CHAPTER 14

ROOT INDUCTION OF *PINUS SYLVESTRIS* L. HYPOCOTYL CUTTINGS USING SPECIFIC ECTOMYCORRHIZAL FUNGI *IN VITRO*

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1. INTRODUCTION

Scots pine (*Pinus sylvestris* L.) is one of the most widely distributed coniferous species in the world, with a natural range that stretches from Spain to large areas in Siberia (Sarvas, 1964). Genetic improvement of Scots pine by means of conventional breeding is hampered by the long generation time of the species, which is characterized by progressive transition from juvenile to the reproductive mature phase.

Traditionally, the production of clonal material for Scots pine has been based on grafting. However, in seed orchards established using grafting it takes more than 15 years to produce a sufficient number of seeds (Antola, 1990). In vitro micropropagation of Scots pine using axillary buds induced on seedlings (Supriyanto & Rohr, 1994) or cotyledons excised from germinated embryos as explants has succeeded at the research level (Häggman et al., 1996). However, in practice the rooting phase has proven problematic particularly because of genotypic variation in the ability to form roots and of increased potential for plagiotrophic growth (Häggman et al., 1996).

Formation of adventitious roots on Scots pine has been studied using hypocotyl cuttings in vitro. Depending on the experimental condition, less than 50% of the non-treated cuttings form roots during a 2 months culture period. Treatments with auxin (Grönroos & von Arnold, 1988) and activated charcoal (Grönroos & von Arnold, 1985) can result in both faster rooting and higher rooting frequencies. However, although these methods can induce rooting, results can be variable depending on the culture medium and on timing and concentration of auxin application. Root elongation is also inhibited under some culture conditions, even though the number of cuttings with roots is increased.

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In nature, Scots pine lives in symbiosis with specific ectomycorrhizal (ECM) fungi. In this unique interaction, nutrients taken up by the fungus are exchanged for carbohydrates derived from the host plant. The fungi may also release specific plant growth regulators usable to plant roots. The fungal hyphae form a mantle around the short roots of Scots pine and also penetrate between epidermal and cortical cells forming a highly branched structure called a Hartig net (Smith & Read, 1997). The structure of plant roots is strongly modified by ECM symbiosis. Establishment of symbiosis inhibits root hair elongation (Béguiristain & Lapeyrie, 1997; Ditengou et al., 2000) but, conversely, it can stimulate the formation of lateral roots (Karabaghli-Degron et al., 1998; Tranvan et al., 2000; Niemi et al., 2006). In Scots pine, as in other pine species, mycorrhizas are characterized by dichotomous branching of the short roots (Duddridge & Read, 1984; Kaska et al., 1999).

The importance of ECM fungi in the growth and morphology of the roots has resulted in increased interest to use them as promoting agents in adventitious rooting (Niemi et al., 2004). We have developed an in vitro method to induce adventitious root formation in Scots pine hypocotyl cuttings by inoculating them with specific ECM fungi (Niemi et al., 2002a,b). This method has a potential to be used for root induction of micropropagated shoots in vitro. Our method describes the use of specific ECM fungi (*Pisolithus tinctorius* and *Paxillus involutus*) for root induction on Scots pine and may need modifications for use with other host and ECM fungus combinations.

2. EXPERIMENTAL PROTOCOL

2.1. Material

1. Scots pine (*Pinus sylvestris* L.) seeds.
2. Fungal mycelium of the ECM fungi *Pisolithus tinctorius* and *Paxillus involutus.*
3. Sterile water and calcium hypochlorite [$Ca(OCl)_2$].
4 Sterile filter paper (Schleicher and Schuell 595), glass jars (150 mL), Petri dishes (9 and 14 cm Ø), parafilm.

2.1.1. Sterile Culture Media

Germination of seeds. 0.7% water agar.

