ENDOPHYTES IN THE BUDS OF SCOTS PINE (*PINUS SYLVESTRIS* L.)

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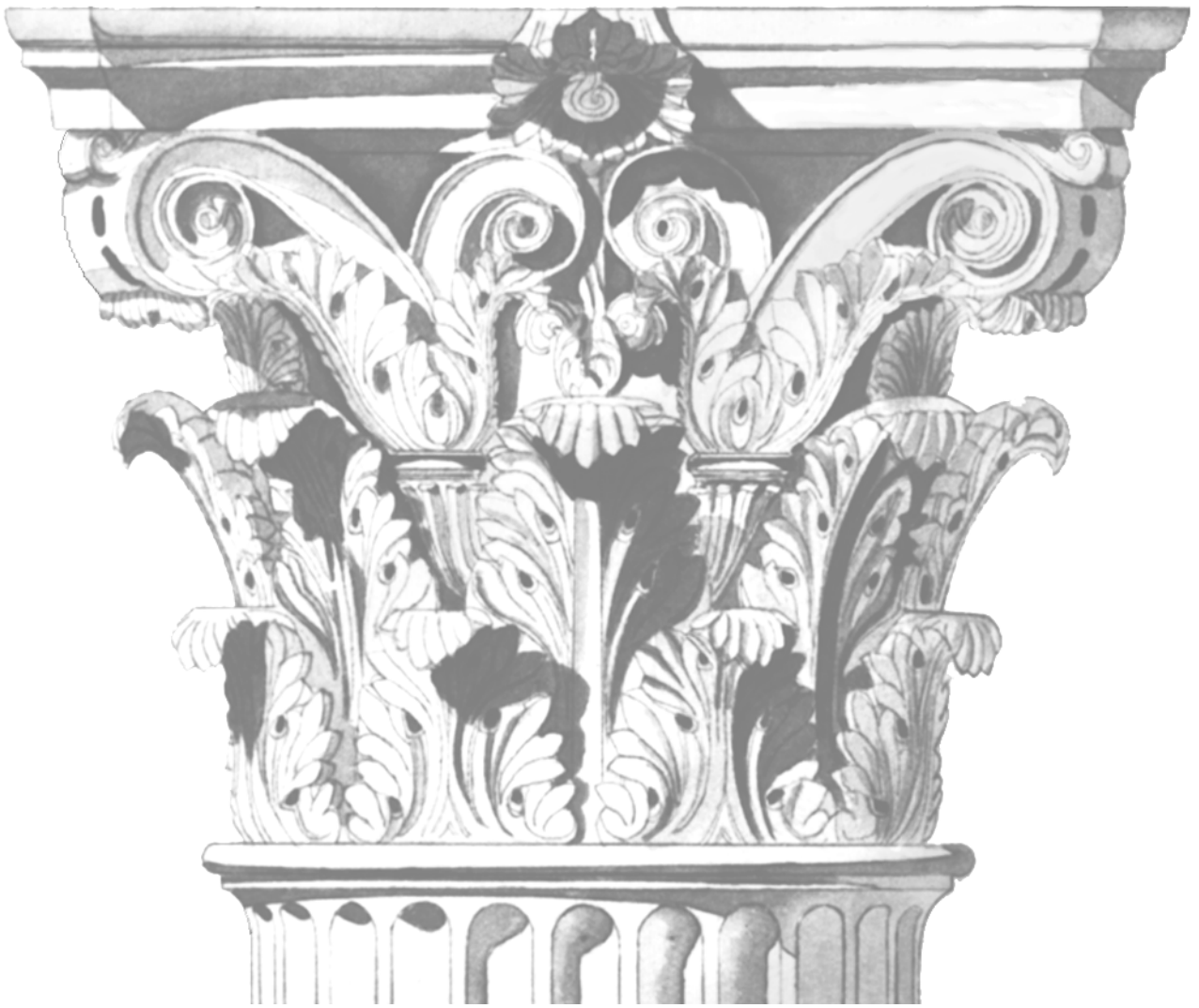

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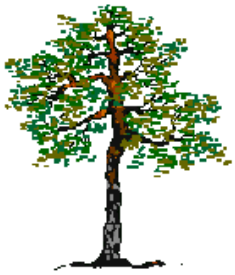
Abstract

Although microbes are generally found as endophytes in many plant tissues, the plant shoot meristems have been considered virtually sterile. Plant tissue culture, which utilizes mostly the meristems, has nevertheless given numerous references to microbial existence in these tissues. Since the bud-derived tissue cultures of Scots pine (*Pinus sylvestris* L.) also become easily occupied by microbes, microbial presence was considered to be one potential cause of the low regeneration capacity of tissues from mature trees. The origin of the microbes occurring in tissue cultures of buds of Scots pine was established in this study. One of the microbes, classified as *Hormonema dematioides* (*Ascomycota*), was localized in the scale tissues of a bud. Several other microbes were detected, and were associated especially with the meristematic tissues of Scots pine buds. This group was comprised of the yeast *Rhodotorula minuta*, and bacteria belonging to *Methylobacterium* spp., the *Pseudomonas fluorescens* subgroup, and a *Mycobacterium* sp. These endophytes were located particularly in the outermost cells of meristems, in the epithelial cells of resin ducts, and in the cells of the developing stem, in the vicinity of the meristems. These endophytes were less frequently found in the vascular tissue or in the intercellular spaces of cells, which are typical locations for the previously known endophytic bacteria.

The meristem-associated endophytes were discovered to affect growth of pine tissues, and some of them produced substances that are suitable as precursors for phytohormone synthesis. Additionally, pure cultures of both bacterial and fungal endophytes showed antagonism *in vitro* against pathogens. When the correlation between the presence endophytes and the degeneration of bud-derived tissue cultures of Scots pine was studied, it was discovered that the endophytes grew uncontrollably once a tissue culture was initiated from the bud. A high level of chitinase production was also detected in these tissue cultures, which was considered to be indicative of a defense reaction. However, the endophytes were not found to colonize excessively in every tissue, but all bud-derived tissue cultures of Scots pine eventually degenerate. Therefore, it was concluded that the endophytes may not exclusively be considered responsible for the degeneration of the cultures.

Keywords: biological control, buds, endophytes, meristem, Pinus sylvestris L., regeneration capacity, tissue culture



Tunturin kyljessä,
puurajan tuolla puolen
niin vanha ja yksinäinen mänty,
että se on merkitty karttaan.

Beside the top of the mountain
high above the forest line
There stands a pine, so old and lonely
that it has been marked in a map.

-Risto Rasa

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Oulu, June 2001 Anna Maria Mattila

Abbreviations

BAP	6-benzylamino purine
BLAST	Basic Local Alignment Search Tool
2,4-D	2,4-dichlorophenoxyacetic acid
D1	tissue culture medium modified by Hohtola
DTT	dithiothreitol
LB	Luria Bertani broth
LC-MS	liquid chromatography mass spectrometry
PDA	potato dextrose agar
rDNA	ribosomal DNA
RDP	Ribosomal Database Project
SEM	scanning electron microscopy
TLC	thin-layer chromatography

List of original papers

The thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I Pirttilä AM, Laukkanen H, Pospiech H, Myllylä R, Hohtola A (2000) Detection of intracellular bacteria in the buds of Scotch pine (*Pinus sylvestris* L.) by in situ hybridization. Appl. Env. Microbiol. 66: 3073-3077.

II Pirttilä AM, Pospiech H, Laukkanen H, Myllylä R, Hohtola A (2001) Detection of two fungal species in pine buds with different modes of endophytic life. Manuscript, submitted for publication.

III Pirttilä AM, Pospiech H, Laukkanen H, Myllylä R, Hohtola A (2001) Dynamics of pine endophytes during the growth season and dormancy. Manuscript, submitted for publication.

IV Pirttilä AM, Joensuu P, Pospiech P, Jalonen J, Hohtola A (2001) Biological activity of the pine bud endophytes. Manuscript.

V Pirttilä AM, Laukkanen H, Hohtola A (2001) Chitinase production in pine callus (*Pinus sylvestris* L.): a defense reaction against endophytes? Manuscript, submitted for publication.

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Preface

In physiology and biochemistry, the research has been from the beginning more advanced in the field of animal sciences, especially of humans, in comparison to plants. Although functionally and structurally there are as many differences as similarities between animals and plants, the plant cell has in many respects been dealt with as an animal cell with a chloroplast. However, in addition to cell wall and photosynthesis, plants differ from animals in several ways, of which the most important is probably their generally sessile life-style. This ‘steadiness’ of plants has caused pressure to create different strategies to survive. A plant, and especially a tree, has to receive nutrition, to suffer the seasonal effects, and to survive pathogenic attacks continually.

