Early microbial community development in stems of *Picea abies* inoculated with characterised decay fungi, non-decay fungi and bacteria

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Abstract

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Norway spruce stems were deliberately wounded and inoculated with different combinations of decay fungi, non-decay fungi and bacteria, all of them typical of wounded spruce. The decay fungi were *Stereum sanguinolentum* and *Heterobasidion annosum*. They were inoculated alone or in combinations with *Ascocoryne cylichnium*, *Nectria fuckeliana*, an unidentified yeast-like fungus and strains of the bacteria *Bacillus pumilus* and *Enterobacter agglomerans*.

The endophytic fungi *Neobulgaria premnophila* and *A. cylichnium* were already present in most stems of the experimental trees before inoculation. The inoculated stems were efficiently colonised by these fungi and bacteria, which then persisted over the whole experimental period of 5 years. The strains of *A. cylichnium* and/or *N. premnophila* were characterised in detail by analysing their fatty acid and sterol compositions.

The upward spreading of the decay fungi *S. sanguinolentum* and *H. annosum* was extensive in the trees felled one and two years after inoculation but less extensive after three years and, from trees felled after five years, these fungi were absent. The different microbe combinations inoculated together with these fungi did not markedly affect the spreading of these fungi. The mixture including *N. fuckeliana*, *A. cylichnium*, a yeast-like fungus and the bacteria, when inoculated together with *S. sanguinolentum*, suppressed invasion by the latter.

The bacteria most frequently isolated were Gram-positive *Bacillus* species, primarily the inoculated *B. pumilus*. Other species frequently found were *B. subtilis* and *B. cereus*. The predominant Gram-negative bacteria were *Enterobacter agglomerans* (inoculated), *E. sakazakii*, and fluorescent and yellow pigmented strains of *Pseudomonas*. The Gram-positive bacteria were able to utilise pectate, cellulose and hemicellulose, whereas some of the Gram-negative bacteria showed lipolytic activity and the ability to grow on pine resins.

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List of publications

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals.

I HALLAKSELA, A-M., 1993: Early interactions of *Heterobasidion annosum* and *Stereum sanguinolentum* with non-decay fungi and bacteria following inoculation into stems of *Picea abies*. Eur. J. For. Path. **23**, 416-430.

II MÜLLER, M. ; HALLAKSELA, A-M., 1994: Variation in combined fatty acid and sterol profiles of *Ascocoryne*, *Nectria* and *Neobulgaria* -strains isolated from Norway spruce. Eur. J. For. Path., in press.

III HALLAKSELA, A-M. ; VÄISÄNEN, O. ; SALKINOJA-SALONEN, M., 1991: Identification of *Bacillus* species isolated from *Picea abies* by physiological tests, phage typing and fatty acid analysis. Scand. J. For. Res. **6**, 365-377.

IV HALLAKSELA, A-M. ; SALKINOJA-SALONEN: M., 1992. Bacteria inhabiting artificially inoculated xylem of *Picea abies*. Scand. J. For. Res. **7**, 165-175.

Preface

Most of this work was carried out at the Finnish Forest Research Institute, Department of Forest Ecology. Prof. Tauno Kallio guided me towards the world of microbes and trees. I thank him for this and remember fondly the intense discussions about microbial life in wounded trees and about life in general. My warm thanks also to Prof. Aino Käärik for introducing me to the world of identification of decay fungi. I wish to thank Prof. Timo Kurkela for providing me with good working facilities throughout this work. My sincere thanks go to Leena Suominen, MSc. for our first steps together in developing identification methods for bacteria inhabiting living trees.

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1. Literature review

1.1 Introduction

Thinning and clear-cutting operations carried out in the forest open new infection channels and nutrient resources for wood-decay microbes by producing fresh stump surfaces and wounds on roots and stems of growing trees. This makes for easy invasion and promotes establishment of pathogenic decay fungi in healthy forest areas. These primary infections also increase the risk of decay microbes invading healthy trees via root contacts and grafts (STENLID 1986 ; PIRI et al. 1990). Some very harmful pathogenic fungi (e.g. *Heterobasidion annosum*) clearly benefit from the activity of man in the forests (ROSNEV 1980).

The interest in damage caused by microbial decay in managed forests has increased since mechanised thinning methods and all-year-round fellings have become common practice. Felling trees with cutting machines often results in injuries to trees along the strip roads. In particular, thinning carried out during summer time increases decay losses by exposing damaged trees to invasion by microbes. On the other hand, reducing the thinning episodes per rotation may increase the risk that many trees with large decay volumes are left in the forest.

Bark is the tree's barrier against microbes. When the tree is wounded deep into the xylem, it is easily invaded by microbes. The microbes spread via the air in summer time and are also transmitted by insects invading wounded trees. To understand the importance of avoiding injuries, it is necessary to know what happens in trees after wounding and microbe invasion.

1.2 The tree as an environment for microbial growth

1.2.1 Anatomy of conifer xylem

The channels which transport water and nutrients within the xylem of the living conifer are also routes for microbes. These passages of compact xylem tissue are composed of long, and slender tracheids with few parenchyma cells. As much as 90 % of the conifer wood consists of vertically oriented dead tracheids with thick lignified walls (WILSON and WHITE 1986).

The parenchyma cells in conifers are situated in rays (horizontal conducting elements transporting sap and periodically storing reserve materials). The ray parenchyma cells have thin walls and retain their living protoplasts for some years. They die in the transition from sapwood to heartwood. The upper and lower margins of each ray are formed from 1-3 rows of ray tracheids (WILSON and WHITE 1986).

Walls of tracheids and parenchyma cells have various types of pits. Pits are formed between adjacent cells, opposite to each other, so that they form a pit-pair, in which only a thin pit membrane separates the two cells. Pits allow movement of water and nutrients between adjacent cells. Bordered pits develop between adjacent tracheids. The membrane between adjacent cells in bordered pits has two parts: a thickened central region (torus) surrounded by a thinner margo. The margo is flexible, and when a large difference in pressure occurs across the pit-pair, the torus is forced against the border, effectively sealing the pit. Such sealed pits are said to be "aspirated". Simple pits without aspiration apparatus are situated between longitudinal tracheids and ray parenchyma cells (WILSON and WHITE 1986 ; BRETT and WALDRON 1990).

In addition to the above mentioned, there are conducting ducts which produce resin. The production of resin is an essential response to invading microbes. These ducts occur regularly in conifers such as *Picea*, *Pinus* and *Larix*, though in *Picea* they may be less numerous than in the others. They comprise vertically oriented ducts scattered throughout the wood, and horizontally oriented radial ducts arranged in specialised fusiform rays. Traumatic resin ducts are occasionally formed as a response to injury to the tree. The vertical resin ducts are separated from the surrounding axial tracheids by a parenchymatous sheath, the so-called epithelium.

This living epithelium soon becomes thickened, especially in *Picea*, a change which probably marks the end of its secretory function (WILSON and WHITE 1986).

The cell wall of a mature tracheid is composed of various layers. The middle lamella and primary wall make up the compound between the secondary walls of adjacent cells. The secondary wall consists of three layers designated as S_1, S_2 and S_3. The S_3 is the innermost, located next to the cell cavity (lumen). The layers are distinct from each other because of different microfibrillar orientations around the cell axis in each layer (WILSON and WHITE 1986).

1.2.2 Chemical composition of xylem cells

The amount of readily degradable substrates such as soluble sugars, lipids and other primary metabolites, is important to invading microbes. These substrates occur in relatively small amounts and are located almost exclusively within the parenchyma in living functional sapwood. The dominant available carbon sources are relatively refractory in a living tree. The major structural polymeric components of woody cell walls are cellulose, hemicelluloses and lignin (RAYNER and BODDY 1988).

Cellulose is the major component of the secondary walls of xylem. It comprised 40-45 % of dry weight in most wood species (SJÖSTRÖM 1993). Cellulose is the predominant renewable carbon compound in the biosphere and it serves as the most important carbon and energy source for wood-inhabiting microbes. The structure of cellulose is regular. The linear polymer is composed of repeated glucose units linked by ß-1,4-glycosyl bonds. The disaccharide cellobiose, rather than glucose, is the basic structural unit of cellulose. It is the major product of hydrolysis of cellulose by cellulolytic enzymes of bacterial and fungal origin (ERIKSSON et al. 1990).

Besides cellulose, coniferous wood contains considerable amounts of polysaccharides such as hemicellulose, usually 20 -30 % of dry weight (SJÖSTRÖM 1993). Hemicellulose is composed of both linear and branched heteropolymers of D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid. Most hemicelluloses contain from two to six of these sugars. The most important hemicelluloses in conifers are galactoglucomannans, arabinoglucuronoxylan and arabinogalactan (ERIKSSON et al. 1990).

Pectic polysaccharides are important in the young primary wall but pectic compounds comprise only 1 % or less of mature wood (WILSON and WHITE 1986). They are situated in the middle lamella and the primary wall. They are covalently linked to phenols, cellulose and protein. Pectic polysaccharides are rich in galacturonic acid, rhamnose, arabinose and galactose (BRETT and WALDRON 1990).

Lignin and hemicellulose form a complex lignin-hemicellulose matrix which encloses the cellulose fibrils, thus forming a rigid protective skeleton for the secondary walls of wood cells against microbial degradation. Lignin is also located in the thin layer, the middle lamella, which combines adjacent cells into a compact tissue (ZABEL AND MORRELL 1992). Furthermore, lignin regulates water transport in plants by decreasing the permeability of cell walls (HIGUCHI 1985). Conifers normally contain lignin at 26-32 % of the cell wall compounds (SJÖSTRÖM 1993). As to chemical structure, lignin is a heterogeneous polymer consisting of phenylpropane units. Lignin in conifers consists mostly of coniferyl subunits (SJÖSTRÖM 1993).

1.2.3 The moisture and gas content of the tree xylem

The pressure of the moving water columns in the xylem is negative most of the time. Sooner or later, the water columns break and this causes the tracheary elements to become filled with gas, a phenomenon called embolism. This loss of water from the conducting channels is the beginning of formation of heartwood or traumatic "air-block" tracheids (ZIMMERMANN 1983).

The xylem of conifers is divided into two parts: the living sapwood and the dead heartwood. The development of sapwood into heartwood, which takes place in a relatively narrow transitional zone, involves a number of co-ordinated changes. In softwoods, the bordered pits become aspirated. During aspiration, the storage starch of vertical and ray parenchyma is mobilised and utilised as secondary metabolites (WILSON and WHITE 1986) such as resin, acids, polyphenols and pigments (HILLIS 1987). Secondary metabolites, such as free and esterified sterols and steryl glucosides, are involved in the formation of heartwood of *Pinus sylvestris* (SARANPÄÄ 1990). These substances become deposited on the cell walls and in the pit cavities and the lumen of the wood cells. They form plugs in tracheids and reduce the permeability of the wood to water. In many softwoods, the heartwood becomes markedly drier than the sapwood. However, the greater the vitality of the

tree, the lower the tendency of the living cells to form heartwood (WILSON and WHITE 1986 ; HILLIS 1987).

The lumina of broken cells after injury are immediately filled with air. The injured xylem tracheids become "air-blocked" and sealed off from the water-conducting area by aspiration (WILSON and WHITE 1986). These air-blocked cells dry. The extent of drying depends on how quickly the vertical withdrawal of water from damaged tracheids by hydrostatic tension takes place (ZIMMERMANN 1983). Through the destroyed sapwood in a wounded tree, it is easy for a decay fungus to reach the more favourable heartwood. The low water content and higher gas content of the heartwood favour the growth of specialised fungi in a standing tree, even when containing fungistatic or fungitoxic chemicals (BODDY and RAYNER 1983 ; SHORTLE and OSTROFSKY 1983). From the injured area, two inwards-tapering regions of air-accessed tissue may be formed, one region above and another below a wound made into the main stem. The tapered region above the injury is somewhat greater because of the upward flow of water (RAYNER and BODDY 1988).

1.3 Hypotheses of microbial succession and tree response

There are two major hypotheses concerning interaction between invading microbes and tree defence reactions in the wounded living tree at the beginning of the decay process. These hypotheses were presented by SHIGO (1979, 1984) and RAYNER and BODDY (1988). Additional details were presented by DUJESIEFKEN et al. (1991).

SHIGO (1979, 1984) stated that micro-organisms invading the living tree have the greatest survival advantage when they attack wounds in a sequential manner (succession). He named three stages of succession. The first stage includes all processes associated with host response to wounding. The host response is based on boundaries formed by the tree cells. These boundaries isolate the injured xylem tissues, and thus resist the onward spread of microbes. In the next stage of succession, micro-organisms surmount the anatomical and chemical protection barriers and invade the xylem. These pioneer invaders are usually bacteria, yeasts and non-decay fungi. Small-spored, non-decay pioneer fungi (*Phialophora* spp.) may be present alone in sapwood or with bacteria (SHORTLE and COWLING 1978a). These pioneer fungi are able to modify phenolic substances for phenol-intolerant decay fungi (SHIGO and SHARON 1968 ; SHIGO and SHARON 1970 ; SHORTLE and COWLING 1978 b). Finally, the decay fungi decompose xylem because it is no longer protected

by the products of sapwood parenchyma cells (SHORTLE and COWLING 1978 b ; SHIGO 1979).