Media for fungal cultures. Modified Melin Norkrans (MMN1) medium (Marx, 1969) supplemented with 10 g L^{-1} glucose and 1.5% (w/v) agar according to Heinonen-Tanski & Holopainen (1991) (Table 1). The pH of the medium is adjusted to 5.8 with 1 N NaOH before autoclaving.

Media for rooting of hypocotyl cuttings. Modified Melin Norkrans medium (MMN2) (Marx, 1969) that differs from MMN1 medium containing 200 mg L^{-1} glucose and 2% (w/v) agar, and 250 mg L^{-1} $(NH_4)_2HPO_4$ instead of NH_4Cl. The pH of the medium is adjusted with 1 M HCl to 5.7 before autoclaving.

Table 1. *Composition of Melin Norkrans medium (MMN1) as modified by Heinonen-Tanski and Holopainen (1991).*

Component	mg L^{-1}
KH_2PO_4	500
NH_4Cl	250
$CaCl_2 \times 2\ H_2O$	66
NaCl	25
$MgSO_4 \times 7H_2O$	150
Thiamine HCl	0.1
$FeCl_3 \times 6H_2O$	0.8

2.2. Method

All the steps are carried out in aseptic conditions in a laminar flow hood. Steps 2.2.1. and 2.2.2. should be started approximately the same date.

2.2.1. Sterilization and Germination of Seeds

1. Surface-sterilize seeds for 20 min in 100 mL of 2% (w/v) [$Ca(OCl)_2$] solution.
2. Rinse seeds 10 times with sterile water.
3. Transfer seeds with sterile tweezers onto 0.7% water agar in glass jars.
4. Germinate seeds for 3 weeks in a growth chamber at 24 ± 2°C providing a 16-h photoperiod (140–150 µmol m^{-2} s^{-1}).

2.2.2. Cultivation of Fungal Mycelium

1. Cut an agar plug of mycelium (5 mm Ø) from the edge of a culture of the ECM fungi *Pisolithus tinctorius* or *Paxillus involutus*.
2. Place mycelial agar plugs onto MMN1 agar in petri dishes (9 cm Ø) and seal dishes with parafilm.
3. Cultivate mycelia for 3 weeks at 21°C in darkness.

2.2.3. Inoculation of Hypocotyl Cuttings

1. Cover the surface of the MMN2 agar with moist sterile filter paper (14 or 9 cm Ø depending on the size of the hypocotyl).
2. Prepare hypocotyl cuttings from 3-week-old seedlings by cutting the stem approximately 5 mm above the root collar.
3. Lay the cutting horizontally on the filter paper covering the surface of the agar (Figure 1).
4. Cut a mycelial agar plug (5 mm Ø) from the edge of the fungal culture and place it beside the base of the cutting (Figure 1).
5. Cover the fungal mycelium and the base of the cutting with a semicircular piece of moist sterile filter paper. This will help prevent desiccation.
6. Seal the petri dishes with parafilm, place them at approximately a 70-degree slant and incubate in the growth chamber under the conditions described for seed germination (2.2.1. above).

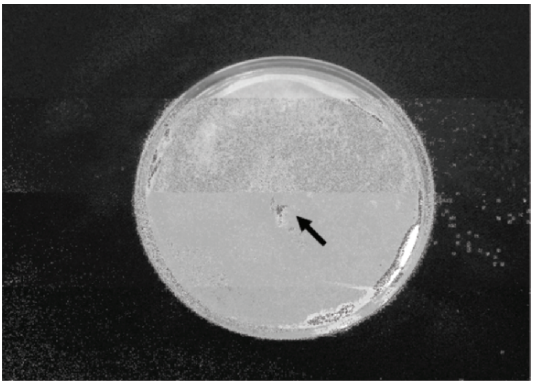

Figure 1. *Hypocotyl cutting of Scots pine is laid horizontally on MMN rooting medium covered by filter paper and inoculated with the mycelial agar plug (arrow).*