Microbes are common companions to plants in comparison to animals, in which microbes are mainly inhabiting the tissue surfaces but almost never tissue interiors, unless under pathogenic attack. In plants, the first reports of microbial symbiosis were made at the end of the 19th century when mycorrhiza and rhizobia were found in association with plant roots. Since then, plant roots have been found to undergo constant interactions with microbes. In addition to the nitrogen-fixing rhizobia of the leguminous plants, the rhizoplane bacteria have a close relationship with plants. Many of them colonize the epidermal, and some even in the internal nonsymbiotic root tissues of various non-leguminous plants. However, especially the meristems of plant aerial tissues have until recently been considered virtually sterile, regardless of the wide reporting of microbes in the roots.

1 Introduction

1.1 Endophytes

The term endophyte is defined as ‘an organism inhabiting plant organs that at some time in its life, can colonize internal plant tissue without causing apparent harm to the host’ (Petrini 1991). In literal translation, the word endophyte is derived from Greek: ‘endo’ >< ‘endon’ meaning within, and ‘phyte’ >< ‘phyton’ meaning plant. Since the first report (de Bary 1866) endophytes have been discovered in high numbers within different tissues of various plants. Besides the mycorrhizal roots, fungal endophytes have been most studied in grasses and tree leaves (Redlin & Carris 1996). Bacterial endophytes have most frequently been detected in the nonsymbiotic root and vascular tissues of several non-leguminous plants (Hallman *et al*. 1997, James & Olivares 1997).

1.1.1 Root-associated symbionts

Mycorrhiza are symbiotic root structures formed between fungi and the majority of plants. The plant receives nutritional ions such as phosphate and ammonium from the mycorrhizal fungi, and provide it with sugars in return (Smith & Read 1997). Whereas mycorrhizal fungi may be regarded as endophytic organisms to some extent and their mycorrhizal part fit in the endophytic definition, they are frequently left out from discussions concerning endophytes. The external hyphae of mycorrhiza emanate far into the soil surrounding the infected root tips, and therefore mycorrhizal fungi reside only partly inside the plant tissue. In this way they may be considered different from a typical endophyte (Chanway 1996). Additionally, some of the mycorrhizae are obligate endosymbionts, which is not a requirement for an endophytic organism (McInroy & Kloepper 1994).

Other well-studied root symbiotic organisms are rhizobia, bacteria belonging to the genera of *Rhizobium, Sinorhizobium, Bradyrhizobium,* and *Azorhizobium*. Rhizobia form

nodules on roots of many leguminous plants. These bacteria are capable of nitrogen fixation by an oxygen-sensitive enzyme, nitrogenase. As a result of the symbiotic interaction, a protein called leghemoglobin is produced in order to protect the nitrogenase from oxygen. The fixed nitrogen is utilized by the plant, and in return the plant provides rhizobia with dicarboxylic acids (Mylona *et al*. 1995). A symbiosis very similar to rhizobia is formed between bacteria belonging to the genus *Frankia* and plants of almost 200 species (Doyle 1998). This actinorhizal symbiosis differs from the rhizobial one mainly with respect to the fact that the nitrogenase is protected inside bacterial vesicles (Pawlowski & Bisseling 1996). Symbiotic interactions have also been detected between cyanobacteria such as *Anabaena* or *Nostoc*, and the cycads, the water fern *Azolla* and the flowering plant *Gunnera* (Doyle 1998).

Although in principle, all the bacterial endosymbionts of plants fit well within the endophytic definition, they usually are considered as a group of their own. Differing from endosymbionts, endophytes are not necessarily symbiotic, and some of them may even become pathogenic under defined conditions (James & Olivares 1997, Olivares *et al*. 1997). In addition, not all the endophytes reside obligately in the plant, as some of them are clearly facultative, which means that they are well capable of surviving in the soil (Baldani *et al*. 1997). While nearly all the endosymbiotic bacteria reside intracellularly in the plant tissues, most root endophytes have adopted an intercellular location (Hallman *et al*. 1997, Reinhold-Hurek & Hurek 1998).

1.1.2 Root-associated bacterial endophytes

Bacteria belonging to the genera *Azospirillum*, *Herbaspirillum*, *Acetobacter*, and *Azoarcus* are found as endophytes of many graminaceous plants, mostly from the tropical regions. The ability to colonize the root interior, to survive only poorly in the soil, and to fix nitrogen in association with these plants is a characteristic of all these bacteria (Baldani *et al*. 1997).

Bacteria of the genus *Azospirillum* have been found in association with many cereals and forage grasses grown both in temperate and tropical climates (Baldani *et al*. 1997). Although these bacteria are regarded more as being rhizospheric bacteria colonizing mainly the elongation and root hair zones of roots, some strains of both *A. lipoferum* and *A. brasilense* are either facultatively or obligately endophytic (Baldani *et al*. 1997, James & Olivares 1997). Strains of *A. brasilense* can colonize plant tissues differentially; some strains live only on root surfaces, whereas others colonize cortical intercellular spaces or even the vascular tissue (James & Olivares 1997). Besides the ability of fixing nitrogen, both *A. brasilense* and *A. lipoferum* can produce auxin (Costacurta & Vanderleyden 1995).

As determined by its host selection *Acetobacter diazotrophicus* is another restricted bacterium found in high numbers mainly in the roots, stems, and leaves of sugar cane. *A. diazotrophicus* has also been detected in *Pennissetum purpureum*, sweet potato (*Ipomoea batatas*), coffee (*Coffea arabica*), and pineapple (*Ananas comosus*) (James & Olivares 1997, Tapia-Hernández *et al*. 2000). *A. diazotrophicus* is a nitrogen-fixing bacterium

which, as with the two *Azospirillum* species, produces the phytohormones auxins and gibberellins (Bastián *et al.* 1998). Because the bacterium survives poorly in soil, it is considered an obligate endophyte, colonizing the intercellular spaces of sugar cane stem parenchyma tissue (Baldani *et al*. 1997). It has also been detected within the xylem vessels (James *et al*. 1994).

The genus *Herbaspirillum* contains an unusual group of endophytes in the respect that these bacteria may become pathogenic to their host under certain conditions. *H. rubrisubalbicans* has been identified in association with sugar cane, sorghum, rice, palm trees, and a C4 grass *Miscanthus*, whereas *H. seropedicae* has been demonstrated within roots of 13 members of the *Gramineae*, as well as in the stem of sugar cane (James & Olivares 1997). Both of these species are obligate endopytes and capable of nitrogen fixation. However, they may cause red stripe disease symptoms on leaves of some sorghum cultivars, although those caused by *S. seropedicae* are very mild. Additionally, *H. rubrisubalbicans* causes mottled stripe disease on sugar cane leaves (James & Olivares 1997). Varieties of sugar cane that grow on areas fertilized with mineral N, are susceptible to the disease (Baldani *et al*. 1996). Both of these bacteria preferentially colonize xylem rather than the intercellular apoplast, which has been found to be connected with the disease progress (James *et al*. 1997, Olivares *et al*. 1997). *H. seropedicae* is another root-associated bacterium capable of producing auxins and gibberellins (Bastián *et al.* 1998). Because *H. seropedicae* has been isolated from seeds of cereals, it may be transmitted through seeds into new plants. Due to the detection of the bacteria in micropropagated plants, a vegetative transfer is another option (Baldani *et al*. 1997).

Nitrogen-fixing bacteria belonging to the genus *Azoarcus* are found mainly in roots of Kallar grass (*Leptochloa fusca*) in the intercellular spaces, xylem vessels, and dead root cells (Hurek *et al*. 1994). *Azoarcus* has been demonstrated to spread systemically within the plant via the xylem vessels (Hurek *et al*. 1994). In addition to the plant roots, this bacterium has been discovered in close interaction with a rhizosphere fungus (Hurek *et al*. 1997).

Because some rhizobacteria are in close association with plant roots, colonizing mainly the root epidermis but also capable of spreading throughout the whole plant, the line between endophytic bacteria and rhizobacteria is obscure. There are several bacterial species, in addition to the most well studied root endophytes, which have been isolated from grasses. Whereas certain species of the genera *Burkholderia* and *Klebsiella* are preferentially regarded as endophytes (Baldani *et al*. 2000, Palus *et al*. 1996), *Pantoea agglomerans* (Remus *et al*. 2000), *Alcaligenes faecalis* (You & Zhou 1989), and a few other bacteria belonging to the genera *Pseudomonas*, *Enterobacter* and *Bacillus* (Lindberg *et al.* 1985, Persello-Cartieaux *et al.* 2001, Watanabe *et al.* 1987) are considered rhizobacteria. All these bacteria are less studied, but connected by their ability to fix nitrogen (James & Olivares 1997).

In addition to the root endophytes of grasses discussed above, endophytic bacteria have been detected in citrus trees and grapevine (Bell *et al.* 1995a, Gardner *et al.* 1982). Bacteria belonging to the genera *Pseudomonas*, *Enterobacter*, *Bacillus* and *Corynebacterium* were found in the xylem of lemon roots, and the xylem tissues of grapevine stem contained bacteria belonging to the genera *Pseudomonas*, *Enterobacter*, *Pantoea* and *Rhodococcus* (Bell *et al.* 1995a, Gardner *et al.* 1982). Some of these bacteria

exhibited antagonistic effects towards other bacteria. *E. agglomerans*, *Rahnella aquatilis* and *P. corrugata* isolated from grapevine were able to control phytopathogenic strains of *Agrobacterium vitis* (Bell *et al.* 1995b, Gardner *et al.* 1982).

