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KAROLIINA NIEMI

Ectomycorrhizal Fungi and Exogenous Growth Regulators in Vegetative Propagation of Scots Pine *(Pinus sylvestris L.)*

Doctoral dissertation

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ABSTRACT

Background Methods applicable to the vegetative propagation of Scots pine (*Pinus sylvestris* L.) are grafting and the production of *in vivo* cuttings, as well as *in vitro* organogenesis and somatic embryogenesis. Low and genotype-dependent rooting hampers cutting production and organogenesis, while precocious maturation and difficulties in germination and subsequent acclimatisation *ex vitro* are the main obstacles in somatic embryogenesis.

Symbiotic ectomycorrhizal (ECM) fungi have been proposed to be possible agents to stimulate rooting of conifers. This is mostly due to the ability of several fungi to produce and secrete indole-3-acetic acid (IAA). Evidence for the involvement of polyamines in the fungus–root interaction has also recently started to accumulate.

Materials and methods The ability of specific ECM fungi to produce IAA and polyamines *in vitro* and to affect formation and growth of the roots on *in vitro* and *in vivo* Scots pine cuttings was studied. The role of exogenous indole-3-butyric acid (IBA), the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA), as well as different polyamines, in the fungus-plant interaction during root and mycorrhiza formation was also investigated.

The interaction between ECM fungi and somatic embryogenesis of Scots pine was studied during proliferation, maturation and conversion. To specify the role of polyamines in the differentiation and maturation of somatic embryos, the maturation media were supplemented with exogenous spermidine and a putative inhibitor of spermidine synthesis.

Results Inoculation increased the rooting and subsequent root growth of *in vitro* hypocotyl cuttings. The rooting responses of the *in vivo* cuttings to ECM fungi were more complicated and highly dependent on the fungus and plant genotypes. ECM fungi varied greatly in their ability to produce IAA and polyamines *in vitro*. Although both auxins and polyamines were involved in the root–fungus interaction, IAA or polyamine concentrations in the culture filtrate did not correlate with the rooting percentages of the inoculated cuttings. Root stimulation was not related to mycorrhizal activity of the fungus either.

The reactions of the proliferating embryogenic cell lines to inoculation depended on the fungus and Scots pine genotypes. Germination of the somatic embryos and subsequent acclimatisation to the *ex vitro* conditions was stimulated by the ECM fungus *Pisolithus tinctorius*. The fungus was also able to form mycorrhizas with somatic embryo plants *in vitro*. Exogenous spermidine and the inhibitor of spermidine synthesis, in particular, stimulated embryo development of the cell line studied, and this stimulation was associated with reduced proliferation growth of the embryogenic cultures and decreased concentration of endogenous free polyamines.

Conclusion Application of ECM fungi was beneficial in the adventitious rooting of *in vitro* and *in vivo* cuttings and the germination of somatic embryos. The positive effects of the fungi on root development were not completely explained by their ability to produce IAA or certain polyamines, or their ability to form mycorrhizal structures, but the interaction was highly dependent on both the Scots pine and fungus genotypes. This specificity allows studies to be carried out on genotype-dependent factors relating to the fungus-root interaction, and sets a considerable challenge on the practical use of ECM fungi in vegetative propagation.

Universal Decimal Classification: 582.47, 631.466, 631.532, 581.14, 577.175.1
CAB Thesaurus: *Pinus sylvestris*; ectomycorrhizas; plant growth regulators; auxins; polyamines; needle fascicles; adventitious roots; rooting; somatic embryogenesis; vegetative propagation; acclimatization

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Karoliina Niemi

ABBREVIATIONS

ABA	abscisic acid
BA	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
DCR	tissue culture medium developed for Douglas-fir
Dw	dry weight
ECM	ectomycorrhiza/ectomycorrhizal
Fw1	fresh weight at the beginning of the experiment
Fw2	fresh weight at the time of harvest
Fw2/Fw1	average growth ratio
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
MES	2-(*N*-morpholino)ethanesulfonic acid
MGBG	methylglyoxal bis(guanylhydrazone)
MMN	modified Melin–Norkrans medium
NAA	1-naphthaleneacetic acid
NPA	N-(naphthyl)phthalamic acid
PEG	polyethylene glycol
SAM	S-adenosylmethionine
SEM	scanning electron microscope
TIBA	2,3,5-triiodobenzoic acid
VAM	vesicular-arbuscular mycorrhiza

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers referred to in the text by Roman numerals **I-VI**. Additional unpublished data is also presented.

I Niemi, K., Salonen, M., Ernstsen, A., Heinonen-Tanski, H. & Häggman, H. 2000. Application of ectomycorrhizal fungi in rooting of Scots pine fascicular shoots. *Canadian Journal of Forest Research* 30: 1221-1230.

II Niemi, K., Vuorinen, T., Ernstsen, A. & Häggman, H. 2002. Ectomycorrhizal fungi and auxins in root formation on *in vitro* Scots pine hypocotyl cuttings. *Submitted.*

III Niemi, K., Häggman, H. & Sarjala, T. 2002. Effects of diamines on the interaction between ectomycorrhizal fungi and adventitious root formation on Scots pine *in vitro*. *Tree Physiology* (in press).

IV Niemi, K., Krajnakova, J. & Häggman, H. 1998. Interaction between embryogenic cultures of Scots pine and ectomycorrhizal fungi. *Mycorrhiza* 8: 101-107.

V Niemi, K., Sarjala, T., Chen, X. & Häggman, H. 2002. Spermidine and methylglyoxal bis(guanylhydrazone) affect maturation and endogenous polyamine content of Scots pine embryogenic cultures. *Submitted.*

VI Niemi, K. & Häggman, H. 2002. *Pisolithus tinctorius* promotes root development and further forms mycorrhizal structures in Scots pine somatic embryos *in vitro*. *Submitted.*

CONTENTS

1 INTRODUCTION

1.1 Importance of Scots pine

Scots pine (*Pinus sylvestris* L.) is the most widely distributed conifer in the world, with a natural range that stretches from Spain to large areas in Siberia (Sarvas 1964). In Finland, where the species is dominant on more than 60 % of the forest land area (Finnish Statistical Yearbook of Forestry, 2000), its distribution extends up to North-Lapland. Furthermore, Scots pine is successfully cultivated outside its natural distribution, for example, in France, North-West Germany and North America.

Scots pine is an important raw material for the building, furniture and joinery industries, as well as for paper production. Its importance for the forest industry, and concern about the loss of genetic diversity due to intensive logging, were the main reasons for initiating breeding programmes for Scots pine. In Finland, the first plus trees were selected immediately after the Second World War, and the establishment of seed orchards for the production of seeds with high genetic and physiological quality was started somewhat later (Sarvas 1953; Oskarsson 1995). Through traditional breeding based on phenotypic selection, both the viability and growth of Scots pine have been increased, and wood quality has been expected to improve (Mikola 1992; Venäläinen et al. 1994). At the same time, high genetic diversity of the bred material has been maintained successfully (Muona and Harju 1989).

Genetic improvement of Scots pine by means of conventional breeding is hampered by the long generation time of the species, which is characterised by progressive transition from juvenile to the reproductive mature phase. In natural stands, Scots pine starts to produce seeds at the age of 8 to 20 years depending on the individual (Sarvas 1964), and in seed orchards it still takes more than 15 years before a sufficient number of seeds is produced (Antola 1990). Therefore, techniques such as marker-aided selection and gene engineering, combined with effective vegetative propagation methods, are needed in order to accelerate breeding of Scots pine.

1.2 Vegetative propagation of Scots pine

1.2.1 Propagation methods applicable to Scots pine

Traditionally, the production of clone material for pines has been based on grafting (Slee and Spidy 1970; Sweet and Thulin 1973; Bhumibhamon 1978). For example, all the Scots pine seed orchards in Finland have been established using grafts (Bhumibhamon 1978). Grafting avoids the problematic rooting phase but, on the other hand, there may be incompatibility between stock and scions (Slee and Spidy 1970; Sweet and Thulin 1973). Furthermore, the high expense and need for specific skills make only small-scale propagation possible.

The applicability of *in vivo* cutting production and *in vitro* methods (organogenesis and somatic embryogenesis) to the vegetative propagation of Scots pine has been intensively studied. Both organogenesis (e.g. Jain et al. 1988; Chalupa 1989; Supriyanto and Rohr 1994; Häggman et al. 1996) and *in vivo* cutting production (e.g. Salonen 1990; Aronen et al. 1996) are based on separate induction of shoots and adventitious roots. In practise, the rooting phase has proved problematic particularly because the ability of the genotypes to form roots varies considerably (Chalupa 1989; Salonen 1990; Aronen et al. 1996; Häggman et al. 1996). The rooted plants may subsequently suffer from a plagiotropic growth habit (Aronen et al. 1996; Häggman et al. 1996). Despite this, both methods have proved applicable to Scots pine. For example, some genotypes produced via organogenesis have shown a dramatically shortened juvenile phase, and started to form megastrobili and microstrobili already at the ages of three and four years, respectively (Häggman et al. 1996).

Production of somatic embryos, i.e. embryos from somatic (vegetative) cells, has the most potential for mass propagation of the genotypes with important traits. The formation of somatic embryos with shoot and root apices occurs as a result of development phases characteristic of zygotic embryogenesis (reviewed by Tautorus et al. 1991). Somatic embryogenesis has been reported to succeed more or less completely in several pine species (e.g. Gupta and Durzan 1987; Lainé and David 1990; Jones and van Staden 1995; Klimaszewska and Smith 1997), and this is also the case with Scots pine (Hohtola 1995; Keinonen-Mettälä et al. 1996; Häggman et al. 1999; Lelu et al. 1999). However, there are still great problems in all the development phases, maturation and conversion placing the major limits on the development of somatic embryogenesis.

In this review of the literature, attention is focused on *in vivo* cutting production and somatic embryogenesis. In the chapters dealing with factors relating to the adventitious rooting of cuttings and application of ectomycorrhizal (ECM) fungi in adventitious rooting, reference is also made to the literature on organogenesis.

1.2.2 *In vivo* cutting production

1.2.2.1 Plant growth regulators involved in adventitious rooting

Indole-3-acetic acid (IAA) is the best-known natural auxin in higher plants. It does not only occur in free form, but also as amide and ester conjugates. It has been suggested that these biologically inactive forms serve as a storage of IAA, from which free, active IAA can be released by hydrolysis (Hangarter and Good 1981; Sztein et al. 2000). The activity of IAA in plant development depends on the ratio of IAA to other plant growth regulators, the sensitivity of cells to IAA, as well as IAA concentration gradient due to transport (reviewed by Normanly 1997). IAA and other auxins are transported in plants by means of mass flow in the vascular tissues and an energy-requiring polar transport system. Specific inhibitors, such as 2,3,5-triiodobenzoic acid (TIBA) and N-(naphthyl)phthalamic acid (NPA) (Lomax et al. 1995), can

prevent auxin polar transport. However, TIBA for example may also affect ion absorption and respiratory metabolism in treated tissues, which complicates its use in studies on IAA transport (Jacobson and Jacobson 1981).

Auxins affect the development and growth of roots in two distinct ways. On the one hand they promote lateral root formation (Baser et al. 1987; Karabaghli-Degron et al. 1998; Kaska et al. 1999), while on the other hand they inhibit root elongation (Karabaghli-Degron et al. 1998; Ditengou and Lapeyrie 2000). Adventitious root formation is characterised by auxin-mediated enhancement of root induction, but subsequent root emergence and elongation may be prevented if the auxin treatment is continued (reviewed by Blakesley et al. 1991b). These interdependent phases of the rooting process appear to follow the changes in the endogenous IAA pool: free IAA accumulates at the base of the shoot before the first root initiation events and, the subsequent decrease in the concentration of IAA leads to early cell divisions and organisation of root primordia (e.g. Heloir et al. 1996; Hausman et al. 1997; Nag et al. 2001). Reduction in the endogenous cytokinin concentration concomitantly with IAA accumulation, as reviewed by Blakesley (1994), and inhibition of the rooting process due to exogenous cytokinin (McCown 1988) suggest that an increase in the auxin/cytokinin ratio is associated with successful rooting.

The polyamines spermidine and spermine, and their precursor diamine putrescine (referred to here as a polyamine), are molecules with positive charges at physiological pH, and therefore may interact with negatively charged nucleic acids, phospholipids and proteins. Polyamines, which occur in relatively high concentrations in plant cells compared to, for example, auxins and cytokinins, have been implicated in the regulation of growth and morphogenesis, including root formation (reviewed by Galston and Flores 1991; Bagni et al 1993). Putrescine appears to play an essential role in root induction because its concentration increases soon after the transfer of the *in vitro* shoots to rooting medium containing indole-3-butyric acid (IBA, an auxin that resembles IAA) (Hausman et al. 1994; Kevers et al. 1997; Nag et al. 2001). Furthermore, supplementation of putrescine into the medium has promoted root formation even in the absence of exogenous auxin (Hausman et al. 1994; Kevers et al. 1997).

1.2.2.2 Shoot induction

Both long (e.g. Boeijink and van Broekhuizen 1974; Hare 1974; Browne et al. 1997a; Fries and Kaya 1997) and fascicular shoots (e.g. Boeijink and van Broekhuizen 1974; Kossuth 1978; Salonen 1990; Aronen et al. 1996; Browne et al. 1997b; Browne et al. 2000) are suitable material for *in vivo* cutting production of pines. The formation of needle fascicles (also referred to as needle bundles, dwarf shoots, spur shoots, short shoots) is a characteristic vegetative feature of pine (*Pinus* spp.) species. These shoots, which have reduced growth, consist of scales, a short stem and needles and a small bud between the needles (Sarvas 1964). This bud can be induced to elongate, and finally used as a cutting (here referred to as a fascicular shoot). Buds are induced by means of pruning (Boeijink and van Broekhuizen 1974; Browne et al.

1997b; Browne et al. 2000) or cytokinin spraying (Kossuth 1978; Salonen 1990; Aronen et al. 1996) and, as a result of successful treatment, considerably more cuttings can be obtained than when using long shoots. A relatively low concentration of fertilisers and regulation of the light conditions may further enhance fascicular shoot formation, whereas cold treatment of the donor plant may increase shoot elongation (Browne et al. 2000).

1.2.2.3 Adventitious root formation of shoots

Application of exogenous auxin, usually in the form of IBA or 1-napthaleneacetic acid (NAA), is a widely successful practise in the rooting of *in vitro* and *in vivo* pine cuttings. Improved rooting due to auxins can be seen as an increased number of shoots with roots and/or of roots per shoot (e.g. Hare 1974; Grönroos and von Arnold 1988; Salonen 1990; Greenwood and Weir 1995; Diaz-Sala et al. 1996; Browne et al. 1997a; Goldfarb et al. 1998; Browne et al. 2000). *In vitro* hypocotyl cuttings treated with auxins have also rooted more rapidly than non-treated ones (Grönroos and von Arnold 1988; Greenwood and Weir 1995; Goldfarb et al. 1998). Root formation of the *in vivo* cuttings can be further affected by pre-harvest treatment of the donor plants. With jack pine (*Pinus banksiana* Lamb.), growing donor plants at a relatively low fertiliser level followed by 2-6 weeks' cold storage resulted in increased rooting potential of cuttings (Browne et al. 2000).

