PRODUCTION OF ECTOMYCORRHIZAL *PICEA ABIES* - *HEBELOMA CRUSTULINIFORME* SEEDLINGS FOR ECOLOGICAL STUDIES: EFFECTS OF SYNTHESIS TECHNIQUES ON THE MORPHOLOGY OF THE SYMBIOSIS

I. BRUNNER

Swiss Federal Institute for Forest, Snow and Landscape Research (WSL)
8903 Birmensdorf
Switzerland

ABSTRACT. Various *in vitro* synthesis techniques were applied to produce numerous ectomycorrhizal *Picea abies* - *Hebeloma crustuliniforme* seedlings which could be later used for experiments in the field of environmental research. Included synthesis methods are the Erlenmeyer flask technique, the cuvette technique, and the growth pouch technique. A mantle and a Hartig net were present in all synthesized and naturally grown symbioses. Stable features were the white color of the mantle, its two-layered structure, and the structure of the Hartig net. A few characters such as the thickness of the mantle and the development of the Hartig net formation were variable due to the synthesis method. The techniques suitable for producing ectomycorrhizal seedlings are the Erlenmeyer flask technique using vermiculite and peat moss and the growth pouch technique: the first technique because of sterility and good ectomycorrhizal formation within approximately two months, the second technique because of direct observation of ectomycorrhizal formation within one to two months and complete removing of ectomycorrhizae including extramatrical mycelia.

1. Introduction

Ectomycorrhizae are an integral part of most trees in temperate and boreal zones. They arise when mycelia of an ectomycorrhizal fungus contact young later roots in the soil. The ectomycorrhizal association supplies nutrients and water from the soil to the host plant in return for carbohydrates. Pollution and other stresses can affect the symbioses directly by influencing growth and physiology of the root and ectomycorrhizal associate, or indirectly by influencing carbon allocation within the plant or by altering soil chemical or physical conditions (Dighton and Jansen, 1991). From this generalized model, it can be predicted that a reduction in carbohydrates to the roots could inhibit development or physiological activities of ectomycorrhizae, or that damage to the roots, ectomycorrhizae, and ectomycorrhizal mycelia reduces nutrient absorption from the soil and nutrient uptake via the symbioses.

Recent experiment studies of the influence of pollutants on forest ecosystems have used ectomycorrhizal trees under controlled conditions in aseptic or semiaseptic environments (e.g. Jentschke *et al.*, 1991a; Jentschke *et al.*, 1991b; Andersen and Rygielwicz, 1991). Potential pollutants can not only affect mycelial uptake and translocation of selected nutrients within the ectomycorrhizal plants, contents of selected elements in needles and roots, transpiration and respiration rate, or carbon allocation, but they also can influence appearance and morphology of ectomycorrhizae. Altered morphological features of ectomycorrhizae due to pollutants include branching index (Meyer, 1987), abundance of intracellular hyphal infections in root cortical cells (Blaschke, 1986), abundance of metacutis formations (Blaschke, 1986), amount of fine root production (Wöllmer and Kottke, 1990), ratio of dead *versus* living fine roots (Wöllmer and Kottke, 1990), and frequency of ectomycorrhizae (Blaschke and Weiss, 1990; Wöllmer and Kottke, 1990). These data strongly suggest that it is essential to know the effect of the synthesis technique on ectomycorrhizal morphology before the symbiosis is exposed to potential pollutants. Ectomycorrhizal trees can either be used for experiments directly in their synthesis vessels, or if

necessary from the experimental design, they can be transferred into different containers. An experiment of the later type is presented in this issue (Ochs *et al.*, 1992).

Previous studies with the system *Picea abies* (L.) Karst. - *Hebeloma crustuliniforme* (Bull. ex St. Amans) Quél. have been carried out to characterize ectomycorrhizae synthesized with various *in vitro* techniques (Brunner, 1991) and naturally grown ectomycorrhizae (Brunner *et al.*, 1991). The objectives of the present study were to test the suitability of various *in vitro* synthesis techniques to produce ectomycorrhizal seedlings with ectomycorrhizae that most closely resemble those found in nature. These symbioses would be considered to be the most valuable to use in further ecological studies, as their similar morphologies would tend to suggest similar physiological response to pollutants or other environmental variables. The *in vitro* methods include the Erlenmeyer flask technique of Melin (1922), the cuvette technique similar to that described by Schüepp *et al.* (1987), and the growth pouch technique (Fortin *et al.*, 1980).

