Root Associated Fungi of Conifer Seedlings and Their Role in Afforestation of Agricultural Land

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Abstract

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The aim of the present thesis was to study root-related, mycological aspects of afforestation of former agricultural land with conifer seedlings, focusing on mycorrhizal, endophytic and pathogenic fungi, and factors influencing their interactions with roots. Mycorrhizal colonisation of root systems is an important factor in determining seedling vigour, and consequently, survival and growth after their outplanting on agricultural land. Investigations in forest nurseries demonstrated that cultivation systems of *Pinus sylvestris* and *Picea abies* seedlings significantly affect the mycorrhizal colonisation of root systems. Bare root cultivation of pine and a containerised polyethylene roll system for spruce provide the most suitable conditions for abundant mycorrhizal colonisation of roots. Artificial inoculation of roots with selected ectomycorrhizal fungi at outplanting was also evaluated as a method to improve establishment of conifer seedlings on agricultural land. The results demonstrated that seedlings inoculated with certain ectomycorrhizal fungi had significantly higher survival and better growth during the first two growing seasons than non-inoculated seedlings. However, these effects appear to be short-lived since, even with relatively costly, labour-intensive inoculation methods, it was difficult to manipulate mycorrhizal communities over time, and their composition was ultimately largely governed by environmental conditions of the planting sites. In other studies, it was also demonstrated that the planting environment determines the fungal communities in decayed conifer seedling roots since different fungi were detected in forest nurseries, afforested clear-cuts and agricultural land. The common occurrence of pathogenic fungi in all planting environments indicated the potential risk of root diseases and consequently the need for accurately assessing plant health before outplanting. The potential for biochemical control of root pathogens was investigated through isolation and characterisation of new antifungal depsipeptides produced by the actinomycete *Kutzneria* sp. 744. The endophytic nature of the organism indicated a possible application for biological control of root pathogens. Combinations of different sampling strategies and detection methods revealed high diversities of fungi associated with both healthy and decayed roots. Mycorrhizal fungi were predominantly detected in healthy roots and pathogenic fungi predominantly in decayed roots while endophytes showed high ecological plasticity and were common in both healthy and decayed roots of conifer seedlings.

Keywords: afforestation, agricultural land, ectomycorrhiza, endophytes, forest nurseries, inoculation, *Pinus sylvestris*, *Picea abies*, root pathogens, seedling cultivation systems.

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Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Menkis, A., Vasiliauskas, R., Taylor, A. F. S., Stenlid, J. & Finlay, R. (2005) Fungal communities in mycorrhizal roots of conifer seedlings in forest nurseries under different cultivation systems, assessed by morphotyping, direct sequencing and mycelial isolation. *Mycorrhiza.* In press.

II. Menkis, A., Vasiliauskas, R., Taylor, A. F. S., Stenström, E., Stenlid, J. & Finlay, R. (2005) Fungi in decayed roots of conifer seedlings from forest nurseries, afforested clearcuts and abandoned farmland. *Plant Pathology*. In press.

III. Menkis, A., Vasiliauskas, R., Taylor, A. F. S., Stenlid, J. & Finlay, R. Afforestation of abandoned farmland with conifer seedlings inoculated with three ectomycorrhizal fungi. Submitted manuscript.

IV. Menkis, A., Allmer, J., Vasiliauskas, R., Lygis, V., Stenlid, J. & Finlay, R. (2004) Ecology and molecular characterization of dark septate fungi from roots, living stems, coarse and fine woody debris. *Mycological Research 108*, 965-973.

V. Broberg, A., Menkis, A., Vasiliauskas, R. Kutzneride 1 - 4, new depsipeptides from the actinomycete *Kutzneria* sp. 744 inhabiting mycorrhizal roots of *Picea abies* seedlings. Submitted manuscript.

Introduction

The living roots of trees harbour diverse communities of symbiotic fungi with the interactions forming a continuum from pathogenic to mutualistic associations (Wilcox, 1983). These communities influence a diverse range of biological processes, such as root diseases and their biological control, and the development of endophytic and mycorrhizal symbioses. Survival, establishment and the health and growth of seedlings are largely dependent on the activity of these fungi, which constitute a large component of microbial biomass in tree roots and therefore may be essential in determining afforestation success.

Socioeconomic issues and challenges of afforestation of former agricultural land

Afforestation of poorly productive former agricultural land is an important issue in rural development of new member states within the European Union and several projects have been initiated that are financially supported by the EU (European Commission press release, 2004). Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* [L.] Karst.) are the most common tree species throughout the hemiboreal forests of north-eastern Europe (Larsson, 2001) and are therefore predominantly used in afforestation of agricultural land. In Lithuania alone, around half a million hectares of former agricultural land is intended for afforestation, aiming to ensure sustainable land use. However, afforestation of old fields is often difficult and largely depends on site quality. The cultivation of agricultural crops, use of machinery, fertilization and liming have changed the physical and chemical properties of the soils. In addition, the composition of the soil microbial communities has been markedly altered. Consequently, routine forestry practices are frequently inappropriate for afforestation of such areas and special approaches and planting techniques are usually required.

Natural regeneration on former agricultural land is frequently poor and self seeded trees tend to occur in clumps, while sowing is not successful due to heavy competition from ground vegetation (Hytönen, 1995). Afforestation is commonly carried out with nursery seedlings but their survival and development can be rapidly compromised by numerous agents of damage, especially in the absence of appropriate beneficial fungi colonising seedling roots. However, roots of outplanted seedlings are frequently found to be lacking these beneficial symbionts (Halonen & Laiho, 1991).

Fungal associations in living tree roots

Mycorrhizal associations

The term *mycorrhiza* (”fungus-root”; Greek: *mykes* = fungus, *rhiza* = root), a mutualistic association formed between specialised soil fungi and roots of higher plants, was first described late in the 19th century by the forest pathologist Frank (1885). Based on the fossil evidence, it is believed that original evolution of

terrestrial plants about 400 million years ago was possible only through a mutualistic partnership similar to the mycorrhizal habit of currently existing plants (Pyrozynski & Malloch, 1975; Simon, 1993; Wilkinson, 2001). About 90% of the world's present species of vascular plants belong to families that are characteristically mycorrhizal (Smith & Read, 1997). Mycorrhizal symbiosis is important in woody plants, especially on marginal habitats (Harley, 1969; Smith & Read, 1997).