The hypothesis of RAYNER and BODDY (1988) promoted consideration of primary importance of the physiological factors. The activity of a microbe depends on environmental conditions, including availability of water, oxygen and other gases, and temperature. For example, the decay fungi are temporarily suppressed by bacteria and non-decay fungi in wood having a high moisture content but become dominant in drier conditions (SHORTLE and OSTROFSKY 1983). The decay fungi show different abilities to grow under different moisture conditions. *Stereum sanguinolentum*, for instance, grows well in dry phenol-enriched heartwood tissue, while *Coriolus versicolor* survives better in sapwood (SHORTLE and OSTROFSKY 1983). Nevertheless, at very low water contents of wood, certain species of non-decay fungi survive better than decay fungi (GRIFFITH and BODDY 1991). Many different types of microbes co-exist in the initial decaying process but their activity fluctuates depending on environmental changes.

DUJESIEFKEN et al. (1991) stressed the importance of parenchyma cell activity as a compartmentalisation factor. In hardwood species, the tree reacts against microbial invasion by secreting fibrillar material from living parenchyma cells through the pit membranes into adjacent vessels. This wound-induced fibrillar synthesis is inactive in many hardwood species during the dormant season because the activity of parenchyma cells depends greatly on temperature. This reaction is not important in conifers because the closure of their bordered pits is based on a pressure difference and no temperature-dependent parenchyma activities are involved (DUJESIEFKEN et al. 1991).

1.4 Defence strategies of a living tree

1.4.1 Constitutive defence

The anatomical structures of different cell elements form the major constitutive defence strategy of a living tree. The anatomy of wood favours microbial growth in some directions more than in others, in particular by facilitating access in the vertical and radial directions, whilst preventing tangential spread (RAYNER and BODDY 1988). The hypothesis of SHIGO (1979, 1984) promoted the importance of compartmentalisation of tree cells as a result of tree wounding. The tissues which

prevent the spread of invaders are called "walls" and it was suggested that they are of four distinctive types, termed walls 1, 2, 3 and 4 in order of increasing resistance to fungi. According to SHIGO's compartmentalisation concept (1979, 1984) wall 1 consists of occlusions (tyloses, resin) of axial elements (vessels, tracheids) and hence limits the vertical spread of microbes. This wall is the weakest; it does not restrict the growth or movement of microbes in the cavity of tracheids (RAYNER and BODDY 1988). Wall 2 is a purely physical barrier, made up by the dense cell walls in annual rings, and limits inward radial spread. Wall 3 consists of sheets of ray cells, which impose a living discontinuous barrier to tangential spread. However, the damaged tissue of ray cells favours the invasion of microbes radially along ray cells deeper into the xylem (RAYNER and BODDY 1988). Walls 1,2 and 3 exist in the tree prior to injury and infection by microbes. Production of wall 4 starts after invasion of microbes into wood cells. After wounding, the cambium begins to form this new protective wall, which separates wood present at the time of injury or infection from new wood that forms subsequently. The wall is both a physical and a chemical barrier (SHIGO 1984).

One example of an anatomical factor accounting for the effectiveness of compartmentalisation is the size of different transporting elements (xylem vessels or tracheids). ECKSTEIN et al. (1979) have reported that trees with few, small vessels are compartmentalised strongly and the trees having many large vessels formed walls less effectively. Furthermore, SMITH and SHORTLE (1993) reported that such small vessels loose less water and hence microbe invasion is slower.

1.4.2 Induced defence

Wounding the living tree xylem leads to processes similar to heartwood formation (HILLIS 1987). The water loss by embolism and aspiration by bordered pits in the injured cells are the primary events which lead to formation of a reaction zone (RAYNER and BODDY 1988). The reaction zone is the barrier between healthy sapwood and damaged dry xylem. This zone is formed by extractives produced by living host cells. The production of extractives is induced by mechanical injury alone or by microbes. The host parenchyma cells of sound sapwood gradually die via a transition zone (some cell layers between the healthy sapwood and the reaction zone) leading to reaction zone formation (BJÖRKMAN ET AL. 1949 ; SHAIN 1971, 1979). The terms "transition zone" and "reaction zone" have been combined by some authors into "column boundary layer" (CBL) (SHORTLE and SMITH 1990). The reaction

zone is considered to be the site of a dynamic interplay between host and microbe and is situated primarily in the same area as the compartmentalisation wall 4, but can also occur at any point where microbes break the anatomical wall. On the outside of this interplay area are the healthy intact sapwood cells and inside is the damaged aspirated xylem, which forms a suitable area for microbial growth (SHIGO and HILLIS 1973 ; SHAIN 1979 ; BODDY and RAYNER 1983 ; RAYNER and BODDY 1988).

Parenchyma cells in the reaction zone die as penetrating microbes advance, probably as a result of altered metabolism in the transition zone. During the microbial penetration, ethylene production increases and phenols accumulate in the wounded area. This limits the growth of invading microbes but may not necessarily stop them. STENLID and JOHANSSON (1987) have shown that the transition zone phenolics are the strongest inhibitors of conidial germination of *H. annosum*, while sapwood phenolics are the weakest. This dry transition zone is also characterised by a decrease in starch content and simultaneous increase in phenolic compounds (JOHANSSON and STENLID 1985).

Most conifers have developed a defence strategy based principally on polyphenols, such as stilbenes, lignans and terpenoids, while the strategy of decay-resistant durable hardwoods (*Castanea, Eucalyptus*) is based on tannins (SCALBERT 1992). Much of the research in this field has been concerned with the interaction between stilbenes and the pathogenic fungus *H. annosum*. POPOFF et al. (1975) have suggested that glucosidation of phenolics could be a mechanism by which the fungus may eliminate phenolic toxicity. This was supported by STENLID and JOHANSSON (1987) who showed that the amount of phenolic glucosides in *H. annosum* -infected wood was larger than in the other wood zones. WOODWARD and PEARCE (1988a) have shown that the phenolic compounds, stilbene (1,2-diphenylethene)-glucosides (astringin, rhaponticin), are constitutive in the Sitka spruce bark tissue. The wounded bark tissue under fungal contact appears to induce the release of stilbene aglycones (astringenin and isorhapontigenin) from the glucosides. These aglycones are more effective antifungal compounds than the constitutive glucosides, but even these stilbene aglucones were inactivated by laccase-producing white-rot fungi such as *S. sanguinolentum* and *H. annosum* and they become oxidised or polymerised. Also, LINDBERG et al. (1992) stated that the amount of stilbenes decreased following infection *in vivo*. A weak pathogen, *Phaeolus schweinitzii*, was not able to inactivate aglucones. Approximately simultaneously with the inactivation, the cell wall alterations (phenolic decomposition, suberization, cell wall thickening) become detectable in tissues underlying the wound (WOODWARD and PEARCE 1988b).

Norway spruce bark contains a high level of phenolic compounds other than stilbenes. Synergism may occur between various bark extractives, making the mixture more toxic than the added effects of the individual compounds (LINDBERG et al. 1992). In addition to interactions in the bark, the reaction between stilbenes and fungi may take place in deep xylem wounds because fungal attack may induce the tree to produce stilbenes in tissues such as sapwood where the compounds would not normally occur (HART 1981). It seems that decay resistance is a multifunctional phenomenon and it is unlikely that a specific substance can be singled out as being solely responsible for the resistance of wood to microbial invasion (HART and SHRIMPTON 1979).

As an example of the interaction between decay fungi and tree defence mechanisms, CWIELONG and HÜTTERMANN (1989) presented a theory that *H. annosum*, when growing inside the trunk of the living tree, actively degrades lignin in order to get access to other wood components. During lignin degradation, too, toxic phenols are liberated, against which the fungus has to protect itself. For this protection, it needs oxygen to detoxify the phenolic compounds by polymerization. This protection is possible for *H. annosum* because oxygen content is highest in the heartwood and, during the cool months, this apparently favours the growth of this decay fungus (CWIELONG and HÜTTERMANN 1989).

1.5 Microbes participating in community development in wounded trees

1.5.1 Non-decay fungi

A wide variety of fungi occurring in wood probably utilise only those easily assimilable substrates such as simple sugars, starch, proteins, etc., since they are either incapable of degrading the relatively refractory structural components of the woody cell walls, or possess only a limited ability to do so. These fungi are called non-decay fungi. Although assimilable substrates are available in a living wood, they are rapidly depleted as decomposition proceeds. Even so, there remains the possibility of a continued existence of non-decay organisms due to the formation of associations with decay fungi. The non-decay fungi will utilise the supply of assimilable substrates produced by decay fungi attacking wood lignocellulose (RAYNER and BODDY 1988).

The non-decay fungi are predominantly Ascomycotina, Deuteromycotina and Zygomycotina species. Some of them stain the wood, either by releasing pigmented substances or because of pigments in their hyphal walls as in the "blue-staining" species of *Ceratocystis*, which invade freshly felled or damaged wood of conifers (KÄÄRIK 1980). These fungi produce characteristically wedge-shaped columns of stain resulting from their colonisation of the radially oriented ray parenchyma which contain the requisite food reserves. "Blue-staining" and, for example *Nectria*-type fungi, benefit from wounds made by man or animals. They generally cause no significant structural damage in wood (ROLL-HANSEN and ROLL-HANSEN 1980b), although stressed young Norway spruce has been reported to have been killed by the blue-staining fungus *Ophiostoma polonicum* (CHRISTIANSEN and SOLHEIM 1990).

A number of wood-inhabiting non-decay fungi are termed "dematiaceous" because of their melanised hyphal walls or spores (ELLIS 1971). These fungal species, e.g. *Rhinocladiella*, *Leptodontium*, can be found in wood at various stages of decomposition, where they cause gray or black discoloration. They may also grow in relatively undecayed interaction zones between mutually exclusive thalli of decay species. When occupying the interaction zones, these fungi can be regarded as relic populations squeezed by the decay fungi into regions where the latter are inactive. Alternatively, their situation in interaction zones may provide them with an opportunity for utilisation of the metabolic products of the decay fungi (RAYNER and BODDY 1988).

Some of the non-decay fungi live within wood without causing any symptoms to the host and thus can be considered as endophytes (PETRINI 1992). One interesting group of these fungi includes certain species of *Ascocoryne* and *Neobulgaria*, which inhabit the heartwood of living spruce trees (ETHERIDGE 1970 ; ROLL-HANSEN and ROLL-HANSEN 1979 b).

1.5.2 Soft-rot fungi

Soft-rot fungi degrade cellulose and hemicelluloses and also lignin. The rate of decay caused by these fungi in conifer wood is low and the extent of the degradation small as compared to the decay fungi (ERIKSSON et al. 1990). In conifers, hyphae of soft-rot fungi colonise ray parenchyma cells and grow from the rays into

the lumina of tracheids (LIESE 1970). Their characteristic mode of attack on woody cell walls is the formation of localised erosion grooves and series of cavities in the S_2 layer. A series of cavities results from repeated penetration of fine hyphae, cessation of hyphal growth, and subsequent cavity formation (HALE and EATON 1985).

A number of Ascomycotina and Deuteromycotina are able to form soft rot. Two important examples of the prominence of soft-rot fungi under circumstances where decay fungi are inhibited are water-saturated wood and preservative-treated wood (RAYNER and BODDY 1988). For example, species such as *Graphium*, *Phialophora* and *Rhinocladiella* were isolated from living spruce (HALLAKSELA 1984b) and from pine poles treated with BIS-salt (KALLIO and HALLAKSELA 1975). These soft-rot fungi are masked by decay fungi in living spruce but can develop more extensively under certain conditions such as in preservative-treated pine poles. A *Phialophora*-type fungus has been reported to modify wood to make it a more favourable substrate for decay fungi (SHORTLE and COWLING 1978b).

1.5.3 Decay fungi

Wood-decay fungi are a group consisting of brown-rot and white-rot fungi. They are characterised by their ability to degrade either wood cellulose or lignin or both. These, mostly Basidiomycotina fungi, are the main degraders of wood, and their enzymatic capacities provide them with the potential to colonise wood at all stages of decomposition.

The white-rot fungi typically degrade cell wall lignin leaving most of the white-coloured cellulose intact. However, these fungi vary considerably with respect to their type of attack on lignin and wood polysaccharides. Furthermore, they demonstrate very different rates of lignin removal. On the basis of these properties, white-rot fungi may be divided into two groups: (i) species degrading all wood components at approximately the same rate, and (ii) those species degrading lignin preferentially. The latter, i.e. selective lignin degraders, typically create localised areas of decay and bleached wood, and are designated as white-pocket or white-mottled rot fungi due to this effect (OTJEN and BLANCHETTE 1986 ; ERIKSSON et al. 1990).

White rot fungi colonise wood quickly and may become established in all cells of the xylem. The ray parenchyma cells are frequently the first to be colonised. Holes

may be numerous in early stages of decay, and hyphal penetration from cell to cell via pit structures or directly through the wall is easily accomplished (LIESE 1970 ; WILCOX 1968). The order in which various amounts of lignin, cellulose, and hemicellulose are degraded is different among species of white-rot fungi and may vary depending on the type of wood substrate being attacked (KIRK and HIGHLEY 1973 ; BLANCHETTE 1984a, b).