Despite the improvements in root formation methods, the problems related to the rooting phase are still the biggest obstacle to the mass production of clone material with pine cuttings. High variation among genotypes in their ability to form roots has proved to be the biggest problem (Boeijink and van Broekhuizen 1974; Salonen 1990; Greenwood and Weir 1995; Fries and Kaya 1997; Aronen et al. 1996; Browne et al. 1997a). It has been proposed that the differences between genotypes are due to the different number of cells able to respond to auxin (Greenwood and Weir 1995), or of rooting-specific receptor sites in the cells (Greenwood and Weir 1995; Greenwood et al. 2001). However, the rooting potential of plants with good responses to auxin also decreases when the donor plant ages (Diaz-Sala et al. 1996; Browne et al. 1997a; Greenwood et al. 2001). The decline, which is reflected in the number of rooted cuttings, as well as rooting time and size (reviewed by Hacket 1988), may eliminate vegetative propagation of desired individuals (reviewed by Greenwood 1987).

1.2.3 Somatic embryogenesis

1.2.3.1 Initiation and proliferation

As with other pine species, embryogenic cultures of Scots pine are usually derived from megagametophyte explants containing precotyledonary-stage embryos with their suspensor cells. On the initiation medium, the isolated megagametophytes are induced to extrude a translucent to light yellow, mucilaginous embryogenic cell mass, which consists of immature somatic embryos with the embryogenic head cell area and suspensor cells. This embryogenic

cell mass proliferates and can be subcultured under low osmotic conditions (Keinonen-Mettälä 1996; Sarjala et al. 1997; Häggman et al. 1999; Lelu et al. 1999). Successful initiation of embryogenic cultures using mature embryos as explants has been reported only once for Scots pine (Hohtola 1995).

The fact that precotyledonary-stage embryos are potentially the best for somatic embryogenesis of pines limits the initiation period to only a few weeks per year (e.g. Becwar et al. 1990; Newton et al. 1995; Häggman et al. 1999; Lelu et al. 1999). Furthermore, initiation frequencies of the embryogenic cultures are low, typically from 1 to 5 % (reviewed by Becwar and Pullman 1995), and highly dependent on the cell line (i.e. genotype). In our previous study (Häggman et al. 1999), several seed families of Scots pine had a capacity to initiate somatic embryogenesis, but only 0.2 to 4 % of the megagametophyte explants within a single seed family produced proliferating embryogenic cell mass.

Media supplemented with both cytokinin (usually 6-benzylaminopurine; BA) and auxin (2,4-dichlorophenoxyacetic acid; 2,4-D) have proven more efficient for initiation and subsequent proliferation than those containing either of the growth regulators alone (Becwar et al. 1990; Jones et al. 1993; Häggman et al. 1999). Proliferation of embryogenic cultures of slash pine (*Pinus elliottii* Engelm.) was, however, improved when the concentration of both BA and 2,4-D was lowered from that used for initiation (Newton et al. 1995). Embryogenic cultures of some seed families of Scots pine proliferated better on the medium lacking all growth regulators (Lelu et al. 1999). Addition of exogenous polyamines to the proliferation medium had no clear effect on the growth of the embryogenic cultures of pines (Li and Huang 1996; Sarjala et al. 1997). However, relatively high concentrations of the polyamine synthesis inhibitors, methylglyoxal bis(guanylhydrazone) (MGBG) and dicyclohexylamine (DCHA), reduced growth and changed the endogenous concentration of polyamines in the embryogenic cell masses (Sarjala et al. 1997). DCHA caused changes in the ratios between putrescine and spermidine, whereas in the presence of MGBG, putrescine was always the main polyamine followed by spermidine and spermine, as in the control cultures. These results indicate that polyamines are involved in the regulation of proliferation growth in Scots pine embryogenic cultures, as earlier reported for several other species (reviewed by Minocha and Minocha 1995).

1.2.3.2 Maturation

Omitting auxin and cytokinin from the medium is necessary for the arrest of proliferation growth and development of the embryos. Therefore, the maturation phase is often preceded by cultivation of the proliferating embryogenic cell mass on the medium without growth regulators, and which is supplemented with activated charcoal to absorb any residual growth regulators. During embryo maturation, exogenous abscisic acid (ABA) is used to prevent proliferation, precocious germination and other abnormalities of somatic embryos in conifers (reviewed by Attree and Fowke 1993). Increased osmolality, concomitantly with the ABA

treatment, favours the early stages of embryo development. Osmotic stress is exerted by non-permeating polyethylene glycol (PEG) or permeating solutes such as mannitol or sorbitol, and by an increased concentration of sucrose or other sugars (Attree and Fowke 1993; Kaul 1995; Li et al. 1998; Garin et al. 2000). With eastern white pine (*Pinus strobus* L.) (Klimaszewska et al. 2000; Garin et al. 2000) and Scots pine (Lelu et al. 1999), the reduced water availability achieved by increasing the gel strength of the medium also promoted maturation. In general, it has been suggested that enhanced maturation under osmotic stress and ABA treatment is related to the increased accumulation of storage compounds, such as triglycerides and proteins, in the embryogenic cultures (reviewed by Attree and Fowke 1993).

The role of polyamines in the maturation of embryogenic cultures is still largely unknown, although transfer of the cultures from proliferation to maturation medium has caused dramatic changes in the endogenous polyamine pool (Santanen and Simola 1992; Minocha et al. 1993; Kong et al. 1998; Minocha et al. 1999). Several studies on conifers have shown that spermidine becomes the predominant polyamine instead of putrescine as embryo development proceeds (Santanen and Simola 1992; Kong et. al. 1998; Minocha et al. 1999). However, somatic embryos of spruces matured normally regardless of a decrease in spermidine concentration due to the low concentration of MGBG. Therefore, it is not known whether spermidine accumulation is a prerequisite for successful maturation (Santanen and Simola 1992; Kong et al. 1998).

Despite the considerable progress in culture techniques and media formulation for maturation, the transition of competent early-stage somatic embryos to mature ones is often incomplete. Therefore, the synchronised maturation of somatic embryos that would germinate is still one of the greatest challenges lying ahead in developing somatic embryogenesis applicable to pines, as well as other coniferous species (Timmis 1998).

1.2.3.3 Conversion

Conversion includes both germination of somatic embryos and subsequent acclimatisation of somatic embryo plants to greenhouse conditions. Mature somatic embryos are germinated on the medium lacking ABA with lowered concentrations of sugar and nutrients. This results in rapid utilization of storage compounds (Attree and Fowke 1993). Successful germination and subsequent growth are greatly dependent on the cell line and quality of the somatic embryos (e.g. Gupta 1995; Garin et al. 1998; Timmis 1998; Häggman et al. 1999; Lelu et al. 1999; Klimaszewska et al. 2000). In the studies of Lelu et al. (1999) and Klimaszewska et al. (2000), the media with an increased gel strength induced development of cotyledonary-stage embryos with a high germination frequency and plant quality, whereas embryos matured on softer media germinated poorly and had an abnormal phenotype. The incompletely maturating embryos may contain insufficient storage reserves or have low tolerance to desiccation, thus, making them incompetent for germination (Timmis 1998).

Following the first report on loblolly pine (*Pinus taeda* L.) (Gupta and Durzan 1987), the acclimatisation of somatic embryo plants to greenhouse conditions by means of a gradual drop in humidity has been attempted with several pine species (e.g. Handley et al. 1995; Newton et al. 1995; Garin et al. 1998; Häggman et al. 1999; Lelu et al. 1999). So far, the only pine species that have been tested in the field are loblolly pine (Becwar and Pullman 1995; Handley et al. 1995) and radiata pine (*Pinus radiata* Don.) (Walter et al. 1998). Large-scale production of radiata pine is already possible, and several clones with desired characters have been propagated using somatic embryos. However, to make the process economically feasible, subsequent multiplication of this species has to occur via cutting production with the somatic embryo-derived plants (Walter et al. 1998). The tests with loblolly pine have also proved promising because survival of somatic embryo seedlings in the field has been high, and the seedlings have phenotypically resembled those germinated from seeds (Becwar and Pullman 1995).

1.3 Mycorrhizal symbiosis of Scots pine

1.3.1 Character of mycorrhizas

In nature, Scots pine always lives in a mutualistic symbiotic association with specific soil fungi. In this unique interaction, called a mycorrhiza, nutrients (e.g. nitrogen and phosphorous) taken up by the fungus are exchanged for carbohydrates derived from the host plant (reviewed by Smith and Read 1997). Classically, mycorrhizas have been divided into several groups primarily based on the way in which the fungi colonise the root system or on the plant species involved (Peterson and Farquhar 1994; Smith and Read 1997). About 90 % of the known vascular plant species form vesicular-arbuscular mycorrhizas (VAM), whereas ectomycorrhizas (ECM) are formed by only 3 % of the species (Carlile and Watkinson 1994). However, the latter group is important in boreal and temperate forests because several economically important tree species from the families Pinaceae (e.g. genera of *Pinus*, *Picea* and *Larix*), Betulaceae and Fagaceae live in ECM symbiosis (Smith and Read 1997).

The number of fungal species involved in ECM association is high. The majority of the species belong to the most advanced groups of the Basidiomycetes (Boletales, Russulales, Telephorales), but there are also several ascomycetes and some zygomycetes. The host plant may have a broad fungus range, and several fungal species may exist concomitantly in the same root system (Smith and Read 1997). However, the specific interactions that occur between individuals in ECM symbiosis appear to depend on both the fungus and plant genotypes (Dixon et al. 1987; Tonkin et al. 1989; Debaud et al. 1995).

1.3.2 Ectomycorrhizas

The root system of pine species consists of distinctive types of root, which differ from each other in growth, structure and life span, as well as in their ability to form mycorrhizas. A main

root, which forms a supporting structure, shows continuous growth. Fine lateral roots (under 2 mm in diameter) differentiate either into long, distributive roots or into short roots with restricted growth. The short roots with a rounded apex and a maximum length of 1 cm are the main targets of colonisation by ECM fungi (Piche et al 1983; Brundrett et al.1990; Brundrett 1991). Colonisation of the main roots (Tranvan et al. 2000) or long roots (Wong et al. 1989) of pine seedlings is also possible.

Formation of a mycorrhiza on the developing short roots starts directly from the colonised parent root (Tranvan et al. 2000). In the case of a new or uncolonised portion of the root system, the hyphae present in the soil are attracted to grow towards the developing roots, after which a complicated series of recognition events is initiated (reviewed by Peterson and Farquhar 1994). Compatible interaction may result in intensive branching of the hyphae, but also in the fusion of hyphal tips (Jacobs et al. 1989). The hyphae first form a mantle (sheath), whose structure in mature mycorrhizas depends on the fungal symbiont (Fig. 1). The mantle has no large contact area with the soil, but it serves both as a site of nutrient and carbohydrate storage and as a mechanical protection against root pathogens (Smith and Read 1997). The hyphae extend out from the mantle into the soil and, in this way, enlarge the soil volume available for water and nutrient absorption (Smith and Read 1997; Agerer 2001). The hyphae of the inner part of the mantle penetrate between epidermal and cortical cells, and form a Hartig net (Fig. 1). This highly branched structure enables effective transfer of nutrients from the fungus to the root cells, and of carbohydrates in the opposite direction (Smith and Read 1997).

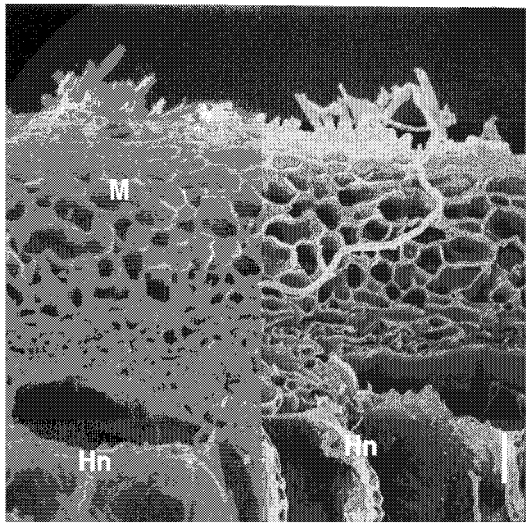

Figure 1. Structure of an ectomycorrhiza. Fungal hyphae cover the short root as a mantle (M) consisting of several cell layers. The hyphae penetrate from the inner part of the mantle into the intercellular space of the root and form a

The structure of plant roots is strongly modified by symbiosis: root hair elongation is inhibited (Béguiristain and Lapeyrie, 1997; Ditengou et al. 2000) but, on the other hand, the formation of lateral roots may be activated (e.g. Rupp et al. 1989; Karabaghli-Degron et al. 1998; Tranvan et al. 2000). The diameter of the short roots may also increase due to altered development of the cortical cells (Laurans et al. 2001). Mycorrhizal association between pines and specific fungi is characterised by dichotomous branching of the short roots which, after repeated branching, may lead to corralloid structures (Slankis 1973; Rupp et al. 1989; Kaska et al. 1999).

1.3.3 Fungal growth regulators related to root and ectomycorrhiza formation

It is now well documented that ECM fungi are able to produce a number of growth regulators *in vitro*. The production of free IAA has been most intensively studied (e.g. Ek et al. 1983; Gay 1986; Ho 1987; Rudawska and Gay 1989; Strzelczyk et al. 1992; Rudawska and Kieliszewska-Rokicka 1997; Scagel and Linderman 1998b), but there are also reports on the release of cytokinins and gibberellins (Strzelczyk and Pokojska-Burdziej 1984; Ho 1987; Strzelczyk et al. 1992), as well as ethylene (Graham and Linderman 1980; Scagel and Linderman 1998b). Synthesis of free IAA is highly dependent on the presence of an exogenous precursor, tryptophan (e.g. Strzelczyk and Pokojska-Burdziej 1984; Rudawska and Kieliszewska-Rokicka 1997; Karabaghli et al. 1998). Production and release of free IAA may also be affected by the pH of the medium (Rudawska et al. 1992; Strzelczyk et al. 1992) and availability of glucose (Gay 1986) or nitrogen (Rudawska and Gay 1989). However, the amount of IAA found in the culture filtrate not only reflects the synthesis of IAA but also its degradation, and therefore, variation between species and strains may also be related to the ability of the fungus to prevent IAA oxidation (Tomaszewski and Wojciechowska 1974).

Slankis (1973) was the first to postulate the relationship between fungal IAA and the morphology of pine mycorrhizas. This so-called hormone theory was based on the findings that exogenous IAA induced dichotomous branching of pine short roots and inhibited root hair proliferation, as occurs during mycorrhiza formation. Later, the use of an IAA-overproducing mutant of *Hebeloma cylindrosporum* Romagnesi made it possible to study the role of fungal IAA more specifically. In *in vitro* studies, the mutant formed more mycorrhizas than the corresponding wild type (Gay et al. 1994; Tranvan et al. 2000), the Hartig net formation was quicker (Tranvan et al. 2000), and the hyphae penetrated deeper. Intensive hyphal penetration by the mutant is thought to be due to modification of the cell wall structure in the cortex by fungal IAA (Gay et al. 1994, Gea et al. 1994; Laurans et al. 2001).

