes adhered to root hairs (Fig. 18).

Discussion

The ectomycorrhizas formed on *P. menziesii* with the dikaryotic isolate (B) of *L. bicolor* had anatomical features typical of a compatible interaction and were used, therefore, for comparison with ectomycorrhizas formed with the other isolates.

Ectomycorrhizas formed with the monokaryon ss7 developed in much the same way as those produced with isolate B, with the formation of a thick mantle and a well-developed Hartig net at maturity. Ectomycorrhizas were produced more sporadically with this isolate than with isolate B. Wong et al. (1989) observed a type I Hartig net with this isolate on *Pinus banksiana*, characterized by shallow penetration of Hartig net hyphae and no separation of cortical cells. The Hartig nets observed with Douglas fir, however, were often type III, with complete separation of outer cortical cells. This mycorrhizal monokaryon also induced the production of darkly staining substances within root cell walls which seemed either not to impede the progress of the Hartig net hyphae or were deposited after Hartig net formation. Hyphae of this isolate were more likely than those of B to accumulate on long laterals, especially laterals giving rise to mycorrhizal short roots.

The monokaryons ss1 and ss5 which were nonmycorrhizal with *Pinus banksiana* (Wong et al. 1989) behaved similarly with roots of Douglas fir in the growth pouch system. Neither isolate formed a mantle or a Hartig net, and only rarely did hyphae accumulate on lateral roots. However, there was a darkening of laterals upon fungal contact, which was presumably due to an accumulation of phenolic substances (Bogar and Smith 1965). This accumulation was so intense that it prevented good resin infiltration of root tissue during processing for light and transmission electron microscopy. This reaction was typical of that reported for incompatible interactions between ectomycorrhizal fungi and other tree species (Malajczuk et al. 1984).

There was a marked difference in the affinity of various isolates of *L. bicolor* towards long and short lateral roots. Specifically, nonmycorrhizal isolates often formed dense accumulations of hyphae on long laterals without subsequent penetration, whereas such accumulation was generally not observed on short laterals. The monokaryotic isolate ss1 had no affinity for short roots but did occasionally form loose hyphal wefts around relatively mature long laterals; these hyphae even became enlarged and exhibited some rudimentary fusion, indicative of two of the initial stages of mantle formation. Isolate ss5, which occasionally accumulated on short laterals, also seemed more attracted by long laterals, although the fungus could not penetrate the root surface. Interestingly, isolates ss7 and B, which were mycorrhizal on short laterals, very rarely accumulated to any degree on long laterals. Wong and Fortin (1990) suggested that differential susceptibility is indirectly dependent on the anatomy of the roots at the time of hyphal contact (see also Wong et al. 1989).

The observations made in this study indicate that the findings of Wong et al. (1989; 1990a, b) with *Pinus banksiana* are demonstrable with another plant species, although in our study the colonization patterns were slightly different with Douglas fir. The monokaryotic isolate ss5, which was weakly mycorrhizal with *Pinus banksiana* was nonmycorrhizal with Douglas fir, al-