Several classes of mycorrhizal relationships are recognised based on the structures formed in roots (Smith & Read, 1997). The most important associations for woody plants in forest ecosystems of the northern hemisphere are called ectomycorrhizas. This association is most characteristic of the plant families *Pinaceae*, *Fagaceae*, *Betulaceae* and others (Meyer, 1973) *i.e.*, the principal tree species of the boreal and temperate forests (Barbour, Burk & Pitts, 1987; Larsson, 2001). Despite the relatively low number (*ca.* 3%) of plant species world wide involved in ectomycorrhizal (ECM) symbiosis (Meyer, 1973; Taylor & Alexander, 2005), an impressively high number of fungal species (5000-6000) has been estimated to date (Harley, 1989; Molina, Massicotte & Trappe, 1992). The fungi are predominantly basidiomycetes (Basidiomycota), in some cases ascomycetes (Ascomycota) with very few zygomycetes (Zygomycota). In a characteristic ectomycorrhiza, the fungus forms a compact sheath or mantle around the rootlet (Fig. 1a), from which hyphae grow inward to the cortex, forming a continuous network (known as the Hartig net) between the cortical cells, and outward to the surrounding soil (Fig. 1b). A fine network of fungal hyphae explores and extracts nutrients from a volume of soil far beyond that directly influenced by the roots themselves. A proportion of these nutrients is translocated through the hyphal network to the short roots. The ECM short roots are the functional units of the symbiosis where exchange of nutrients, carbon and water between the symbiotic partners take place (Smith & Read, 1997). In general, more than 95% of the short roots of boreal forest trees are colonised by ECM fungi (Taylor, Martin & Read, 2000). Ectomycorrhizal colonisation is a prerequisite for normal growth of certain tree species, such as *Pinus* spp. (Read, 1998).

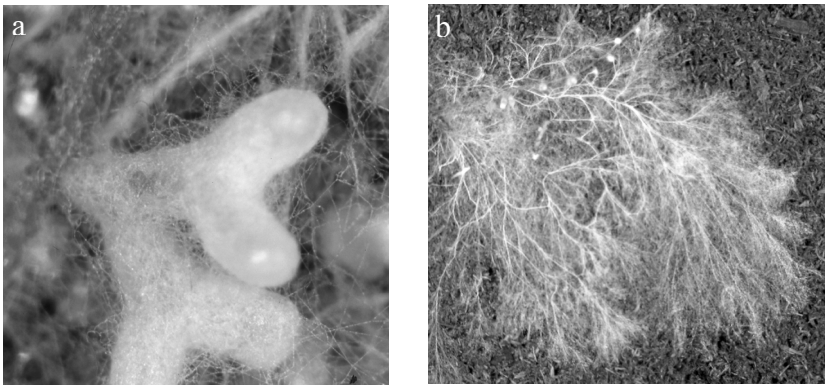

Fig. 1. Vegetative structures of the ectomycorrhizal basidiomycete *Suillus luteus* (L.) Gray on short roots of *Pinus sylvestris* L. a) dichotomously branched root tips covered by the hyphal mantle; b) extensive extraradical mycelium growing out into the soil from the mycorrhizal tips.

Pathogenic associations

A large number of soil fungi are root pathogens, which kill living trees by attacking functional vascular and cambium tissues (Schippers & Gams, 1979). The first symptoms of disease generally appear on the above-ground parts of conifer seedlings as stunted growth, discoloration and loss of the needles (Beyer-Ericson, Damm & Unestam, 1991; Lilja *et al.*, 1992). Lack of fine root development, partial or total death of the root systems and extensive cortical decay are the most common disease symptoms below-ground (Beyer-Ericson, Damm & Unestam, 1991; Lilja *et al.*, 1992). *Cylindrocladium*, *Fusarium*, *Nectria*, *Pythium*, *Phytophthora* and *Rhizoctonia* spp. are the most common root-rotting organisms associated with conifer seedlings (Galaaen & Venn, 1979; Lilja *et al.*, 1992; Lilja & Rikala, 2000; Paper **II**). Most species can infect seedlings at an early stage of development, remain latent and cause disease later in the season or following seedling outplanting when growing conditions stress the plants (Lilja & Rikala, 2000).

In contrast to mycorrhizal fungi, the presence of root pathogens in seedling roots has adverse effects on their survival and growth (Lilja *et al.*, 1992). Root dieback leads to a significant decrease in seedling quality in forest nurseries (Venn, Sandvik & Langerud, 1986; Lilja, Lilja & Poteri, 1988; Unestam, Beyer-Ericson & Strand, 1989; Beyer-Ericson, Damm & Unestam, 1991; Lilja *et al.*, 1992; Kacprzak, 1997; Camporota & Perrin, 1998; Lilja & Rikala, 2000; Hietala, Vahala & Hantula, 2001; Paper **II**). In some cases up to 40% of stock production and up to 93% of outplanted seedlings may be lost (Lilja, 1994; Lilja & Rikala, 2000). Potential root pathogens also persist on planting areas (Perry *et al.* 1987; Wilberforce *et al.*, 2003; Paper **II**) and can readily infect transferred seedlings which are likely to be predisposed to infection due to replanting stress.

Endophytic associations

Fungal endophytes live asymptomatically within plants (intercellularly or intracellularly) for at least part of their life cycle (Petrini, 1991; Wilson, 1995; Saikkonen *et al.*, 1998). Endophytes have been found in all woody plants studied to date and represent numerous fungal species (Sieber, 2002). Many taxa found in roots have darkly pigmented hyphae. Among these, *Phialocephala fortinii* Wang & Wilcox is the most common species (Jumpponen & Trappe, 1998; Addy, Piercey & Currah, 2005; Papers **I**, **II** & **IV**), however the basis of its symbiotic relationship remains ambiguous. It may be a weak pathogen, degrader of senescent root tissue or a mutualist (Harney, Wentworth & Wargo, 1995; Jumpponen, Mattson & Trappe, 1998; Jumpponen, 2001; Sieber, 2002; Addy, Piercey & Currah, 2005; Paper **IV**).

Endophytes live in a habitat which involves continual metabolic interactions between fungus and the host. Substances produced by these organisms may be toxic to plant pathogens or act as repellents against insects or herbivores (Clay, 1989; Calhoun *et al.*, 1992; Schulz *et al.*, 1995; Lane, Christensen & Miles, 2000; Arnold *et al.*, 2003; Findlay *et al.*, 2004). Some endophytes of woody roots may

be selectively antagonistic to plant pathogens and/or pest insects and may be an important source of new biologically active secondary metabolites (Paper **V**).