2. Materials and Methods

2.1. IN VITRO SYNTHESIZED ECTOMYCORRHIZA

Seeds of *Picea abies* were surface sterilized for 30 min in 30% H₂O₂, transferred on water agar plates, and germinated for 2 to 3 weeks. For the Erlenmeyer flask and cuvette techniques, fungal mycelia of *Hebeloma crustuliniforme* were pregrown for 20 days on a shaker in 125 ml Erlenmeyer flasks containing 50 ml modified Melin-Norkrans solution (MMN; Marx and Bryan 1975) including glucose (10 g/l) and malt (3 g/l). Mycelia were homogenized in a blender and introduced into flasks and cuvettes using an inverted pipette. For use in growth pouches, 3 mm discs were removed with a cork borer from mycelia grown on solid MMN media, transferred onto new solid MMN media, and incubated for 5 days. Synthesis experiments were carried out in a growth chamber with a 16 h day period (PAR: 100 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 20°C and 70% humidity. The duration of the experiments varied between 43 and 150 days. Ectomycorrhizal rootlets obtained from the various techniques were fixed in 2.5% glutaraldehyde. A schematic outline of the synthesis techniques is provided in Figure 1.

2.1.1. Erlenmeyer flask technique. Flasks (500 ml) were filled with a vermiculite-peat moss mixture (200 ml-30 ml) or with 250 ml porous glass balls (Schott, Germany; Siran, 2-3 mm in diameter) and with 150 ml MMN-solution and autoclaved. pH after autoclaving ranged from 5.5 in the vermiculite-peat moss mixture to 7.0 in the porous glass balls. Fungal inocula (20 ml) were introduced and allowed to grow for 34 days prior to seedling introduction.

2.1.2. Cuvette technique. Seedlings were pregrown for 34 days in Erlenmeyer flasks (500 ml) containing a vermiculite-peat moss mixture (200 ml-30 ml) and 150 ml MMN-solution. Autoclaved cuvettes of stainless steel (15x12x2 cm) were planted with the seedlings using the substrate from the Erlenmeyers, and inoculated with 20 ml fungal mycelia.

2.1.3. Growth pouch technique. Autoclaved polyethylene pouches including a glass fiber paper with an activated charcoal paper disc attached were filled with 10 ml MMN minus glucose and malt, and one seedling of *P. abies*. The final pH in the system was 6.5 after autoclaving. After 2 months, four to six discs of fungal inocula were placed in the vicinity of the short roots and another 10 ml MMN (including 5 g/l glucose, no malt) was added. Two strips of foam were introduced to provide air space.

2.2. NATURALLY GROWN ECTOMYCORRHIZAE

The procedure of the soil sampling was similar to the method described in Agerer (1986). Within a nearly pure *P. abies* forest, soil samples were cut out with a spade around single or clustered fruiting bodies of *H. crustuliniforme*, placed in a plastic bucket, covered with plastic film to

Figure 1. Schematic outline of the synthesis techniques starting from a pure fungal culture and surface sterilized seeds. Erlenmeyer flask technique with porous glass balls (a) or with a vermiculite-peat moss mixture (b), cuvette technique (c), growth pouch technique (d) (for details see text).

preserve moisture, and transported to the laboratory. Mycelial connections between fruiting bodies and ectomycorrhizae were looked for on the same day by carefully removing soil and plant debris. Roots with ectomycorrhizae connected to the fruiting bodies were excised and fixed in buffered 6% formaldehyde.