Figs. 13–18 Light microscopy of *P. menziesii* roots colonized by ▶ various monokaryons of *L. bicolor*. **Fig. 13** Transverse section of root colonized by the monokaryon ss7. A well-developed mantle (*) incorporating root cap cells (*arrowheads*) has formed. In addition, a Hartig net has formed between epidermal (*e*) and cortical (*c*) cells; *bar* 0.1 mm. **Fig. 14** Higher magnification of root similar to that in Fig. 13. The mantle (*) and Hartig net (*arrowheads*) are evident. Some cortical cell walls have thickened and have stained intensely (*double arrowheads*) (*RC* root cap cells, *E* epidermal cells, *C* cortical cells); *bar* 0.01 mm. **Fig. 15** Longitudinal section of long root colonized by the monokaryon ss5. Most of the surface hyphae have been lost during tissue processing but a few (*arrowheads*) have remained; *bar* 0.1 mm. **Fig. 16** Portion of root colonized by the monokaryon ss5. A few hyphae (*arrowheads*) have accumulated along a root hair (*RH*); *bar* 0.01 mm. **Fig. 17** Longitudinal section of root colonized by the monokaryon ss1. The root surface (*arrowheads*) appears largely free of hyphae; *bar* 0.1 mm. **Fig. 18** Surface of root colonized by the monokaryon ss1 showing a few vacuolated hyphae (*arrowheads*) adjacent to a root hair (*RH*); *bar* 0.01 mm