However, there are also several studies that criticise the classical hormone theory. For example, although Rudawska and Kieliszewska-Rokicka (1997) observed an overall positive correlation between the ability of *Paxillus involutus* (Batch.) Fr. strains to produce IAA and form mycorrhizas *in vitro*, some exceptional strains were also found. In a semi-hydroponic system, inoculation of Scots pine with the ECM fungus *Laccaria bicolor* (Maire) Orton resulted in mycorrhiza formation with a concomitant decrease in the concentration of free IAA in short

roots (Wallander et al. 1992; 1994). Under the same culture conditions, the IAA overproducing mutant of *H. cylindrosporum* did not differ from the wild type in regard to root modification or mycorrhiza formation (Nylund et al. 1994). These findings show that culture conditions have a significant impact on the interaction between fungal IAA and mycorrhiza formation. They also suggest that, in addition to IAA, several other factors characteristic of the specific fungal species or strain may regulate root morphology and mycorrhiza formation.

Scagel and Linderman (1998a; 1998b) studied whether *in vitro* IAA or ethylene production capacity of the ECM fungi is related to changes in the root morphology of three coniferous species. Although, the results proved to be specific for the interaction between certain fungi and tree species, a general positive correlation was found between *in vitro* ethylene production and several morphological responses in the seedlings (Scagel and Linderman 1998a; 1998b). Ethylene production also correlated with root IAA concentration, which may indicate that the influence of ethylene partly occurs via activation of IAA synthesis (Scagel and Linderman 1998b). On the other hand, several studies have demonstrated that ethylene is the main trigger of dichotomous branching, and IAA acts indirectly by inducing ethylene production (Rupp and Mudge 1985; Rupp et al. 1989, Kaska et al. 1999).

Ethylene synthesis is closely associated with polyamine metabolism because spermidine and spermine are derived from the same precursor, S-adenosylmethionine (SAM), as ethylene. In general, a high proportion of SAM is directed to polyamine synthesis in actively dividing cells, and ethylene synthesis gradually increases with increasing age of the cell (reviewed by Kushad and Dumbroff 1991). Information has recently started to accumulate about the production of polyamines by ECM fungi and the involvement of polyamines in the symbiotic interaction (Zarb and Walters 1994a; 1994b; Fornalé et al. 1999; Kytöviita and Sarjala 1997; Sarjala 1999). ECM fungi are able to release free polyamines into the culture medium *in vitro* (Fornalé et al. 1999; Sarjala 1999). Subsequently, formation of ECM symbiosis can result in changes in the total concentration of polyamines, and also in the ratios between individual polyamines in roots (Kytöviita and Sarjala 1997).

1.4 Ectomycorrhizal fungi in vegetative propagation

The importance of ECM fungi in plant growth has resulted in attempts to use them as promoting agents in vegetative propagation, especially in adventitious rooting. Inoculation with specific fungi has increased the rooting ability and/or subsequent root growth on both *in vitro* hypocotyl cuttings (Gay 1990; Normand et al. 1996; Karabaghli et al. 1998) and micropropagated shoots (David and Faye 1983; Supriyanto and Rohr 1994; Normand et al. 1996). The same positive response has been observed on cuttings *in vivo* (Linderman and Call 1977; Navratil and Rochon 1981). Furthermore, inoculation of the micropropagated shoots *in vitro* has favoured acclimatisation to the conditions *ex vitro* in a greenhouse (Supriyanto and

Rohr 1994; Martins et al. 1996; Normand et al. 1996; Martins et al. 1997; Sudhakara Reddy and Satyanarayana 1998).

There are only two reports on the effects of ECM fungi on the growth of somatic embryo plants. With hybrid larch (*Larix* x *eurolepis* Henry), inoculation *in vitro* resulted in mycorrhiza formation and improved root growth and quality (Piola et al. 1995). In the case of Sitka spruce (*Picea sitchensis* (Bong.) Carr.), mycorrhiza formation occurred only in *ex vitro* conditions (Sasa and Krogstrup 1991), the fungi with the highest mycorrhiza frequency resulting in a slight increase in the shoot and root dry weight.

Promotion of root development and subsequent root growth by the ECM fungi is not necessarily related to the capacity of the fungus to form mycorrhizal structures (Linderman and Call 1977; Normand et al. 1996; Sudhakara Reddy and Satyanarayana 1998). Enhancement without mycorrhiza formation indicates that ECM fungi may release root related metabolites, such as growth regulators, into the rooting medium. IAA is one of the fungal factors implicated in the enhancement of adventitious root formation. However, as is the case for reports on mycorrhiza formation, the reports on root stimulation have been ambiguous (Gay 1990; Normand et al. 1996; Karabaghli et al. 1998). In the study of Normand et al. (1996), over-production of IAA by the *H. cylindrosporum* mutant was not found to favour rooting of the micropropagated shoots and hypocotyl cuttings of maritime pine (*Pinus pinaster* (Ait.) Sol.), whereas with micropropagated shoots of Scots pine the benefit was remarkable. Furthermore, Karabaghli et al. (1998) determined IAA production both in the ECM fungus *Laccaria bicolor* and bacterium *Pseudomonas fluorescens*, but only the former organism stimulated the rooting (%) and subsequent root branching on hypocotyl cuttings of Norway spruce (*Picea abies* (L.) Karst.).

2 AIMS OF THE STUDY

This study was a part of the larger project called "Application of biotechnology for genetic research and gene conservation of forest trees", carried out at the Punkaharju Research Station of the Finnish Forest Research Institute. Its practical aim was to elucidate whether ECM fungi could be used as promoting agents in the vegetative propagation of Scots pine. More specific emphasis was placed on the possible role of exogenous growth regulators, as well as those produced by the fungi, in the interaction between Scots pine and ECM fungi.

The specific aims of the study were:

1. To determine the ability of ECM fungi to improve root initiation and subsequent growth of the roots formed both on *in vitro* hypocotyl cuttings (**II, III**) and *in vivo* fascicular shoots (i.e. cuttings) (**I**).

2. To determine whether *in vitro* IAA (**I, II**) and polyamine (**III**) production by ECM fungi is related to root formation, and whether exogenous IBA, the auxin transport inhibitor TIBA (**II**) or polyamines (**III**), affect the fungus–cutting interaction.

3. To study the role of ECM fungi in somatic embryogenesis, i.e. in proliferation (**IV**), maturation (unpublished data) and conversion (**VI**). The effects of exogenous spermidine and MGBG, a putative inhibitor of spermidine synthesis, on maturation were also investigated (**V**).

3 MATERIALS AND METHODS

3.1. Ectomycorrhizal fungi

3.1.1 Fungal strains and inoculation techniques

The ECM fungi used in the study were originally isolated from basidiocarps growing in Scots pine forests (Table 1). Fungal mycelia were maintained on modified Melin–Norkrans agar medium (MMN1) supplemented with 55.5 mM glucose (Marx 1969; Heinonen-Tanski and Holopainen 1991) in the culture collection of the University of Kuopio, Finland. The abbreviations of the medium in the individual studies were mMN (**I**), MN (**IV**) and MMN1 (**II-III, VI**). The strain numbers, as well as the collections from which the fungal strains were originally obtained, are listed in Table 1.

Table 1. Fungal species and strains used in studies **I-IV** and **VI**.

Fungal species /strains	Strain number[1]	Original collection	Study
Laccaria bicolor	89	Dept. of Forest Pathology and Mycology Swedish University of Agricultural Sciences, Sweden	**IV**
Laccaria proxima	92	Dept. of Microbiology and Ecology, University of Lund, Sweden, strain 90003	**IV**
Paxillus involutus, H	7	Prof. Veikko Hintikka, Dept. of Biology, University of Helsinki, Finland (isolated in Helsinki, Finland)	**I-IV**
Paxillus involutus, K	88	University of Kuopio, Finland (isolated in Kolari, Finland)	**I**
Paxillus involutus, O	87	University of Kuopio, Finland (isolated in Oulu, Finland)	**I**
Pisolithus tinctorius	13	Dept. of Forest Pathology and Mycology University of Agricultural Sciences, Sweden, strain 1984a	**I-IV, VI**
Suillus variegatus, 1	68	University of Kuopio, Finland (isolated in Kuopio, Finland)	**IV**
Suillus variegatus, H	5	Prof. Veikko Hintikka, Dept. of Biology, University of Helsinki, Finland (isolated in Helsinki, Finland)	**IV**

[1] The number of the strain in the culture collection of the University of Kuopio, Finland.

Hypocotyl cuttings (**II-III**) and embryogenic cultures (**IV, VI**) were inoculated by means of mycelial agar plugs, which were 5 mm in diameter and cut from the edge of 4-week-old cultures on MMN1 agar medium. In the control cultures of the hypocotyl cuttings (**II-III**) and somatic embryos (**VI**), fresh MMN agar plugs were substituted for the mycelial plugs. On the proliferation (**IV**) and maturation (**V**) media, the non-inoculated cultures grew in the absence of an agar plug. In the *in vivo* rooting experiment (**I**), the mycelia were cultivated for 4 weeks in liquid MMN1 medium supplemented with 41.6 mM glucose and 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES). The mycelium was homogenized in water, and mycelium slurries were used for the inoculations. The viability of the mycelium after homogenisation was investigated, and no marked damage was observed in the mycelium. However, the variation in the inoculum potential between the individual isolates was not tested

before the rooting experiment. The non-inoculated cuttings were rooted directly after the IBA treatment (**I**).

3.1.2 Analysis of IAA and polyamines produced by the fungi

IAA production by the fungi was determined after 3- (**I-II**) and 5- (**I**) weeks' cultivation in liquid MMN1 (**I**) or MMN2 (**II**) medium supplemented with 41.6 mM glucose. In Study **I**, IAA production was analysed in the medium buffered with MES and supplemented or not with 1 mM L-tryptophan, a precursor of IAA. The medium without tryptophan and MES was used in Study **II**. Both free and conjugated IAA were analysed in the fungal culture filtrates (**I-II**) and the mycelia (**II**). IAA in the extracts was determined by HPLC (Waters, Milford, USA), after which the IAA samples were methylated, silylated, and finally analysed by gas chromatography–mass spectrometry (GC-SIM-SM) (Hewlett Packard GC type 5890 and MSD type 5870, **I** or Fisons Instruments GC type 8060, FisonsV6 Platform II MS, **II**).

For the polyamine analysis, the mycelia were cultivated for 3 weeks in liquid MMN2 medium supplemented with 41.6 mM glucose (**III**). Free and conjugated (soluble and insoluble) polyamines in the mycelia and culture filtrates were extracted in $HClO_4$ according to Fornalé et al. (1999) and Sarjala and Kaunisto (1993). Polyamines in the crude and hydrolyzed extracts were dansylated and determined by HPLC (Merck/Hitachi, Germany) as described by Sarjala and Kaunisto (1993).

3.2 Plant material and experimental designs

3.2.1 Rooting of *in vivo* cuttings

Study **I** was carried out to test whether (1) the level of adventitious rooting on Scots pine *in vivo* cuttings inoculated with ECM fungi is related to the level of IAA produced by the fungi *in vitro*, and (2) the level of adventitious rooting on Scots pine cuttings inoculated with ECM fungi varies with host genotypes.

Forty nine Scots pine (*Pinus sylvestris* L.) seedlings (i.e. genotypes) representing two seed families from southern Finland (3-year-old seedlings) and four families from northern Finland (2-year-old seedlings) were sprayed with BA to induce fascicular bud development. After an artificial growing season and short day–cold treatment, the elongated fascicular shoots (i.e. cuttings) were excised from the main shoot of the donor plants, and the bases of the cuttings were treated with IBA over night. Next day, the cuttings were inoculated with one strain of *Pisolithus tinctorius* and three strains of *Paxillus involutus* (Table 1) by dipping the bases into the fresh mycelium slurry and planted in an autoclaved, non-fertilized mixture of *Spaghnum*-peat (VAPO, Finland), Scots pine bark humus and perlite, (5:3:2, pH 4.3). The cuttings were rooted in a greenhouse under 90 % relative humidity and natural light (from April to August) The temperature of the benches was kept over 15 °C. Rooting was examined

7, 10, 14, and 20 weeks after planting and, at the same time, the rooted cuttings were transferred into fertilized 100 % *Spaghnum*-peat (pH 4.3, Finnpeat M6, VAPO, Finland) in a greenhouse under ambient conditions (from May to September). Three weeks later, the cuttings that had rooted within 7, 10 or 14 weeks were fertilized with 0.2 % 5-Superex fertilizer (Kekkilä, Finland). All the rooted cuttings were harvested 24 weeks after planting.

3.2.2 Rooting of *in vitro* hypocotyl cuttings

For Studies **II-III** on adventitious rooting, open-pollinated seeds of Scots pine originating from Konginkangas in central Finland (63°N; 26°E) were surface-sterilised with calcium hyphochlorite and germinated for 17 days on water agar in aseptic conditions under a 16-h photoperiod (130-150 µmol m^{-2} s^{-1}). Hypocotyl cuttings were prepared from the germinated seedlings by cutting the stem about 5 mm above the root collar. Rooting was carried out on MMN2 rooting medium (Marx 1969) supplemented with 1.1 mM glucose.

Study **II** consisted of four individual experiments to determine whether the rooting response of hypocotyl cuttings to ECM fungi is (1) related to the level of IAA produced by the fungi *in vitro* and (2) altered by exogenous application of IBA or TIBA (Table 2). In experiments 1 and 2, the cuttings were pre-treated by immersing the basal 0.5 cm of the cuttings in aqueous solutions of IBA or TIBA, whereas in experiment 3 the MMN2 rooting medium was supplemented with TIBA. In experiments 1-3, the cuttings were inoculated with a mycelial agar plug of *Pisolithus tinctorius* or *Paxillus involutus* H (Table 1). In experiment 4, the bases were pre-treated with IBA or the pooled filtrate obtained from 3-week-old fungal cultures in liquid MMN2 medium with 41.6 mM glucose, and rooting was carried out in the absence of the fungi (Table 2). The pooled culture filtrate was the same from which IAA was analysed (**II**). The cuttings were rooted for 6 weeks in a growth chamber at 25±2 °C under a 16-h photoperiod (140-150 µmol m^{-2} s^{-1}). In experiment 4, the concentration of free IAA in the hypocotyls was determined after different treatment periods (Table 2). IAA was extracted and analysed in the same way as for the fungal cultures (**II**).

Table 2. Treatment and inoculation descriptions from experiments 1-4 in Study **II**.