White-rot fungi are widely distributed throughout all the major groups of higher basidiomycetes, and are also found amongst Ascomycotina (GILBERTSON 1980). They are common in degradation of hardwood, but some important white-rot species also attach conifers, e.g. *Heterobasidion annosum* (Fr.) Bref. and *Stereum sanguinolentum* (Alb. & Schw. ex Fr.) Fr. These two fungi are in fact the most important pathogens in Norway spruce in southern Finland (HALLAKSELA 1984b).

H. annosum selectively removes lignin and/or attacks all wood components simultaneously in the same substratum (HARTIG 1878 ; ERIKSSON et al. 1990). This decay-fungus is an active pathogen being able to invade intact sapwood without preconditioning. The primary infection route is via recently exposed wood. The spore infection may take place through wounds (ISOMÄKI and KALLIO 1974) or through stump surfaces created during thinning or clear-cutting (RISHBETH 1951a). The secondary infections of standing trees are generally caused by the fungus spreading from neighbouring infected stumps or trees via root contacts (RISHBETH 1951b ; KANGAS 1952 ; STENLID 1986 ; PIRI et.al. 1990).

Another important white-rot fungus in conifers is the wound pathogen *S. sanguinolentum*. At the beginning of degradation, the coloration of wood caused by *S. sanguinolentum* is reddish-brown, progressing to a white pocket rot, and finally, by coalescence of these pockets, resulting in a white stringy rot (CARTWRIGHT and FINDLAY 1958). It is typical for this fungus, that in the beginning of the decay process, the colour change depends more on the interaction reactions between the fungus and tree than on breakdown of any wood components. Whilst being particularly common in recently cut logs and branches, *S. sanguinolentum* can also cause extensive infection and heart rot in standing trees (HALLAKSELA 1984b ; SOLHEIM and SELÅS 1986).

Brown-rot fungi typically cause an extensive breakdown of wood polysaccharides, i.e. cellulose and hemicellulose. Lignin loss is slight, but there is considerable lignin modification (NILSSON 1985 ; BLANCHETTE 1991). Thus, degraded wood takes on a red-

brown and friable appearance (CARTWRIGHT and FINDLAY 1958). At the cell level, the cellulolytic enzymes or other agents produced by brown rot fungi apparently are able to diffuse throughout the secondary wall. These highly diffusible cellulolytic enzymes degrade cellulose at considerable distances from the hyphae (WILCOX 1968 ; ERIKSSON et al. 1990). ERIKSSON et. al (1990) stated that the cell walls appear intact, but cellulose is depleted, and only the lignified framework remains. Strength properties were reduced significantly after only a slight weight loss. In the early stages of decay, the cells do not appear altered.

The total number of currently recognised brown-rot species, all of which are Basidiomycotina, comprise only about 6 % of known wood-rotting Basidiomycotina. Important examples of brown-rot fungi are the *Coniophora* species causing decay in mature spruces (NOROKORPI 1979).

1.5.4 Yeasts

The unicellular form of the yeasts enables rapid dissemination through aqueous environments and efficient uptake of soluble, easily assimilable nutrients. Hence it might be expected that they are most abundant in the very initial decay processes and at last stages of decomposition, and suppressed when decay fungi are active. There is, however, also the possibility that their lack of penetrative ability could be overcome by the formation of associations with basidiomycetes, and there is evidence that this can take place during early stages of colonisation (BLANCHETTE and SHAW 1978).

Although the Homobasidiomycetes generally do not have yeast phases, there are indications that these may exist occasionally (RAYNER and COATES 1987). Bud-like hyphal branches can develop in a variety of wood-decaying Homobasidiomycetes subjected to high carbon dioxide levels, e.g. *Stereum gausapatum* (RAYNER and BODDY 1988). These bud-like hyphal branches or oidia are typical in malt agar cultures of *Phlebia gigantea* and *Bjerkandera adusta* isolated from spruce for example (HALLAKSELA 1977). A yeast phase is also advantageous when the colonisation is associated with an insect vector. Yeasts and other fungi associated with bark beetles appear to play an important role in enabling these beetles to overcome tree defences (LEUFVEN 1991).

1.5.5 Bacteria

As with fungi, bacteria can exist both as unicellular and mycelial forms. Unicellular forms are most common but there are also examples of actinomycetes with mycelial thallus. The small size and limited penetrative ability of unicellular bacteria make them suited for occupation of aqueous environments (RAYNER and BODDY 1988). GREAVES (1971) placed the bacteria that colonise wood into four categories: (i) bacteria that utilise the cell contents of rays and affect the liquid-permeability of wood but do not alter the strength, (ii) bacteria that attack the cell walls directly, (iii) antagonistic bacteria that are inhibitory to other micro-organisms that colonise wood, and (iv) bacteria that are associated with other micro-organisms in wood and contribute to the decomposition process. Greaves' categories are not narrowly limited; same bacteria types may be able to use many of these strategies in different circumstances.

Bacteria appear to first invade the ray parenchyma cells. They utilise the cell contents and may attack the walls of the parenchyma cells (GREAVES 1969). Bacteria move into adjacent cells quickly via the cross-field pits between ray parenchyma cells and tracheids (LEVY 1975). The lytic action of bacteria has been observed (GREAVES 1969) in pit chambers at the annulus region in *Pinus* cell wall, causing perforations in the outer margins of the bordered pits. Bacteria accumulate in the pit chambers, attaching to the microfibrils of the margo and causing a breakdown of the membrane. In samples of *Pinus monticola* from the field, bacteria had destroyed the margo completely, leaving remnants of the torus (ERIKSSON et al. 1990). Large increases in permeability to liquids, caused by bacteria, have been reported for wood kept in water storage (HIGHLEY and LUTZ 1970). Bacteria may be pioneers in the wood decay process, because they seem to be able to colonise fresh sapwood despite its high water content. They may be deposited in sapwood through the many tiny wounds found on all tree stems or they may be spread by the transpiration stream following root injury (SHORTLE and COWLING 1978a). The pectic enzymes form the "spreading factor" that enables some bacteria to move through the host via the pectin-rich tissue, e.g. in ray parenchyma. The bacterial invasion depends not only on the degradation of pectin but also on the efficiency of bacterial enzymes which inactivate host defence reactions (GOODMAN et al. 1986).

Wood compounds, even lignin, can be degraded directly by bacteria (ZIMMERMANN 1990). Bacteria are able to attack the lignified cell walls of beech or pine sapwood submerged in lakes, although even then, the delignification is slight (SCHMIDT et al.

1987). However, bacteria can cause erosion of the tracheid or fiber walls. DANIEL and NILSSON (1986) have reported mixed populations of bacteria attacking birch and pine wood. In birch, all secondary cell wall layers were degraded, and attack on parts of the middle lamella and primary wall begins once the S_1 layer has been removed. Other patterns of direct cell wall attack are cavitation and tunnelling. These features have been identified in wood colonised by mixed cultures of bacteria (NILSSON and DANIEL 1983) The tunnelling bacteria attach themselves to the S_3 layer and produce a hole in the S_2, forming small cavities with pointed ends. In advanced stages of decay, the tunnels coalesce and the middle lamella and outermost S_3 layer are the only ones that remain. A remarkable characteristic of these bacteria is their ability to attack impregnated wood (DANIEL and NILSSON 1985).

Many bacteria may be antagonistic to fungi and act as biocontrol agents. Bacteria have been reported to lyse zoospores of the root pathogen *Phytophthora cinnamomi* encysted on the ectomycorrhizal mantle. It has been suggested that mycorrhizosphere microbes are the primary agents reducing the activity of *P. cinnamomi* zoospores and thus limit root infection (LINDERMAN and PAULIZ 1990). The bacteria isolated from the soils of boreal mixed forests in Ontario were investigated for their potential to control *Armillaria*, the most important root disease of mature and immature tree species in Ontario. Isolates of *Pseudomonas fluorescens* and *Bacillus* spp. were the most effective inhibitors of mycelial growth of *A. ostoyae in vitro* (DUMAS 1992). Bacterial populations can be dense in the wetwood of living trees.

Bacterial wetwood is formed when intrastem gas pressure is elevated (MURDOCH et al. 1983). The reduced oxygen level and formation of methane in wetwood of elm and other tree species have been shown to be of microbial origin (ZEIKUS and WARD 1974).

The two groups of organisms, bacteria and filamentous fungi, can be considered to undergo minimal competition with each other because of their different size and different ability to penetrate wood cells. Interactions between them may take place under special circumstances where their normally separate habitats overlap. Such a situation occurs in wounded sapwood, which is inhabited by large numbers of bacteria and fungi (SHORTLE and COWLING 1978a ; HALLAKSELA 1984a). The bacteria may play important roles in wood decomposition. Bacteria may themselves be capable of degrading wood components (RAYNER and BODDY 1988) and they increase the permeability of tracheids to fluid movement and to aeration (RAYNER and BODDY

1988), facilitating penetration of decay fungi from sapwood into heartwood. As stated above, bacteria have a limited capacity to degrade lignified wood cell walls. However, degradation of lignocellulosic materials, especially in nature, is not normally carried out by one organism but rather by a mixed population of organisms, in which bacteria participate (ERIKSSON et al. 1990). When bacteria and decay fungi simultaneously invade a wounded spruce, the bacteria spread ahead of the decay fungi in the xylem (HALLAKSELA 1984a).

1.5.6 Viruses

There are several difficulties in research on virus infections in forest trees. In many cases, it has proved difficult to transmit viruses back to the tree species from which they were isolated. There may be several explanations for this. First, woody plants are, in general, difficult to inoculate with viruses. Second, the latent period from the time of inoculation to the appearance of symptoms due to viruses may be long. Additionally, many viruses recovered from trees are likely to be root-restricted, or occur at the highest concentration in the roots. The viruses in woody plants may also occur at relatively low concentrations. And finally, certain viruses require helper proteins or viruses for successful transmission. However, many viral infections have been described in *Pinus*, *Picea* and *Betula* trees, causing diseases such as chlorotic needles, stunting or chlorotic spots in leaves, as well as root diseases (NIENHAUS and CASTELLO 1989).

1.6 Attack strategies of microbes

There are four distinct phases in the occupation and utilisation of woody resources by microbes: arrival, establishment, utilisation and exit (BODDY 1992). Prior to colonisation, microbes arrive at the woody substratum as propagules. These propagules may lie dormant until a suitable substratum becomes available, or may be transmitted into wood when carried actively or passively by animals (SWIFT and BODDY 1984 ; MALLOCH and BLACKWELL 1992). If many propagules arrive on the same substratum, they have to compete with each other for territory and nutrients and even propagules of the same species may compete with each other because of somatic incompatibility mechanisms (RAYNER et al. 1984).

With respect to nutrient utilisation, three distinct classes of behaviour can be identified amongst wood inhabitants: (i) passive occupation, in which the presence of the organism affects neither the resource itself nor other inhabitants; (ii) assimilation of woody substratum either directly or via extracellular enzyme action; (iii) utilisation of substrates derived from other inhabitants (RAYNER and BODDY 1988).

Microbes fill the different types of niches available by adopting different invasion strategies: ruderal, stress-tolerant and combative (COOKE and RAYNER 1984 ; BODDY 1992).

1.6.1 Ruderal strategies

Microbes exhibiting ruderal strategies arrive early at exposed resources and disappear early when more combative competitors arrive. They are thus favoured by disturbances which make new habitat available for colonisation. With wood, such disturbance results from wounding or felling of a standing tree. A common feature of disturbance-initiated colonisation is the abundance of non-decay fungi at early stages of community development. Many of these fungi readily produce large numbers of asexual spores, and are capable of assimilating only non-refractory substrates (RAYNER and BODDY 1988).

Although the bacteria and non-decay fungi may function as pioneers or preconditioning agents, decay fungi can develop in the absence of any other microbe both during natural colonisation and following inoculation (BODDY and RAYNER 1984). For example, pioneer decay fungi such as *S. sanguinolentum* and *Cylindrobasidium evolvens*, whose colonisation does not require pre-conditioning, are also clearly favoured by disturbance of tree cells and predominate at early stages of community development. They have rapid mycelial extension rates, and colonise a wide variety of tree species. They have little decay capacity and decline when the more combative species become dominant (RAYNER and BODDY 1988). A major difference in community structure between two disturbance environments, a stump surface and a standing wounded tree, is that the former consists initially of numerous small decay columns reflecting colonisation by prolific numbers of spores, and in the latter, only a few species are involved (HALLAKSELA 1977, 1984a).

1.6.2 Stress-tolerant strategies

Stress-tolerant microbes are characterised by their ability to colonise under conditions which prevent or limit potential competitors. Some environments impose stresses such as unfavourable moisture level, aeration, temperature and pH, production of toxic compounds, and restrictions on nutrient availability (RAYNER and BODDY 1988). Unfavourable moisture/aeration regimes commonly impose selective conditions and may be particularly critical in a standing tree. Stress-tolerant species may use different strategies during invasion. In heartwood, stress conditions may be imposed by extractives and gaseous conditions. Decay fungi such as *S. sanguinolentum* are ecologically specialised, being able to invade heartwood and possessing strong stress-tolerant characteristics (RAYNER and BODDY 1988).