The role of ectomycorrhizal fungi in forest nurseries and in the field

Seedling production

Ectomycorrhizal colonisation of root systems is an important factor in determining seedling vigour, and consequently quality (Smith & Read, 1997). Apart from nutritional benefits to their hosts, some mycorrhizal fungi can enable seedlings to withstand high soil temperatures (Marx & Bryan, 1971) and increase resistance to drought (Parke, Linderman & Black, 1983). Of practical importance to nursery management, some mycorrhizal fungi can protect roots against certain pathogens (Sinclair, Sylvia & Larsen, 1982; Sampagni, Perrin & Le Tacon, 1985; Stenström, Damm & Unestam, 1997) and consequently can improve growth of the seedlings (Smith & Read, 1997).

However, commercial nursery seedlings generally either lack mycorrhizal fungi or have a very limited flora associated with their root systems. Species of the genera *Thelephora*, *Laccaria*, *Suillus*, *Amphinema*, *Hebeloma* and *Phialophora* are the most common colonisers of conifer seedling roots (Thomas & Jackson, 1979; Wilcox & Wang, 1987b; Grogan, O'Neill & Mitchell, 1994; Kernaghan, Sigler & Khasa, 2003; Paper **I**). The extent of colonisation may depend on several factors: the cultivation system, soil conditions and management practices. Fumigation, soil disturbance and high rates of pesticide and fertilizer application may inhibit formation of ectomycorrhizal roots (Väre, 1990; O'Neill & Mitchell, 2000; Laatikainen & Heinonen-Tanski, 2002; Kernaghan, Sigler & Khasa, 2003). High pH also has a strong inhibitory effect on ECM development (Cordell & Marx, 1994; Sundari & Adholeya, 2003).

Artificial inoculation with mycorrhizal fungi may eliminate potential mycorrhiza deficiency and improve outplanting performance of seedlings. Therefore, several techniques have been developed to inoculate nursery seedlings with specific ECM species (Trappe, 1977; Danielson, Visser & Parkinson, 1984; Marx *et al.*, 1984; Castellano, Trappe & Molina, 1985; Kuek, Tommerup & Malajczuk, 1992; Castellano, 1994). However, these methods require additional production efforts and costs. Another approach was investigated in the study described in Paper **I**, namely to gain better knowledge of how different cultivation systems promote natural ECM colonisation of roots, and by what taxa of fungi.

Performance of seedlings after outplanting

Ectomycorrhizal fungi are practically ubiquitous in natural forests (Taylor, Martin & Read, 2000), and ectomycorrhiza are probably formed by the species best suited to the prevailing conditions (Mikola, 1973). Therefore, when new species are introduced they are likely to have little chance to survive in competition with the indigenous fungal community. On areas without existing ECM inoculum (*e.g.* abandoned agricultural land) the situation is different (Hacskaylo, 1973). For

example afforestation with exotic pine species in many countries failed, until the appropriate mycorrhizal symbionts were introduced (Hatch, 1936; Briscoe, 1959; Gibson, 1963; Madu, 1967; Bjorkman, 1970; Mikola, 1970; Marx, 1980).

Colonisation of roots by particular ECM fungi, as the consequence of particular cultivation practices in forest nurseries (*e.g.* Paper **I**) or achieved by artificial inoculation, both in the nursery (Trappe, 1977) or in the field (Dunabeitia *et al.*, 2004; Paper **III**), may significantly promote survival, establishment and growth of young trees in newly established forest plantations (Perry, Molina & Amaranthus, 1987; Kropp & Langlois, 1990; Stenström, Ek & Unestam, 1990; Le Tacon *et al.*, 1994; Haselwandter & Bowen, 1996; Garbaye & Churin, 1997; Pera *et al.*, 1999; Ortega *et al.*, 2004; Paper **III**). The main mechanisms behind this improvement are thought to be enhanced uptake of water and nutrients through a greatly increased root-absorbing surface (Hatch, 1937; Smith & Read, 1997), increased longevity and growth of roots (Chilvers & Gust, 1982; Wilcox, 1996), and protection against environmental stress factors such as drought, pathogens and heavy metal pollution (Chakravarty & Unestam, 1985; Colpaert & Vanassche, 1992; Morin, Samson & Dessureault, 1999; Van Tichelen, Colpaert & Vangronsveld, 2001; Ortega *et al.*, 2004).

Aims of this study

The overall aim of the work described in this thesis was to study root-related, mycological aspects of afforestation of former agricultural land with conifer seedlings

More specifically, the objectives were:

- to investigate the mycorrhizal and pathological status of seedling roots in forest nurseries and after their outplanting
- to determine the impact of different cultivation systems and different planting environments on fungal colonisation of roots
- to provide more detailed information about root-inhabiting fungi by combining different sampling approaches and/or different identification methods
- to investigate the role of ectomycorrhizal inoculation on survival and growth of seedlings in the field
- to determine the identity and ecology of endophytic taxa associated with both healthy and decayed roots
- to investigate new biochemical means for controlling root pathogens

Materials and Methods

Study sites and sampling

Figure 2 shows the study sites where pine (*Pinus sylvestris* L.) and spruce (*Picea abies* [L.] Karst.) seedlings were sampled for the studies presented in this thesis.

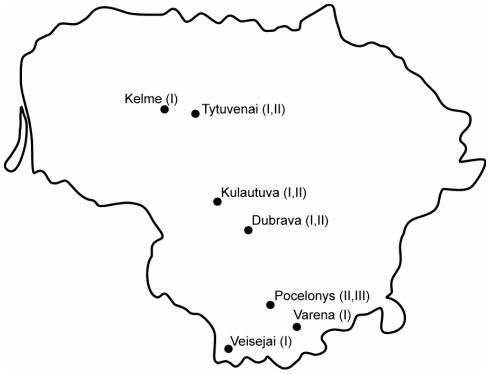

Fig. 2. Map of Lithuania showing the location of seven study areas. Roman numerals in brackets refer to the respective papers.

The study described in Paper **I** was carried out in six forest nurseries utilising different cultivation systems (Fig. 3). In this study, a total of 330 pine and 330 spruce seedlings were sampled. Mycorrhizal roots of each tree species were collected using two approaches: i) in April 2001, high numbers of root tips (*ca.* 10%) from a small number of plants (30) were collected in an intensive sampling; ii) in April 2002, a small number of root tips (20) from a high number of plants (300) was collected as an extensive sampling. In total, 18166 and 12000 root tips were collected in the intensive and extensive sampling approaches, respectively.