Experiment	Treatment	Duration of the treatment	Inoculation
1	0, 5, 10, 50 µM IBA in H_2O	24 h	mycelial/ fresh agar plug
2	0, 5, 10 µM TIBA in H_2O	24 h	mycelial / fresh agar plug
3	0, 5, 10 µM TIBA added to MMN2 rooting medium	–	mycelial / fresh agar plug
4	1) MMN2 solution with 41.6 mM glucose 2) 10 µM IBA in MMN2 solution with 41.6 mM glucose 3) Pooled culture filtrate of *Pisolithus tinctorius* (MMN2 with 41.6 mM glucose) 4) Pooled culture filtrate of *Paxillus involutus* H (MMN2 with 41.6 mM glucose)	5, 24, 72 h	fresh agar plug

To determine how TIBA affects the growth of the fungi, the mycelia were cultivated on MMN2 medium supplemented with 55.5 mM glucose and the same concentrations of TIBA as used in the rooting experiment 3. The radial growth of the fungi growing in the dark at 22 ± 2 °C was followed for 3 weeks.

In Study **III**, the effects of (1) exogenous polyamines and (2) a seedling forming mycorrhiza on the fungus-cutting interaction were studied. Rooting was carried out on MMN2 rooting medium supplemented with 0, 0.1 or 0.5 mM cadaverine or putrescine. The cultures were inoculated with two mycelial agar plugs. When cuttings produced roots in the presence of the seedling, the seedling and mycelial plugs were placed on the medium 1 week before the cutting. The cuttings were rooted in a growth chamber at 22 ± 1 °C under a 16-h photoperiod (130-140 μmol m^{-2} s^{-1}). The number of cuttings with roots was determined for the first time after 4-weeks' culture. At the same time, the cuttings were fertilized with MMN2 liquid medium without glucose. Rooting was continued for 5 more weeks, after which the cuttings and seedlings were harvested (**III**).

The effects of cadaverine and putrescine on the growth of the fungi were studied by cultivating the mycelia on MMN2 medium supplemented with 41.6 mM glucose and the same concentrations of cadaverine and putrescine as in the rooting experiment. The radial growth of the fungi growing in the dark at 21 ± 1 °C was followed for 3 weeks.

3.2.3 Embryogenic cultures

3.2.3.1 Initiation of embryogenic cultures

For studies on somatic embryogenesis (**IV-VI**), embryogenic cultures were derived from one-year-old immature seed cones collected from the open-pollinated, elite Scots pine clones growing in the Punkaharju clone collection (61°48′ N; 29°17′ E). The cultures were initiated using immature female gametophytes, including immature zygotic embryos with suspensor tissues. The inoculation, induction and proliferation of the embryogenic cultures were carried out according to Sarjala et al. (1997) and Häggman et al. (1999) on the slightly modified DCR medium, originally used for Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Gupta and Durzan 1985; Becwar et al. 1990). The cell lines used were K779/1, K884/9 and K1009/1 in Study **IV**, K818/7 in Studies **V** and **VI**, and K818/9, K818/10, K884/6 and K884/7 in Study **VI**.

3.2.3.2 Proliferation of embryogenic cultures

Study **IV** was carried out to determine (1) whether the addition of ECM fungi to the proliferation media influences the growth of embryogenic cell masses, and (2) how embryogenic cultures and ECM fungi in direct contact interact with each other. Pieces of embryogenic cell masses were weighed (Fw 1 = initial fresh weight) and transferred onto DCR proliferation medium. After 2 weeks' cultivation in the dark at 21 °C, they were inoculated

with six different ECM fungi (Table 1) by placing a mycelial agar plug at a distance of about 1 cm from the embryogenic cell masses. The dual cultures were incubated for 2 weeks in the dark, after which the cell masses were weighed (Fw2 = fresh weight at the time of harvest) for determination of the growth ratio (Fw2/Fw1). However, some dual cultures were continued for a longer time in order to observe the interaction between the embryogenic cells and ECM fungi.

3.2.3.3 Maturation of embryogenic cultures

Study **V** was performed to determine whether (1) addition of spermidine or MGBG to the maturation medium alters the development of somatic embryos, and (2) possible changes in embryo development are related to fluctuations in endogenous polyamine concnetrations. Furthermore, the effects of an ECM fungus alone and in the presence of spermidine or MGBG on embryo development were studied (unpublished data).

After weighing (Fw1), the embryogenic masses of cell line K818/7 were transferred for 2 weeks onto the DCR medium supplemented with ABA and PEG. This treatment was followed by 2 weeks' cultivation on the same medium without PEG, after which the cultivation was continued for 2 more weeks on the growth regulator-free DCR medium. Media were supplemented or not with 10 µM spermidine or 1 mM MGBG (**V**). The inoculation with *Pisolithus tinctorius* was carried out in concomitance with transfer onto the ABA-containing and hormone-free media by placing a mycelial agar plug at a distance of about 1.5 cm from the embryogenic cell masses (unpublished data). The embryogenic tissues were incubated for 2, 4 or 6 weeks at 25 ± 1 °C with a 16-h photoperiod (about 30 µmol m^{-2} s^{-1}). At the time of harvest, the cell masses were weighed (Fw2) for determination of the growth ratio (Fw2/Fw1), and the number of cell masses producing embryos and of embryos per cell mass were scored. Endogenous free polyamines and moisture content (Dw/Fw, %) of the embryogenic cultures were determined. Polyamines were analysed according to Sarjala et al. (1997) and Sarjala and Kaunisto (1993).

3.2.3.4 Conversion of mature somatic embryos

In Study **VI**, the effects of an ECM fungus on (1) germination of somatic embryos *in vitro*, and (2) *ex vitro* establishment of somatic embryos plants were studied. The ability of the fungus to form a mycorrhizal symbiosis with *in vitro* germinated embryos was also investigated.

Mature somatic embryos representing five different cell lines were germinated on half-strength DCR medium (**VI**). A mycelial agar plug cut from the culture of *Pisolithus tinctorius* (Table 1) was placed at a distance of about 2 cm from the base of the embryos. This prevented direct contact between the fungus and the embryos. Germination was carried out in a growth chamber under a 16-h photoperiod (140-150 µmol m^{-2} s^{-1}), and was examined after 4 and 8 weeks' cultivation. In June-July, the germinated embryo plants of two cell lines were adapted to *ex*

vitro conditions in a greenhouse by transplanting them to a non-fertilized mixture of peat (pH 3.8, VAPO, Finland) and perlite, (2:1). Re-inoculation with *Pisolithus tinctorius* was carried out by placing a mycelial agar plug close to the developing root system. Control embryo plants were planted without any treatment. The somatic embryo plants were fertilized once with 0.2 % 5-Superex fertilizer (Kekkilä, Finland), and they were grown for 18 to 25 weeks depending on the cell line (**VI**).

The somatic embryo plants of two cell lines were transferred onto MMN2 medium supplemented with 7.3 mM sucrose and re-inoculated with *Pisolithus tinctorius* (Table 1) (**VI**). For mycorrhiza formation, the mycelial agar plug was placed close to the radicle. The dual cultures were carried out for 7 weeks under the same conditions as during germination.

3.3 Analysis of growth and mycorrhiza formation

The number of rooted cuttings and of adventitious roots per cutting were scored at the time of harvest both *in vitro* (**II-III**) and *in vivo* (**I**). The length and fresh weight of the roots and the number of lateral roots were determined from cuttings (**I-III**), seedlings (**III**) and somatic embryo plants (**VI**). Also the fresh weight of the shoots was determined (**I-III, VI**). The number of root tips covered by a mycelial mantle in inoculated cuttings (**I-III**), seedlings (**III**) and somatic embryo plants (**VI**) was evaluated by means of dissecting (**I-III, VI**) microscopy. The thickness of the mantle and Hartig net formation were further examined by means of scanning electron (SEM) (Honegger 1985) (**I, III**) or light microscopy (**VI**). Control cuttings *in vivo* (**I**) and somatic embryo plants *ex vitro* (**VI**) were also assessed for mycorrhizal colonisation.

3.4 Statistical analyses

Rooting percentages of the cuttings *in vivo* (**I**) were analysed by the χ^2 test with Bonferroni correction (Zar 1984; Altman 1991). The data on growth of the fungi and plants were analysed with the t-test or analysis of variance (ANOVA) combined with Tukey's honestly significant difference test. If the data were not distributed normally, a non-parametric Mann-Whitney *U* test or Kruskal-Wallis test, combined with Mann-Whitney *U* test with Bonferroni correction (Zar 1984; Altman 1991), was used (**I-VI**). Because the individual experiments in Studies **II** and **III** were carried out separately, no statistical comparisons were made between the experiments. All statistical analyses were conducted with SPSS/PC (versions 7.5 and 9.0).

4 RESULTS

4.1 IAA and polyamines produced by the fungi

In the absence of L-tryptophan, considerably more IAA was found in the culture filtrate of *Pisolithus tinctorius* than in that of the *Paxillus involutus* strains (**I-II**). The addition of 1 mM tryptophan to the medium induced IAA production in all four fungus strains (**I**). In Study **I**, the culture medium MMN1 was buffered with MES to keep the pH at 5.4 to 5.7, whereas the pH of the non-buffered MMN2 medium used in Study **II** fell to 4.1 and 3.4 due to growth of *Pisolithus tinctorius* and *Paxillus involutus* strain H, respectively. The concentration of IAA in the buffered culture medium (**I**) of both fungi was higher than that in the non-buffered one (**II**). Most of the IAA present in the 3-week-old mycelium (**II**) was in the conjugated form, whereas in the culture filtrate at least half of the total IAA was in free form (**I-II**).

Spermidine was the main polyamine in the mycelium of *Paxillus involutus* H and *Pisolithus tinctorius* (**III**). *Paxillus involutus* H also contained a high concentration of putrescine (about 110 nmol g^{-1} FW), whereas in the mycelium of *Pisolithus tinctorius* traces of both cadaverine and putrescine (under 10 nmol g^{-1} FW) were found. Putrescine was the main polyamine in the culture filtrate of *Paxillus involutus* H. In contrast, *Pisolithus tinctorius* released large amounts of an unknown polyamine with a retention time between that of N-methyl putrescine and cadaverine. The culture filtrates contained no conjugated polyamines, and the concentration of free polyamines was lower than that in the mycelium, except for spermine in the case of *Paxillus involutus* H (**III**).

4.2 Adventitious root formation on *in vivo* cuttings

Approximately 37 % of the cuttings across all genotypes and treatments formed roots within the 24 weeks' rooting period *in vivo* (**I**). IBA treatment combined with inoculation resulted in a twofold increase (from 33 to 64 %) in the rooting percentage of the cuttings originating from northern Finland. In contrast, there was no difference in root initiation between the non-inoculated and inoculated cuttings within the southern origin. Most of the northern seed families rooted better when any of the four fungus strains was present, whereas the rooting of one southern seed family was reduced by the presence of the fungi. Root formation of some seed families was accelerated by most of the fungus strains, and the faster rooting correlated with the number of roots per cutting and total root length. Despite the positive interactions between several cutting genotypes and ECM fungi, mycorrhiza formation was very random. Some root tips covered by a hyphal mantle were observed both in inoculated and non-inoculated cuttings growing in fertilized non-sterile peat (**I**).

Root stimulation by the fungus strain was not correlated with the concentration of IAA in the culture filtrate of the strain. Instead, rooting and subsequent root growth caused by the fungi proved to be highly dependent on the specific interaction between the Scots pine and fungus

genotypes (Fig. 2). Great genotypic variation in rooting was also found in the non-inoculated cuttings treated only with IBA (**I**).

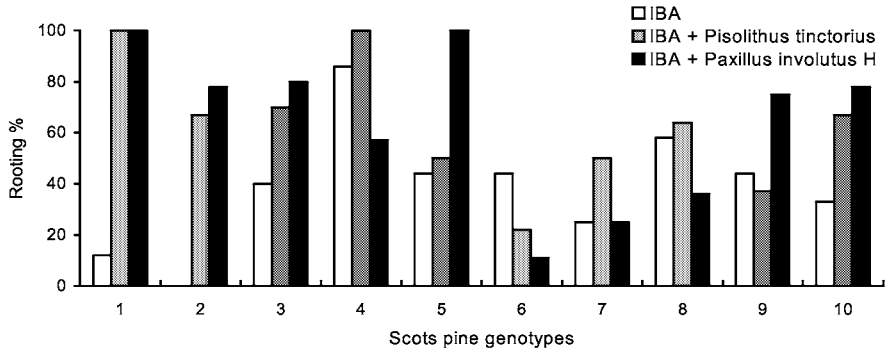

Fig. 2 Effects of *Pisolithus tinctorius* with high IAA production and *Paxillus involutus* strain H with low IAA production on root formation on the IBA-treated *in vivo* Scots pine cuttings representing 10 example genotypes.

4.3 Adventitious root formation on *in vitro* hypocotyl cuttings

4.3.1 Root initiation

The rooting percentage of the H_2O-treated cuttings was increased by inoculation with *Pisolithus tinctorius* or *Paxillus involutus* H in the 6-week experiment (**II**). In Study **III**, the fungi accelerated root initiation by increasing the number of cuttings with roots within the first 4 weeks. At the time of harvest, i.e. at the 9th week of the experiment, the reactions of the cuttings depended on whether they were growing with or without a seedling: in the presence of the seedling, the rooting percentage of the inoculated cuttings treated with H_2O was about 90 and that of the non-inoculated ones 68, whereas in the absence of the seedling no difference was observed (**III**).

Cadaverine and putrescine in the medium retarded root initiation of the non-inoculated cuttings growing without a seedling (**III**). In contrast, the combined cadaverine (0.5 mM) and *Pisolithus tinctorius*, and the putrescine (0.5 mM) and *Paxillus involutus* H treatments resulted in a higher number of cuttings with roots within the first 4 weeks than the inoculation alone. When a seedling was present, both cadaverine and putrescine slightly accelerated rooting of the non-inoculated cuttings, as did 0.5 mM cadaverine on the inoculated cuttings (**III**).

Treatment of the bases with IBA or TIBA, as well as supplement of 5 µM TIBA to the medium, increased the percentage of non-inoculated cuttings with roots (**II**). A similar response to IBA and 5 µM TIBA was observed on the cuttings inoculated with *Pisolithus tinctorius*. Despite this, inoculation with *Paxillus involutus* H always resulted in the highest

rooting percentage. Addition of 10 μM TIBA to the medium proved to be too high for both non-inoculated and inoculated cuttings (**II**).

The treatment with the 3-week-old culture filtrate of *Paxillus involutus* H induced rooting in a higher number of cuttings than that with the culture filtrate of *Pisolithus tinctorius* (**II**). In general, culture filtrates caused lower rooting percentage than inoculation with the same fungi. However, the number of cuttings with roots increased with increasing length of the culture filtrate treatment (5, 24 or 72 h). This was also the case with the IBA treatments. Treating the cutting bases with IBA, but not with the culture filtrate of the fungi, increased the IAA concentration of the hypocotyls (**II**).

4.3.2 Subsequent growth and mycorrhiza formation of the roots formed

Inoculation improved growth of the roots by increasing the length of the adventitious roots and number of lateral roots per root, as well as the fresh weight of the root system. Improved root growth and the presence of the fungal mycelium in the roots increased the root/shoot ratio (**II-III**). As was the case for rooting, cadaverine increased the positive effects of *Pisolithus tinctorius* on growth of the roots formed (**III**). In contrast, TIBA in the medium inhibited root elongation and lateral root formation of the cuttings inoculated with *Pisolithus tinctorius*, and a decreasing trend was also observed on the cuttings inoculated with *Paxillus involutus* H.