2.3. ANALYSES OF ROOT MATERIAL

Ectomycorrhizal rootlets were dehydrated in alcohol, embedded in glycol-methacrylate, longitudinally sectioned (1.5 μm), and stained with Giemsa for chitinoïd material (Clark, 1981). Drawings were made with the aid of a Wild drawing tube. Morphological structures of mantle and Hartig net were drawn from the middle portions of central longitudinal sections. Anatomical structures were drawn from emanating hyphae, outer and inner layers of the mantle, and Hartig net using the middle portions of tangential longitudinal sections. Photographs of ectomycorrhizae were taken with the aid of a Leitz dissecting photomicroscope. Longitudinal fractures of ectomycorrhizal roots were obtained after freezing specimens in liquid nitrogen, freeze-fracturing, and partially freeze-drying for low-temperature scanning electron microscopy following the method described in Müller *et al.* (1991) and Scheidegger *et al.* (1991), and applied to ectomycorrhizae in Brunner and Scheidegger (1992).

3. Results

3.1. DESCRIPTION OF SYNTHESIZED AND NATURALLY GROWN ECTOMYCORRHIZAE

3.1.1. *Morphology.* First order lateral root tips are up to 7 mm long and 0.3-0.5 mm thick, straight to slightly coiled, cylindrical, with rounded apices (Figs. 2, 3). Root tips and apices are white, or bright yellow to orange-brown due to translucent cortical cells. Hyphal veils often connect several root tips in a row. Surfaces are smooth to fine fibrous, often covered with densely interwoven and emanating hyphae. Rhizomorphs are to be observed only in naturally grown symbioses.

3.1.2. *Mantles.* The surface of the mantles consists of a loose to dense prosenchymatous web with often branched hyphae. Hyphal cells are 6-40 μm long, 2-5 μm broad, and clamp connections are present. Mantles are composed of two distinct layers (Fig. 4). The outer layer is prosenchymatous with hyphae loosely to closely interlaced (Fig. 5). Prosenchymatous elements are 2-4 μm broad, and clamp connections are present. The inner layer close to the epidermal cells is synenchymatous with hyphae closely interlaced (Fig. 5). Synenchymatous elements vary in size with hyphal cells 2-7 μm broad. Clamp connections were not observed. Mantles vary in thickness from 5 to 40 μm . Zones of metacutizations can occur and surround the apical meristem.

3.1.3. *Hartig nets.* Hartig nets are composed of distinct fingerlike structures (palmettes), penetrating up to the endodermis (Fig. 5). One or as many as three hyphal cell rows are between the epidermal cortical cells; hyphal cells and lobes of palmettes are 2-6 μm broad.

3.1.4. *Emanating hyphae.* Emanating hyphae are frequently present, thin-walled, smooth, hyaline and often branched. Hyphal cells are up to 70 μm long and 2-4 μm broad, clamp connections are present (Fig. 5). Hyphae often form loose to densely interwoven veils.

3.1.5. *Rhizomorphs.* Rhizomorphs are fibrous, white, up to 150 μm in diameter, consisting of a bundle of tightly interwoven and undifferentiated hyphae. Hyphal cells are 2.5-5 μm broad, clamp connections are present.

Figures 2-4. Photographs of *Picea abies* - *Hebeloma crustuliniforme* ectomycorrhizae. Figure 2. Naturally grown ectomycorrhiza. Scale bar = 0.5 mm. Figure 3. Ectomycorrhiza synthesized with the cuvette technique 46 days after fungal inoculation. Scale bar = 0.5 mm. Figure 4. Low-temperature scanning electron micrograph of a longitudinal fractured ectomycorrhiza synthesized with the growth pouch technique 46 days after fungal inoculation. An inner (IM) and outer mantle (OM) and a Hartig net (arrowheads) between rhizodermal cells (RD) and cortical cells (CC) are present. Scale bar = 10 μ m.

Figure 5. Line drawings of longitudinal sections through *Picea abies* - *Hebeloma crustuliniforme* ectomycorrhizae synthesized with the Erlenmeyer flask technique including a vermiculite-peat moss mixture. Longitudinal sections show the basal (a), the middle (b), and the apical portion (c). Tangential sections show emanating hyphae (d), the structure of the outer (e) and inner layer of the mantle (f), and the structure of the Hartig net (g). Scale bar = 10 μ m.

3.2. STABLE FEATURES

Stable features of all synthesized and naturally grown ectomycorrhizae are the presence of mantles, Hartig nets, and emanating hyphae (Tab. 1). In addition, the color of the mantles is white. Mantles are always composed of two layers with an outer prosenchymatous and an inner synenchymatous layer. Hartig nets are formed of tightly interlaced synenchymatous palmettes with maximum penetration up to the endodermis (Tab. 1).