The sampling areas described in Paper **II** included three bare root nurseries and tree clear-cuts adjacent to them, and one area of afforested farmland at Pocelonys (Fig. 2). In total, 240 pine and 240 spruce seedlings with symptoms of root rot were collected in July 2003. From each root system, three to five main lateral roots were selected randomly and from each of these a single *ca.* 5 mm long segment was sampled at the zone of advancing decay.

The study site described in Paper **III** was abandoned farmland (Fig. 2) which was afforested with 8000 pine and 8000 spruce seedlings that had been inoculated prior to outplanting with three ECM fungi. The three inoculation treatments and the control treatment were arranged in 16 plots for each tree species throughout the four hectares of afforestation area. Sampling was carried out in October 2003 and 2004. Each year, five pine and spruce seedlings were collected from each plot and 20 root tips were randomly sampled from the root system of each plant. In total, 320 plants and 6400 root tips were collected during this study.

Fig. 3. Cultivation systems of *Pinus sylvestris* and *Picea abies* seedlings in forest nurseries (Paper **I**) – localities shown in Figure 2: bare root a) outdoor (Dubrava, Kelme, Kulautuva, Veisejai (pine only)) and b) greenhouse (Varena (pine only)); containerised systems c) plastic tray (Tytuvenai) and d) polyethylene rolls (Varena and Veisejai (both spruce only)).

Material described in Papers **IV** and **V** originated from studies of Papers **I** and **II**. Additional material described in Paper **IV** was sampled during studies of other authors (Lygis, Vasiliauskas & Stenlid, 2004; Lygis *et al.*, 2004; Vasiliauskas *et al.*, 2004; Allmer *et al.*, 2005).

Identification of fungi

Mycorrhizal morphotyping

Morphotyping – morphological and anatomical identification of mycorrhizal root tips. In the studies of Papers **I** and **III** mycorrhizal tips were identified by the presence of a mantle, external hyphae or rhizomorphs, the absence of root hairs, a slightly swollen apex and, in pine, dichotomous branching of the fine roots. In the absence of macroscopic mycorrhizal features, sections were made of root tips using a razor blade to verify the presence of a Hartig net. Root squashes were used to examine the mantle, hyphae and rhizomorphs microscopically. Each morphotype was examined and compared with available illustrative materials

(Agerer, 1986-1988; Agerer *et al.*, 1996-1998). Only morphotypes matching published descriptions were given taxonomical names. The morphotypes, which did not match any of these descriptions, were classed as unidentified, grouped accordingly to morphological characters, and given a descriptive name.

Mycelial isolation

Pure culture isolation of fungi from tree roots is an important approach with which to explore the fungal diversity of culturable species and is the basis for traditional fungal taxonomy. In the studies described in this thesis, root-inhabiting fungi were isolated from both mycorrhizal root tips (**I**) and segments of decayed main lateral roots (**II**) of *P. sylvestris* and *P. abies* seedlings. Prior to isolation, roots were carefully washed in tap water, surface sterilized for 15 – 60s in 33% hydrogen peroxide and rinsed three times in autoclaved, deionised water. For initial isolation, roots were placed on nutrient medium: mycorrhizal roots tips (**I**) onto modified Melin Norkrans agar (Marx, 1969), while segments of decayed roots (**II**) were plated onto three different types of agars, – 2% water agar, vegetable juice agar (Barklund & Unestam, 1988) and Hagem agar (Stenlid, 1985). In addition, apple tissue was used as an intermediate nutrient source for isolation of fungi from segments of decayed roots (Hansen *et al.*, 1979). Inoculated systems were checked daily and any outgrowing mycelia were immediately subcultured on fresh agar medium.

All isolated fungi were separated into groups based on mycelial morphology. For identification, representative cultures from each morphological group were analysed by sequencing the internal transcribed spacer (ITS) of the ribosomal DNA using the fungal-specific primer ITS1-F (Gardes & Bruns, 1993) and universal primer ITS4 (White *et al.*, 1990). A culture of an actinomycetous endophyte (**V**) which was isolated during the study in Paper **I** was identified by sequencing of the 16S region of the ribosomal DNA using the primers 27F and 1492R (Lane, 1991). Extraction of DNA, amplification and sequencing followed established methods (Rosling *et al.*, 2003; Rangel-Castro, Levenfors & Danell, 2004). In addition, representatives of sporulating cultures that had not been taxonomically defined by sequencing were sent for morphological identification to the Central Bureau of Fungal Cultures (CBS) in Utrecht, the Netherlands.

Direct sequencing

Direct sequencing of fungal DNA from roots is a sensitive method for the detection of potentially all root-inhabiting fungi, in particular species that are usually overlooked by isolation (Egger, 1995; Horton & Bruns, 2001; Kernaghan, Sigler & Khasa, 2003). In the studies of this thesis, the method was extensively used to identify fungi from both mycorrhizal root tips (**I**) and segments of decayed roots (**II**). Extraction of DNA, amplification and sequencing followed the same methods as described for fungal cultures. If amplification gave only one DNA band per sample (confirming that all DNA came from one source only), the product was used for sequencing. Multiple-banded PCR products were separated on 2% agarose gels and gel plugs were cored from the bands with pipette tips.

Separated bands were re-amplified with universal primers ITS1 (internal to ITS1-F) and ITS4 and the resulting single-banded products were sequenced in both directions using the same primers as for PCR amplification.

Identity of sequences

Databases at both GenBank (Altschul *et al.*, 1997) and at the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala were used to determine the identity of sequences (**I**, **II**, **IV** & **V**). The criteria used for deciding on the taxon or genus of a given strain were its intra- and interspecific ITS/16S sequence similarity to those present in the databases. For each taxon an individual approach was taken, as the extent of ITS/16S variation differs from species to species or genus to genus. In most cases intraspecific ITS similarity for the sequenced fungi was 98–100%, and the similarity within genera varied between 90–97%. The interspecific 16S similarity for the sequenced actinomycete was 98%.

Results and Discussion

Impact of cultivation system upon mycorrhizal colonisation of roots

The study described in Paper **I** investigated the possibility of achieving an abundant mycorrhizal colonisation of conifer seedling roots by exploring the existing natural mycorrhization in forest nurseries. The investigation revealed that the extent of mycorrhizal colonisation depends to a large extent on the cultivation system. In pine, colonisation was highest in the nursery outdoor bare root system (Fig. 3a), where 47.9% of the roots were mycorrhizal, while in spruce the highest colonisation was found in the polyethylene rolls (71.0%) (Fig. 3d). The lowest colonisation was observed in bare root greenhouse seedlings of pine (19.4% of roots colonised) (Fig. 3b), and in spruce, grown as outdoor bare root seedlings (35.3%). These results demonstrated that selection of a proper cultivation system in forest nurseries may yield seedling material with a high extent of mycorrhizal colonisation, which is known to increase seedling vitality (Herrmann *et al.*, 1992; Genere, 1995; Krasowski *et al.*, 1999), and consequently may improve survival and growth of seedlings following their replanting (Smith & Read, 1997; Paper **III**).