Pisolithus tinctorius was able to form mycorrhizas *in vitro* (**II, III**), whereas *Paxillus involutus* H grew near to the root systems of both cuttings (**II, III**) and seedlings (**III**) without forming any mycorrhizal structures. *Pisolithus tinctorius* formed a hyphal mantle over the lateral roots of both cuttings (**II-III**) and seedlings, but Hartig net formation was observed only in the seedling roots (**III**). The presence of cadaverine in the medium (**III**) and treating the cutting bases with IBA (**II**) increased the number of root tips covered by the hyphae of *Pisolithus tinctorius*. Furthermore, cadaverine improved the growth of the fungus. In contrast, TIBA had a dose-dependent, negative effect on the radial growth and mycorrhiza formation of *Pisolithus tinctorius*. The growth of *Paxillus involutus* H was not disturbed by the inhibitor (**II**).

4.4 Embryogenic cultures

4.4.1 Proliferation of embryogenic cell masses and conversion of somatic embryos

Reactions of the proliferating embryogenic cultures (**IV**) and mature embryos (**VI**) to inoculation were dependent on the Scots pine cell lines (i.e. genotypes) and fungus strains. Growth of the slowly proliferating cell line, determined as the Fw2/Fw1 ratio, was increased by two of the six fungi tested, whereas the fungi had no or negative effect on the growth of the rapidly proliferating cell lines. Depending on the fungus strain, the embryogenic cultures either remained translucent to light yellow in colour (the same as the control cultures) and were slightly covered by the fungal hyphae, or became brown and partly necrotic. In the latter case, the fungus grew as a thick hyphal mantle over the embryogenic tissues. No clear correlation

between colour changes of the embryogenic cell masses and changes in the growth ratios was observed (**IV**).

The speed and frequency of germination in cell lines capable of germinating was increased by *Pisolithus tinctorius* (**VI**). However, the fungus had no effect on subsequent growth of the embryo plants *in vitro*. In contrast, adaptation to *ex vitro* conditions in the greenhouse was improved, and this occurred without mycorrhiza formation (mantle and Hartig net) by the fungus. Cultivation on MMN2 medium *in vitro* resulted in mycorrhiza formation between somatic embryo plants and *Pisolithus tinctorius*. The number of mycorrhizal root tips was high and the mantle consisted of several cell layers. Furthermore, the fungus penetrated the lateral roots and formed a Hartig net between the cortical cells (**VI**).

4.4.2 Development and maturation of somatic embryos

Maturation of the embryogenic cell line K818/7 was improved by most of the MGBG and spermidine treatments (**V**), as well as the combined treatments with *Pisolithus tinctorius* and MGBG or spermidine. In contrast, the presence of *Pisolithus tinctorius* alone on the growth regulator-free DCR medium decreased the level of maturation on the embryogenic cultures (unpublished data). Enhanced maturation, which was observed as a higher number of cell masses with embryos and/or higher number of embryos per cell mass, correlated positively with the reduced growth ratio (Fw2/Fw1). Decreasing levels of free polyamines at the end of the experiment were also associated with enhanced maturation. In the control cultures with a low number of maturating embryos, putrescine accumulated during the last 4 weeks. In contrast, a reduction in putrescine was observed during the last 2 weeks in the treatments promoting maturation. Furthermore, the reduction in the spermidine and spermine concentrations was stronger than that in the non-treated control cultures (**V**).

5 DISCUSSION

5.1 Ectomycorrhizal fungi and auxins in rooting

The role of different ECM fungi in adventitious root formation was studied using *in vivo* cuttings pre-treated with IBA (**I**) and *in vitro* hypocotyl cuttings as explants (**II-III**). Rooting and subsequent root growth of the *in vitro* cuttings was favoured by inoculation with specific ECM fungi (**II-III**), which is consistent with earlier studies on *in vitro* hypocotyl cuttings of conifers (Gay 1990; Normand et al. 1996; Karabaghli et al. 1998). The type and strength of the reactions of *in vivo* cuttings to fungal inoculation were much more complicated. In general, cuttings originating from northern Finland benefited from fungal inoculation, which may be partly related to lower age of their donor plants compared to that of the southern cuttings (Salonen 1991, Browne et al. 1997a). However, the rooting responses of single northern genotypes to specific fungus strains were not only positive, but also negative or similar with the IBA-treated controls (**I**). Similarly, auxin-treated *in vivo* cuttings of miniature rose (*Rosa* sp.) (Scagel 2001) and mountain laurel (*Kalmia latifolia*) (personal communication) have shown cultivar-dependent rooting reactions to VA and ericoid mycorrhizal fungi, respectively. These results show that a fungus and plant may interact specifically already before the roots have emerged.

Several ECM fungi are able to synthesise free IAA *in vitro*, even though production is greatly dependent on the supply of exogenous tryptophan (e.g. Strzelczyk and Pokojska-Burdziej 1984; Gay et al. 1989; Rudawska and Kieliszewska-Rokicka 1997). Induction of *in vitro* IAA synthesis (both free and conjugated forms) caused by tryptophan was also clear in all four ECM fungus strains of Scots pine (**I**). The ability of *Pisolithus tinctorius* to produce high amount of IAA even in the absence of tryptophan (**I-II**), indicates a good ability to metabolise endogenous tryptophan to IAA (Gay et al. 1989; Rudawska et al. 1992) and/or the presence of a tryptophan-independent IAA synthesis pathway (reviewed by Sztein et al. 2000). Production of IAA and its release from the mycelium have been shown to decrease with increasing acidity of the culture medium (Rudawska et al. 1992; Strzelczyk et al. 1992). This might explain the considerably lower IAA concentration in the non-buffered MMN2 medium (**II**) than in the buffered MMN1 medium (**I**). Slightly different nutrient concentrations in MMN1 (for maintenance of the mycelium) and MMN2 (for mycorrhiza formation) media might also affect IAA synthesis. Whether continuous cultivation of *Pisolithus tinctorius* and *Paxillus involutus* strain H had reduced their ability to produce IAA remains unclear.

The culture filtrates of three *Paxillus involutus* strains contained different amounts of IAA (**I**), which is in agreement with earlier reports on a genotype-dependent production of IAA by ECM fungi (e.g. Ho 1987; Rudawska and Kieliszewska-Rokicka 1997; Scagel and Linderman 1998b). In Study **I**, the IAA concentration in the culture filtrate of *Paxillus involutus* H decreased between the 3rd and 5th culture weeks, which may be due to enhanced IAA degradation. The decrease took place despite the production of dark pigments which have been suggested to prevent IAA oxidation (Tomaszewski and Wojciechoeska 1974).

The efficiency of exogenous free auxins as root stimulators is well-known (e.g. Grönroos and von Arnold 1988; Salonen 1990; Diaz-Sala et al. 1996; Browne et al. 1997a), and free IAA produced by the ECM fungi has also been implicated in root enhancement (Gay 1990; Normand et al. 1996; Karabaghli et al. 1998). With Scots pine cuttings, both exogenous free IBA (**I-II**) and ECM fungi (**I-III**) promoted root formation, but no clear correlation between IAA production by the fungus and root stimulation was found. For example, inoculation with *Paxillus involutus* H, which produced a relatively low amount of IAA resulted in as good or even better rooting than that with the high IAA-producer *Pisolithus tinctorius* (**I-III**). Furthermore, treatment with the culture filtrate of the 3-week-old *Paxillus involutus* H for a period as short as 5-72 h resulted in better rooting than the corresponding treatment with the culture filtrate of *Pisolithus tinctorius* (**II**). This indicates that, in addition to free IAA, other characteristics by the ECM fungi may play an important role in root formation. Higher root formation due to inoculation than to the short treatments with IBA or fungal culture filtrates (**II**) indicates that a continuous, low release of root related compounds by the fungi favoured formation and growth of the roots, as suggested by Karabaghli et al. (1998).

Certain treatments with the auxin transport inhibitor TIBA increased rooting percentage of the non-inoculated Scots pine *in vitro* cuttings and of those inoculated with *Pisolithus tinctorius* (**II**). This enhancement might be due to localised accumulation of free IAA at the base, as suggested by Kaska et al. (1999). In general, effects of TIBA on rooting have been variable (Batten and Goodwin 1981; Garcia-Comez et al. 1994; Marks 1996), and also the mechanisms through which TIBA exerts an effect on the fungus-plant interaction are still unclear. Tranvan et al. (2000) suggested that TIBA reduced mycorrhiza formation by inhibiting transport of free IAA from the fungus, whereas Karabaghli-Degron et al. (1998) reported that TIBA blocked transport of the fungal free IAA in the roots. In Study **II**, root growth and mycorrhiza formation of Scots pine caused by *Pisolithus tinctorius* was reduced by TIBA. However, the reduced growth of the fungal mycelium showed that the effects of TIBA are complicated and not only related to IAA metabolism (**II**), as earlier reported by Jacobson and Jacobson (1981).

So far, attention in the fungus-root interaction has been focused on free IAA. IAA conjugates, some of which are transportable compounds (reviewed by Normanly 1997), are also involved in root formation (reviewed by Blakesley 1994). In general, they have not proved to be as good root stimulators as free IAA, but the efficiency has depended on the form of the conjugate (Pence and Caruso 1984). Furthermore, increased level of the endogenous IAA conjugates has been associated with a reduced ability of the cutting to form roots (Blakesley 1991a). This study showed that depending on the fungal strain, IAA conjugates form an essential part of the total IAA pool in the culture filtrate (**I-II**) and mycelium of the ECM fungi (**II**). Therefore, conjugated forms of IAA may also be involved in the specific plant-fungus interaction during root formation.

The results obtained for Scots pine rooting (**I-III**) do not diminish the importance of free IAA produced by the fungi in root formation, but they do suggest that specific fungal strains, e.g. *Paxillus involutus* H (**I-III**) may have other characteristics that play a major role in stimulating

root initiation. Synergistic effect with free IAA is also highly possible. This is supported by the fact that the combined IBA and fungal treatment was more beneficial for rooting of several Scots pine genotypes than the IBA treatment alone (**I**).

5.2 Ectomycorrhizal fungi and polyamines in rooting

Polyamines are potential agents involved in the ECM fungus-plant interaction during root formation. Putrescine has been repeatedly implicated in adventitious rooting (Hausman et al. 1994; Kevers et al. 1997; Nag et al. 2001) and, on the other hand, polyamines have been shown to play an important role in the interaction between plants and mycorrhizal fungi (El Ghachtouli et al. 1995; El Ghachtouli et al. 1996; Kytöviita and Sarjala 1997). In the present study, *Pisolithus tinctorius* and *Paxillus involutus* H differed from each other in the production of cadaverine and putrescine (**III**). Cadaverine, synthesised by *Pisolithus tinctorius*, has been earlier found in the mycelium of some ECM fungi (Zarb and Walters 1994b). In contrast to IAA (**I-II**), polyamine conjugates were not found in the culture filtrate of the fungi (**III**), thus indicating that polyamine conjugates are not transported across the plasma membrane (Fornalé et al. 1999).

El-Ghachtouli et al. (1995) observed an increased mycorrhiza frequency on pea roots as a consequence of exogenous polyamines. On Scots pine hypocotyl cuttings, exogenous putrescine and *Paxillus involutus* H, as well as cadaverine and *Pisolithus tinctorius*, had a synergistic effect on root formation. The latter combination also favoured root growth and mycorrhiza formation (**III**). Cadaverine is not found in the endogenous polyamine pool of Scots pine (e.g. **III**; **V**; Sarjala and Kaunisto 1993; Sarjala 1996), but was formed by *Pisolithus tinctorius* (**III**), which indicates that it probably stimulated the cutting-*Pisolithus tinctorius* interaction via the fungus. The enhanced fungal growth caused by cadaverine might result in more rapid and extensive contact with the cutting. However, because polyamine metabolism is known to be connected to auxin metabolism in rooting (Hausman et al. 1994; Heloir et al. 1996; Kevers et al. 1997), a possible synergistic effect of exogenous cadaverine (**III**) and IAA (**I-II**) produced by *Pisolithus tinctorius* on rooting and mycorrhiza formation is also possible. Whether the unknown compound released by *Pisolithus tinctorius* is involved in the fungus-root interaction, will be studied in the future.

The absence of the synergistic effect of exogenous putrescine and *Paxillus involutus* H after root initiation might be associated with an increased production of putrescine with increasing age of the fungus (**III;** Fornalé et al. 1999; our preliminary unpublished data). If putrescine had any role in the subsequent interaction between *Paxillus involutus* H and the growing root system, the putrescine produced by the fungus was probably enough to maintain this interaction.

5.3 Relationship between root enhancement and mycorrhiza formation

It is possible that the copious release of IAA by *Pisolithus tinctorius* favoured mycorrhiza formation *in vitro* (**II-III**), as earlier reported with other fungi (e.g. Gay et al. 1994, Rudawska and Kieliszewska-Rokicka 1997; Tranvan et al. 2000). The role of auxin is supported by the observations that IBA treatment increased mycorrhiza formation by *Pisolithus tinctorius* (**II**). *Paxillus involutus* H formed no mycorrhizal structures *in vitro* (**II-III**), which may indicate that the fungus had lost its ability to form a mycorrhiza under the conditions used. Regardless of this, *Paxillus involutus* H had a high ability to enhance rooting and subsequent root growth *in vitro* (**II-III**). Similar positive rooting responses were observed on *in vivo* cuttings of Scots pine without any clear mycorrhiza formation (**I**). These results, together with those obtained with *in vivo* cuttings of other woody plant species (Linderman and Call 1977; Scagel 2001) suggest that root stimulation is not necessarily related to mycorrhizal activity of the fungus. However, the presence of the fungus, even without mycorrhiza formation, was extremely important, because inoculated *in vitro* Scots pine cuttings rooted and grew better than those treated with the culture filtrate of the same fungi (**II**). This stimulation is certainly a result of several fungal characteristics, such as the release of growth regulators and modification of the rooting medium (e.g. pH and nutrient composition) (Smith and Read 1997). In non-aseptic conditions, the ECM fungi may also favour root growth by changing microbe population in the soil (Fitter and Garbaye 1994).

5.4 Exogenous polyamines and polyamine synthesis inhibitors in somatic embryogenesis

In embryogenic cultures, endogenous polyamine concentrations fluctuate concomitantly with somatic embryo development. Therefore, attempts have been made to improve somatic embryogenesis by adding certain exogenous polyamines or polyamine synthesis inhibitors to the medium (**V**; Santanen and Simola 1992; Minocha et al. 1993; Sarjala et al. 1997; Kong et al. 1998). Proliferation growth of the Scots pine embryogenic cultures was not affected by exogenous 10 μM spermidine, and the polyamine synthesis inhibitor MGBG reduced growth only when supplied at a high concentration (5 mM) (Sarjala et al. 1997). On maturation media, the addition of 10 μM spermidine and especially 1 mM MGBG dramatically decreased proliferation growth of Scots pine cultures. Decreasing growth was correlated with increased embryo maturation (**V**). The inhibition in proliferation due to MGBG supports the results obtained with spruce embryogenic cultures (Santanen and Simola 1992; Kong et al. 1998). However, the concentration suitable to Scots pine inhibited not only tissue growth but also embryo development of spruce species (Santanen and Simola 1992; Kong et al. 1998). These results indicate that a species differ in sensitivity to MGBG (**V**; Sarjala et al. 1997) and, furthermore, that this compound may at certain concentrations either directly or indirectly promote somatic embryo differentiation.