1.1.3 Fungal endophytes

When compared to the bacterial endophytes, the research of endophytic fungi has a long history and their diversity among plants has been found to be considerably large. Endophytic fungi were reported for the first time in grasses (de Bary 1866) and in trees in *Picea canadiensis* (Lewis 1924). Since then, fungal endophytes have been found in the leaves, bark, and xylem of almost all plant species examined (Petrini 1986, Carroll 1988, Redlin & Carris 1996, Schulz *et al.* 1993).

The endophytic fungi of grasses are well studied, especially species belonging to *Acremonium*, found within the grass tall fescue (*Festuca arundinacea*). Other well-known endophytic fungi are members of the genus *Epichloë* and their asexual relatives of *Neotyphodium*, infecting the *Bromus* grass species (Siegel *et al.* 1987, White 1987). These fungi have interested researchers because they are found responsible for intoxication of cattle grazing on the endophyte-infected grass (Fletcher & Harvey 1981). The substances causing the intoxication are considered to be the lolitrem family of neurotoxic indole-diterpenoid alkaloids produced by the fungi (Gallagher *et al.* 1984). Some of the same substances that are responsible for the intoxication have been found important for bioprotection of the plant from insect herbivores (Bush *et al.* 1982, Siegel *et al.* 1990). The endophytes are beneficial for the plant also in increased growth (Groppe *et al.* 1999) and improved persistence under drought stress (Elmi & West 1995, West *et al.* 1993). However, they may have a negative impact on the flowering of the host (Groppe *et al.* 1999, Schardl *et al.* 1997).

The endophytic fungi of grasses are located within the intercellular spaces of the host (Hinton & Bacon 1985). When the metabolic activity of the *Acremoniu*m endophytes was studied in ryegrass, it was found to be highest in the leaf sheath, followed by the mature leaf, and the lower and upper parts of the emerging leaf (Herd *et al.* 1997). There was virtually no activity present in the roots, and it was also low in the core of the stem (Herd *et al.* 1997). When the oldest mature leaves were compared with the younger ones, however, they contained less activity of the fungal endophytes. Endophytic activity also decreased once the size of the tissues increased (Herd *et al.* 1997).

The grass endophytes are intimate and perennial, exhibiting a close matching with the life cycle of their host. Their life cycle within the plant may result in vertical or horizontal transmission, or in a mixture of these two transmission forms (Schardl *et al.* 1997). An example of a pure vertical transmission pattern is the asexual *Neotyphidum* species, which propagate clonally in flowering meristems, ovules, and seeds, and in this way infect the seedlings of the mother plants (Schardl *et al.* 1997). Certain *Epichloë* species, on the other hand, have acquired the horizontal transmission strategy and produce contagious spores on their sexual structure surrounding the grass flag leaf sheath. As soon as the fungal structure called stroma are produced, the inflorescence of the affected tiller

ceases development (Schardl *et al.* 1997). The following suppression of seed production is known as 'choke disease'. In the third case, the fungi choke only a few flowering tillers, but leave the majority of them unaffected, being mostly transmitted in the seeds (Shardl *et al.* 1997).

Besides in grasses, fungal endophytes have been discovered to a great extent in trees, particularly within the leaves. The interactions between fungi and trees are less known in detail, but the endophytic fungal diversity has been found to be very large in trees. The species richness of fungi has been extensively studied especially in conifers (Barklund & Kowalski 1996, Deckert & Peterson 2000, Hata & Futai 1996, Helander *et al.* 1994, Kowalski 1993, Müller & Hallaksela 1998, Sieber *et al.* 1999, Sieber-Canavesi & Sieber 1993).

As many as 110 different fungal species have been isolated from their coniferous hosts (Petrini 1986). In that study, the majority of the fungi were observed infrequently or only once (Petrini 1986). Regardless of the species richness, some host specificity has been discovered among the fungal endophytes. The specificity may depend on the taxonomic position of the host (Hata & Futai 1996) and on the fungal species (Sherwood-Pike *et al.* 1986). The fungus *Rhabdocline parkeri* is absolutely host specific, having Douglas fir (*Pseudotsuga menziesii*) as its only host (Sherwood-Pike *et al.* 1986). In contrast, *Sclerophoma pythiophila* has been isolated from needles of both *Pinus sylvestris* (Kowalski 1993) and *Picea abies* (Müller & Hallaksela 1998), and can therefore be termed a host-neutral endophyte.

Within a conifer, the amount and diversity of endophytes may vary with the age of the needles (Hata *et al.* 1998). Fungal endophytes are virtually absent from needles after emergence and grow most abundantly in the old needles (Deckert & Peterson 2000, Hata *et al.* 1998, Helander *et al.* 1994, Kowalski 1993). Time of the year has been found to affect the isolation frequency of certain endophytic species, while most fungi are unaffected by the season (Hata *et al.* 1998, Kowalski 1993). The occurrence of endophytic fungi in different positions on the needle has been determined in several studies. However, the results have produced variable data (Deckert & Peterson 2000, Hata *et al.* 1998, Helander *et al.* 1994, Kowalski 1993). At the microscopic level, fungal endophytes have mostly been localized intercellularly in the leaf tissue in conifers and in other trees like in grasses (Johnson & Whitney 1989, Suske & Acker 1989, Viret & Petrini 1993, Yang *et al.* 1994). When infection by the endophyte *Discula umbrinella* was studied in beech leaves (*Fagus sylvatica*), colonization was primarily observed on the abaxial side of the leaf where conidial germ penetrated the outer edges of the guard cells (Viret & Petrini 1993).

Although conifer leaves are the best studied tissues in which fungi exist, fungi have been detected in other trees and tissues as well. *Tryblidiopsis pinastri* appears to be both species and organ specific, inhabiting mainly the bark tissues of Norway spruce branches (Barklund & Kowalski 1996, Kowalski & Kehr 1992, Müller & Hallaksela 2000). Fungal endophytes have been found within the wood and bark tissues of several tree species (Bettucci *et al.* 1999, Collado *et al.* 1999, Fisher & Petrini 1990, Petrini & Fisher 1990, Müller & Hallaksela 2000, Kowalski & Kehr 1992). However, when compared to the bark tissue, endophytic fungi are rarely found in the wood (Barklund & Kowalski 1996, Bissegger & Sieber 1994, Kowalski & Kehr 1992, Wilson & Carroll 1994). An exception

is the endophyte *Ceratocystis erinaceus*, which can colonize the heartwood of oak (*Quercus robur* L.) (Bohár 1996).

1.2 The endophytic role

The endophytic fungi of grasses appear to have a pronounced role in biological protection of the host by production of antibiotic substances (Bush *et al.* 1997). Similar effects have been suggested for many fungal species that inhabit trees (Calhoun *et al.* 1992, Findlay *et al.* 1997, Johnson & Whitney 1994, Peláez *et al.* 2000, Rodrigues *et al.* 2000, Strobel & Hess 1997). As with their fungal counterparts, many root-associated bacteria act also to protect the host from pathogens (Sturz *et al.* 2000). The rhizobacteria are the best studied in this respect, and they apply different strategies to control the pathogens; by producing antibiotics (Lambert *et al.* 1987), siderophores (Kloepper *et al.* 1980) or by nutrient competition (Lockwood 1990) and indirectly through the induction of systemic acquired resistance of the host (Tuzun and Kloepper 1994).

In addition to the protective function, grass endophytes affect the growth and survival of the plant, although the means of these effects have not yet been determined (Elmi & West 1995, Groppe *et al.* 1999, Hill *et al.* 1996). One fungal endophyte, *Colletotrichum* sp. can produce the plant growth hormone indole-acetic acid (Lu *et al.* 2000). Production of auxins and gibberellins is also typical for many *Rhizobium* species and for the root-associated endophytic bacteria *Azospirillum* sp., *Acetobacter diazotrophicus*, and *Herbaspirillum seropedicae* (Bastián *et al.* 1998, Costacurta & Vanderleyden 1995, Lambrecht *et al.* 2000, Rademacher 1994). However, apart from the roots, the importance of the microbial production of phytohormones has been evaluated to be low, and the significance of these products for the plant has remained ambiguous (Lambrecht *et al.* 2000, Rademacher 1994, Tuomi *et al.* 1993).