Although profound differences were observed in mycorrhizal colonisation, moderate similarity was recorded in mycorrhizal communities between pine and spruce, and among different cultivation systems, indicating a low host and site specificity of many mycorrhizal fungi occurring in forest nurseries. Moreover, the study revealed many mycorrhizal species *e.g. Rhizopogon* spp., *Suillus* spp., *Tomentella* spp. and *Phialophora finlandia* Wang & Wilcox that also form associations with trees under forest or field conditions. Among these, *P. finlandia* was the most common for both tree species and in all cultivation systems. The

ability to form mycorrhizal symbioses with both ECM and ericoid hosts (Wang & Wilcox, 1985; Wilcox & Wang, 1987a, b; Ursic & Peterson, 1997; Monreal, Berch & Berbee, 1999; Vrålstad, Myhre & Schumacher, 2002) and common occurrence in forest nurseries (Ursic & Peterson, 1997; Ursic, Peterson & Husband, 1997; Kernaghan, Sigler & Khasa, 2003; Paper **I**) as well as in forest ecosystems (Tedersoo *et al.*, 2003) indicate a certain ecological plasticity of the fungus which might have a positive impact on vitality and establishment of outplanted seedlings. Apart from mycorrhizal and endophytic fungi, the study (**I**) also revealed the presence of root pathogens, *e.g. Nectria radicicola* Gerlach & L. Nilsson or *Fusarium oxysporum* Schltdl.. Nevertheless, as investigated plants showed no apparent disease symptoms, it is possible that disease development in roots was restricted by the presence of mycorrhizal fungi (Chakravarty & Unestam, 1987; Duchesne, 1994).

The combination of different sampling strategies and different detection methods yielded a high fungal diversity in mycorrhizal roots (**I**). Certain morphotypes were revealed only by intensive root system analyses, while others were only detected with increasing numbers of examined plants. Morphotyping allowed preliminary detection of mycorrhizal species, whereas isolation enabled the additional detection of many other (endophytic and pathogenic) root associated fungi. Nevertheless, direct sequencing revealed the highest diversity of both mycorrhizal and non-mycorrhizal fungi as up to four distinct taxa were detected in a single root tip. The overlap between isolation and direct sequencing was low (*ca.* 14%) as each of the methods was more or less specific in detecting particular functional groups of fungi: direct sequencing was best for mycorrhizal basidiomycetes, while isolation was good for detecting ascomycetous endophytes. Both isolation and direct sequencing were valuable methods for studying fungal communities in mycorrhizal roots, whereas morphotyping was a fast and reliable method for assessment of the presence or absence of mycorrhizal colonisation.

Fungi colonising decayed roots of conifer seedlings

The study described in Paper **II** demonstrated that different fungi colonise, and presumably cause, root-rot of conifer seedlings in different types of planting environment: forest nurseries; afforested clear-cuts and abandoned farmland (Fig. 4). The roots of outplanted seedlings thus had to be rapidly colonised, in twelve weeks, by indigenous soil fungi of clear-cuts and agricultural land. The common occurrence of many root-rot fungi *e.g. Fusarium* spp., *Nectria* spp., *Chalara* spp. and other species in different planting environments indicated a potential risk of root disease. Accurate, early assessment of plant health in the nursery is of considerable practical importance since weakened seedlings are likely to be more susceptible to infections following transfer to the field, especially due to recent replanting stress.

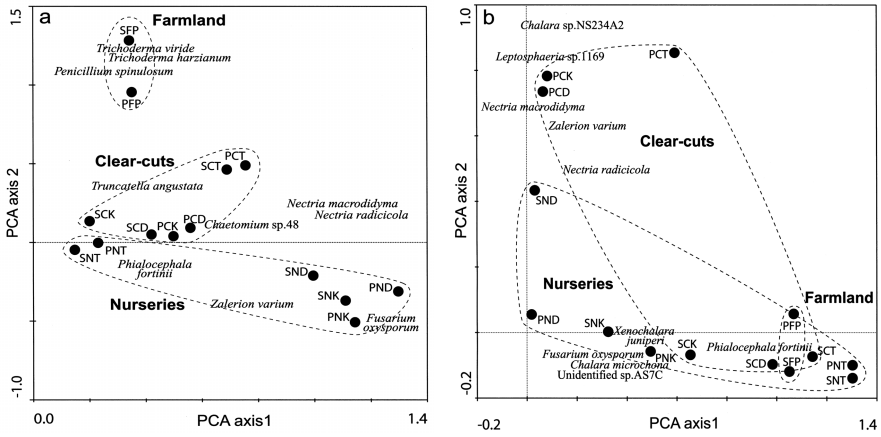

Fig. 4. First and second axes of a Principal Component Analysis of fungal communities inhabiting decayed roots of conifer seedlings (PNT – *Pinus sylvestris*; SNT – *Picea abies*) in different planting environment (PNT – nurseries; PCT – clear-cuts, PFP – farmland) and their respective localities (PND – Dubrava; PNK – Kulautuva; PNT – Tytuvenai; PFP – Pocelonys) assessed by: a) mycelial isolation followed by ITS rDNA sequencing; b) direct ITS rDNA sequencing. Taxonomic names show the ten most common fungi detected by each of the methods.

In study **II**, pure culture isolation and direct sequencing provided complementary data that was necessary for a complete description of the fungal communities colonising decayed roots of conifer seedlings. Figure 4 shows that the use of either of these methods alone would have resulted in very different descriptions of the fungal community composition. Fungi detected by direct sequencing were only seldom or never isolated into pure culture indicating that some of them might be unculturable. The most common fungi isolated in forest nurseries were *Fusarium* spp., in clear-cuts – *Nectria* spp., and in abandoned farmland, – *Penicillium* spp. and *Trichoderma* spp. In contrast to isolation, the most common taxa detected by direct sequencing were different in all planting environments and included the endophyte *Phialocephala fortinii* Wang & Wilcox and *Chalara* sp. NS234A2. The exact role of *P. fortinii* in root dieback is unknown, but there is a possible shift from endophytic to pathogenic behaviour along with changes in health and resistance of a tree (**IV**).