A reduction in the endogenous free polyamine pool appears to favour development of somatic embryos (**V**; Meijer and Simmonds 1988; Santanen and Simola 1992; Minocha et al. 1993; Nabha et al. 1999). In some coniferous species, a dramatic decline in the free putrescine pool

and a consequent increase in the spermidine/putrescine ratio in the whole cell mass (Santanen and Simola 1992; Kong et al. 1998) or in developing somatic embryos (Minocha et al. 1999) precedes formation of globular stage embryos. In Scots pine embryogenic cultures, the inducing effects of MGBG and spermidine treatments was probably related to a general decrease in the endogenous free polyamine pool, because a transient increase in the spermidine/putrescine ratio during the first 2 weeks was observed both in the control and treated cultures (**V**).

The reduction in the endogenous putrescine pool caused by MGBG was surprising (**V**) because, as a putative inhibitor of spermidine and spermine synthesis, MGBG would have been expected to increase it. Spermidine related decrease in the free polyamine pool indicates the existence of feed back regulatory mechanisms (reviewed by Tiburcio et al. 1997). Non-specific effects of both MGBG and spermidine have also been observed in proliferating Scots pine embryogenic cultures (Sarjala et al. 1997) and, in the case of MGBG, during embryo maturation of some spruce species (Santanen and Simola 1992; Kong et al. 1998). Although conjugate formation was not studied (**V**), the findings do show that the supply of exogenous polyamines or polyamine synthesis inhibitors does not necessarily correlate with changes in the endogenous polyamine pool.

5.5 Ectomycorrhizal fungi in somatic embryogenesis

So far, application of different ECM fungi in somatic embryogenesis has been limited to the inoculation of germinated somatic embryo plants of Sitka spruce (Sasa and Krogstrop 1991) and hybrid larch (*Larix* x *eurolepis* Henry) (Piola et al. 1995). In the present study with Scots pine, germination of somatic embryos and *ex vitro* acclimatisation of somatic embryo plants were improved by inoculation with *Pisolithus tinctorius* (**VI**). Moreover, this study showed that specific ECM fungi may have positive effects on proliferation (**IV**) and maturation (unpublished data) of the embryogenic cultures. Similar to the case with adventitious rooting (**I**), the reactions were highly dependent on the specific interaction between the Scots pine and fungus genotypes (**IV**, **VI**).

The release of growth regulators and polyamines by specific ECM fungi might favour or inhibit the proliferation growth of single Scots pine cell lines (**IV**), and the release of IAA by *Pisolithus tinctorius* might be one reason for low maturation of the somatic embryos when MGBG and spermidine were absent (unpublished data). However, the results obtained from the studies on IAA or polyamine production by the fungi cannot be applied as such, because growth regulators were analysed in the MMN1 (**I**) and MMN2 (**II-III**) media, which had much lower concentration of nutrients and sugar than the proliferation (**IV**) and maturation media (**V**). In order to find out why MGBG or spermidine and *Pisolithus tinctorius* had a synergistic effect on maturation (unpublished data), studies are now being carried out on the growth and production of polyamines by the fungus on maturation medium in the absence and presence of these compounds.

High concentrations of sugar (Duddriddge 1986) and a changed nitrogen/phosphorous ratio (Brun et al. 1995) are known to unbalance the mycorrhizal interaction, and this might be one reason for the negative reactions of some of the cell lines on the proliferation media (**IV**). An imbalance between the fungus and somatic embryos was also evident in our preliminary study on germination performed on half-strength DCR medium. On this medium, the mycelium of both *Pisolithus tinctorius* and *Paxillus involutus* H (unpublished data) grew aggressively over the plant when they were in contact with a somatic embryo. Similarly, Sasa and Krogstrop (1991) observed that ECM fungi grew over Sitka spruce somatic embryos with short radicles *in vitro*. In Study **VI**, the germination of Scots pine somatic embryos was successful when direct contact between the fungus and the germinating somatic embryo was prevented. Subsequent inoculation of the germinated somatic embryo plants on MMN2 medium with a low nitrogen/phosphorus ratio (Brun et al. 1995) and low sugar concentration enabled positive interaction and mycorrhiza formation *in vitro*. In addition to the nutrient composition of the medium, this study together with that of Sasa and Krogstrop (1991) demonstrated the importance of the developmental stage of the somatic embryo plant in balanced interaction with the fungus.

Acclimatisation of the somatic embryo plants to *ex vitro* conditions was improved by *Pisolithus tinctorius*, although inoculation in these conditions did not result in mycorrhiza formation (**VI**). Facilitated adaptation has also been evident on rooted *in vitro* shoots that were inoculated before transfer to *ex vitro* conditions (Supriyanto and Rohr 1994; Martins et al. 1996; Normand et al. 1996; Martins et al. 1997). In the study of Martins et al. (1996), the percentage of mycorrhizal root tips correlated positively with the survival of the micropropagated European chestnut (*Castanea sativa* Mill.) plants, whereas in the case of maritime pine, the degree of positive responses during acclimatisation depended on both the plant and fungus genotypes (Normand et al. 1996).

5.6 Possibilities for practical application

Inoculation with specific ECM fungi proved to be a potential tool to improve adventitious root formation and subsequent root growth of *in vitro* and *in vivo* Scots pine cuttings. The studies on rooting showed that auxins play a regulatory role in the interaction between specific ECM fungi and Scots pine during root and mycorrhiza formation. Despite this, production of free IAA by the fungus cannot be used as the only criterion when selecting fungi for rooting studies. Polyamines were also involved in the fungus–root interaction *in vitro*, and the effects of an exogenous polyamine appeared to be dependent on the fungal strain and its ability to produce this compound. This, together with the results on the *in vivo* study carried out with different Scots pine genotypes, showed that rooting responses depend on both the Scots pine and fungus genotypes. ECM fungi may have different strain-specific factors associated with rooting, which alone or synergistically result in positive responses in compatible Scots pine genotypes. Several *in vivo* cutting genotypes rooted better as a result of combined IBA and fungal treatment than that of IBA alone, which was evidence of a synergistic effect on rooting, and may be a useful technique in further rooting studies. In addition to growth regulators

produced by the fungi, strain-specific effects on rooting soil, as well as the carbohydrate and growth regulator status of the cuttings may be involved in the root-fungus interaction. On the other hand, the possibility that fungus strains release compounds, e.g. cytokinins or some conjugated IAA forms, that may counteract the positive rooting effects, cannot be ruled out when fungus strains are tested for rooting.

To make ECM inoculations feasible in large-scale *in vivo* cutting production for Scots pine, the number of genotypes with positive rooting responses should be continuously high. This could be achieved by pre-inoculating the rooting soil with several root-promoting fungus strains. However, to ensure that one fungus strain does not reduce the activity of another, their interactions should be carefully studied before performing inoculations. Regardless of the inoculation method, i.e. dipping or soil inoculation, large amounts of fungal mycelium is needed. Therefore, efficient techniques for the mass production of the fungal mycelium should be developed for the large-scale use of ECM fungi.

Propagation using cuttings is useful only when the growth of the rooted cuttings in the field is comparable to that of normal seedlings. Mycorrhiza formation does not appear to be necessary for root stimulation on *in vitro* and *in vivo* Scots pine cuttings. However, the further survival and subsequent growth may be improved by true ECM structures. Therefore, it would be important to study whether (1) the same fungi which increased the rooting of Scots pine cuttings also improve acclimatisation in the field, and (2) whether the formation of a mantle and Hartig net favours the acclimatisation process.

The reactions of the proliferating embryogenic cultures were dependent on the Scots pine cell line and ECM fungus. These results, together with those obtained from the maturation study, suggest the possibility to improve the growth and development of embryogenic cultures using compatible cell line–fungus strain interaction. However, as with cuttings, inoculation is worthwhile only if it provides positive responses across a wide range of genotypes. Because the use of mixed inoculum is more difficult in embryogenic cultures, it would be essential to find single strains causing positive reactions in a high number of cell lines.

The germination of somatic embryos representing several cell lines was stimulated by *Pisolithus tinctorius* when direct contact between the symbiotic partners was prevented. A direct contact leading to mycorrhiza formation proved possible only after the embryos had germinated and had been transferred to medium with a low nutrient concentration. In practise, the high concentration of nutrients needed for somatic embryogenesis places limits on the use of ECM fungi before germination. Therefore, the most potential phases in which ECM fungi could be applied are germination of mature somatic embryos and adaptation of the somatic embryo plants to *ex vitro* conditions in a greenhouse.

6 CONCLUSIONS

In this study with Scots pine, specific ECM fungi improved root initiation, germination, and subsequent growth of the roots in the vegetatively propagated material (i.e. *in vivo* fascicular shoot cuttings and *in vitro* somatic embryos) and *in vitro* hypocotyl cuttings. The positive rooting responses were observed both in the absence and presence of mycorrhizal structures, suggesting that root enhancement by the fungus is not necessarily associated with its mycorrhizal activity. ECM fungi varied in their ability to produce free and conjugated IAA, as well as different polyamines. Although both IAA and certain polyamines were involved in the fungus-root interaction, no clear correlation between production of these compounds and root stimulation by the fungi was found. The importance of polyamines in plant differentiation and the fungus-plant interaction was emphasised in the maturating embryogenic cultures. Embryogenic cultures in the proliferation phase were also able to interact with ECM fungi, which suggests the presence of some kind of recognition mechanisms before the root primordium emerges. As was the case in root development, the embryogenic cultures of different Scots pine genotypes reacted to ECM fungi specifically, showing that the interaction between Scots pine and ECM fungi is a complicated, genotype-dependent phenomenon.

7 REFERENCES

Agerer, R. (2001) Exploration types of ectomycorrhizae. A proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. *Mycorrhiza* 11: 107-114.

Altman, DG. (1991) Practical statistics for medical research. Chapman & Hall, London. 611 p.

Antola, J. (1990) Working party to plan new seed orchards. In: Napola, J. (ed.) *Annual report for forest tree breeding in Finland 1989.* Auranen, Forssa. pp. 26-27.

Aronen, T., Häggman, H. and Salonen, M. (1996) Rooting of Scots pine fascicular shoots by *Agrobacterium rhizogenes*. *Forest Genetics* 3: 15-24.

Attree, S.M. and Fowke, L.C. (1993) Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. *Plant Cell Tissue and Organ Culture* 35: 1-35

Bagni, N., Altamura, M.M., Biondi, S., Mengoli, M. and Torrigiani, P. (1993) Polyamines and morphogenesis in normal and transgenic plant cultures. In: Roubelakis-Angelakis, K.A. and Tran Thanh Van, K. (eds.). *Morphogenesis in plants*. Plenum Press, New York. pp. 89-111.

Baser, C.M., Garret, H.E., Mitchell, R.J. and Cox, G.S. (1987) Indolebutyric acid and ectomycorrhizal inoculation increase lateral root initiation and development of container-grown black oak seedlings. *Canadian Journal of Forest Research* 17: 36-39.

Batten, D.J. and Goodwin, P.B. (1981) Auxin transport inhibitors and the rooting of hypocotyl cuttings from etiolated mung-bean *Vigna radiata* (L.) Wilczek seedlings. *Annals of Botany* 47: 497-503.

Becwar, M.R., Nagmani, R. and Wann, S.R. (1990) Initiation and embryogenic cultures and somatic embryo development in loblolly pine *Pinus taeda*. *Canadian Journal of Forest Research* 20: 810-817.

Becwar, M.R. and Pullman, G.S. (1995) Somatic embryogenesis in loblolly pine (*Pinus taeda* L.). In: Jain, S.M., Gupta, P.K. and Newton, R.J. (eds.). *Somatic embryogenesis in woody plants, vol. 3 – Gymnosperms*. Kluwer Academic Publishers, Dordrecht. pp. 287-301.

Béguiristain T. and Lapeyrie, F. (1997). Host plant stimulates hypaphorine accumulation in *Pisolithus tinctorius* hyphae during ectomycorrhizal infection while excreted fungal hypaphorine controls root hair development. *New Phytologist* 136: 525-532.

Bhumibhamon, S. (1978) Studies on Scots pine seed orchards in Finland with special emphasis on the genetic composition of the seed. *Communicationes Instituti Forestalis Fenniae* 94: 1-118.

Blakesley, D. (1994) Auxin metabolism and adventitious root initiation. In: Davis, T.D. and Haissig, B.E. (eds.) *Biology of adventitious root formation*. Plenum Press, New York. pp. 143-154.

Blakesley, D., Weston, G.D. and Elliot, M.C. (1991a) Endogenous levels of indole-3-acetic acid and abscisic acid during rooting of *Cotinus coggygria* cuttings taken at different times of the year. *Plant Growth Regulation* 10: 1-12.

Blakesley, D., Weston, G.D. and Hall, J.F. (1991b) The role of endogenous auxin in root initiation. Part I: Evidence from studies on auxin application, and analysis of endogenous levels. *Plant Growth Regulation* 10: 341-353.

Boeijink, D.E. and van Broekhuizen J.T.M. (1974) Rooting of cuttings of *Pinus sylvestris* under mist. *New Zealand Journal of Forest Science* 4: 127-132.

Browne, R.D., Davidson, C.G. and Enns, S.M. (2000) Improvements in asexual multiplication procedures for jack pine (*Pinus banksiana*). *New Forests* 19: 259-278.

Browne, R.D., Davidson, C.G., Steeves, T.A. and Dunstan, D.I. (1997a) Effects of ortet age on adventitious rooting of jack pine (*Pinus banksiana*) long-shoot cuttings. *Canadian Journal of Forest Research* 27: 91-96.

Browne, R.D., Davidson, C.G., Steeves, T.A. and Dunstan, D.I. (1997b) Rooting of proliferated dwarf shoot cuttings of jack pine (*Pinus banksiana*). *Canadian Journal of Forest Research* 27: 97-101.

Brun, A., Chalot, M., Finlay, R.D. and Söderström, B. (1995). Structure and function of the ectomycorrhizal association between *Paxillus involutus* (Batsch) Fr. and *Betula pendula* Roth. I. Dynamics of mycorrhiza formation. *New Phytologist* 129: 487-493.

Brundrett, M., Murase, G. and Kendrick, B. (1990) Comparative anatomy of roots and mycorrhizae of common Ontario trees. *Canadian Journal of Botany* 68: 551-578.

Brundrett, M. (1991) Mycorrhizas in Natural Ecosystems. In: *Advances in ecological research*, vol. 21. Academic Press Limited. pp. 171-313.

Carlile, M.J. and Watkinson, S.C. (1994) The Fungi. Academic press Ltd., London. 482 p.