3.3. VARIABLE FEATURES

Variable features of synthesized ectomycorrhizae are the thickness of the mantles and the development of the Hartig net formation (Tab. 1). Thick mantles have been observed only in naturally ectomycorrhizae and in synthesis with the Erlenmeyer flask technique using vermiculite and peat moss. In other techniques, the thickness of the mantles is slight or varies from thin to thick (Tab. 1). Poorly developed Hartig nets have been observed only in ectomycorrhizae synthesized with the Erlenmeyer flask technique using porous glass balls and with the cuvette technique (Tab. 1). Zones of metacutizations are inconsistently present only in ectomycorrhizae synthesized in growth pouches. Rhizomorphs have been observed only in naturally grown ectomycorrhizae (Tab. 1).

4. Discussion

All of the *in vitro* synthesis techniques in this study using tree seedlings of *Picea abies* and the fungus *Hebeloma crustuliniforme* produced ectomycorrhizae. These techniques are well established and often used. The Erlenmeyer flask technique has been used mainly as an aseptic method to synthesize known or unknown ectomycorrhizal fungi with a putative host tree (e.g. Kottke, 1986) or to study the ultrastructure of the symbioses (e.g. Kottke and Oberwinkler, 1986). The semiaseptic cuvette technique has been used to monitor hyphal growth of endomycorrhizae (e.g. Miller *et al.*, 1989), and the semiaseptic growth pouch technique to study the ability to form ectomycorrhizae (e.g. Brunner *et al.*, 1990) as well as ultrastructure and ontogeny of ectomycorrhizae (e.g. Massicotte *et al.*, 1990; Brunner and Scheidegger, 1992). The potential and limitations of the growth pouch method for the study of ectomycorrhizae have been summarized by Fortin *et al.* (1983).

An ectomycorrhizae, by definition, must have a mantle and a Hartig net (Harley and Smith, 1983). These features were present in all synthesized and the naturally grown symbioses in this study. Stable features in all ectomycorrhizae of *P. abies* - *H. crustuliniforme* in this work are the white color of the mantle and its two-layered structure composed of a prosenchymatous outer and a synenchymatous inner mantle. In addition, the structure of the Hartig net of closely interlaced palmettes and its penetration depth up to the endodermis are also stable features. Only a few characters differ among synthesizing techniques and therefore count as variable features. Such characters are the thickness of the mantle and the development of the Hartig net.

A thin mantle and a poor Hartig net developed in both the Erlenmeyer flasks with porous glass balls as the substrate and in the cuvettes. This suggests that development of the ectomycorrhizae might have been slowed. Studies with the growth pouch technique have demonstrated that the ontogenetic development of the *P. abies* - *H. crustuliniforme* ectomycorrhizae follows a characteristic pattern of steps (Brunner and Scheidegger, 1992). First, emanating hyphae contact root hairs and root surfaces, and later their morphology alters immediately after contact to become short-celled with few clamp connections, highly branched, and unornamented. Second, hyphae on the root surfaces form an inner synenchymatous mantle. A loose hyphal envelope forms as well, constituting an outer prosenchymatous mantle. Simultaneously, root cap cells become invaded and penetrated by fungal hyphae and their remnants are incorporated into the inner mantle. Third, a Hartig net begins to form in basal portions of developing ectomycorrhizae and the fungal mantle becomes more dense and thick. Fourth, growth of the root slows down and hyphae of the Hartig net invade the cortex toward the meristematic apex. This whole process may take

about six weeks. As a result, ectomycorrhizal development in the flasks with porous glass balls and the cuvettes was probably still in the third step and was not fully completed at the time of harvest. Reasons for this delay might be the high pH due to a loss of alkaline siliceous residues of the substrate (Erlenmeyer flasks with porous glass balls) and the relatively short synthesis duration (cuvettes).