Microbial promotion of plant growth may be the outcome of several additional factors besides the production of phytohormones. For example, although found capable of producing hormones, mycorrhiza benefit the plant mainly by providing it with nutritional ions such as nitrogen or phosphorus (Hanley & Greene 1987, Syvertsen & Graham 1999, Strzelczyk & Pokojska-Burdziej 1984, Wullschleger & Reid 1990). Another example of an indirect plant growth promotion is nitrogen fixation, which has been considered to be the main function in the rhizobial and actinorhizal symbioses. Almost all root endophytes fix also nitrogen (Baldani *et al.* 1997). However, the benefit of their nitrogen fixing ability for the plant has not indisputably been demonstrated (James 2000). Therefore, until now the only endophytic function which is considered distinctively beneficial for the plant appears to be the protection of the host against pathogens. Because not all endophytes are responsible of producing antagonistic substances, their role is yet to be discovered. Nevertheless, it appears that the function of an endophyte may be composed of several diverse factors that may together have a positive influence on the plant.

1.3 Microbes in plant tissue culture

Although microorganisms are not unusual guests inside plants, they have mostly been detected in the root and stem tissues of plants. Besides the foliar endophytic fungi, the reports of endophytes outside the xylem have been uncommon in the upper parts of plants. A general theory has been that plant shoot internal tissues are essentially free of microorganisms, and that the most sterile tissues would be the meristems (Leifert *et al.* 1994). Plant tissue culture, which commonly utilizes the meristems, has nevertheless given numerous references to microbial existence in these tissues (Holland & Polacco 1994, Leifert *et al.* 1994, Tanprasert & Reed 1997).

1.3.1 Contamination of tissue cultures

The presence of microbes in plant tissue cultures has usually been explained to be the result of an insufficient surface sterilization process, or laboratory contamination (Danby *et al.* 1994, Leifert *et al.* 1994). Because plant surfaces contain large populations of microbes (Andrews & Harris 2000), bacterial cells may be protected in the protrusions and caverns of the plant surface from being in contact with the chemicals that are used in the surface sterilization (Leifert *et al.* 1994). Laboratory contaminants may be introduced into tissue cultures from the air near the edge of a laminar flow cabinet, from the dust on the surface of culture vessels and from the condensation water on the rim of culture containers (Danby *et al.* 1994). Furthermore, mites and thrips have been proposed to mediate fungal and bacterial contamination in the tissue cultures (Leifert *et al.* 1991). Inside the tissue cultures, microbes may kill the plant tissue or reduce its growth mainly by inducing changes to the composition of the growth medium (Leifert & Waites 1992). These changes include reduction or increase in pH, depletion of sucrose from the medium, and production of herbicidic substances (Leifert *et al.* 1989, Leifert *et al.* 1994). Bacterial contamination has been considered to be a special problem, because the bacteria may remain latent and not become visible at any stage of the culture (Leifert *et al.* 1994).

1.3.2 Theory of endogenous origin of the microbes

Although microbes appearing in plant tissue cultures are often obvious contaminants originating from outside the cultures, some scientists have suggested that some of them may be endophytes originating from the plant tissue itself (Cassells 1991, Holland 1997, Holland & Polacco 1994). The importance of bacteria belonging to the genus *Methylobacterium* has especially been addressed in this respect (Holland 1997, Holland & Polacco 1994). These bacteria may be associated with plant nitrogen metabolism by means of the bacterial urease enzyme (Holland & Polacco 1992). Furthermore, the beneficial effects proposed for *Methylobacteria* in plant tissue involve production of

vitamin B12 and cytokinins, and removal of methanol and other metabolic wastes from plants (Basile *et al.* 1985, Holland 1997, Holland & Polacco 1994). Regardless of these theories, the existence and location for any of the microbes found in tissue cultures has not been ascertained in the plant tissue (Leifert *et al.* 1994).

1.3.3 Tissue culture of Scots pine (Pinus sylvestris L.)

In tissue cultures of woody plants, browning or blackening of the culture is a typical feature. The phenomenon is constantly detected also in the tissue cultures derived from shoot tips of mature Scots pine (*Pinus sylvestris* L.) (Laukkanen *et al.* 1997). In general, browning of a tissue culture is caused by oxidation of phenols which occurs as a result of cellular disorganization (Lee & Whitaker 1995). The browning is accompanied by lipid peroxidation and lignification of cells in tissue cultures of Scots pine (Laukkanen *et al.* 2000a). These reactions are symptoms of high oxidative stress (Laukkanen *et al.* 2000a) and characteristic features of the plant defense reaction (Lee & Whitaker 1995, Keller *et al.* 1996). The defense system of a plant is comprised of a large amount of different enzymes, of which chitinases are the largest group. Chitinases are especially interesting because their substrates do not originate from plants, but from fungal cell walls and the exoskeleton of arthropods, organisms of which many are plant pathogens and pests (Collinge *et al.* 1993).

The bud-derived tissue cultures of Scots pine also become easily occupied by microbes, especially when initiated from winter buds (Hohtola 1988). Typically visible microbial growth appears in a week in the cultures (Hohtola 1988). A bacterium belonging to the genus *Mycobacterium* has earlier been characterized from the tissue cultures of Scots pine and found to interfere with growth of pine seedlings (Laukkanen *et al.* 2000b).

2 Outline of the present study

This study is part of a larger project aiming to enable micropropagation of Scots pine from callus originating from shoot tips of mature trees. The tissue deterioration, which occurs in the bud-derived tissue cultures of Scots pine has been considered earlier in several studies. The biochemical reactions leading to brown, degenerated callus tissue have been elucidated and found to be characteristic of plant defense reactions. In this study, the correlation between the tissue deterioration and frequent microbial presence in the tissue cultures was investigated. The main goal for this work was set to:

1. Determine the potential origin of the microbes in the pine tissue by use of *in situ* hybridization.

Additional goals were set, after microbes were found originating from the pine tissue, to:

2. Identify the microbes.
3. Describe the possible function of the endophytes.
4. Ascertain the microbial role in the deterioration of tissue cultures.

3 Materials and methods

3.1 Pine tissue culture

Buds of Scots pine (*Pinus sylvestris* L.) were collected from healthy-looking trees growing on a natural stand in Oulu (65°N;25°30'E). The buds and seeds were surface sterilized for 1 min in 70% ethanol and for 20 min in 6% calcium hypochlorite. After rinsing, the brown bud and seed scales were removed aseptically. The apices with some needle primordia were placed on a modified Murashige and Skoog medium (D1) which was supplemented with 0.3 mg l^{-1} BAP (6-benzylamino purine) and 1 mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid) (Hohtola 1988, Murashige & Skoog 1962).

When tissue cultures were initiated in order to study chitinase production in the calli, ammonium nitrate (2 mM) was the inorganic nitrogen source in D1 medium, the sucrose concentration was 2%, and arginine and glucose were omitted. Seed embryos were separated from megagametophytes and placed on K medium (Krogstrup 1986). Samples for protein isolation were taken on days 0, 4, 12, 28 and 35, and for *in situ* hybridization on day 10 from the start of the culture. Additionally, a long-term culture of embryogenic calli originating from immature embryos of *Pinus nigra* was grown as a control (Salajová *et al.* 1999) on DCR medium (Gupta & Durzan 1985).

3.2 Isolation of the microbes

Bud-derived tissue cultures were visually observed weekly for the presence of microbes and transferred to fresh medium every second week. All bacterial, and representatives of recurrently occurring fungal populations found in the cultures were chosen for further investigation. The bacterial or fungal cells were transferred onto Luria Bertani (LB) plates or onto fresh D1 medium for cultivation, and a pure culture for each isolate was obtained. The effectiveness of the surface sterilization was tested with all isolates, as

described in II. Detailed data on microbial occurrence in the tissue cultures, as well as designations for the isolates, are shown in Table 1.

Table 1. A summary of the bud collections performed for the tissue cultures, and of the microbes isolated in this study.

collection area	collection month	number of buds	all microbes %[a]	isolates[b]	publication
Oulu	May	160	16.7	F^B, G^B, H^B, I^B, J^B	I
Oulu	March	240	40	L^F, M^F, K^B, N^B, P^B	II, III
Oulu	June	180	8.3	T^F	II

[a] The percentage of tissue cultures where any microbes were detected in relation to the number of all tissue cultures initiated

[b] B, bacterial F, fungal isolate

3.3 DNA extraction, PCR, cloning, and sequencing of 16S and 18S ribosomal DNA

Bacterial DNA was isolated according to Birnboim and Doly (1979) and Bollet *et al.* (1991), and the isolation of fungal DNA was performed as described by Rogers and Bendich (1994). Bacterial 16S ribosomal DNA (rDNA) was amplified with universal primers described by Jalava *et al.* (1995) and the primers for amplification of fungal 18S rDNA were obtained from White *et al.* (1990). The PCR products were cloned (Sambrook & Russel 2001) and sequenced (Abi Prism 377 DNA Sequencer, Perkin Elmer).

3.4 Database search and molecular phylogenetic analysis

The phylogenetic analyses were performed with the closest relatives of the isolates. The sequences were aligned by using ClustalW (Thompson *et al.* 1994), and the gaps were manually excluded from the alignment. A distance matrix was created with the DNADIST program of PHYLIP (Felsenstein 1989), from which the tree topology was built by the neighbor-joining method in the program NEIGHBOR. The confidence for individual branches of the resulting tree was estimated by performing 1,000 bootstrap replicates by using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSUS.