The access to wood may result from active pathogenesis as in the case of *H. annosum*. In pine roots, this pathogen grows in the outer part of the sapwood and kills the cells in the cambial zone. However, *H. annosum* infection through intact root bark is not possible (LINDBERG and JOHANSSON 1991). Stem decay in pine seldom spreads higher than 1m above ground. The growth of *H. annosum* in roots of Norway spruce and other species susceptible to butt rot is somewhat different from that in pine. In thin roots, all tissues are invaded, but in larger roots, fungal growth is usually restricted to their central parts by the initiation of a reaction of the living sapwood. Once established in the root system of spruce, the fungus grows towards the trunk and infects the valuable lower bole of the tree. Decay can reach considerable heights in spruce; recorded heights up to 11m have been reported from Nordic countries (STENLID 1986).

Another stress-tolerance strategy is the latent invasion of microbes from injuries into xylem cells where they remain dormant as mycelial fragments or spores (CHAPELA and BODDY 1988). Later, associated with the loss of sapwood function, these propagules colonise the xylem tissue by actively growing mycelium leading to an initiation of the active phase of decay (RAYNER and BODDY 1986). The colonisation of larger volumes of wood by decay fungi occurs only when susceptibility of the tree to infection is high (CHAPELA and BODDY 1988).

1.6.3 Combative strategy

Combative strategy is favoured by the lack of both disturbance and stress. Many decay fungi attain dominance at middle to late stages of community development. They can be regarded as combative because of their capacity to defend themselves in modified substratum, or invade those regions occupied by other organisms (RAYNER and BODDY 1988).

In Norway spruce, *S. sanguinolentum* is commonly found both as stress-tolerant pioneer and, in advanced decay, as combative decayer (ISOMÄKI and KALLIO 1974 ; NOROKORPI 1979 ; ROLL-HANSEN and ROLL-HANSEN 1980a). Some other first pioneer decayers such as *C. evolvens* and *Sistotrema brinkmannii* (HALLAKSELA 1984a ; ROLL-HANSEN and ROLL-HANSEN 1980a) can be replaced, e.g. by *Resinicium bicolor* (KATÓ 1967 ; WHITNEY 1978) and *Climatocystis borealis* (SCHÖNHAR 1969 ; HALLAKSELA 1984b) which are more tolerant in modified substratum. Combative fungi, e.g. *Coniophora* sp., are common in old spruces (BJÖRKMAN et al. 1964 ; NOROKORPI 1979).

1.7 Summarised hypothesis of early microbial community development

During the process of invasion of the trunk, microbes have to overcome selective conditions, such as unfavourable aeration, lack of assimilable nutrients, presence of mechanical barriers (walls) and toxic chemicals produced by the host. The high free-water content causes unfavourable aeration, suppressing the growth of decay fungi for example. The free-water content is highest in sapwood and becomes gradually lower in the radial direction through the transition zone inwards to the reaction zone and to the embolised broken cells of the heartwood (SHAIN 1971 ; SHORTLE and OSTROFSKY 1983). The reaction zone acts as a sealant layer limiting the spread of drying. This zone divides xylem into two environments regarding aeration: the water-filled sapwood unfavourable to fungi, and the aspirated tissue which is more favourable for fungal growth (RAYNER 1986). The success of invading microbes is also affected by endophytic fungi (*Ascocoryne*) inhabiting the xylem of the living trees prior to wounding (ETHERIDGE 1970). These endophytes promote or prevent the invasion of other microbes (HUSE and VENN 1993).

Bacteria are usually found in the inner sapwood (transition zone) and sometimes in the reaction zone (SHAIN 1971 ; HALLAKSELA 1984a). This indicates that they are

capable of living and moving in cells with high water content and that they are resistant to inhibitory chemicals (phenols, resins). Bacteria spread ahead of the front of decay fungi, but also overlap with these (HALLAKSELA 1984a). Some bacteria are small enough (e.g. *Pseudomonas*) to move in the water stream of spruce xylem, whereas others (*Bacillus*) are unable to move from cell to cell without degradation of tracheid elements such as bordered pits (GREAVES 1971 ; LIESE and SCHMIDT 1986). The increase in permeability might be the primary role of bacteria in facilitating the withdrawal of liquids from cells and in initiating cell wall degradation (GREAVES 1971). The radial spread of decay fungi might be favoured by bacteria preconditioning ray cells. The bacteria and other early colonisers such as sporulating non-decay fungi (*Phialophora*) modify phenolic substances in the reaction zone (SHORTLE and COWLING 1978b). All these primary degradations modify wood xylem sufficiently for the decay fungi to break down the main part of the cell walls (lignin and cellulose).

The initial area of drying, reaction zone and compartmentalisation of xylem cells may exist only for a limited time because the decay fungi are able to pass through this zone under suitable conditions (RAYNER and BODDY 1988). The reaction zone can be degraded by microbes while external healthy sapwood is converted into a new reaction zone (BODDY and RAYNER 1983 ; PEARCE 1987).

This proposed temporal succession is based on the observations that non-decay fungi and bacteria are consistently isolated from the discoloured margin area closest to intact wood, whereas decay fungi are isolated from regions which are visibly undergoing decay (SHORTLE and OSTROFSKY 1983 ; CHAPELA and BODDY 1988 ; RAYNER and BODDY 1988). The above mentioned sequence is more related to spatial differences than temporal succession of bacteria, non-decay fungi and decay fungi.

2. Purpose of the study

The first investigations in this study concerned the stems of Norway spruce (*Picea abies* (L.) H. Karsten) which were simultaneously inoculated with various different combinations of decay and non-decay fungi and associated bacterial species (I). The growth and survival of the microbes over the subsequent five-year period were then followed in order to characterise their interactive effects on microbial population development (I). Special attention was paid to bacteria which were observed to be present in the xylem of wounded spruce ahead of the advancing front of wood-decay fungi or discoloration (HALLAKSELA 1984a, I, III and IV). The possible biocontrol role of non-decay fungi and bacteria were discussed. The identification of decay- and non-decay fungi was based on morphological characteristics (HALLAKSELA 1977), and a more detailed identification of *Ascocoryne*, *Neobulgaria* and *Nectria* strains was based on combined fatty acid and sterol profiles (II).

In order to gain a better understanding of interactions between fungi and bacteria in early decay process in living trees, it is important to identify bacteria participating in these processes. In this study, methods for characterisation of clinical bacteria were modified for the characterisation of bacteria inhabiting the xylem of living trees (III, IV). These bacteria were analysed for their ability to utilise resin and hydrolyse wood components (IV).

3. Materials and methods

3.1 Trees

In May 1981, twelve 80-year-old Norway spruce trees of healthy appearance and with diameters of about 36 cm were chosen within an area of 0.5 ha in a spruce stand in Ruotsinkylä, Finland (60°22'N, 25°0'E). These twelve trees were inoculated with mixtures of fungi and bacteria and later sampled after two, three and five years. In May 1986, four additional trees were inoculated and sampled a year later. Four other reference trees were not inoculated but sampled at the end of the sampling period (I).

3.2 Microbes and preparation of inocula

The following fungi were used: the decay fungi *H. annosum* and *S. sanguinolentum*, the non-decay fungi *Nectria fuckeliana* Booth., *Ascocoryne cylichnium* (Tul.) Korf. and an unidentified white yeast-like fungus. The inoculated bacteria were *Bacillus pumilus* and *Enterobacter agglomerans* (I). All these strains were originally isolated from wounded Norway spruce (HALLAKSELA 1984a). A mixed inoculum consisting of a homogenised suspension of one strain of the decay fungus (either *S. sanguinolentum* or *H. annosum*) and of one, two or four other microbial cultures was prepared (Table 1/I).

3.3 Inoculation of spruce stems

For inoculation, 16 holes equally distributed around the circumference of the trunk were made with an increment borer into the stem at three levels: 1, 3 and 5 meters above the ground; consequently 3 x 16 holes were made in each experimental tree. A core from each hole was taken for isolation of the indigenous microbes occurring in the wood prior to inoculation. A 3 ml aliquot of each microbial mixture (Table 1/I) was injected into each hole as aseptically as possible, and the holes were sealed with grafting wax (I).

3.4 Sampling and analysis of microbes

Individual trees were felled one, two, three and five growing seasons after inoculation. Discs 5 cm thick were cut immediately above the inoculation level and at 20 cm intervals to a height of 100 cm. From each disc, slivers of sapwood, outer heartwood, and inner heartwood were taken aseptically from positions in the same vertical plane as the inoculation bore holes (I). The wood slivers were incubated in petri dishes on 1.25 % malt agar and the resulting fungal cultures identified by morphological characteristics as described by HALLAKSELA (1977) or by whole-cell fatty acid analysis modified for this purpose (II). The wood slivers for bacterial isolations were homogenised in 20 ml of sterile water and plated on four different

media (III). The bacteria were identified on the basis of cell morphology, physiological and biochemical properties (III, IV), phage typing (III) and whole-cell fatty acid analysis (III, IV). The ability of isolated bacteria to utilise wood components was investigated on modified media with cellulose, pectin or pine resin fractions (IV).

3.5 Statistical methods

The effect of inoculation level in the stem (1, 3 and 5 m) on the invasion of the decay fungus was statistically analysed by Analysis of Variance and Covariance with Repeated Measures (BMDP 2V), the time related effect on colonisation (1, 2, 3, and 5 years) and the effect of fast- and slow-growing strains of decay fungus were analysed by MGLH Analysis of Variance one-way (SYSTAT), the effect of different microbe combinations was analysed by Stepwise Logistic Regression (BMDP LR) (I).

4. Results and discussion

4.1 The microbial colonisation of spruce stems before and after inoculation

4.1.1 The non-decay fungi

The heartwood of living healthy spruce trees is often inhabited by endophytic microbes (ROLL-HANSEN and ROLL-HANSEN 1979b ; HUSE 1981). Most spruce stems investigated in this study were already colonised by *A. cylichnium* and *Neobulgaria premnophila* before inoculation (Fig. 1/I). The prevalence of the endophytes in the trees was less extensive two years after the inoculation but more extensive again in the trees investigated after three and five years (Fig. 2/I). The investigated part of the stem of most trees was colonised by one or other of these fungi, regardless of the microbial composition of the inoculum site and the height of the inoculum in the stem. Introduction of *A. cylichnium* did not affect the frequency of endophytic

N. premnophila at any sampling time. This situation was maintained over the five-year study period (Fig. 1 and Fig. 2/I).

A. cylichnium and *N. premnophila* are examples of endophytic fungi which inhabit the heartwood of sound living trees but do not cause decay (ETHERIDGE 1970 ; ROLL-HANSEN and ROLL-HANSEN 1979a ; HUSE 1981 ; HALLAKSELA 1984a). *Ascocoryne* is able to grow at a lower oxygen concentration than the decay fungi (METZLER et al. 1993). It was observed to invade the tree through wounds in the roots (ETHERIDGE 1970), and *N. premnophila* also seems to colonise in the same way. Both fungi benefited greatly from the tree being exposed to stress by introducing 48 bore holes/ tree (I). BODDY (1992) also stated that spreading of fungi already present in the wood of the living tree increased after wounding or other kinds of disturbance.

The preliminary identification of *A. cylichnium*, *N. premnophila* and *N. fuckeliana*, based on hyphal and conidial morphology (HALLAKSELA 1977), was confirmed by discriminant analysis of their combined fatty acid and sterol profiles (II). *A. cylichnium* (I, P, S in Fig.1B/II), *N. premnophila* (W in Fig.1B/II) and *N. fuckeliana* (E in Fig.1B/II) were recognisable as clearly distinct species, although reisolated strains displayed heterogeneity. The fatty acid profiles were specific and different for *A. cylichnium* and *N. premnophila*, although these species were morphologically similar. The *A. cylichnium* strains (Fig.2/II) reisolated from inoculated experimental trees were different as compared to strains isolated from non-inoculated reference trees. Three trees (R, S, U in Fig. 2/II) out of the four reference trees each contained an endemic chemotype of *A. cylichnium* strains. The *A. cylichnium* spectrum of the experimental trees (Fig.2/II) was heterogeneous regardless of whether this fungus had been detected in the tree prior to inoculation or not. Thus the fungus may have been present, even if not found, prior to inoculation, or the wounding may have favoured colonisation of the fungus, for example via the roots.

N. fuckeliana was repeatedly found during the five-year post infection period but only at the lowest (1m) wound area. It had no effect on the growth of the decay fungi (I). ROLL-HANSEN and ROLL-HANSEN (1979a, 1980b) also found that *N. fuckeliana* was wound-oriented, but they observed a greater vertical spread of the fungus than in this study. DELATOUR (1990) reported that *N. fuckeliana* reduced the growth of *H. annosum* in Norway spruce. The reisolated *N. fuckeliana* (Fig. 3/II) strains differed from the inoculated strain despite the fact that no indigenous infection by *N. fuckeliana* was found prior to inoculation. The diversity among the strains of the *A. cylichnium*, *N. premnophila* and *N. fuckeliana* found in this study can possibly be

explained by the much larger numbers of wounds in this study and possible variations in the defence ability of spruce trees against these fungi, allowing several strains to invade some trees.