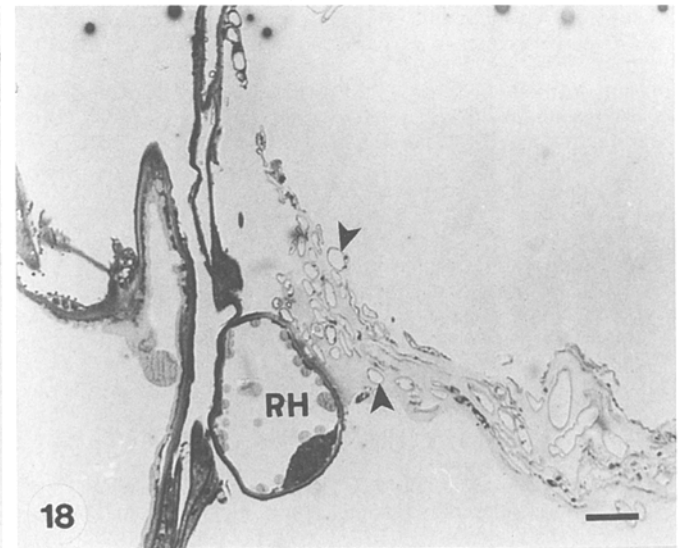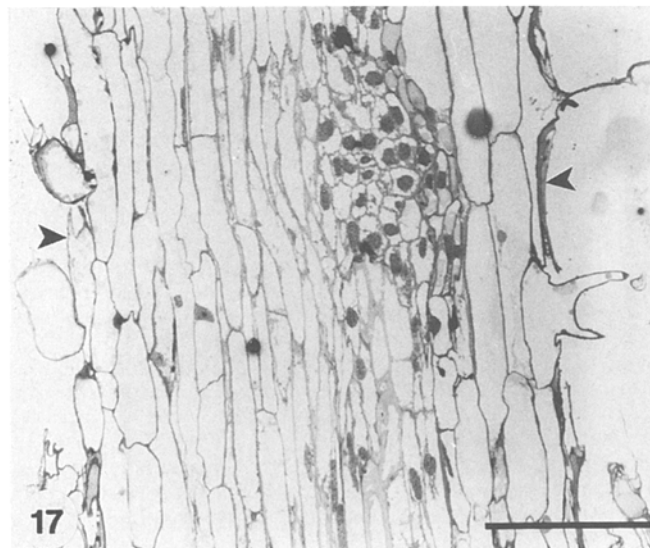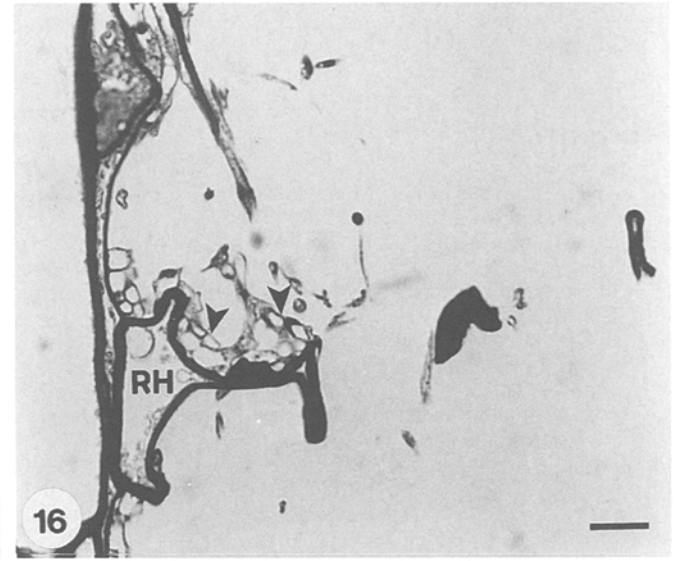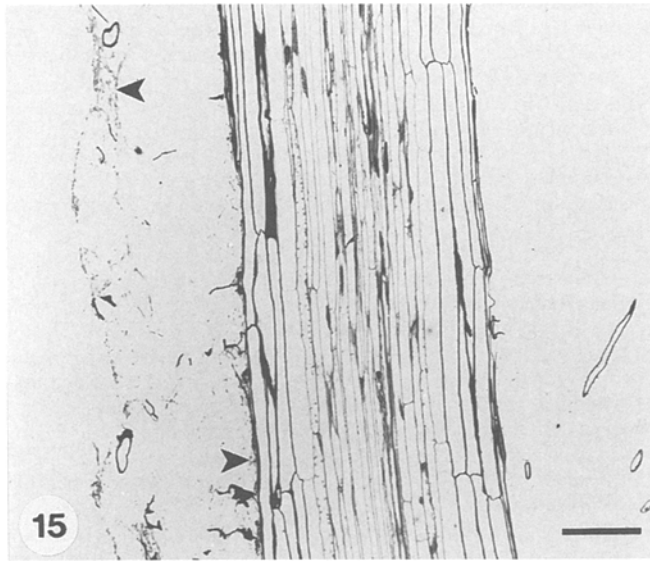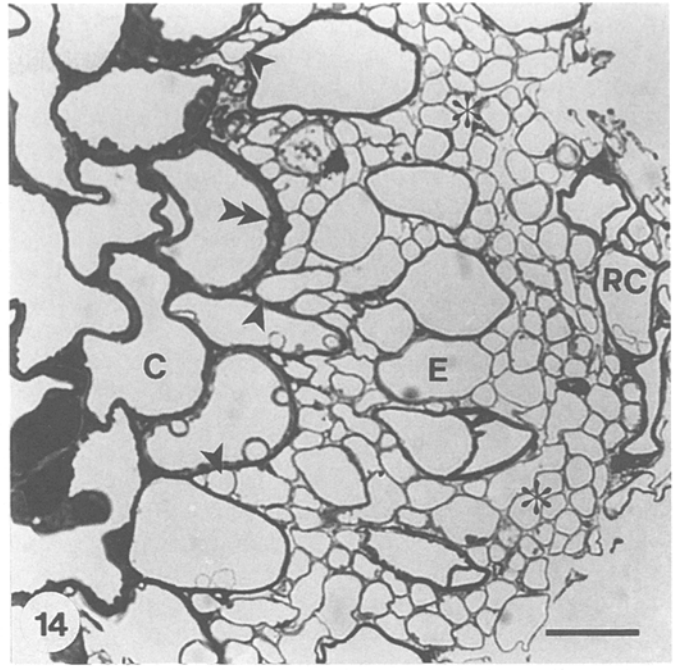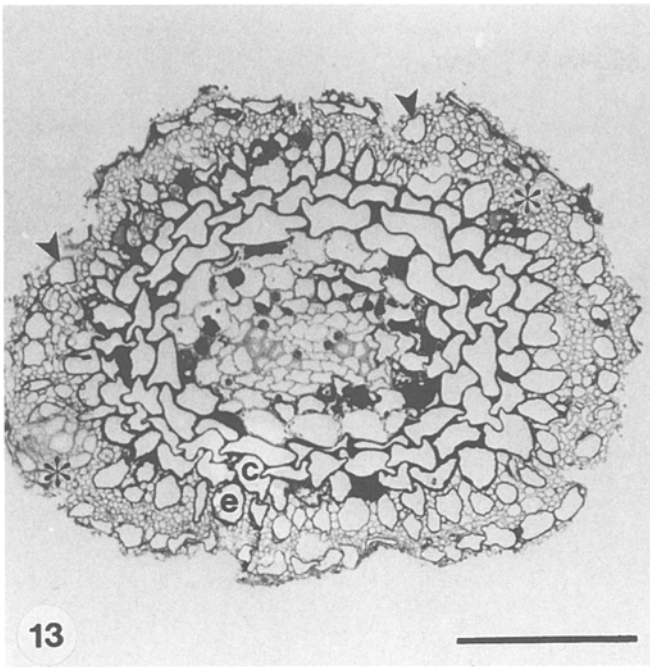
though hyphae accumulated on long laterals. Isolate ss7, on the other hand, was even more vigorously mycorrhizal on Douglas fir, forming a thicker mantle and more extensive Hartig net than with *Pinus banksiana*.

It is evident from this and previous studies (Wong et al. 1989, 1990b) that some monokaryotic isolates of *L. bicolor* are capable of initiating ectomycorrhiza formation. Similar results have been obtained with monokaryotic isolates of *Hebeloma cylindrosporum* inoculated onto *Pinus pinaster* (Ait.) Sol. (Debaud et al. 1988) and with *Pisolithus arhizus* on *Pinus pinaster* and *Pinus banksiana* (Lamhamedi et al. 1990). This is in contrast to results obtained with *Suillus granulatus* (Fr.) Kuntze in which monokaryotic mycelium failed to initiate ectomycorrhizas with *Pinus pinaster* (Ducamp et al. 1986). It is evident that ectomycorrhiza formation involves a complex series of events (Peterson and Farquhar 1994) undoubtedly controlled by several fungal and plant genes. Further studies with monokaryotic isolates should contribute to a better understanding of the genetic control of ectomycorrhiza development.

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