3.5 Identification of the microbes

Physiological tests for the identification of the bacterial and fungal isolates were performed by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

3.6 *In situ* hybridization experiments

3.6.1 Design, synthesis and labeling of the probes

Oligonucleotide probes complementary to the unique regions of 16S and 18S rDNA of the isolates were designed by using the programs 'Sequence Match' and 'Probe Match' of the Ribosomal Database Project (RDP) (Maidak *et al.* 1999). A summary of the oligonucleotides created is presented in Table 2. The probes were found to contain at least one mismatch with all accessible 16S and 18S rDNA sequences obtained through the RDP and Basic local alignment search tool (BLAST) (Altschul *et al.* 1990, Maidak *et al.* 1999). The oligonucleotides were labeled with digoxigenin (DIG Oligonucleotide 3'-End Labeling Kit, Roche). Because all the probes were targeted towards the 16S or 18S rDNA from which an untranslated rRNA is produced, during in situ hybridization the probes would recognize the ribosomal RNA of the microbial cell. Therefore, the amount of the rRNA-targeted probe should reflect the cell number and metabolic activities of the microbes, as suggested by DeLong *et al.* (1989).

Table 2. A summary of probes used in the study, and of formamide concentrations determined for specific hybridization of the probes.

probe	sequence 5'->3'	positions[a]	target	formamide (%)
E11	AGCCATGCAGCACCTGTCTC	1065 – 1045	eubacteria	20
MB	AGCGCCGTCGGGTAAGA	1388 - 1371	methylobacteria	30
MY2	CCCATGAAGGCCGTAGTCCT	220-201	*Mycobacterium* sp.[b]	35
PSG17	GCCGCTGAATCAGGGAGCAA	103-84	*Pseudomonas agarici* subgroup	45
PS5	GCAGAGTATTAATCTACAACC	468 - 448	*P. fluorescens* subpgroup	35
HD13	TCCTTCCGGACAAGGTGATGAAC	1506-1484	*Hormonema dematioides*	45
RM6	TGAGTCATTAAAAACCTCATC	1064-1044	*Rhodotorula minuta*	40

[a] 16S rDNA positions corresponding to sequence of *Escherichia coli*, 18S rDNA positions corresponding to sequence of *Saccharomyces cerevisiae*

[b] *Mycobacterium* sp. isolated by Laukkanen *et al.* (2000b)

3.6.2 Determination of hybridization stringency

The hybridization stringency was determined using 'whole cell' hybridization as described by DeLong *et al.* (1989) and confirmed by dot blot hybridization as described by Wagner *et al.* (1994). When bacterial isolates were studied, *Lactobacillus delbrüeckii* subsp. *lactis*, and *Escherchia coli* DH5α were used as reference strains. In addition, chloroplasts isolated according to Oku and Tomita (1976) from pine needles were used as controls in the whole cell hybridization. When the fungal isolates were studied, *Saccharomyces cerevisiae* L40, *Hymenoscypha ericae* Read 100, *Phialophora finlandia* UAMH 8322 and *Phialocephala fortinii* SE 24 were used as reference strains.

In the whole cell hybridization protocol, the cells were fixed (DeLong *et al.* 1989) and attached onto slides coated with 3-aminopropyltriethoxysilane by baking at 55°C overnight. Prior to hybridization the slides were processed as described in DeLong *et al.* (1989). The slides were then treated with proteinase K and hybridized. The stringency of the hybridization was adjusted by gradually increasing the formamide concentration (in 5% intervals) (Wagner *et al.* 1994) in the hybridization buffer, which contained 3× SET (450 mM NaCl, 60 mM Tris-HCl [pH 7.5] and 3 mM EDTA), Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin), 0.02% tRNA, 0.02% polyadenylic acid, 10% dextran sulfate, 50 mM ditiothreitol (DTT), and the digoxigenin labeled oligonucleotide probe (0.5 ng/µl). After hybridization, the slides were washed (I) and the hybrids were detected using the DIG Nucleic Acid Detection Kit (Roche). The specific formamide concentration for each probe are presented in Table 2.

For dot blot hybridization, DNA was extracted from the isolates and reference strains (Birnboim & Doly 1979, Bollet *et al.* 1991, Rogers & Bendich 1994). About 1 µg of the DNA was pipetted and immobilized on a nylon membrane (Amersham) at 120°C for 1/2 hour. The membrane was placed in a hybridization bag, and a hybridization buffer containing 3× SET, 0.1% sodium lauroyl sarcosine, 0.02% sodium lauryl sulfate, 1% blocking reagent (Roche), 0.01% polyadenylic acid, and formamide at a probe-specific concentration without a probe was added. Prehybridization was performed at 37°C for 2 hours, after which the probe was added to a concentration of 1 µg/ml, and the hybridization was continued overnight at 38°C. The membrane was washed (I) and processed (DIG Nucleic Acid Detection Kit, Roche).

3.6.3 Collection of specimens

In order to perform a distribution study on the fungal and bacterial presence in the buds of pine, buds were collected from different areas in Northern Finland. In addition, the occurrence of endophytes was studied in pine buds throughout the year. Flowers and seeds of pine were studied for the presence of endophytes, as well. A summary of all these specimens used for *in situ* hybridization experiments is presented in Table 3.

In order to study how endophytes react once a tissue culture is initiated from the buds, callus samples were additionally prepared for *in situ* hybridization. Ten samples were

taken from both bud-derived and embryo-originating callus after 10 days of growth. Additionally, two samples of *P. nigra* callus were prepared.

Table 3. A summary of specimens collected for the in situ hybridization experiments.

collection area	coordinates	no. of trees	specimen type	specimens per tree	probes hybridized	month
Oulu	(65°00'N;25°30'E)	3	bud	5	E11, MB, PS5	2
Tyrnävä	(64°45'N;26°00'E)	3	bud	5	E11, MB, PS5	2
Sodankylä	(67°30'N;27°00'E)	3	bud	5	E11, MB, PS5	2
Oulu	(65°00'N;25°30'E)	3	bud	5	E11, MY2, PSG17, HD13, RM6	3
Sodankylä	(67°30'N;27°00'E)	3	bud	5	E11, MY2, PSG17, HD13, RM6	3
Oulu	(65°00'N;25°30'E)	4	bud	1	all	1-12
Oulu	(65°00'N;25°30'E)	5	inflorescence	1	all	7
Oulu	(65°00'N;25°30'E)	5	seed	1	all	11
Rovaniemi	(66°50'N;25°00'E)	4	male flower	1	all	7

3.6.4 Sample preparation

Pine seeds were allowed to imbibe water overnight before sterilization and fixation. The nonsterile specimens (buds, flowers and seeds) were surface sterilized as described in 3.1. After rinsing, the scales of buds and seeds were removed aseptically. Buds longer than 2 mm, female inflorescences, and seeds were dissected longitudinally. The callus samples were dissected aseptically into pieces of 2×3 mm or smaller. The specimens were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M NaH_2PO_4/Na_2HPO_4 (pH 7.4) at 4°C overnight. The fixed specimen were dehydrated, cleared through an ethanol *t*-butanol series, and embedded in paraffin (Merck).

3.6.5 In situ hybridization

The paraffin-embedded specimens were sectioned longitudinally. The sections were baked on silane-coated slides and paraffin was removed by xylene. The probes used in each experiment are presented in Table 3. In addition, a control without a probe was included every time for reduction of the background from the hybridization signal. Regarding the callus samples, the probes E11, RM6 and HD13 were hybridized. The slides were treated prior to hybridization, as described in 3.6.2., and hybridized under maximum stringency conditions (Section 3.6.2. and Table 2). The detection was performed with the DIG Nucleic Acid Detection Kit (Roche), after which the slides were

viewed under bright field illumination. Negative and positive control (sense and antisense) hybridizations with an RNA-probe targeted towards the plant 25S rRNA (Wanner & Gruissem 1991) were also performed for every specimen type. Amount of the hybridization signal was evaluated visually by comparing the sample with the background control.

3.7 DNA staining

The bud sections were treated with RNAse prior to DNA staining (I), and ethidium bromide was applied (10 µg/ml). The staining took place in the dark for 15 min. The slides were rinsed in water and 70% ethanol, air dried in the dark, and viewed immediately under immersion oil. For fungal hyphae the RNAse treatment was omitted, and the staining was performed for two hours in the dark.

3.8 Light microscopy

The samples were viewed with Fluor objectives on an Optiphot-2 Photomicroscope (Nikon). For detection of fluorescing ethidium bromide, an Episcopic-fluorescence Attachment EF-D (Nikon), a mercury lamp, and the Nikon filter set UV-1A were used.

3.9 Scanning electron microscopy (SEM)

Callus samples were obtained after 42 days of growth, fixed, processed as described in (V), and viewed in a Jeol JEM 100B scanning electron microscope.