Although only a small portion of vascular plants have been examined for the presence of endophytes, almost every host examined has proved to harbour such fungi (CAROLL 1992). Some examples of antagonistic behaviour of endophytes against pathogens have been presented. The take-all disease of wheat caused by the root pathogen *Gaeumannomyces graminis* var. *tritici* in host roots can be suppressed by a "sterile red fungus", an endophytic basidiomycetous fungus (DEWAN and SIVASITHAMPARAM 1988). In the green needles of Norway spruce, the fungi *Lophodermium piceae* and *Tiarosporella parca* obviously can be considered as endophytes, because they can live for years without causing symptoms. *L. piceae* completes its development when the needle starts dying due to abiotic or biotic factors (STEPHAN and OSORIO 1993). Such kinds of needle fungi, the endophyte *Lophodermium conigenum* for example, may prevent the virulent pathogen *Lophodermium seditiosum* invading needles of *Pinus sylvestris* (MINTER 1981).

4.1.2 The inoculated decay fungi

The rapid invasion by *S. sanguinolentum* and *H. annosum* during the first two years after inoculation was followed by a regressive later phase (Fig. 3/I), possibly reflecting a successful defence reaction of the tree towards the invading decay fungi introduced via relatively small wounds that were immediately sealed. In a similar study, PAWSEY and STANKOVICOVÁ (1974) reported that the growth of *S. sanguinolentum* introduced via small wounds was suppressed in inoculated sealed *Picea abies* stems. GRAMSS (1992) has shown experimentally that a vital living tree can actively kill even pathogenic decay fungi.

The spread of *H. annosum* or *S. sanguinolentum* showed large individual differences between the experimental trees, preventing observations of any significant time dependent changes (Fig. 3/I). Only one microbe combination suppressed the invasion of *S. sanguinolentum*. It was a mixture of *N. fuckeliana*, *A. cylichnium*, the yeast-like fungus and bacteria (Fig.4/I). The spread of *H. annosum* was not affected

by any of the experimental inoculum mixtures. In all cases, *H. annosum* and *S. sanguinolentum* isolated from directly above the inoculation point belonged to the same somatic compatibility group as the inoculated strain, indicating that the inoculated strain was not replaced by wild strains.

One year after inoculation, a typical brown coloration was detected in the boundary zone between sap- and heartwood and, later, in the outer heartwood of trees inoculated with *S. sanguinolentum*. No colour change was detected in the trees inoculated with *H. annosum* after one year, but typical violet reaction zones were visible in trees investigated two, three and five years after inoculation. These colour changes suggest that *S. sanguinolentum* and *H. annosum* had been present in the wood but were not later detectable (Fig.3/I). *S. sanguinolentum* has been observed to be the predominant stem decay pathogen of *Abies balsamea* (SHORTLE and OSTROFSKY 1983). It appears that dry heartwood is relatively resistant against *S. sanguinolentum* due to a combination of increased phenol content and decreased water content. If heartwood becomes wet, protection weakens, followed later by loss of water-soluble phenols as the wood dries and the decay accelerates. Decay fungi can cause both an initial wetting and a subsequent drying during pathogenesis. Once a decay fungus has established itself in a living tree, its development may not necessarily follow continuous expansion but may also include stages of regression (SHORTLE and OSTROFSKY 1983).

The inoculated microbes were observed to grow in a narrow vertical zone from the inoculum point (HALLAKSELA 1984a) without much horizontal spread and more spread upwards than downwards. There is a dynamic interplay between the decay fungus, the conducting system of the tree and air access initially caused by wounding. This interplay may continue as long as the tissues do not become permanently sealed off. The formation of dry zones is at least partly due to withdrawal of water from damaged tracheids by hydrostatic tension. Similarly, mycelial growth is more rapid upwards than downwards because of upwards water flow (RAYNER and BODDY 1988).

4.1.3 The role of bacteria in microbe interactions

After inoculation, several bacterial species other than those inoculated (*B. pumilus* and *E. agglomerans*) were found in the trees. The bacterial species most frequently

isolated after inoculation were Gram-positive *Bacillus* species, primarily *B. pumilus*. Other frequent species were *B. subtilis* and *B. cereus*. The predominant Gram-negative bacteria were *E. agglomerans*, *E. sakazakii*, and fluorescent and yellow pigmented strains of *Pseudomonas*. Whole-cell fatty acid analysis was used in this study as a tool to group the bacteria isolated from the living Norway spruce. It was found that the currently available database enabled a good identification of *Bacillus* species from spruce (Table 2 and Fig. 2/III) whereas much less success was obtained with the Gram-negative bacteria (Table 3 and Fig. 1/IV).

All the inoculated trees in this study were colonised by bacteria. The inoculated bacteria (*B. pumilus* and *E. agglomerans*) were well represented in each tree (Fig. 5/I, Tables 1 and 5/III, Table 2/IV). The inoculated species of bacteria were found in the proximal part of the moist sapwood and ahead of the advancing front of the decay fungi or discoloration. No other microbes were isolated from those areas (HALLAKSELA unpubl.). The bacterial invasion was extensive vertically and laterally from the inoculated wounds. These inoculated bacteria were not found either in the reference trees or in the experimental trees prior to inoculation (Fig. 1/I). In general, the amount of bacteria in the stems increased greatly after inoculation (compare Fig.1/I and Fig.5/I). The bacterial species composition of the trees was similar, regardless of the inoculation mixture or the species of decay fungus (either *H. annosum* or *S. sanguinolentum*). *B. pumilus* was most often accompanied by *B. subtilis*, *B. cereus* and occasionally *B. circulans* although the latter three species were not included in the inoculation mixtures (Fig. 5/I ; Table 5/III). The entry of bacteria seemed to be through the wounds resulting from the bore holes and, subsequently, both inoculated and some of the contaminant bacteria, such as *E. sakazakii*, *Pseudomonas* species and *Acinetobacter*, readily invaded the inner sapwood and heartwood.

These bacteria have been found in some other woody environments. *Bacillus* were reported in living red maple trees altered by decay (PARKER and SHORTLE 1992 unpubl.) and *Pseudomonas* species in alder logs inoculated with shiitake (RAASKA and MATTILA-SANDHOLM 1991), in the root tissue of Norway spruce (TIEDEMANN and HÜTTERMANN 1989) and in soil of boreal mixed forest in Ontario (DUMAS 1992).

The results obtained indicate greater defence reactions of the host tree towards the inoculated decay fungi than towards bacteria. Bacteria may aid the decay fungi in the initial steps of infection of the wounded tree. For example, bacteria are more successful in surviving in moist sapwood than the decay fungi (GREAVES 1971), as

has also been shown in this study. Hydration increases the accessibility of wood cell components to the hydrolytic enzymes secreted by the bacteria (GOODMAN et al. 1986). Bacteria isolated from spruces were able to secrete enzymes capable of degrading cell wall components such as pectin (*B. pumilus*), xylan (*B. subtilis, B. pumilus, B. circulans*) and cellulose (*B. subtilis* and *B. circulans*) (Table 2/III , Table 4/IV). Bacteria may contribute to the initial degradation of crystalline cellulose, which is then further broken down by the multicomponent enzyme complex produced by fungi (ERIKSSON et al. 1990). In this study it was found that fluorescent *Pseudomonas* and *Acinetobacter* spp., isolated from living Norway spruce, were able to grow on antifungal wood resins (Table 4/IV), which may assist sensitive fungi to overcome the natural defence barriers of the tree.

Direct interaction between bacteria and decay fungi can result in either a stimulation or an inhibition of growth of the fungal partner (BLANCHETTE and SHAW 1978 ; SHORTLE et al. 1978). Bacteria may associate with mycorrhizal fungi and function as mycorrhization helper bacteria. GARBAY and DUPONNOIS (1992) showed that the *Bacillus* and *Pseudomonas* strains isolated from sporocarps or the ectomycorrhizal mantel of *Laccaria laccata* stimulated the ectomycorrhizal symbiosis between Douglas fir and *L. laccata*. They stated that mycorrhization helper bacteria mainly acted by controlling the extension of the fungus into the rhizospheric soil and on the rhizoplane before mycorrhiza formation.

4.1.4 Early microbial community development

In the present study (I), the endophytic fungi *A. cylichnium* or *N. premnophila*, which were present before inoculation in heartwood of the experimental spruces, regressed in two years when the invasion of *H. annosum* and *S. sanguinolentum* was greatest. In contrast, HUSE and VENN (1993) in Norway described the inhibition of *H. annosum* by endophytic *Ascocoryne* spp. within after one year of inoculation in spruce. In this study, the endophytes were again common in all investigated trees after three and five years when decay fungi were suppressed. In contrast, GRAMSS (1992) found endophytic fungi only occasionally in vital living spruces and in no more than 13 % of stressed trees.

The presence of *A. cylichnium* or *N. premnophila* prior to inoculation does not seem to have affected the invasion of the inoculated decay fungi over five years (Figs. 1/I and Fig. 3/I). *H. annosum* and *S. sanguinolentum*, were common both in trees

colonised with *A. cylichnium* and/or *N. premnophila* (e.g. trees 14, 15 and 11/I) as well as in trees almost devoid of these endophytes (e.g. tree 1 level 1m and tree 13 level 5m /I). DELATOUR (1990) reported that the inoculated non-decay fungi *Phialophora* sp. or *Scytalidium* sp. were not able to restrain the spread of *H. annosum* into Norway spruce.

One growing season post infection, *H. annosum* and *S. sanguinolentum* were found at the boundary between sap- and heartwood. They were in close contact with bacteria but not with endophytes, which were situated in heartwood. At that time the decay fungus was favoured by aeration caused by wounding and natural embolism. Two years post infection, the decay fungi and the endophytes met each other in deeper heartwood. In order to get there decay fungi have to resist heartwood extractives and the moisture content must be modified favourably. At the end of the experimental period, the decay fungi were suppressed by endophytes, bacteria, or the inhibition of the host. The bacterial flora and endophytic *A. cylichnium* and *N. premnophila* were maintained over the whole experimental period of five years. The picture of the tree microbial diversity is incomplete, because a part of the microbes may have been lost because of the artificial growth media used in the laboratory for the isolation of microbes.

Conclusions

The spatial locations and movements of the microbes were clarified by monitoring the growth and survival of the microbes over the five-year period subsequent to the inoculation.

In this study, a large number of inoculation bore holes, a total of 48 in each tree, contributed to the stress of the tree and the strong bacterial and endophytical infection at the end of the experimental period. This raises the question how long microbes could maintain the trees without decay or whether the trees could become subject to *S. sanguinolentum* decay in the future. The analysis of a tree in this study was a "destructive" one, in the sense that a piece of tree can only be analysed once. This implies that, in order to monitor the growth of the microbes as a function of time, different stems had to be used.

In conclusion, microbes were found to be located in different spatial parts of the tree xylem favoured by the physiological conditions. The regions occupied by bacteria were the inner sapwood nearest the moist living cells. The decay fungi were situated just behind the bacteria at the boundary of the sapwood and heartwood at the beginning of the decay process, and the endophytic fungi had invaded deep into the heartwood. One idea in this study was to test the possibility of using bacteria and non-decay fungi as biocontrol agents against decay fungi in standing trees. However, the results show that it was impossible to influence the later distribution and speciation of inoculated microbes in the living tree. In the case of a small wound, which was sealed, the anatomical, physiological and chemical defence of the spruce tree powerfully counteracted the establishment of the decay fungi without major harm to the tree. However, the wounded trees supported a large population of bacteria and endophytic fungi 3 and 5 years post infection, when the decay fungi were suppressed or had disappeared. This raises the question whether the influence of the inoculated microbes on the decay fungi was of a long term nature .

The persistence of *B. pumilus* and *E. agglomerans* and their comigrants at the distal end of the discoloration, followed by the endophytic fungi *A. cylichnium* and *N. premnophila* may be indicative of mutualism. Among the bacteria were cellulolytic, xylanolytic and pectinolytic strains and utilisers of wood resin, explaining their ability to colonise wood. Bacteria like these may constitute a synergistic element in the consortium with endophytic fungi.

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Early interactions of Heterobasidion annosum and Stereum sanguinolentum with non-decay fungi and bacteria following inoculation into stems of Picea abies

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Abstract

Different combinations of two decay fungi (*Heterobasidion annosum, Stereum sanguinolentum*), two non-decay fungi (*Nectria fuckeliana, Ascocoryne cylichnium*), a yeast-like fungus and two bacteria (*Bacillus pumilus, Enterobacter agglomerans*) all of spruce origin, were inoculated into stems of 16 healthy-looking *Picea abies*. Growth and survival of the microbes were followed over the subsequent five years. The rapid upward vertical spread of the decay fungi during the first two years was followed by a later regression and loss of these decay fungi by the fifth year.