Chalupa, V. (1989) Micropropagation of *Larix decidua* Mill. and *Pinus sylvestris* L. *Biologia Plantarum* 31: 400-407.

David, A. and Faye, M. (1983) Influence of auxin and mycorrhizal fungi on the *in vitro* formation and growth of *Pinus pinaster* roots. *Plant and Soil* 71: 501-505.

Debaud, J.C., Marmeisse, R. and Gay, G. (1995) Intraspecific genetic variation in ectomycorrhizal fungi. In: Varma A. and Hock, B. (eds.) *Mycorrhiza: Structure, function, molecular biology and biotechnology*. Springer-Verlag, Heidelberg. pp. 79-113.

Diaz-Sala, C., Hutchison, K.W., Goldfarb, B. and Greenwood M.S. (1996) Maturation-related loss in rooting competence by loblolly pine stem cuttings: The role of auxin transport, metabolism and tissue sensitivity. *Physiologia Plantarum* 97: 481-490.

Ditengou, F.A., Béguiristain, T. and Lapeyrie, F. (2000) Root hair elongation is inhibited by hypaphorine, the indole alkaloid from the ectomycorrhiza fungus *Pisolithus tinctorius*, and restored by indole-3-acetic acid. *Planta* 211: 722-728.

Ditengou, F.A. and Lapeyrie, F. (2000) Hypaphorine from the ectomycorrhizal fungus *Pisolithus tinctorius* counteracts activities of indole-3-acetic acid and ethylene but not synthetic auxins in eucalypt seedlings. *Molecular Plant Microbe Interaction* 13: 151-158.

Dixon, R.K., Garret, H.E. and Stelzer, H.E. (1987) Growth and ectomycorrhizal development of loblolly pine progenies inoculated with three isolates of *Pisolithus tinctorius*. *Silvae Genetica* 5-6: 240-245.

Duddridge, J.A. (1986) The development and ultrastructure of ectomycorrhizas. IV. Compatible and incompatible interactions between *Suillus grevillei* (Klozsch) Sing. and a number of ectomycorrhizal hosts *in vitro* in the presence of exogenous carbohydrates. *New Phytologist* 103: 465-471.

Ek, M., Ljungquist P.O. and Stenström, E. (1983) Indole-3-acetic acid production by mycorrhizal fungi determined by gas chromatography-mass spectrometry. *New Phytologist* 94: 401-407.

El Ghachtouli, N., Martin-Tanguy, J., Paynot, M. and Gianinazzi, S. (1996) First report of the inhibition of arbuscular mycorrhizal infection of *Pisum sativum* by specific and irreversible inhibition of polyamine biosynthesis or by gibberellic acid treatment. *Federation of European Biochemical Societies Letters*: 385: 189-192.

El Ghachtouli, N., Paynot, M., Morandi, D., Martin-Tanguy J. and Gianinazzi, S. (1995) The effect of polyamines on endomycorrhizal infection of wild-type *Pisum sativum*, cv. Frisson (nod^+ myc^+) and two mutants (nod^- myc^+ and nod^- myc^-). *Mycorrhiza* 5:189-192.

Finnish Statistical Yearbook of Forestry (2000). Savela Y. (ed.), Gummerus Kirjapaino Oy, Jyväskylä. 365 p.

Fitter, A.H. and Garbaye, J. (1994) Interactions between mycorrhizal fungi and other soil organisms. *Plant and Soil* 159: 123-132.

Fornalé, S., Sarjala, T. and Bagni, N. (1999) Endogenous polyamine content and metabolism in the ectomycorrhizal fungus *Paxillus involutus*. *New Phytologist* 143: 581-587.

Fries, A. and Kaya, Z. (1997) Genetic control of rooting ability of lodgepole pine cuttings. *Forest Science* 43: 582-588.

Galston, A.W. and Flores, H.E. (1991) Polyamines and plant morphogenesis. In: Slocum, R.D. and Flores, H.E. (eds.). *Biochemistry and physiology of polyamines in plants*. CRC Press, Boca Raton, FL. pp. 175-186.

Garcia-Gomez, M.L., Sánchez-Romero, C., Barceló-Munoz, A., Heredia, A. and Pliego-Alfaro, F. (1994) Levels of endogenous indole-3-acetic acid and indole-3-acetyl-aspartic acid during adventitious rooting in avocado microcuttings. *Journal of Experimental Botany* 45: 865-870.

Garin, É., Bernier-Cardou, M., Isabel, N., Klimaszewska, K. and Plourde, A. (2000). Effect of sugars, amino acids, and culture techniques on maturation of somatic embryos of *Pinus strobus* on medium with two gellan gum concetrations. *Plant Cell Tissue and Organ Culture* 62: 27-37.

Garin, É., Isabel, N. and Plourde, A. (1998) Screening of large numbers of seed families of *Pinus strobus* L. for somatic embryogenesis from immature and mature zygotic embryos. *Plant Cell Reports* 18: 37-43.

Gay, G. (1986) Effect of glucose on indole-3-acetic acid production by ectomycorrhzal fungus *Hebeloma hiemale* in pure culture. *Physiologie Végetalé* 24: 185-192.

Gay, G. (1990) Effect of the ectomycorrhizal fungus *Hebeloma hiemale* on adventitious root formation in derooted *Pinus halepensis* shoot hypocotyls. *Canadian Journal of Botany* 68: 1265-1270.

Gay, G., Normand, L., Marmeisse, R., Sotta, B. and Debaud, J.C. (1994) Auxin overproducer mutants of *Hebeloma cylindrosporum* Romagnesi have increased mycorrhizal activity. *New Phytologist* 128: 645-657.

Gay, G., Rouillon, R., Bernillon, J. and Favre-Bonvin, J. (1989) IAA biosynthesis by the ectomycorrhizal fungus *Hebeloma hiemale* as affected by different precursors. *Canadian Journal of Botany* 67: 2235-2239.

Gea, L., Norman, L., Vian, B. and Gay, G. (1994) Structural aspects of ectomycorrhizas of *Pinus pinaster* (Ait.) Sol. formed by and IAA overproducing mutant of the fungus *Hebeloma cylindrosporum* Romagnesi. *New Phytologist* 128: 659-670.

Goldfarb, B., Hacket, W.P., Furnier, G.R., Mohn, C.A. and Plietzsch, A. (1998) Adventitious root initiation in hypocotyl and epicotyl cuttings of eastern white pine (*Pinus strobus*) seedlings. *Physiologia Plantarum* 102: 513-522.

Graham, J.H. and Linderman, R.G (1980) Ethylene production by ectomycorrhizal fungi, *Fusarium oxysporum* f. sp. *pini*, and by aseptically synthesized ectomycorrhizae and *Fusarium*-infected Douglas-fir roots. *Canadian Journal of Microbiology* 26: 1340-1347.

Greenwood, M.S. (1987) Rejuvenation of forest trees. *Plant Growth Regulation* 6: 1-12.

Greenwood, M.S, Cui, X. and Xu, F. (2001) Response to auxin changes during maturation-related loss of adventitious rooting competence in loblolly pine (*Pinus taeda*) stem cuttings. *Physiologia Plantarum* 111: 373-380.

Greenwood, M.S and Weir, R.J. (1995) Genetic variation in rooting ability of loblolly pine cuttings: effects of auxin and family on rooting by hypocotyl cuttings. *Tree Physiology* 15:41-45.

Grönroos, R. and von Arnold, S. (1988) Initiation of roots on hypocotyl cuttings of *Pinus sylvestris*, with emphasis on direct rooting, root elongation, and auxin uptake. *Canadian Journal of Forest Research* 18: 1457-1462.

Gupta, P.K. (1995) Somatic embryogenesis in sugar pine (*Pinus lambertiana* Dougl.). In: Jain, S.M., Gupta, P.K. and Newton, R.J. (eds.). *Somatic embryogenesis in woody plants, vol. 3 – Gymnosperms*. Kluwer Academic Publishers, Dordrecht. pp. 197-205.

Gupta, P.K. and Durzan, D.J. (1985) Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). *Plant Cell Reports* 4: 177-179.

Gupta, P.K. and Durzan, D.J. (1987) Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. *Bio/Technology* 5: 147-151.

Hacket, W.P. (1988) Donor plant maturation and adventitious root formation. In: Davis, T.D., Haissig, B.E. and Sankhla, N. (eds). *Adventitious root formation in cuttings*. Dioscorides Press, Portland, Oregon. pp.11-28.

Häggman, H.M, Aronen, T.S. and Stomp, A.-M. (1996) Early-flowering Scots pines through tissue culture for accelerating tree breeding. *Theoretical and Applied Genetics* 93: 840-848.

Häggman, H., Jokela, A., Krajnakova, J., Kauppi, A., Niemi, K. and Aronen, T. (1999) Somatic embryogenesis of Scots pine: cold treatment and characteristics of explants affecting induction. *Journal of Experimental Botany* 50: 1769-1778.

Handley, L.W., Becwar, M.R., Chesick, E.E., Coke, J.E., Godbey, A.P. and Rutter, M.R. (1995) Research and development of commercial tissue culture systems in loblolly pine. *Tappi Journal* 78: 169-175.

Hangarter, R.P. and Good, N.E. (1981) Evidence that IAA conjugates are slow-release sources of free IAA in plant tissues. *Plant Physiology* 68: 1424-1427.

Hare, R.C. (1974) Chemical and environmental treatments promoting rooting of pine cuttings. *Canadian Journal of Forest Research* 4: 101-106.

Hausman, J.F., Evers, D., Kevers, C. and Gaspar, T. (1997) Internal controls of root induction in poplar shoots raised *in vitro*. *Angewandte Botanik* 71: 104-107.

Hausman, J.F., Kevers, C. and Gaspar, T. (1994) Involvement of putrescine in the inductive rooting phase of poplar shoots in vitro. *Physiologia Plantarum* 92: 201-206.

Heinonen-Tanski, H. and Holopainen, T. (1991) Maintenance of ectomycorrhizal fungi. In: Norris, J.R., Read, D.J. and Varna, A.K. (eds.) *Methods in microbiology, vol 23, Techniques for the study of mycorrhizas*. Academic Press, London. pp. 413-422.

Heloir, M.C., Kevers, C., Hausman, J.-F. and Gaspar, T. (1996) Changes in the concentration of auxins and polyamines during rooting of *in vitro*-propagated walnut shoots. *Tree Physiology* 15: 515-519.

Ho, I. (1987) Comparison of eight *Pisolithus tinctorius* isolates for growth rate, enzyme activity, and phytohormone production. *Canadian Journal of Forest Research* 17: 31-35.

Hohtola, A. (1995) Somatic embryogenesis of Scots pine (*Pinus sylvestris* L.). In: Jain, S.M., Gupta, P.K. and Newton, R.J. (eds.). *Somatic embryogenesis in woody plants, vol. 3 – Gymnosperms.* Kluwer Academic Publishers, Dordrecht. pp. 269-285.

Honegger, R. (1985) Scanning electron microscopy of the fungus - plant cell preparative techniques. *Transcription of British Mycological Society* 84: 530-533.

Jacobs, P.E., Peterson, R.L. and Massicotte, H.B. (1989) Altered fungal morphogenesis during early stages of ectomycorrhiza formation in *Eucalyptus pilularis. Scanning Electron Microscopy* 3:249-255.

Jacobson, A. and Jacobson, L. (1981) Inhibitory effects of 2,3,5-triiodobenzoic acid on ion absorption, respiration and carbon metabolism in excised barley roots. *Plant Physiology* 67: 282-286.

Jain, M.S., Newton, R.J. and Soltes, E.J. (1988) Induction of adventitious buds and plantlet regeneration in *Pinus sylvestris* L. *Current Science* 57: 677-679.

Jones, N.B. and van Staden, J. (1995) Plantlet production from somatic embryos of *Pinus patula. Journal of Plant Physiology* 145: 519-525.

Jones, N.B., van Staden, J. and Bayley, A.D. (1993) Somatic embryogenesis in *Pinus patula. Journal of Plant Physiology* 142: 366-372.

Karabaghli, C., Frey-Klett, P., Sotta, B., Bonnet M. and Le Tacon, F. (1998) *In vitro* effects of *Laccaria bicolor* S238N and *Pseudomonas fluorescens* strain BBc6 on rooting of de-rooted shoot hypocotyls of Norway spruce. *Tree Physiology* 18:103-111.

Karabaghli-Degron, C., Sotta, B., Bonnet, M., Gay, G. and Le Tacon, F. (1998) The auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) inhibits the stimulation of *in vitro* lateral root formation and the colonization of the tap-root cortex of Norway spruce (*Picea abies*) seedlings by the ectomycorrhizal fungus *Laccaria bicolor. New Phytologist* 140: 723-733.

Kaska, D.D., Myllylä, R. and Cooper, J.B. (1999) Auxin transport inhibitors act through ethylene to regulate dichotomous branching of lateral root meristems in pine. *New Phytolologist* 142: 49-58.

Kaul, K. (1995) Somatic embryogenesis in eastern white pine (*Pinus strobus* L.). In: Jain, M.S., Gupta, P.K. and Newton, R.J. (eds.). *Somatic embryogenesis in woody plants, vol. 3 – Gymnosperms.* Kluwer Academic Publishers, Dordrecht. pp. 257-268.

Kevers, C., Bringaud, C., Hausman, J.F. and Gaspar, T. (1997) Putrescine involvement in the inductive phase of walnut shoots rooting *in vitro. Saussurea* 28: 47-57.

Keinonen-Mettälä, K., Jalonen, P., Eurola, P., von A§rnold, S. and von Weissenberg, K. (1996) Somatic embryogenesis of *Pinus sylvestris. Scandinavian Journal of Forest Research* 11: 242-250.

Klimaszewska, K. and Smith, D.R. (1997) Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. *Physiologia Plantarum* 100: 949-957.

Klimaszewska, K., Bernier-Cardou, M., Cyr, D.R. and Sutton, B.C.S. (2000). Influence of gelling agents on culture medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L. *In Vitro Cellular and Developmental Biology-Plant* 36: 279-286.

Kong, L., Attree, S.M. and Fowke, L.C. (1998) Effects of polyethylene glycol and methylglyoxal bis(guanylhydrazone) on endogenous polyamine levels and somatic embryo maturation in white spruce (*Picea glauca*). *Plant Science* 133: 211-220.

Kossuth, S.V. (1978) Induction of fascicular bud development in *Pinus sylvestris* L. *HortScience* 13: 174-176.

Kushad, M.M. and Dumbroff, E.B. (1991) Metabolic and physiological relationships between the polyamine and ethylene biosynthetic pathways. In: Slocum, R.D. and Flores, H.E. (eds.). *Biochemistry and physiology of polyamines in plants.* CRC Press, Boca Raton, FL. pp. 77-92.

Kytöviita, M-M. and Sarjala, T. (1997) Effects of defoliation and symbiosis on polyamine levels in pine and birch. *Mycorrhiza* 7:107-111.

Lainé, E. and David, A. (1990) Somatic embryognesis in immature embryos and protoplasts of *Pinus caribea. Plant Science* 69: 215-224.