3.10 Extraction of proteins and immunoblotting

The callus samples (0.5 g) were homogenized in liquid nitrogen followed by use of an electric grinder in 2 ml of extraction buffer comprised of 50 mM Tris-HCl (pH 8.65), 1 mM DTT and 25% (w/v) insoluble polyvinyl polypyrrolidone. The homogenates were placed on ice for 40 min. After centrifugation, the supernatant fluid was filtered through glass fiber (Whatman) and 0.22 µm PVDF filters. Finally, the samples were concentrated by centrifuging for 1 hour (2 500 × g) at +4°C using Ultrafree CL filters (Millipore).

Protein electrophoresis and western blotting were performed as described in Laukkanen *et al.* 1999. Polyclonal antibodies raised against *Beta vulgaris* chitinases class II and IV were used at a dilution of 1:200 to recognize chitinases in the pine tissues.

3.11 Tissue culture experiments

3.11.1 Testing of antibiotics in the growth medium

Various antibiotics were tested at different concentrations (III) against *R. minuta*, *H. dematioides, Mycobacterium* sp., *P. synxantha*, and *M. extorquens*. Carboxin and miconazole nitrate were effective against *R. minuta* and *H. dematioides* at concentrations of 50 mg l^{-1} and 10 mg l^{-1}, respectively. Streptomycin and tetracycline inhibited bacterial growth at concentrations of 50 mg l^{-1} and 10 mg l^{-1}, respectively. D1 media containing effective concentrations of either miconazole nitrate and carboxin, or streptomycin and tetracycline, or no antibiotics (control), were then prepared. Tissue cultures were started from pine buds on the media (24 explants per medium type) as described in 3.1. The cultures were observed weekly for two months, and transferred to a fresh medium every second week.

3.11.2 Conditioned test

Cells of *M. extorquens*, *P. synxantha*, or *R. minuta* were transferred to 250 ml liquid D1 media containing the plant growth regulators BAP and 2,4-D (section 3.1). The three strains were transferred similarly to 250 ml D1 medium without the growth regulators. The microbes were grown to stationary phase (4 days) and removed from the media by centrifugation. The media were filtered and antibiotics were added at effective concentrations (IV). Fresh D1 media were prepared with and without growth regulators, and each conditioned medium was then mixed with fresh medium (1:1 v/v) and plated (IV). Basal D1 media with or without growth regulators and containing the antibiotics were also prepared (IV).

Tissue cultures were started from pine buds on the media (12 - 16 explants per medium type) as described in 3.1. The cultures were observed weekly for two months, and transferred to a fresh medium every second week.

3.12 Mass spectrometric analyses

Cells of *M. extorquens*, *P. synxantha*, or *R. minuta* were transferred to 100 ml LB medium. The microbes were grown to stationary phase and removed from the media by centrifugation. The pH of the media was adjusted to 8-9 and the media were extracted three times with dichloromethane. The remaining aqueous solutions were acidified to pH 2.5 and extracted three times with diethyl ether. The ethereal extracts were evaporated to 1.5 ml and analyzed with a mass spectrometer (IV).

The acidic aqueous solution was adjusted to pH 7.5-8 and extracted three times with water-saturated *n*-butanol. The *n*-butanol extract was evaporated, resuspended in 1.5 ml methanol and analyzed with a mass spectrometer. Part of the *n*-butanol fraction was subjected to thin-layer chromatography (TLC) in a solvent system of isopropanol/benzene/ammonia (4:1:1, v/v/v) and detected using UV light. The bands, which were eluted with zeatin riboside were scraped from the plates, extracted in ethanol, filtered, and analyzed using a mass spectrometer.

A negative control of LB medium and a positive control of LB medium in which indole-acetic acid, gibberellic acid (GA_3), zeatin, and zeatin riboside had been added were processed as described above.

3.13 Testing of antagonistic effect

Cells of *M. extorquens*, *P. synxantha*, *Pseudomonas* sp. isolate K, and *R. minuta* were transferred to 5 ml LB medium, and cells of *Mycobacterium* sp. and *H. dematioides* to 5 ml D1 medium. The microbes were grown to stationary phase and the cells were removed from 1.5 ml of the culture by centrifugation and filtering. In addition to the microbes under study, reference strains of *Staphylococcus aureus*, *E. coli*, and *Klebsiella oxytoca* were grown. The reference strains and microbes under study were plated in LB or D1 top-agar. The plates were punched, and media in which the strains under study had been grown were added in the holes. Fresh LB and D1 broths were also added in the holes as controls. Once the microbes had grown on the plates, the inhibition zones around the holes were measured (IV).

Six strains of *Gremmeniella abietina* (A-type strains HU1.6, HR3, KR and B-type strains KAI 1.2, KAI 1.3, SUP 1.2) were transferred to potato dextrose agar (PDA) plates, and strains of *Hymenoscypha ericae* Read 100, *Phialophora finlandia* UAMH 8322, and *Phialocephala fortinii* SE 24 to 1/2 MMN plates. The microbes under study were transferred around the fungal reference strain. When the microbes had grown on the plates, inhibition zones between the strains were measured (IV).

4 Results

4.1 Identification of the microbes

The microbes which were found in the tissue cultures of Scots pine (*Pinus sylvestris* L.), were classified according to the physiological and morphological characteristics as *Methylobacterium extorquens* (isolate F), *Pseudomonas synxantha* (isolates G, H, I, J), *Pseudomonas* sp. (isolates K, N, P), *Hormonema dematioides* (isolates L, M) and *Rhodotorula minuta* (isolate T). Neighbor-joining trees showing the phylogenetic positions of the isolates are shown in (I), (II) and (III).

4.2 Origin of the microbes

Bacteria belonging to the genera *Methylobacterium*, *Pseudomonas fluorescens* subgroup and *Mycobacterium,* and the anamorphic pink yeast *Rhodotorula minuta* were detected in the pine bud tissues by *in situ* hybridization using the eubacterial probe E11 and the probes MB, PS5, MY2 and RM6, respectively (I, II, III). The microbes were located in the buds particularly in the cells of scale primordia, in the outermost cells of the meristems and in the cells of the developing stem, right below the meristems. The microbes were also found in the epithelial cells of the resin ducts, but less frequently in the vascular tissue or in the intercellular spaces of cells. The positive and negative controls of antisense and sense 25S rRNA hybridized as expected (I, II, III, V).

The *Pseudomonas* sp. (isolate K) and *Hormonema dematioides* were generally not found in the living tissues of pine buds (II, III). However, *H. dematioides* was detected on the interior of the bud scale using light microscopy, primarily at the tips of the outermost scales (II).

4.3 Distribution of the endophytes within pine buds

The distribution of pine endophytes, bacteria belonging to the genera *Methylobacterium*, *Pseudomonas fluorescens* subgroup and *Mycobacterium,* and the yeast *Rhodotorula minuta*, was studied in buds of three trees at different sample areas. Each of these microbes was detected in every tree sampled, except for the *Mycobacterium* sp. which was only found in buds of five of the six trees. A summary of the results (I, II, III) is shown in Figure 1.

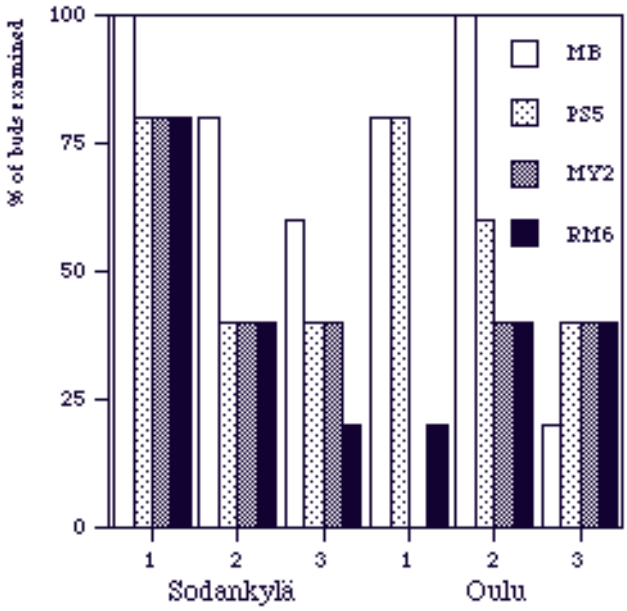

Fig. 1. Detection of *Methylobacterium* spp., *Pseudomonas fluorescens* subgroup, *Mycobacterium* sp., and *Rhodotorula minuta* in pine buds by *in situ* hybridization. The bud specimens (5 per tree) were collected from three trees in each of the two sample areas. The graph represents the percentage of bud specimens hybridizing positively with the probes MB, PS5, MY2, and RM6, respectively, from each tree examined.