Many features of ectomycorrhizae are dynamic and change gradually (e.g. thickness and color of mantles, formation of rhizomorphs) with the developmental stage (Egli and Kälin, 1990). Other factors in addition to the age of a symbiosis which might be decisive for the development of certain morphological characters include gas exchange, fungal strains, temperature, moisture or irradiation (Marks and Foster, 1973). However, the use of closed *versus* open culture vessels did not influence ectomycorrhizal morphology significantly in the present study. Also the absence of the complex interactions in soil ecosystems between other fungi, bacteria, animals, and roots might not influence the morphology distinctly since synthesized *versus* naturally grown ectomycorrhizae had a similar morphology as shown in the present study and stated earlier by others (Kottke, 1986; Weiss, 1988).

One technique suitable for producing ectomycorrhizal seedlings for further experimentation is in Erlenmeyer flasks using vermiculite and peat moss as a substrate. This is due to the sterility of the material and the formation of ectomycorrhizae in approximately two months. Unfortunately, direct observation of the ontogenetic stage of the symbioses is not possible using this method, and experience must show when the trees are ready to harvest. In addition, removal of the ectomycorrhizal seedlings must be done carefully, and damage to ectomycorrhizae and roots can still occur. Extramatrical mycelium will likely be torn from ectomycorrhizal rootlets. The growth pouch technique is also suitable for production of large numbers of ectomycorrhizal seedlings. In contrast to seedlings grown in flasks, roots, ectomycorrhizae, and extramatrical mycelium can be removed from the growth pouches more or less completely. Direct observation of ectomycorrhizal development allows pinpointing of the best moment of sampling the seedlings. Formation of ectomycorrhizae takes approximately one to two months. A disadvantage is the infertility of the system.

Acknowledgements

I thank Mrs. M. Zollinger for assistance in synthesizing the material, Mrs. B. Schneider for sectioning and staining the ectomycorrhizas, Dr. C. Scheidegger for freeze-fracturing of the rootlets, and Mrs. M. J. Sieber for correcting the English text.

References

- Agerer, R. (1986) 'Studies on ectomycorrhizae. II. Introducing remarks on characterization and identification', *Mycotaxon* **24**, 473-492.
- Andersen, C.P. and Rygiewicz, P.T. (1991) 'Stress interactions and mycorrhizal plant response: Understanding carbon allocation priorities', *Environm. Pollut.* **73**, 217-244.
- Blaschke, H. (1986) 'Vergleichende Untersuchungen über die Entwicklung mykorrhizierter Feinwurzeln von Fichten in Waldschadensgebieten', *Forstw. Cbl.* **105**, 477-487.
- Blaschke, H. and Weiss, M. (1990) 'Impact of ozone, acid mist and soil characteristics on growth and development of fine roots and ectomycorrhiza of young clonal Norway spruce', *Environm. Pollut.* **64**, 255-263.
- Brunner, I. (1991) 'Comparative studies on ectomycorrhizae synthesized with various *in vitro* techniques using *Picea abies* and two *Hebeloma* species', *Trees* **5**, 90-94.
- Brunner, I., Amiet, R. and Schneider, B. (1991) 'Characterization of naturally grown and *in vitro* synthesized ectomycorrhizas of *Hebeloma crustuliniforme* and *Picea abies*', *Mycol. Res.* **95**, 1407-1413.
- Brunner, I., Brunner, F. and Miller, O.K. Jr. (1990) 'Ectomycorrhizal synthesis with Alaskan *Alnus tenuifolia*', *Can. J. Bot.* **68**, 761-767.