Application of ectomycorrhizal fungi in afforestation of abandoned agricultural land

One potential problem facing outplanted seedlings on agricultural land is the lack of mycorrhizal fungi, therefore in the study described in Paper **III** the effect of artificial inoculation on survival and growth of *P. sylvestris* and *P. abies* seedlings was investigated. A novel, non-destructive filter paper inoculation method developed from Chilvers, Douglass & Lapeyrie (1986) enabled production of large amounts of high quality vegetative inoculum of selected fungi: *Cenococcum geophilum* Fr., *Hebeloma crustuliniforme* (Bull.) Quél. or the *Piceirhiza*

bicolorata mycobiont. The inoculation procedure enabled application of standardized amounts of inoculum at outplanting by wrapping each root system in a filter paper containing ectomycorrhizal mycelia, enclosure in a damp layer of peat-sand mixture and final wrapping in an outer paper towel. This was expected to create favourable, semi-sterile conditions for root mycorrhization as the roots were temporarily separated from the bulk soil allowing colonisation by the inoculated fungi without competition from other soil fungi.

The investigation revealed that during two growing seasons, seedlings inoculated with *C. geophilum* and the *P. bicolorata* mycobiont showed significantly higher survival and better growth compared with non-inoculated seedlings. Although the target mycorrhizas of both *C. geophilum* and *P. bicolorata* were regularly found on inoculated seedlings, the dominant mycorrhizas were different and in many cases represented taxa commonly observed in forest nurseries (**I**). Interestingly, seedlings inoculated with either of these two fungi showed increased overall mycorrhizal colonisation of roots implying a synergistic effect of root colonisation by different mycorrhizal fungi under suitable environmental conditions. Moreover, *C. geophilum* and the *P. bicolorata* mycobiont were also observed on *H. crustuliniforme* inoculated and non-treated seedlings showing independent colonisation and suitability for the particular field conditions. By contrast, *H. crustuliniforme* was less suitable for the particular site conditions as the inoculation treatment did not result in a positive effect on either tree species, and the fungus was completely absent from the site after two growing seasons.

Furthermore, the study showed importance of tree species in picking up the mycorrhizal species as the seedlings of pine and spruce were in most cases colonised by the different fungi. Thus, success of mycorrhizal inoculation in the field largely depends on the fungus, host tree, and ecological conditions of the soil which to a great extent regulate mycorrhizal colonisation at a given site (McAfee & Fortin, 1985). The study also demonstrated that with relatively labour intensive methods and suitable fungi for ectomycorrhizal inoculation it is possible to achieve a positive effect on seedling survival and growth during the first two years. However, such effects could be temporary since it was hard to manipulate the mycorrhizal community over longer periods under field conditions.

Dark septate endophytes in healthy and decayed trees

In conifer seedlings, a common isolation of dark septate (DS) fungi from mycorrhizal roots (**I**) and frequent detection by direct sequencing in decayed roots (**II**) indicated that 1) DS fungi were always present in seedling roots regardless of the health status of the tree; 2) in healthy roots they may persist latently or resemble endophytes in life style; 3) in decayed roots they might act as decomposers, however, it remains unclear whether DS fungi can weaken or even cause death of the tree and 4) only appropriate combinations of different detection