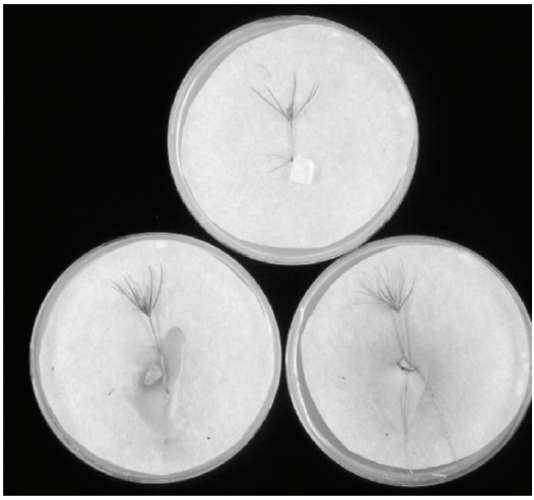

Figure 2. *Rooted hypocotyl cuttings of Scots pine after 6 weeks on MMN2 medium. Pisolithus tinctorius (bottom left) and Paxillus involutus (bottom right) have induced elongation of adventitious roots and primary needles compared to non-inoculated cutting (top center).*

Adventitious roots will appear after 2–3 weeks. After 6 weeks, under these incubation conditions, approximately 40% of the non-inoculated cuttings and 80–100% of cuttings inoculated with *Pisolithus tinctorius* or *Paxillus involutus* will have formed roots. The fungi will also enhance elongation of adventitious roots and primary needles (Figure 2). Indications of mycorrhiza formation include the hyphal mantle covering short roots (Figure 3A,B) and penetration of hyphae between root cortical cells (Figure 3B).

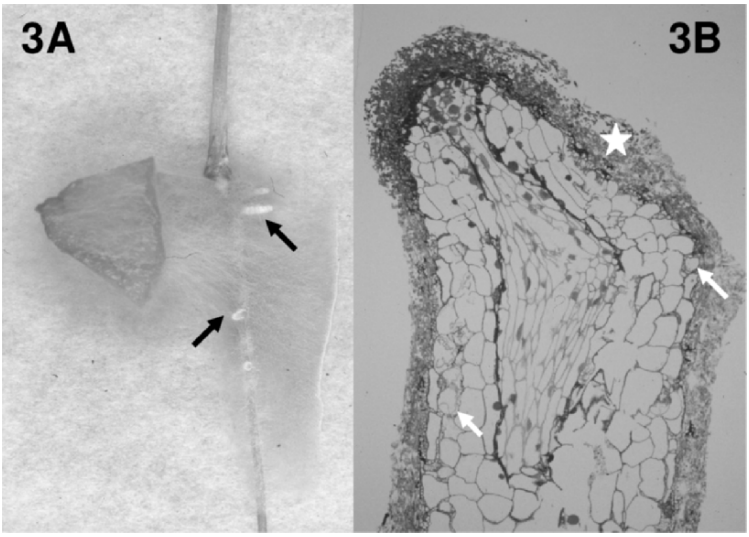

Figure 3. *A) The hyphae of P. tinctorius cover lateral roots as a mantle (arrows). B) A light micrograph of the lateral root that has started to branch dichotomously due to P. tinctorius. The fungal hyphae cover a lateral root (star) and penetrate between epidermal cells (arrows).*

3. CONCLUSIONS

Ectomycorrhizal fungi, such as *Pisolithus tinctorius*, can be used to promote adventitious rooting in Scots pine hypocotyl cuttings using the protocol outlined above. This protocol may need modifications for use with other host and ECM fungus combinations. Other ECM as well as arbuscular mycorrhizal fungi and ericoid mycorrhizal fungi have also been shown to improve adventitious root formation. Current and future research on the hormonal interactions between ECM fungi and their host plants will help to elucidate the mechanisms underlying this rooting response to ECM inoculation. Information from this research will be useful for optimizing adventitious root formation during tissue culture and conventional cutting propagation.

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