4.4 Seasonal study

The distribution studies with the pine endophytes (*Methylobacterium* spp., *Pseudomonas fluorescens* subgroup, *Mycobacterium* sp., and *R. minuta*) using the eubacterial probe E11 and the probes MB, PS5, MY2 and RM6, respectively, were performed from buds of dormancy break. When the bud samples were studied by *in situ* hybridization further along bud development, an increase in the hybridization signal was detected in the bud samples collected close to the bud break. However, in the bud specimens taken during the

bud break, the hybridization signal decreased uniformly. In developing needles, the amount of ribosomal transcripts of the microbes was very low in the mesophyll tissue, although a distinct signal was detected in the still dividing tissue. Fully developed needles were devoid of any hybridization signal. The signal decreased also in the apical meristem during the bud break. When newly developed buds were examined, intense hybridization signals were detected in the tissues surrounding the resin ducts, while the overall signal was low. Additionally, a weaker signal was present in the vascular tissues of the newly developed bud. Later in bud development, strong signals were detected in the meristems and in the tissues surrounding them, but not in the vascular tissues or in the base of the bud. When the bud differentiation was completed, the amount of ribosomal transcripts of the microbes was at a similar level to that detected in the buds of dormancy break. Partly overlapping the bud dormancy, a decrease, and later a disappearance was observed in the hybridization signal. Regardless of the exceptionally warm temperatures in November and December 2000, the microbial metabolic activity was suppressed during these months. The activity was recovered differentially in the samples from January of 1998 and 2001 (III).

When the flowers were studied for the presence of endophytes, strong hybridization signal was detected in the sporogenous cells of male flowers with the eubacterial probe E11 and probe RM6, specific for *Rhodotorula minuta*. A weaker signal was present in the megasporangium of the female inflorescences with the probes E11, MB (for *Methylobacterium* spp.), PS5 (for a *P. fluorescens* subgroup), MY2 (for *Mycobacterium* sp.) and RM6. The seeds gave an intense signal after hybridizing with the five probes, but the results were not considered reliable because of a strong background signal (III).

4.5 Biological activity of the endophytes

When endophytes were eliminated from the bud-derived callus of Scots pine by antibiotics and antimycotics, a profound effect was observed. The explants maintained their shoot tip-like appearance for a longer period on the antimycotic medium than did the calli on the control media. In the tissue cultures grown on the antibiotic medium, the tissues remained significantly smaller and maintained the shoot tip-like appearance, producing only a narrow layer of callus on the surface (III).

The effects on tissue morphology and callus production, described above, were not observed in tissues grown on the conditioned media, although these media contained antibiotics as well. When the bud-derived calli were grown on conditioned media, browning which is typically observed in tissues originating from woody plants began at a later stage than when calli were grown on the control media. This effect was more evident on hormone-free media. However, the tissues remained generally smaller on the conditioned media (IV). The only exception was the hormone-free medium that was conditioned with *Rhodotorula minuta*, on which the explants grew significantly larger than the controls (IV).

Hormone production by the endophytes was studied by mass spectrometry. However, gibberellins, auxins, or cytokinins were not detected in the fractions studied. Instead,

Methylobacterium extorquens and *Rhodotorula minuta* were found to produce adenine and adenine derivatives such as adenine ribose and adenine deoxyribose (IV).

The antagonism of the endophytes was studied in cultures, and *Hormonema dematioides* was found to inhibit the growth of two strains of *Gremmeniella abietina* (HR3 and KR) and *Hymenoscypha ericae* Read 100. A slight inhibition in the growth of *G. abietina* strain KAI 1.2 was also observed (IV). The rest of the endophytes that were studied produced antagonistic substances towards *H. dematioides* (IV).

4.6 Chitinase production in callus of *Pinus sylvestris*

When class IV and II chitinase production was studied in the callus of Scots pine (*Pinus sylvestris* L.) by immunoblotting, 12 days of growth appeared to be the turning point. The type IV chitinases were produced in pine calli, especially after 12 days of growth. In contrast, the class II chitinases were present until 12 days of callus growth, after which their production diminished. When the endophytic fungus of pine bud scales, *Hormonema dematioides* was studied for its own chitinase production, some proteins belonging to the same size range as in the callus fraction of *P. sylvestris* were detected. Chitinases were not detected in the callus originating from immature embryos of *Pinus nigra* with either of the antibodies used (V).

4.7 Endophytes in callus of *Pinus sylvestris*

When the presence of endophytes was examined by *in situ* hybridization in the callus samples, they were detected in tissue cultures of *P. sylvestris* (originating from both buds and embryos) but not of *P. nigra* (originating from immature embryos). Bacterial endophytes were detected with probe E11 in 95%, and *Rhodotorula minuta* in 40% (in 50% of bud-derived and in 30% of embryo-originating cultures) of all the *P. sylvestris* callus samples. *Hormonema dematioides* was detected in two bud-originating tissue cultures with probe HD13. There was also one bud-derived callus of *P. sylvestris* that was devoid of microbes. Biofilms were detected inside calli originating from both buds and embryos of *Pinus sylvestris* by *in situ* hybridization. Biofilms were also detected on the surface of the bud-derived callus using scanning electron microscopy (V).

5 Discussion

5.1 The endophyte concept

Microbes have frequently been isolated from tissue cultures of several plant species (Holland & Polacco 1994, Leifert *et al.* 1994, Tanprasert & Reed 1997). They have generally been believed to be contaminants originating from outside the tissues, causing constant losses in commercial and scientific plant tissue culture laboratories (Boxus & Terzi 1987, 1988, Leifert *et al.* 1994). Inconsistently, fungal and bacterial endophytes have been isolated from plant leaf and root tissues using similar sterilization methods as in plant tissue culture. This contradiction has not been addressed, perhaps because of different approaches among the scientists. However, in this study it was demonstrated by *in situ* hybridization that most of the microbes found in the bud-derived tissue cultures of *Pinus sylvestris* L. originate from the plant tissue (I, II, III).

Based on the definition, all the microbes, which were found in pine tissues may be considered endophytes. Because the endophytic concept is very broad, it may cover diverse organisms that have different functions, locations and statuses in the plant tissue. Therefore, endophytes may be symbiotic as well as latent, or opportunistic pathogens (An *et al.* 1992, Olivares *et al.* 1997, James & Olivares 1997). Bacteria belonging to the *Pseudomonas agarici* subgroup and especially the *Pseudomonas* sp. K which was isolated from pine tissue cultures, were detected only rarely in the pine bud tissues by *in situ* hybridization (III). Another microbe which was generally not detected in the living bud tissues of pine is *Hormonema dematioides*. However, *H. dematioides* was found frequently to colonize the scale tissues of a bud (II). Although *H. dematioides* may therefore be considered an endophyte, this does not exclude the possibility that *H. dematioides* was pathogenic towards its host in other tissues. The rest of the microbes that were isolated from pine tissue cultures, or close relatives of these microbes were recurrently detected in the living tissues of pine buds by *in situ* hybridization (I, II).

5.2 Location and dynamics of the endophytes

While *Herbaspirillum seropedicae* has been isolated from seeds of cereals and micropropagated plants of sugar cane, and is probably therefore found also in the buds, *H. seropedicae* has been demonstrated to colonize mostly intercellularly or to reside in the xylem, which is also typical for other root-associated endophytes (Chanway 1996, Hallman *et al.* 1997). The endophytes which were associated with meristems of pine buds were located intracellularly, and rarely in the vascular tissues (I, II, III). In addition, they were not found in pine needles (III), as some of the root endophytes may be found within grass leaves (James & Olivares 1997). Because the nitrogen fixation ability of pine bud endophytes was not determined in this study, they may not be compared to the root-associated endophytes in this respect. However, their location is different from that of the root-associated endophytes, and they may therefore represent a new type of endophytic microorganism.

The detection of the meristem-associated endophytes in abundance in the epithelial cells of resin ducts, especially of newly developed buds, was an intriguing finding (I, III). The resin was suspected to represent a chemotactic signal for the microbes, and therefore their chemotaxis to pine resin was tested (III). Although only *P. synxantha* showed slight orientation, the resin may still play a role for these endophytes. The relationship between the fungal endophytes and plant has been addressed in this respect by Schulz *et al.* (1999). They determined that a significantly higher proportion of endophytic isolates produced herbicidic substances, compared to phytopathogens and fungi isolated from other sources. The host in turn produced secondary metabolites in higher amounts when it was infected by an endophyte, than when infected with a pathogen. Regardless of the high amount of secondary metabolites produced, the endophytic infections remained symptomless. The enzymes responsible for the production of these substances are also activated strongly upon infection by an endophyte, but with less intensity than when the host is infected by a pathogen (Peters *et al.* 1998). A similar effect was observed earlier with the meristem-associated endophyte *Mycobacterium* sp. (Laukkanen *et al.* 2000b). Phenylalanine ammonia-lyase activity increases significantly in pine seedlings when they are cultivated with the *Mycobacterium* sp. compared with the reactions caused by *Pseudomonas fluorescens* or *Mycobacterium chlorophenolicum*.