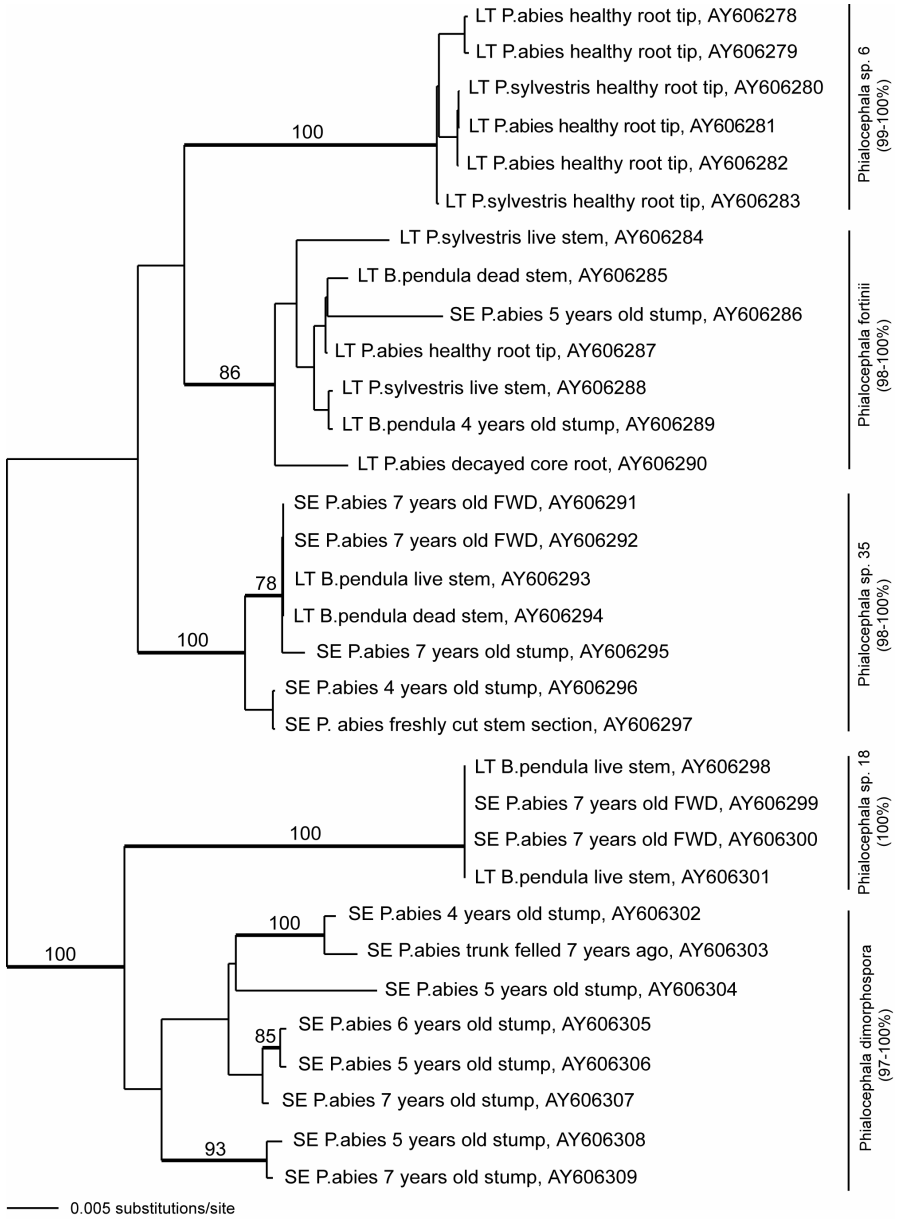

Fig. 5. Neighbour-joining topology (unrooted) of ITS rDNA sequences of dark septate fungi from our collection. In clusters of *Phialocephala fortinii* (a total of 46 strains sequenced) and *Phialocephala* sp. 35 (63 strains sequenced) only representative isolates from distinct ecological niches and of different geographic origin are included, and they do not necessarily represent distinct ITS types. Percentages in the brackets indicate sequence similarity observed within the whole sequenced sample of each species. For each specimen, information on geographical location (LT, Lithuania; SE, Sweden), substrate, and GenBank accession no. is given. Bootstrap values of 75% or higher, based on 1000 replicates are indicated above the branches of the tree.

methods could reveal the presence of DS fungi in healthy and decayed roots. The results suggest that DS fungi may play important ecological roles in determining health and vitality of conifer seedlings. In the study described in Paper **IV,** DS fungi were investigated from a broad number of ecological niches including healthy root tips, decayed coarse roots, live healthy-looking stems, coarse (stumps, snags and logs) and fine (tree branches and tops) woody debris, with the aim of determining their identity and ecology.

The ITS rDNA sequence analysis of 127 strains revealed that all of them had 95-100% homology with identified *Phialocephala* species, and they were thus eligible for assignment to this genus. Moreover, in a neighbour-joining similarity tree all strains studied were grouped into five clusters which possibly represent distinct taxa (Fig. 5). The placement of representatives of each cluster among known *Phialocephala* spp. and other related species by means of heuristic parsimony analysis revealed that representatives of two clusters were *P. fortinii* and *Phialocephala dimorphospora* Kendrick, whereas the remaining three did not cluster with any known species and were therefore defined as *Phialocephala* sp. 6, *Phialocephala* sp. 18 and *Phialocephala* sp. 35. The study (**IV**) thus revealed the presence of new fungal taxa within the genus *Phialocephala*. Among these, *Phialocephala* sp. 6 was associated with healthy conifer seedling roots, *Phialocephala* sp. 35 inhabited coarse and fine woody debris whereas *Phialocephala* sp. 18 occupied a broad ecological niche colonising both living stems of *Betula pendula* Roth. and fine woody debris of *P. abies* (Fig. 5).

Phialocephala dimorphospora was characteristically a degrader of coarse woody debris of *P. abies*. In the case of *P. fortinii*, apart from characteristic isolation from healthy and decayed roots as in many other studies (Wilcox & Wang, 1987a, b; Holdenrieder & Sieber, 1992; Harney, Wentworth & Wargo, 1995; Jumpponen, Mattson & Trappe, 1998; Addy, Hambleton & Currah, 2000; Grünig *et al.*, 2002; Sieber, 2002; Papers **I** & **II**), this study (**IV**) also revealed the presence of the fungus in several new ecological niches including living stems of *P. sylvestris*, dead stems of *B. pendula*, and old stumps of *B. pendula* and *P. abies* (Fig. 5). This study thus demonstrated a significantly higher ecological plasticity of *P. fortinii* than previously detected since, apart from roots, the fungus could also be found in above ground woody parts of living and dead trees. The fact, that *P. fortinii* remains active for several years in wood following the death of the tree supports the hypothesis that the fungus may act as a decomposer of wood in forest ecosystems. Moreover, the ability to cause soft-rot has been shown under laboratory conditions (Sieber, 2002). Despite increasing knowledge about the ecology of DS fungi, at this stage we can only speculate whether their role in trees may change along with host and/or environmental conditions.

New metabolites inhibitory to root pathogens of conifer seedlings

Kutzneria sp. 744 isolated from mycorrhizal root tips of *P. abies* (**I**) suppressed vegetative growth of the tested root pathogens *Pythium undulatum* Petersen; *Ceratobasidium bicorne* Erikss. & Ryv. and *Fusarium avenaceum* (Corda ex Fr.) Sacc. in paired culture on agar media. Moreover, the strain inhibited conidial

germination of *F. avenaceum* in *Kutzneria*-grown culture filtrate. Thus, production of antifungal secondary metabolites was suspected as one of the mechanisms behind the observed inhibitory effect and this option was investigated in the study described in Paper **V** performed in co-operation with Dr. Anders Broberg (Department of Chemistry, SLU). *Kutzneria* strain 744 was cultured in liquid MMN medium (Marx, 1969), and high performance liquid chromatography (HPLC) of the culture filtrate yielded four distinct fractions (Fig. 6) inhibiting the conidial germination of *F. avenaceum* in a microtitre plate assay.

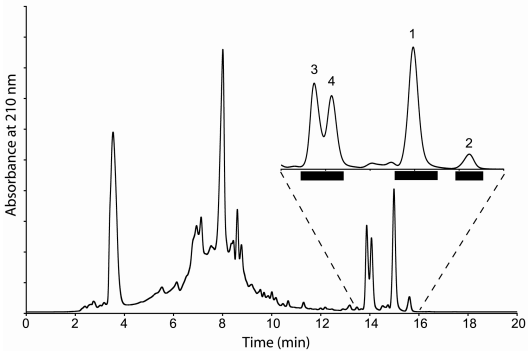

Fig. 6. Chromatogram from isolation of compounds 1-4 with gradient HPLC (C-18 column, 20 × 100 mm, 10–100% aqueous CH_3CN in 10 minutes followed by 10 min at 100% CH_3CN, at 10 mL/min, UV-210) with fractions displaying antifungal activity indicated by black boxes. Compounds 3 and 4 were further purified by isocratic HPLC at 61% aqueous CH_3CN.

Investigation by one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy, supplemented by mass spectrometry (MS), revealed the general structure of the depsipeptides (Fig. 7): cyclo[2-(1-methylcyclopropyl)-D-glycine — (2*S,* 3a*R,* 8a*S*)-6,7-dichloro-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxy-lic acid — 3-hydroxy-D-glutamic acid — *O*-methyl-L-serine — L-piperazic acid — (*S*)-2-hydroxy-3,3-dimethylbutanoic acid]. The 3-hydroxy-D-glutamic acid was present as its *threo*-isomer in compounds 1 and 2, and as its *erythro*-isomer in compounds 3 and 4.