The stems were effectively colonized by the bacteria, *A. cylichnium* and/or *Neobulgaria premnophila* which then persisted over the whole experimental period. The endophytic fungi, *N. premnophila* and *A. cylichnium*, were abundant in the stems of the experimental trees before inoculation.

Key words: *Ascocoryne* – *Nectria* – Bacteria – Competition – Succession – Colonization – Antagonism.

1 Introduction

During the wood decomposition process, which proceeds from the primary microbial attack of a healthy living tree to the final degradation of the cellulytic and lignolytic components, the species composition of the microbial community involved undergoes dynamic changes. Microbial succession has been extensively studied in dead wood substrates such as stumps and logs (Kääärik 1971; Rayner 1977a, b; Gramss 1987; Sexton et al. 1992), but limited information is available on similar succession patterns in the living wood of trees. The sapwood of wounded living sweetgum (*Liquidambar styraciflua* L.) and yellow-poplar (*Liriodendron tulipfera* L.) were found to be primarily colonized by bacteria and non-decay fungi such as *Phialophora, Fusarium* and *Cephalosporium* (Shortle and Cowling 1978a). *Phialophora* differs from the other pioneers in its ability to alter phenolic compounds, thereby permitting the subsequent colonization and growth of decay fungi (Shortle et al. 1971; Shortle and Cowling 1978b). Examples include *Stereum sanguinolentum, Heterobasidion annosum* and *Cylindrobasidium evolvens* which have been reported to be the pioneer decay species in Norway spruce (Roll-Hansen and Roll-Hansen 1980a). The microbial community at this very early stage of the decay process in spruce is mainly composed of these decay fungi, the non-decay fungi *Nectria fuckeliana* or *Ceratocystis* and species of bacteria.

Pioneer decay fungi such as *S. sanguinolentum* and *C. evolvens* and small spored non-decay fungi, which enter the tree through above ground stem wounds, may initially latently invade xylem cells of deciduous trees and then remain dormant as mycelial fragments or spores. Later, associated with the loss of sapwood function, these propagules are triggered and colonization of the xylem tissue by actively growing mycelium leads to an

initiation of the active phase of decay (RAYNER and BODDY 1986). Because the small pore size of tracheids in conifers (HINTIKKA 1973; BAINES et al. 1983) naturally prevents the latent invasion of fungi, thus enzyme production and active hyphal invasion by fungi is a prerequisite during early stages of microbial community development. *S. sanguinolentum*, known to possess a high mycelial extension rate in wounded healthy xylem, is regarded as a pioneer colonizer in a wide variety of coniferous tree species (HALLAKSELA 1984b; RAYNER and BODDY 1988).

Earlier studies into the fate of microbes in decaying wood of living trees have dealt with trees naturally infected or trees experimentally inoculated with a single fungal strain (SHORTLE and COWLING 1978a; ROLL-HANSEN and ROLL-HANSEN 1980a; RAYNER and BODDY 1988). The effect of simultaneously inoculated combinations of microbes have not been addressed.

This study was initiated from the earlier observation on the presence of bacteria (*Bacillus pumilus, Enterobacter agglomerans*) in the xylem of wounded Norway spruce ahead of the advancing front of wood decay fungi (HALLAKSELA 1984a; HALLAKSELA et al. 1991). In the present investigation the stems of living spruce trees were simultaneously inoculated with various different combinations of decay and non-decay fungi and associated bacterial species. The growth and survival of the microbes over the subsequent five year period were then followed in order to characterize their interactive effects on microbial population development. In two earlier studies the bacterial populations in these inoculated trees and their ability to hydrolyse wood components were reported (HALLAKSELA et al. 1991; HALLAKSELA and SALKINOJA-SALONEN 1992).

2 Materials and methods

2.1 Trees

In May 1981, 16 healthy looking 80-year-old Norway spruce [*Picea abies* (L.) H. Karsten] trees [mean diameter at breast height (1.3 m) was 36 cm ± 6 cm] were chosen within a 0.5 ha area of spruce stand in Ruotsinkylä, Finland (60°22′ N, 25°0′ E). Of these experimental trees, twelve were inoculated with mixtures of fungi and bacteria and later sampled after two, three and five years. The four remaining reference trees that were not inoculated were sampled at the same time as the inoculated trees. These reference trees were taken only once at the end of the sampling period. In May 1986, four additional trees were inoculated and sampled a year later.

2.2 Microbes and inocula preparation

The following fungi and bacteria were studied: the fungi included a slow and fast growing strain of each of the decay fungi *Heterobasidion annosum* (Fr.) Bref., Ha70 and Ha61, and *Stereum sanguinolentum* (Alb. & Schw. ex. Fr.) Fr., Ss96 and SsL50. The growth rates had been measured in living heartwood of Norway spruce. The non-decay fungi studied were *Nectria fuckeliana* Booth, Nf96 (Nf), *Ascocoryne cylichnium* (Tul.) Korf, Ac70 (Ac), a white yeast-like fungus, ye70 (ye). The bacteria (b) studied were *Bacillus pumilus*, K74A and *Enterobacter agglomerans*, K74B. All these fungal and bacterial strains were originally isolated from wounded Norway spruce (HALLAKSELA 1984a).

The fungal strains were each inoculated into 1 % malt extract medium in 100 ml Erlenmeyer flasks and incubated unshaken for 2-weeks at room temperature, harvested by filtration through nylon cloth and washed in sterile water. The recovered mycelium of each fungus was then combined and homogenized (Ultra-Turrax) in 25 ml sterile water. The bacteria K74A and K74B were dual inoculated into 10 ml Bacto nutrient broth (Difco) and incubated without agitation at room temperature for 24 h. One ml of this culture was

added to the homogenized fungal suspension and the total was diluted to 30 ml with sterile water. Mixed inocula comprising of one slow or fast-growing strain of the decay fungus (either *S. sanguinolentum* or *H. annosum*) in combination with one, two or four other microbial cultures were prepared as in Table 1.

In order to quantify the microbial biomasses in the inocula, separate individual cultures were similarly prepared and their microbial masses measured using a TOC-5000 Shimadzu total organic carbon analyzer. Biomass expressed as dissolved organic carbon, were as follows: Ha70: 0.08 mg; Ha61: 0.09 mg; Ss96: 0.06 mg; SsL50: 0.02 mg; Nf 0.11 mg; Ac 0.03 mg; ye 0.06 mg. The number of viable bacteria in the inocula varied between 10^6–10^7/ml.

Table 1. **Different microbial mixtures inoculated into bore holes of Picea abies stems.**
Microbes: Ha = *Heterobasidion annosum*, Ss = *Stereum sanguinolentum*, Nf = *Nectria fuckeliana*, Ac = *Ascocoryne cylichnium*, ye = a yeast-like fungus, b = bacteria (*Bacillus pumilus* and *Enterobacter agglomerans*)

Trees no.	Bore hole	Microbes in inocula mixture		Trees no.	Bore hole	Microbes in inocula mixture	
		Decay fungi slow	Other microbial strains			Decay fungi fast	Other microbial strains
	1	Ha70			1	Ha61	
1, 2, 3	2		Nf	4, 5, 6	2		Nf
and 13	3		Ac	and 14	3		Ac
	4		ye		4		ye
	5		b		5		b
	6	Ha70	Nf		6	Ha61	Nf
	7	Ha70	Ac		7	Ha61	Ac
	8	Ha70	ye		8	Ha61	ye
	9	Ha70	b		9	Ha61	b
	10	Ha70	Nf, Ac		10	Ha61	Nf, Ac
	11	Ha70	Ac, ye		11	Ha61	Ac, ye
	12	Ha70	Ac, b		12	Ha61	Ac, b
	13	Ha70	Nf, b		13	Ha61	Nf, b
	14	Ha70	ye, b		14	Ha61	ye, b
	15	Ha70	Nf, Ac, ye, b		15	Ha61	Nf, Ac, ye, b
	16		sterile water		16		sterile water
	1	Ss96			1	SsL50	
7, 8, 9	2		Nf	10, 11,	2		Nf
and 15	3		Ac	12	3		Ac
	4		ye	and 16	4		ye
	5		b		5		b
	6	Ss96	Nf		6	SsL50	Nf
	7	Ss96	Ac		7	SsL50	Ac
	8	Ss96	ye		8	SsL50	ye
	9	Ss96	b		9	SsL50	b
	10	Ss96	Nf, Ac		10	SsL50	Nf, Ac
	11	Ss96	Ac, ye		11	SsL50	Ac, ye
	12	Ss96	Ac, b		12	SsL50	Ac, b
	13	Ss96	Nf, b		13	SsL50	Nf, b
	14	Ss96	ye, b		14	SsL50	ye, b
	15	Ss96	Nf, Ac, ye, b		15	SsL50	Nf, Ac, ye, b
	16		sterile water		16		sterile water

2.3 Inoculation of spruce stems

For inoculation (Table 1) 16 equidistant holes (6 cm deep and 0.5 cm wide) were radially bored into each tree at heights of 1, 3 and 5 meters above ground-level (48 holes/tree). An increment core from each hole was taken for isolation of the indigenous microbes present

in the wood prior to inoculation. A 3 ml aliquot of each microbial mixture was aseptically injected into each hole (c. f. Table 1) and the holes were sealed with grafting wax.

2.4 Sampling and analysis of microbes

Individual trees were felled one, two, three and five growing seasons after inoculation. The felling was carried out in the winter when the air temperature was below 0 °C. Discs, 5 cm thick, were cut immediately above the inoculation plane and at 20 cm intervals to a height of 100 cm as measured from the inoculation plane. The position of each disc in relation to the position of inoculation holes was carefully marked. The discs from uninoculated reference trees were cut at heights 1, 3 and 5 m above ground-level. The discs were sealed in plastic bags, and stored at −20 °C (max. 6 months) prior to the isolation of microbes.

From each disc, slivers of sapwood, and outer and inner heartwood were cut from positions in the same vertical plane as the inoculation bore holes (3 × 16 sampling points per disc) with a flamed knife. In the case of reference trees only 12 slivers per disc were taken. The wood slivers were incubated in petri dishes on 1.25 % malt agar and resulting fungal cultures isolated (NOBLES 1948). The identification of fungi and the isolation and identification of bacteria were carried out as described earlier (HALLAKSELA 1977; HALLAKSELA et al. 1991, 1992; MÜLLER and HALLAKSELA 1994).

Further conformation of the *H. annosum* and *S. sanguinolentum* genotype isolated from the stems was made by detection of somatic incompatibility reactions between the inoculated and isolated strains (RAYNER and TURTON 1982; STENLID 1985).

2.5 Statistical methods

The effect of inoculation level in the stem (1, 3 and 5 m) on the invasion of the decay fungus was statistically analyzed using Analysis of Variance and Covariance with Repeated Measures (BMDP 2V), the time related effect on colonization (1, 2, 3 and 5 years) and the effect of fast- and slow-growing strains of decay fungus were analysed by MGLH Analysis of Variance one-way (SYSTAT), the effect of different microbe combinations by Stepwise Logistic Regression (BMDP LR).

3 Results

3.1 The indigenous microbes

The indigenous microbial flora in the wood of experimental and healthy (no colour change) reference trees prior to inoculation are presented in Figure 1. *A. cylichnium* and *Neobulgaria premnophila* Roll-Hansen & Roll-Hansen were commonly co-isolated from the experimental trees; one or both were present in all trees except two in which infection was restricted to bacteria and yeast (no. 2) or no microorganisms at all (no. 12). *N. premnophila* was not found in reference trees (Fig. 1). Only those indigenous bacteria isolated from the reference trees were identified. A mixed community of yellow and fluorescent *Pseudomonas* was detected only in tree no. 19 (Fig. 1).