- Brunner, I. and Scheidegger, C. (1992) 'Ontogeny of synthesized *Picea abies* (L.) Karst. - *Hebeloma crustuliniforme* (Bull. ex. St. Amans) Quéf. ectomycorrhizas', *New Phytol.* **120**, 359-369.
- Clark, G. (1981) 'Staining procedures', Williams and Wilkins, Baltimore.
- Dighton, J. and Jansen, A.E. (1991) 'Atmospheric pollutants and ectomycorrhizae: More questions than answers', *Environm. Pollut.* **73**, 179-204.
- Egli, S. and Kälin, I. (1990) 'The root window - a technique for observing mycorrhizae on living trees', *Agric. Ecosystems Environ.* **28**, 107-110.
- Fortin, J.A., Piché, Y. and Godbout, C. (1983) 'Methods for synthesizing ectomycorrhizas and their effect on mycorrhizal development', *Plant Soil* **71**, 275-284.
- Fortin, J.A., Piché, Y. and Lalonde, M. (1980) 'Techniques for the observation of early morphological changes during ectomycorrhiza formation', *Can. J. Bot.* **58**, 361-365.
- Harley, J.L. and Smith, S.E. (1983) 'Mycorrhizal symbiosis', Academic Press, London.
- Jentschke, G., Fritz, E. and Godbold, D.L. (1991a) 'Distribution of lead in mycorrhizal and non-mycorrhizal Norway spruce seedlings', *Physiol. Plant.* **81**, 417-422.
- Jentschke, G., Godbold, D.L. and Hüttermann, A. (1991b) 'Culture of mycorrhizal tree seedlings under controlled conditions: Effects of nitrogen and aluminium', *Physiol. Plant.* **81**, 408-416.
- Kottke, I. (1986) 'Charakterisierung und Identifizierung von Mykorrhizen', in G. Einsele (ed.), Das landschaftsökologische Forschungsprojekt Naturpark Schönbuch, Deutsche Forschungsgemeinschaft, Forschungsbericht, Weinheim, pp. 463-485.
- Kottke, I. and Oberwinkler, F. (1986) 'Root-fungus association observed on initial stages of mantle formation and Hartig net establishment in mycorrhizas of *Amanita muscaria* on *Picea abies* in pure culture', *Can. J. Bot.* **64**, 2348-2354.
- Marks, G.C. and Foster, R.C. (1973) 'Structure, morphogenesis, and ultrastructure of ectomycorrhizae', in G.C. Marks and T.T. Kozlowski (eds.), Ectomycorrhizae, their ecology and physiology, Academic Press, New York, pp. 1-41.
- Marx, D.H. and Bryan, W.C. (1975) 'Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*', *For. Sci.* **21**, 245-254.
- Massicotte, H.B., Peterson, R.L., Ackerley, C.A. and Melville, L.H. (1990) 'Structure and ontogeny of *Betula alleghaniensis*-*Pisolithus tinctorius* ectomycorrhizae', *Can. J. Bot.* **68**, 579-593.
- Melin, E. (1922) 'Untersuchungen über die *Larix*-Mykorrhiza. I: Synthese der Mykorrhiza in Reinkultur', *Svensk Bot. Tidskr.* **16**, 161-196.
- Meyer, F.H. (1987) 'Der Verzweigungsindex, ein Indikator für Schäden am Feinwurzelsystem', *Forstw. Cbl.* **106**, 84-92.
- Miller, D.D., Bodmer, M. and Schüepp, H. (1989) 'Spread of endomycorrhizal colonization and effects on growth of apple seedlings', *New Phytol.* **111**, 51-59.
- Müller, T., Guggenheim, G., Duggelin, M. and Scheidegger, C. (1991) 'Freeze-fracturing for conventional and field emission low-temperature scanning electron microscopy: the scanning cryo unit SCU 020', *J. Microsc.* **161**, 73-83.
- Ochs, M., Brunner, I., Stumm, W. and Cosovic, B. (1992) 'Effects of root exudates and humic substances on weathering kinetics', *Water, Air, and Soil Pollut.* (this issue).
- Scheidegger, C., Günthardt-Goerg, M., Matyssek, R. and Hatvani, P. (1991) 'Low-temperature scanning electron microscopy of birch leaves after exposure to ozone', *J. Microsc.* **161**, 85-95.
- Schüepp, H., Miller, D.D. and Bodmer, M. (1987) 'A new technique for monitoring hyphal growth of vesicular-arbuscular mycorrhizal fungi through soil', *Trans. Br. Mycol. Soc.* **89**, 429-435.
- Weiss, M. (1988) 'Ektomykorrhizen von *Picea abies*: Synthese, Ontogenie und Reaktion auf Umweltschadstoffe', Dissertation, München.
- Wöllmer, H. and Kottke, I. (1990) 'Fine root studies *in situ* and in the laboratory', *Environm. Pollut.* **68**, 383-407.