Laurans, F., Pepin, R. and Gay, G. (2001) Fungal auxin overproduction affects the anatomy of *Hebeloma cylindrosporum-Pinus pinaster* ectomycorrhizas. *Tree Physiology* 21:533-540.

Lelu M.-A., Bastien, C., Drugeault, A., Gouez, M.-L. and Klimaszewska, K. (1999) Somatic embryogenesis and plantlet development in *Pinus sylvestris* and *Pinus pinaster* on medium with and without growth regulators. *Physiologia Plantarum* 105: 719-728.

Li, X.Y. and Huang, F.H. (1996) Induction of somatic embryogenesis in loblolly pine (*Pinus taeda* L.). *In Vitro Cellular and Developmental Biology-Plant* 32: 129-135.

Li, X.Y., Hunag, F.H., Murphy, J.B. and Gbur, E.E. (1998) Polyethylene glycol and maltose enhance somatic embryo maturation in loblolly pine. *In Vitro Cellular and Developmental Biology-Plant* 34: 22-26.

Linderman, R. and Call, C. (1977) Enhanced rooting of woody plant cuttings by mycorrhizal fungi. *Journal of American Society of Horticultural Science* 102:629-632.

Lomax, T.L., Muday, G.K. and Rubery PH (1995) Auxin transport. In: Davies, P.J (ed.). *Plant hormones: Physiology, biochemistry and molecular biology*. Kluwer Academic Publishers, Dordrecht. pp. 509-530.

Marks, T.R. (1996) The role of root apex in controlling rhizogenesis *in vitro*. *Plant Growth Regulation* 20: 57-60.

Martins, A., Barroso, J. and Pais, M.S. (1996) Effect of ectomycorrhizal fungi on survival and growth of micropropagated plants and seedlings of *Castanea sativa* Mill. *Mycorrhiza* 6: 265-270.

Martins, A., Casimiro, A. and Pais, M.S. (1997) Influence of mycorrhization on physiological parameters of micropropagated *Castanea sativa* Mill. plants. *Mycorrhiza* 7: 161-165.

Marx, D.H. (1969) The influence of ectotrophic fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* 59: 153-163.

McCown, B.H. (1988) Adventitious rooting of tissue cultured plants. In: Davis, T.D., Haissig, B.E. and Sankhla, N. (eds.) *Adventitious root formation in cuttings*. Dioscorides Press, Portland, Oregon. pp. 289-302.

Meijer, E. and Simmonds, J. (1998) Polyamine levels in ratio to growth and somatic embryogenesis in tissue cultures of *Mediago sativa* L. *Journal of Experimental Botany* 39: 787-794.

Mikola, J. (1992) Forest tree breeding in Finland. *Paper and Timber* 74: 83-89.

Minocha, R., Kvaalen, H., Minocha, S.C. and Long, S. (1993) Polyamines in embryogenic cultures of Norway Spruce (*Picea abies*) and red spruce (*Picea rubens*). *Tree Physiology* 13: 365-377.

Minocha, R., Smith, D.R., Reeves, C., Steele, K.C. and Minocha, S.C. (1999) Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*. *Physiologia Plantarum* 105: 155-164.

Minocha, S.C. and Minocha, R. (1995) Role of polyamines in somatic embryogenesis. In: Bajaj Y.P.S. (ed.) *Biotechnology in agriculture and forestry, vol. 3, Somatic embryogenesis and synthetic seed I*. Springer-Verlag, Berlin. pp. 53-70.

Muona, O. and Harju, A. (1989) Effective population sizes, genetic variability, and mating system in natural stands and seed orchards of *Pinus sylvestris*. *Silvae Genetica* 38: 221-228.

Nabha, S., Lamblin, F., Gillet, F., Laurain, D., Fliniaux, M., David, A. and Jacquin, A. (1999) Polyamine content and somatic embryogenesis in *Papaver somniferum* cells transformed with sam-1 gene. *Journal of Plant Physiology* 154: 729-734.

Nag, S., Saha, K. and Choudhuri, M.A. (2001) Role of auxin and polyamines in adventitious root formation in relation to changes in compounds involved in rooting. *Journal of Plant Growth Regulators* 20: 182-194.

Navratil, S. and Rochon, G.C. (1981) Enhanced root and shoot development of poplar cuttings induced by *Pisolithus* inoculum. *Canadian Journal of Forest Research* 11: 844-848.

Newton, R.J., Mare-Swize, K.A., Magallanes-Cedeno, M.E, Dong, N., Sen, S. and Jain, S.M. (1995) Somatic embryogenesis in Slash pine (*Pinus elliottii* Engelm.). In: Jain, S.M., Gupta, P.K. and Newton, R.J. (eds.). *Somatic embryogenesis in woody plants, vol. 3 – Gymnosperms*. Kluwer Academic Publishers, Dordrecht. pp. 183-195.

Normand, L., Bärtschi, H., Debaud, J.C. and Gay, G. (1996) Rooting and acclimatization of micropropagated cuttings of *Pinus pinaster* and *Pinus sylvestris* are enhanced by the ectomycorrhizal fungus *Hebeloma cylindrosporum*. *Physiologia Plantarum* 98:759-766.

Normanly, J. (1997) Auxin metabolism. *Physiologia Plantarum* 100: 431-442.

Nylund, J.-E., Wallander, H., Sundberg B. and Gay, G. (1994) IAA-overproducer mutants of *Hebeloma cylindrosporum* Romagnesi mycorrhizal with *Pinus pinaster* (Ait.) Sol. and *P. sylvestris* L. in hydroponic culture. *Mycorrhiza* 4: 247-250.

Oskarsson, O. (1995) Silmällä tehty savotta – pluspuiden valinnan historia ja arki. *Metsäntutkimuslaitoksen tiedonantoja* 579: 65 s.

Pence, V. and Caruso, J. (1984) Effects of IAA and four IAA conjugates on morphogenesis and callus growth from tomato leaf discs. *Plant Cell Tissue Organ Culture* 3: 101-110.

Peterson, R.L. and Farquhar, M.L. (1994) Mycorrhizas – Intergrated development between roots and fungi. *Mycologia* 86: 311-326.

Piché, Y., Peterson, R.L. and Ackerley, C.A. (1983) Early development of ectomycorrhizal short roots of pine. *Scanning Electron Microscopy* 3: 1467-1474.

Piola, F., Rohr, R. and von Aderkas, P. (1995) Controlled mycorrhizal initiation as a means to improve root development in somatic embryo plantlets of hybrid larch. *Physiologia Plantarum* 95: 575-580.

Rudawska, M., Bernillon, J. and Gay, G. (1992) Indole compounds released by the ectendomycorrhizal fungal strain MrgX isolated from a pine nursery. *Mycorrhiza* 2: 17-23.

Rudawska, M. and Gay, G. (1989) IAA-synthesizing activity of different ectomycorrhizal fungi in relation to nitrogen nutrition. *Agriculture Ecosystems and Environment* 28: 425-430.

Rudawska, M. and Kieliszewska-Rokicka, B. (1997) Mycorrhizal formation by *Paxillus involutus* in relation to their IAA-synthesizing activity. *New Phytologist* 137: 509-517.

Rupp, L.A. and Mudge, K.W.(1985) Ethephon and auxin induce mycorrhiza-like changes in the morphology of root organ culture of Mugo pine. *Physiologia Plantarum* 64: 316-322.

Rupp, L.A., Mudge, K.W. and Negm, F.B. (1989) Involvement of ethylene in ectomycorrhiza formation and dichotomous branching of roots of mugo pine seedlings. *Canadian Journal of Botany* 67: 477-482.

Salonen, M. (1990) Cytokinin spraying produces more cuttings. In: Napola, J. (ed.) *Annual report for forest tree breeding in Finland 1989.* Auranen, Forssa. pp. 28-29.

Salonen, M. (1991) Rooting of fascicular shoots of pines produced by cytokinin spraying treatments. In: Napola, J. (ed.) *Annual report for forest tree breeding in Finland 1990.* Mestarioffset Oy, Vantaa. pp. 32-33.

Santanen, A. and Simola, L.K. (1992) Changes in polyamine metabolism during somatic embryogenesis in *Picea abies*. *Journal of Plant Physiology* 140: 475-480.

Sarjala, T. (1996) Growth, potassium and polyamine concentrations of Scots pine seedlings in relation to potassium availability under controlled growth conditions. *Journal of Plant Physiology* 147:593-598.

Sarjala, T. (1999) Effect of organic and inorganic nitrogen sources on endogenous polyamines and growth of ectomycorrhizal fungi in pure culture. *Mycorrhiza* 8: 277-281.

Sarjala, T., Häggman, H. and Aronen, T. (1997) Effect of exogenous polyamines and inhibitors of polyamine biosynthesis on growth and free polyamine contents of embryogenic Scots pine callus. *Journal Plant Physiology* 150: 597-602.

Sarjala, T. and Kaunisto, S. (1993) Needle polyamine concentrations and potassium nutrition in Scots pine. *Tree Physiology* 13: 87-96.

Sarvas, R. (1953) Ohjeita pluspuiden valitsemisesta ja ilmoittamista varten. Silva Fennica 80: 9 p.

Sarvas, R. (1964) Havupuut. Werner Söderström Oy, Porvoo. 518 p.

Sasa, M. and Krogstrup, P. (1991) Ectomycorrhizal formation in plantlets derived from somatic embryos of Sitka spruce. *Scandinavian Journal of Forest Research* 6: 129-136.

Scagel, C. (2001) Cultivar specific effects of mycorrhizal fungi on rooting of miniature rose cuttings. *Journal of Environmental Horticulture* 19: 15-20.

Scagel, C. and Linderman, R. (1998a) Influence of ectomycorrhizal fungal inoculation on growth and root IAA concentrations of transplanted conifers. *Tree Physiology* 18: 739-747.

Scagel, C. and Linderman, R. (1998b) Relationships between differential *in vitro* indole-acetic acid or ethylene production capacity by ectomycorrhizal fungi and conifer seedling responses in symbiosis. *Symbiosis* 24: 13-34.

Slankis, V. (1973) Hormonal relationship in mycorrhizal development. In: Marks, G.C. and Kozlowski, T.T. (eds.). *Ectomycorrhizae.* Academic Press Ltd., New York. pp. 231-298.

Slee, M.U. and Spidy, T. (1970) The incidence of graft compatibility with related stock in *Pinus caribea* Mor. Var. *hondurensis* B et. G. *Silva Genetica* 3: 82-90.

Smith, S.E. and Read, D.J. (1997) Mycorrhizal symbiosis. 2nd edn. Academic Press Ltd., San Diego. 605 p.

Strzelczyk, E., Pokojska, A. and Kampert, M. (1992) The effect of pH on production of plant growth regulators by mycorrhizal fungi. *Symbiosis* 14: 201-215.

Strzelczyk, E. and Pokojska-Burdziej, A. (1984) Production of auxins and gibberellin-like substances by mycorrhizal fungi, bacteria and actinomycetes isolated from soil and the mycorrhizosphere of pine (*Pinus silvestris* L.). *Plant and Soil* 81: 185-194.

Sudhakara Reddy, M. and Satyanarayana, T. (1998) Inoculation of micropropagated plantlets of *Eucalyptus tereticornis* with ectomycorrhizal fungi. *New Forests* 16: 273-279.

Supriyanto, M. and Rohr, R. (1994) *In vitro* regeneration of Scots pine (*Pinus sylvestris*) with mycorrhizal roots from subcultured callus initiated from needle adventitious buds. *Canadian Journal of Botany* 72:1144-1150.

Sweet, G.B. and Thulin, I.H. (1973) Graft incompatibility in radiata pine in New Zealand. *New Zealand Journal of Forestry* 3: 82-90.

Sztein, A.E., Cohen, J.D. and Cooke, T.J. (2000) Evolutionary patterns in the auxin metabolism of green plants. *International Journal of Plant Science* 161: 849-859.

Tautorus, T.E., Fowke, L.C. and Dunstan, D.I. (1991) Somatic embryogenesis in conifers. *Canadian Journal of Botany* 69: 1873-1899.

Tiburcio, A.F., Altabella, T., Borrell, A. and Masgrau, C. (1997) Polyamine metabolism and its regulation. *Physiologia Plantarum* 100: 664-674.

Timmis, R. (1998) Bioprocessing for tree production in the forest industry: conifer somatic embryogenesis. *Biotechnology Progress* 14: 156-166.

Tomaszewski, M. and Wojciechowska, B. (1974) The role of growth regulators released by fungi in pine mycorrhizae. In: Carr, D.J. (ed.) *Proceedings of the 8th international conference on plant growth substances*. Hirokawa Publishing Company, Tokyo. pp. 217-227.

Tonkin, C.M., Malajczuk, N. and McComb, J.A. (1989) Ectomycorrhizal formation by micropropagated clones of *Eucalyptus marginata* inoculated with isolates of *Pisolithus tinctorius*. *New Phytologist* 111: 209-214.

Tranvan, H., Habricot, Y., Jeannette, E., Gay, G. and Sotta, B. (2000) Dynamics of symbiotic establishment between an IAA-overproducing mutant of the ectomycorrhizal fungus *Hebeloma cylindrosporum* and *Pinus pinaster*. *Tree Physiology* 20: 123-129.

Venäläinen, M., Annala, M-L., Kosonen, E., Rantanen, H. and Tynkkynen, H. (1994) Plusmäntyjen testausrekisteri ja jalostushyöty. *Metsäntutkimuslaitoksen tiedonantoja* 497. Hakapaino Oy, Helsinki, 89 p.

Wallander, H., Nylund, J.-E., and Sundberg, B. (1992) Ectomycorrhiza and nitrogen effects on root IAA: results contrary to current theory. *Mycorrhiza* 1: 91-92.

Wallander, H., Nylund, J.-E., and Sundberg, B. (1994) The influence of IAA, carbohydrate and mineral concentration in host tissue on ectomycorrhizal development on *Pinus sylvestris* L. in relation to nutrient supply. *New Phytologist* 127: 521-528.

Walter, C., Carson, S.D., Menzies, M.I., Richardson, T. and Carson, M. (1998) Review: Application of biotechnology to forestry – molecular biology of conifers. *World Journal of Microbiology and Biotechnology* 14: 321-330.

Wong, K.K.Y., Piche, Y., Montpetit, D. and Kropp, B.R. (1989) Differences in the colonization of *Pinus banksiana* roots by sib-monocaryotic and dikaryotic strains of ectomycorrhizal *Laccaria bicolor*. *Canadian Journal of Botany* 67: 1717-1726.

Zar, J.H. (1984) Biostatistical analyses, 2nd edn. Prentice-Hall Inc, Englewood Cliffs, New York, 718 p.

Zarb, J. and Walters, D.R. (1994a) The effects of polyamine biosynthesis inhibitors on growth, enzyme activities and polyamine concentrations in the mycorrhizal fungus *Laccaria proxima*. *New Phytologist* 126: 99-104.

Zarb, J. and Walters, D.R. (1994b) The formation of cadaverine, aminopropylcadaverine and N,N bis (3-aminopropyl) cadaverine in mycorrhizal and phytopathogenic fungi. *Letters in Applied Microbiology* 19: 277-280.