3.2 Distribution of non-decay fungi after inoculation

The presence of *A. cylichnium* and *N. premnophila* in the stems of trees at the different sampling times after inoculation with mixed inocula is shown in Figure 2. The frequent occurrence of *A. cylichnium* in trees prior to inoculation caused difficulties in determining the spreading rate of this fungus. In two trees where *A. cylichnium* was initially absent inoculation with this fungus resulted in heavy invasion by *A. cylichnium* or *N. premnophila*

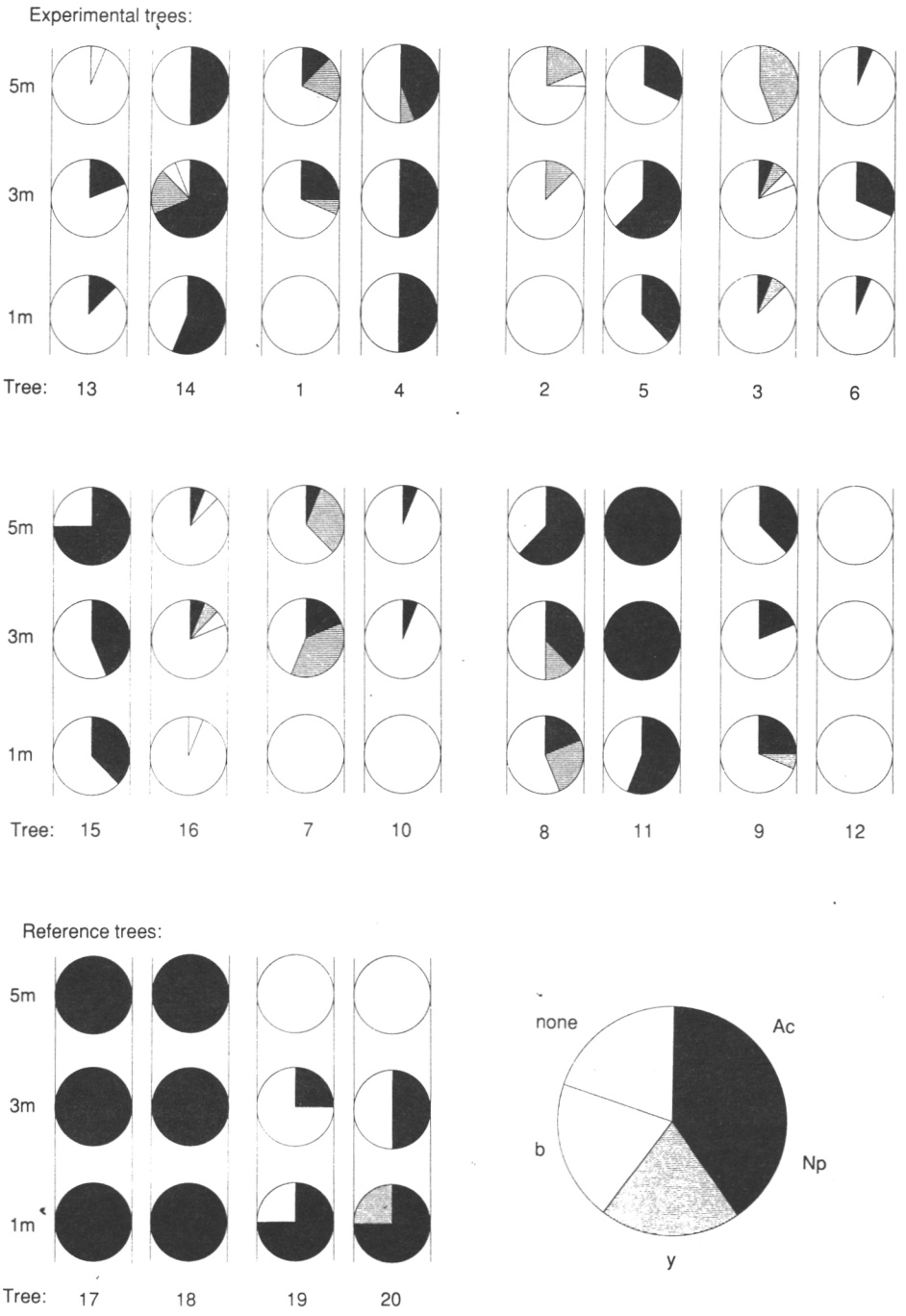

Fig. 1. The indigenous microbes isolated from each stem sampling level (1, 3, 5 m) prior to inoculation. From experimental and reference trees a total of 16 and 4 increment cores were taken at each level, respectively. Microbes: Ac = *Ascocoryne cylichnium*, Np = *Neobulgaria premnophila*, y = yeast-like fungus, b = bacteria, none = no microbes. The numbering of trees is analogous to that in Figures 3 and 4

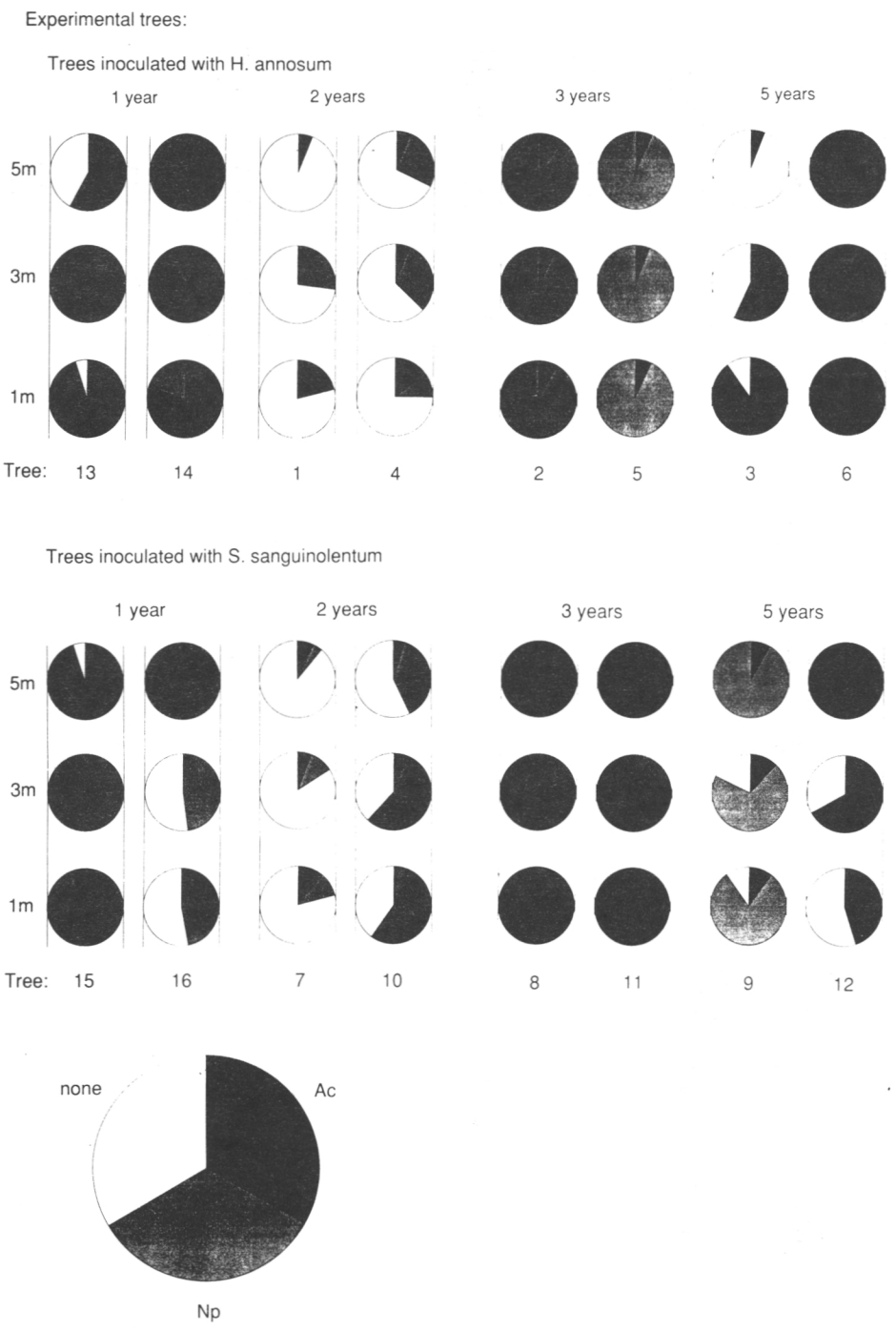

Fig. 2. The frequency of *Ascocoryne cylichnium* and *Neobulgaria premnophila* colonization in the presence of *Heterobasidion annosum* or *Stereum sanguinolentum* in the different sampling positions directly above the respective inoculation points at stem heights of 1, 3 and 5 m one, two, three and five growing seasons after inoculation

in trees 12 and 2, respectively, a year after inoculation. The upward vertical spread of the fungi to a height of at least 1 m in the outer and inner heartwood of the whole stem volume of each respective tree occurred regardless of microbial composition of the inoculum and the height at which the inoculum was introduced into the stem. In trees investigated after two years the spread of these endophytes had regressed but had again increased in trees investigated after three and five years (Fig. 2). Introduction of *A. cylichnium* did not lower the frequency of *N. premnophila* at any of the sampling times studied (Fig. 2). However, *N. premnophila* appeared in the stem of two trees (2 and 14), which were investigated one year after inoculation. In almost all cases the fungus predominating in the stem before inoculation was also the dominant fungus in the fifth sampling season (Figs. 1 and 2).

Of the other inoculated non-decay fungi, *N. fuckeliana* was found after one, two, three and five years, but only in the immediate vicinity of the inoculation points persisting in the sapwood or outer heartwood. The inoculated yeast-like fungus disappeared from all the experimental trees, although some other yeasts were isolated which were morphologically very heterogeneous and differed from the inoculant yeast-like fungus. These fungi are not included in the figures presented.

3.3 Distribution of the inoculated decay fungi

Figure 3 shows how the decay fungi *H. annosum* and *S. sanguinolentum* were distributed in the experimental trees investigated one, two, three and five growing seasons after inoculation.

Although the spread of *H. annosum* introduced into the stems varied greatly between the individual trees, the fungus colonized the stems irrespectively of whether inoculated singly or in combination with other microbes. There were no significant differences between the spread of the slow-growing strain (Ha70) and the fast-growing strain (Ha61). In all cases *H. annosum* isolated from the sampling points directly above the inoculation point belonged to the same somatic compatibility group as the inoculated strain.

In trees investigated one year after the inoculation the mean spread of *H. annosum* was 40 cm and the maximal spread was 1 m (in tree 13 at inoculation levels 3 and 5 m). In trees investigated after two growing seasons the spread of *H. annosum* had not increased and in all cases this fungus was isolated from the boundary between the sap- and heartwood. Subsequently, the infection seemed to regress, since no *H. annosum* was identified in the trees investigated after three and five growing seasons (Fig. 3). No colour change was detected in the trees examined one year after inoculation with *H. annosum* but typical violet reaction zones were visible in trees analysed two, three and five years after inoculation, suggesting that *H. annosum* had earlier been present in the wood but had later disappeared from the trees.

The stem inoculation level did not greatly influence the spread of *H. annosum* although spread in two trees (14 and 4) was significantly decreased at the highest inoculation level ($P = 0.0009$) whereas it was increased in the third tree (13) (Fig. 3). The differences between the responses of individual trees were thus larger ($P = 0.0053$), masking the differences between inoculation levels. The sampling points directly above holes not inoculated with *H. annosum* only rarely became infected. The infection was apparently a result of cross-infection from neighbouring sampling points inoculated with this fungus.

The other decay fungus *S. sanguinolentum* was also able to effectively invade the inoculated trees (Fig. 3). In all cases the isolated fungus belonged to the same somatic compatibility group as the inoculated strains indicating that the inoculated strain had not been replaced by other wild strains. The mean upward growth rate progression of *S. sanguinolentum* increased in the trees investigated during the two first years, but in trees investigated later the level of spread had reduced to the point where after 5 growing seasons only traces of infection remained (Fig. 3). One year after inoculation typical brown

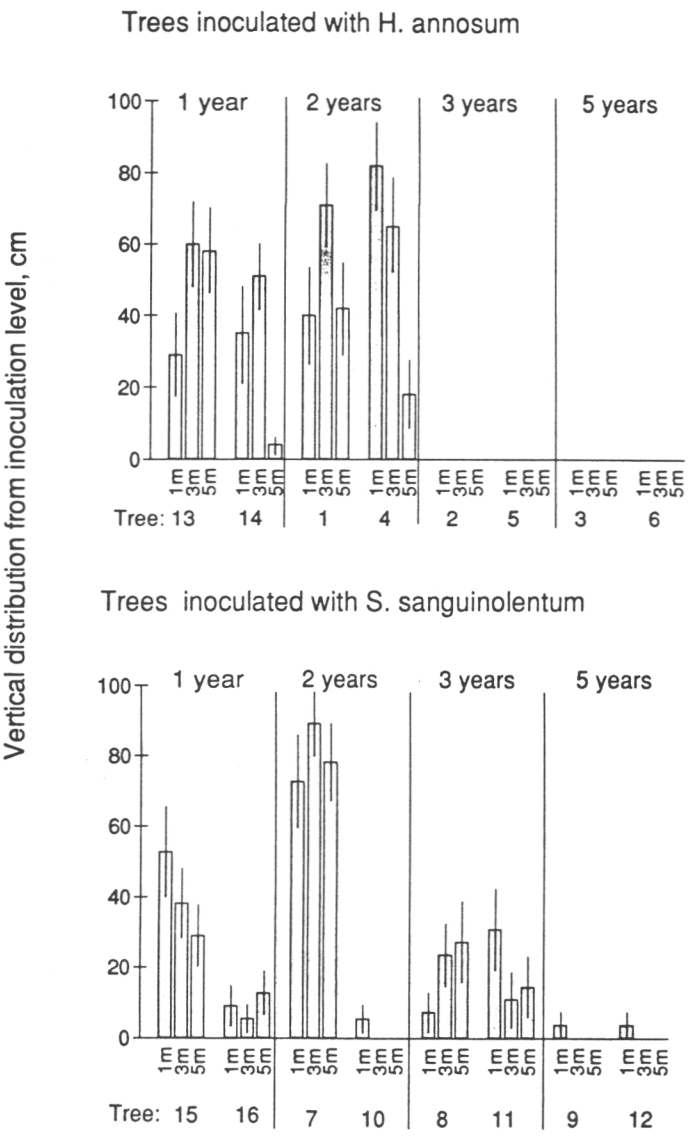

Fig. 3. The mean distribution (± S.E.M.) of the decay fungi, *Heterobasidion annosum* and *Stereum sanguinolentum*, in trees investigated one, two, three and five growing seasons after inoculation at different inoculation levels (1, 3 and 5 m). Each column is a mean of all the 11 microbe combinations which included *H. annosum* or *S. sanguinolentum*

colouration was detected in the boundary zone between sap- and heartwood and later in the outer heartwood of trees inoculated with *S. sanguinolentum*, suggesting that *S. sanguinolentum* had been present in all these trees. However, differences between the years were not statistically significant ($P = 0.065$). The significant variation ($P = 0.000$) in the susceptibility of individual trees to invasion by *S. sanguinolentum* masked the impact of other factors such as inoculation levels ($P = 0.8539$).