The number of meristem-associated endophytes of pine buds or their metabolic activity increased close to the bud break, but diminished during the growth of the bud (III). A decrease in the amount of fungal endophytes during growth was observed earlier with fungal endophytes of grasses (Herd *et al.* 1997). Because the endophytes were no longer found in the needles, it is likely that they were removed from the mesophyll tissue once its growth had stopped, through elimination, or transportation. The vascular tissue can function as a means of transportation for the root endophytes (Hallman *et al.* 1997). However, the possibility that the endophytes were eliminated from the tissues should not be excluded. In that case, the endophytes would be introduced in the newly developed bud from outside the plant, which could also take place through the vascular tissue (III).

Partly overlapping the bud dormancy, a decrease and later a disappearance was observed in the metabolic activity or number of the endophytes (III). Because the endophytes were detected in the same tissues again when the dormancy of the bud was

ending, the reduction occurred most probably in the metabolic activity and not in the number of the microbes. The metabolic activity was suppressed regardless of the exceptionally warm temperatures and again recovered differentially, and therefore both temperature and day length are considered to affect the activity of the microbes (III). This is most probably mediated through the control of the host which is affected by these factors in hardening and dehardening (Junttila 1989, Rohde *et al.* 2000).

5.3 The endophytic role

Although the endophytes *Methylobacterium* spp., members of the *P. fluorescens* subgroup, the *Mycobacterium* sp., and *Rhodotorula minuta* were distinctively associated with growing tissues in the pine buds, their effect on growth of the tissues remains obscure. Because the tissues were smaller and produced less callus when grown on antibiotic medium (III), an explanation could be that the endophytes have a positive effect on growth and callus production of the tissues. However, contradictory results to these were obtained from the conditioned test. Calli grown on conditioned media were smaller than those grown on control media in almost every case, and the only exception was the hormone-free medium conditioned with *R. minuta* (IV). One explanation for all this would be that the antibiotics caused the effects on size and morphology of the tissues. However, when nystatin was used in the control media during the conditioned test, there was a similar effect on morphology of the tissue observed, as with carboxin and miconazole nitrate (data not shown). Tetracycline or streptomycin applied alone in the culture medium had a milder but similar effect on size, callus production and morphology of the tissues than what was observed with these antibiotics together in the medium (data not shown). Because conditioning of the media removed the effects on callus production and shape of the tissues (IV), these effects may not be a consequence of the antibiotic substances after all, but of removal of the endophytes from the tissues.

Another reason for the contradictory results from the antibiotic and conditioned tests would be that the endophytes had consumed nutrients from the medium during the conditioning, and therefore the tissues were smaller on the conditioned media than the controls (IV). However, in previous studies with the *Mycobacterium* sp. this bacterium reduced the growth of pine seedlings (Laukkanen *et al.* 2000b). Therefore, the possibility that the effect of the endophytes on growth is negative should be taken into consideration. Otherwise, the conditioning of the media with the endophytes had an effect on the pine tissues which could be considered positive, because on these media the tissues remained green for a longer period than on the control media (IV).

When the endophytes *Methylobacterium extorquens*, *Pseudomonas synxantha* and *Rhodotorula minuta* were studied for plant hormone production, any well-known phytohormones were not detected (IV). Based on the TLC analysis alone, *M. extorquens* and *R. minuta* could have been concluded to produce cytokinins, because several bands were eluted with the standard of zeatin riboside, and fluoresced in UV light (IV). However, when these bands were analyzed with LC-MS, zeatin or zeatin ribosides were not detected, but adenine ribosides were found in these fractions (IV). Adenine riboside

has earlier been detected in significant amounts in the vascular cambial region of *Pinus sylvestris* (Moritz & Sundberg 1996). In addition, adenine riboside was described from the feeding experiments in *Coffea arabica* cell suspension cultures as a metabolite of adenine (Baumann *et al.* 1994), and in radish cotyledons as a metabolite of zeatin riboside (Tao *et al.* 1991). Therefore, adenine ribosides could act as precursors for cytokinin synthesis. However, whether these bacterial products are beneficial for the plant remains unknown. The occurrence of cytokinins was not studied inside the cells of the endophytes, because zeatin riboside is commonly found to be attached to nucleotides inside bacterial cells (Ajitkumar & Cherayil 1985, Helbach & Klämbt 1981, Janzer *et al.* 1982).

The studies on production of antagonistic substances by the endophytes produced data that is well in accordance with their location in the pine buds. *H. dematioides* inhibited growth of some strains of *Gremmeniella abietina*, which is a fungus pathogenic to *Pinus sylvestris*. Because *H. dematioides* is located in the bud scales that are the outermost parts of the bud, it may readily protect the host from pathogenic attacks. Some strains of *H. dematioides* produce rugulosin, an anthraquinone which exhibits a wide spectrum of biological activity (Calhoun *et al.* 1992). Another member of the *Hormonema* genus produces enfumafungin, which is an antifungal substance (Peláez *et al.* 2000). However, because *H. dematioides* is pathogenic once inoculated into pine (Lieutier *et al.* 1989) the finding that the bacterial endophytes and *R. minuta* produce antagonistic substances towards *H. dematioides*, is consistent with their location inside the buds.

5.4 Endophytes in tissue culture of *Pinus sylvestris*

Browning of the bud-derived tissue cultures of Scots pine is accompanied by lipid peroxidation and lignification of cells (Laukkanen *et al.* 2000a), which are characteristic features of the plant defense reaction (Lee & Whitaker 1995, Keller *et al.* 1996). Endophytes of pine buds were considered a potential cause for the defense reaction, and because chitinases are expressed in response to microbial attack (Collinge *et al.* 1993), their role in the defense reaction was elucidated. Chitinases belonging to class IV and II were present differentially in pine tissue, having 12 days of growth as the turning point (V). Type II chitinases were also produced by *H. dematioides*, and therefore some of the proteins detected in the callus fraction of *P. sylvestris* may actually originate from *H. dematioides*, which frequently occupies the pine tissue cultures (II, V).

The endophytes were found in abundance in half of the tissue cultures of *P. sylvestris*, and biofilms were detected both inside and on the surface of some calli (V). Neither biofilms nor intensive metabolic activity of the endophytes were detected in pine buds during their natural, active growth (III), although a high metabolic activity of the endophytes was detected in buds preparing for growth (III). The endophytes subsequently decreased in either number, or in metabolic activity in the bud tissue during the growth (III). Therefore, the endophytes are probably under strict control in the growing pine bud, but become uncontrollable once the tissue culture is initiated from the bud.

When embryogenic callus of *Pinus nigra* was studied for chitinase production, type II and IV chitinases were not detected (V). Because endophytes or browning were not detected in *P. nigra* callus, there appears to be a correlation between browning, chitinase production, and occurrence of endophytes in the tissues (V). However, it should be noted that there was also one bud-derived callus of *P. sylvestris* which was devoid of endophytes, and the defense reaction and browning can therefore not exclusively be explained as the result of the endophytes.

The bud-derived callus of *Pinus sylvestris* has generally the lowest regeneration ability when compared to mature embryos or especially immature embryos and cotyledons used as the starting material (Häggman *et al.* 1996, Hohtola 1995, Keinonen-Mettälä *et al.* 1996). Because the endophytes were detected in the embryo-originating calli with nearly similar frequency as in bud-derived calli, there are probably additional factors affecting the regeneration ability of Scots pine tissue. However, biofilms and the abundancy of endophytes in the tissues most likely affect the growth of callus by causing a defense reaction. Therefore, it is concluded that endophytes are partly responsible for the degeneration of the bud-derived pine tissue cultures.

6 Conclusions and future prospects

In this research it was demonstrated for the first time, that microbes occurring in tissue cultures originate from the plant tissue itself. One of the microbes, identified as *Hormonema dematioides*, was localized in the bud scales of *Pinus sylvestris* L. A location was established for some of the microbes in meristematic tissues of the bud. This group comprised of *Rhodotorula minuta*, a *Mycobacterium* sp., and bacteria belonging to *Methylobacterium* spp. and *Pseudomonas fluorescens* subgroups. Because these microbes were located intracellularly in the meristematic tissues, they may represent a new type of endophyte.

It was indicated that the meristem-associated endophytes may affect growth of pine tissue. Some of these endophytes produced substances that are suitable as precursors in phytohormone synthesis. The importance of these products for the plant and the nature of their effect on plant growth need additional consideration in the future. According to the *in vitro* studies, most bud endophytes of Scots pine inhibited growth of particular pine pathogens. Therefore, a role for these endophytes could be suggested in protection of the host from pathogens, but this would require additional studies.

A possible connection between endophytes and degeneration of bud-derived tissue cultures of Scots pine (*Pinus sylvestris* L.) was established in this research. The endophytes were discovered to grow uncontrollably once a tissue culture was initiated from the bud, and high level of chitinase production was detected in the bud-derived calli of Scots pine. In contrast, a callus originating from immature embryos of *Pinus nigra* did not show browning, chitinase production, or endophytic occurrence. However, because the endophytes were not detected in abundance in all tissue cultures of Scots pine, the endophytes may not exclusively be considered responsible for the degeneration of the cultures. Therefore, dilemma of the browning phenomenon which occurs in the tissue cultures of Scots pine remains to be solved in the future.

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