All four depsipeptides were isolated and characterised for the first time and thus were named Kutzneride and numbered 1-4, respectively. Assessment revealed that compounds 1-4 possess moderate spore germination inhibition activity against four common root-rotting fungi *Cylindrocladium canadense* J.C. Kang, Crous & C.L. Schoch, *F. avenaceum*, *Fusarium oxysporum* Schlecht. and *Nectria radicicola* Gerlach & L. Nilsson. (Galaaen & Venn, 1979; Lilja *et al.*, 1992; Lilja & Rikala, 2000; Paper **II**) with minimal inhibitory values in the range 500-1000 µg/ml.

Integrated control using both mycorrhizal fungi and mycorrhiza-friendly fungicides proved to be an efficient approach to control root-rot pathogens of conifer seedlings (Chakravarty, Peterson & Ellis, 1990; Chakravarty *et al.*, 1999). In such a system of control, the fungicide provides protection against a particular pathogen at times when environmental conditions are not favourable for activity of the biological control agent. Thus, the natural products *e.g.* isolated and characterised from living organisms co-occurring along with pathogenic fungi (**V**), should be of particular interest as they might be selectively antagonistic towards pathogenic fungi and have only minor effects upon beneficial organisms.

However, the role of Kutzneride 1-4 depsipeptides was not tested using *in vivo* systems and it remains an interesting subject for future work.

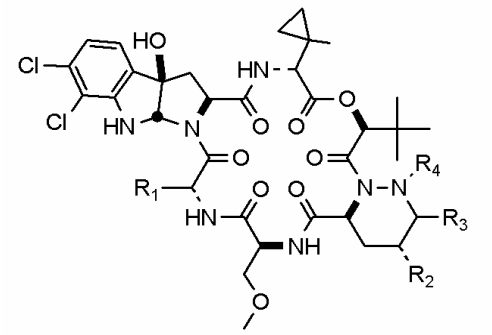

Fig. 7. Basic structure of the actinomycete *Kutzneria* sp. 744-produced depsipeptides 1-4 showing moderate inhibitory properties on germination of conidiospores of common root-rot fungi.

Isolation of *Kutzneria* sp. 744 from healthy-looking mycorrhizal root tips (**I**) and the lack of any negative effect on the growth of *P. sylvestris* and *P. abies* seedlings inoculated with strain 744 suggest that this particular actinomycete grows asymptomatically as an endophyte. Antibiosis by endophytic actinomycetes has been suggested as mode of action to fight pathogenic fungi (Sabaratnam & Traquair, 2002; Tian *et al.*, 2002). Therefore, apart from biochemical control, *Kutzneria* sp. 744 might also be considered for biological control of root pathogens.

Conclusions

Cultivation systems of conifer seedlings in forest nurseries significantly affect the extent of mycorrhizal colonisation of root systems (**I**) and consequently, may influence survival and growth of seedlings after their outplanting. Bare root cultivation of pine and containerised polyethylene roll cultivation of spruce provide most suitable conditions for abundant mycorrhizal colonisation of roots.

As a result of dynamic root colonisation by indigenous soil fungi of afforested sites, different fungi colonise decayed conifer seedling roots in forest nurseries, clear-cuts and agricultural land (**II**). The presence of pathogenic fungi in all planting environments indicates the potential risk of root diseases and consequently the need for accurate assessment of plant health before outplanting.

Afforestation of agricultural land with conifer seedlings artificially inoculated with selected ectomycorrhizal fungi (**III**) can be considered successful since significantly higher survival and better growth was achieved during two growing seasons following plantation establishment. However, this effect appears to be temporary since, even with labour-intensive inoculation methods and high cost inputs, it was difficult to manipulate the mycorrhizal community structure and fungal colonisation of roots over longer periods was largely governed by environmental conditions of the planting site.

A combination of different sampling strategies and detection methods yielded a high diversity of fungi associated with healthy and decayed roots. Mycorrhizal fungi were predominantly detected in healthy roots (**I** & **III**), pathogenic fungi predominantly in decayed roots (**II**) while endophytes showed high ecological plasticity and were common in both healthy and decayed roots (**I**, **II** & **IV**) of conifer seedlings.

Phylogenetic analysis of dark septate fungi (**IV**) from broad ecological niches and of wide geographical origin revealed the presence of three new taxa within the genus *Phialocephala*. This study (**IV**) also broadened available knowledge about *P. fortinii* which, apart from colonising roots, inhabits above ground parts of living and dead trees and may act as a wood decomposer in forest ecosystems.

Isolation and characterisation of new bioactive depsipeptides produced by the actinomycete *Kutzneria* sp. 744 (**V**) and its endophytic nature suggested a potential role of the organism for biochemical and biological control of root pathogens.

Future prospects

The logical continuation of the study published in Paper **I** would be establishment of experimental plantations on former agricultural land using conifer seedlings produced under different cultivation systems. This is an essential step in evaluating whether the differences in mycorrhizal colonisation of roots observed in different forest nurseries have any impact on survival and growth of the plants under field conditions. This would also provide information about whether production of conifer seedlings using selective cultivation systems could be considered as an alternative to artificial mycorrhizal inoculation (**III**).

Certain mycorrhizal species may dominate at the boundaries between forest and agricultural land and therefore may play an important role in natural establishment of forest tree seedlings (Dickie & Reich, 2005). Assessment of mycorrhizal fungi associated with roots of self seeded conifer seedlings on agricultural land distant from the forest edge would provide new information about the presence, extent and species richness, and community composition of mycorrhizal fungi under characteristic field conditions. In addition, this would also provide valuable information about mycorrhizal species as potential candidates for inoculation experiments.

According to recent reports *Phialocephala fortinii* Wang & Wilcox is characterised as a species complex composed of several cryptic species (Grünig *et al.*, 2004; Piercey, Graham & Currah, 2004). Contradictory reports about mycorrhizal, endophytic or pathogenic interactions of *P. fortinii* with tree roots may account for this. To check this hypothesis, materials collected in the studies from Papers **I**, **II** and **IV** could be analysed to identify cryptic species followed by inoculation experiments on seedling roots.

New depsipeptides (**V**) isolated from the ascomycete *Kutzneria* sp. 744 were shown to have a moderate inhibitory effect upon germination of conidiospores of root-rot fungi *in vitro*. Further trials are needed to investigate the usefulness of these metabolites under field conditions. On the other hand, the endophytic nature of the actinomycete producing these substances offers an excellent opportunity to investigate its usefulness for biological control of root pathogens.

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