The invasion of the slow-growing strain of *S. sanguinolentum* was suppressed when inoculated as a mixture including *N. fuckeliana*, *A. cylichnium*, a yeast-like fungus and the

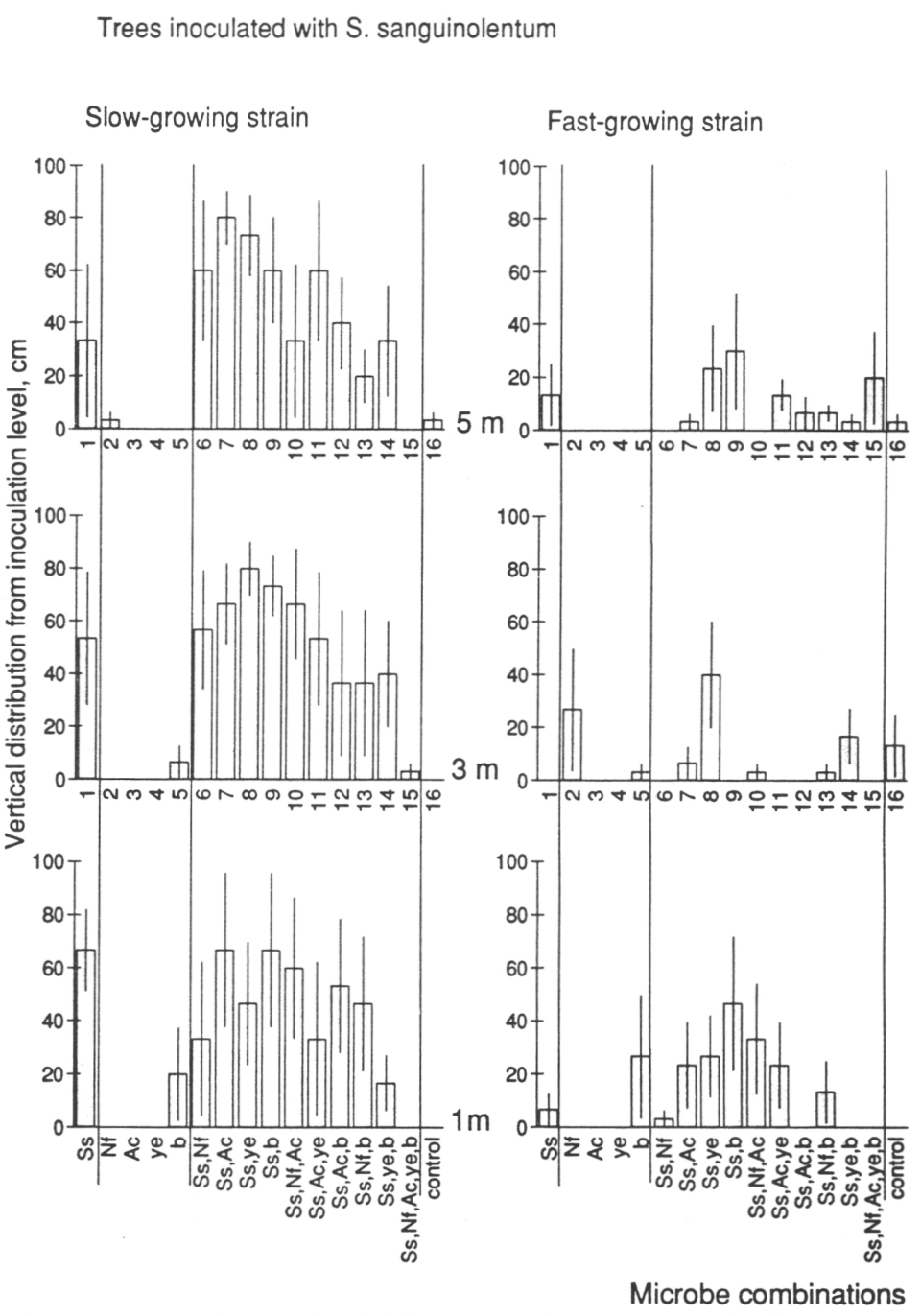

Fig. 4. The mean vertical spread (± S.E.M.) of fast- and slow-growing strains of *Stereum sanguinolentum* in trees investigated over the first three growing seasons. Data for the fifth year are not present due to the very low frequency of isolations of *S. sanguinolentum*. The inoculated microbes are listed in Table 1

bacteria (Fig. 4) as analysed by the Stepwise Logistic Regression analysis (88.9 %) whereas other microbe combinations did not differ in their effect on the spread of *S. sanguinolentum*. Somewhat surprisingly, colonization of spruce trees by the slow-growing strain Ss96 was significantly greater than that of the fast-growing strain SsL50 (P = 0.010), though the variation between individual trees was also high in this case (Fig. 4). Compared to *H. annosum*, the lateral spread of *S. sanguinolentum* was much slower, it being only very occasionally isolated from sampling point above uninoculated bore-holes after 2 and 3 growing seasons (Fig. 4).

3.4 Distribution of bacteria

The spread of bacteria in the spruce trees following inoculation at different heights from ground level is presented in Figure 5. The inoculated bacteria (*B. pumilus* + *E. agglomerans*) were well represented in each tree. After a single growing season these bacteria were found in 86 % of the sampling points although they were originally inoculated in only 38 % of the holes. The upward vertical spread was up to 1 m within a year. In contrast, these bacteria were not found either in the reference trees or in the experimental trees prior to inoculation. This indicates the likelihood of extensive vertical invasion from the holes and lateral invasion from the inoculated sampling points to the uninoculated ones.

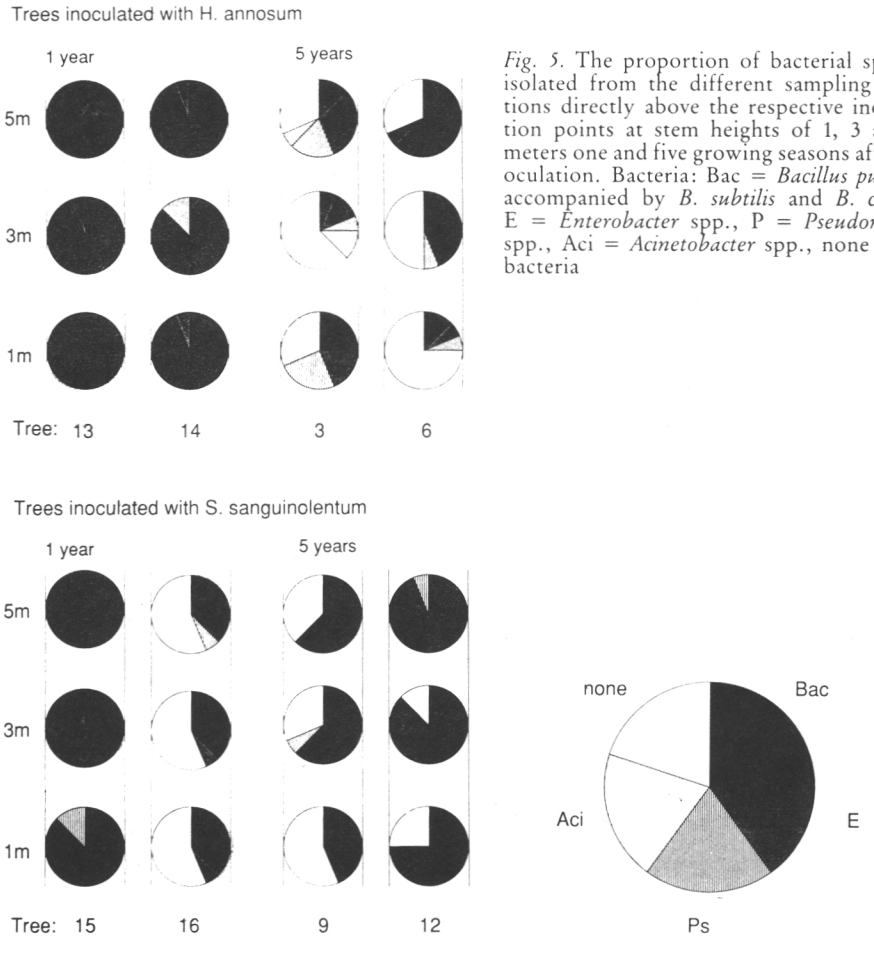

Fig. 5. The proportion of bacterial species isolated from the different sampling positions directly above the respective inoculation points at stem heights of 1, 3 and 5 meters one and five growing seasons after inoculation. Bacteria: Bac = *Bacillus pumilus* accompanied by *B. subtilis* and *B. cereus*, E = *Enterobacter* spp., P = *Pseudomonas* spp., Aci = *Acinetobacter* spp., none = no bacteria

In general, the amount of bacteria in the stems increased greatly after inoculation. There were no significant differences in the bacterial species composition found in the trees inoculated with either *H. annosum* or *S. sanguinolentum*. *B. pumilus* was most often accompanied by *B. subtilis*, *B. cereus* and occasionally *B. circulans* although the latter three species were not included in the inoculation mixtures (Fig. 5; HALLAKSELA et al. 1991).

After five growing seasons bacteria were found in 65% of the bore-holes and had invaded sampling points up to the height of 80 cm above the two lower inoculation levels and 1 m above the upper 5 m inoculation level. Increases in overall bacterial species diversity in trees inoculated with *H. annosum* was reflected by the appearance of *E. agglomerans*, *E. sakazakii* (E), *Pseudomonas* species (P) and *Acinetobacter* (Aci) (Fig. 5) but a decrease in species diversity of *Bacillus* (HALLAKSELA et al. 1991). The entry of bacteria seemed to be through the wounds resulting from the bore holes and subsequently both inoculated and some of the contaminant bacteria readily invaded the inner sapwood and heartwood.

4 Discussion

The heartwood of living healthy trees represents an environment that is seldom sterile (ROLL-HANSEN and ROLL-HANSEN 1979b; HUSE 1981). Most *Picea abies* stems investigated in this study prior to inoculation were colonized by *A. cylichnium* and *N. premnophila*. Over the five years study period these noninoculated species maintained and inoculated *A. cylichnium* was present. *A. cylichnium* and *N. premnophila* are representatives of endophytic fungi which inhabit the heartwood of sound living trees but do not cause decay (ETHERIDGE 1970; ROLL-HANSEN and ROLL-HANSEN 1979a; HUSE 1981; HALLAKSELA 1984a). The presence of *N. fuckeliana* was restricted to the immediate vicinity of the inoculation hole. Earlier studies by ROLL-HANSEN and ROLL-HANSEN (1980a) and HALLAKSELA (1984a) had demonstrated a more rapid invasion by this fungus in open trunk wounds. Many primarily invading nondecay-fungi e. g. *Botryodiplodia excelsea* and *Ceratocystis coerulescens* and die out during few years after wounding living spruces (ROLL-HANSEN and ROLL-HANSEN 1980b).

The rapid invasion by *S. sanguinolentum* and *H. annosum* during the first two years seemed to be followed by a regressive later phase possibly reflecting a successful defense reaction of individual trees towards the invading decay fungi that had been introduced via relatively small wounds made with an increment borer and were then immediately sealed. In a similar study PAWSEY and STANKOVICOVA (1974) reported that the growth of *S. sanguinolentum* introduced via small wounds was suppressed in inoculated and sealed *Picea abies* stems. However, *S. sanguinolentum* is known to be an important pioneer wound decay fungus in natural open scars essentially larger than those of this study (ROLL-HANSEN and ROLL-HANSEN 1980a; HALLAKSELA 1984b). This fungus can continue to cause decay over a period of several decades from open scars of living spruce stems (ISOMÄKI and KALLIO 1974; HALLAKSELA 1984b).

Following infection, *H. annosum* rapidly colonized the trunks of the trees but was unable to permanently establish itself in these trees. However, final conclusions cannot be made because variation in the defence of individual trees and microbes may have affected the results. ROLL-HANSEN and ROLL-HANSEN (1980b) found only few infection and early dying of *H. annosum* in the naturally infected small wounds. *H. annosum* seldom invades stem wounds, but is the most common invasive fungus of root and ground contact wounds (HINDS et al. 1983). The natural mode of infection of *H. annosum* is based on its ability to colonize living roots and grow towards the trunk (STENLID 1986).

The suppressive effect of the inoculum containing *N. fuckeliana*, *A. cylichnium*, the yeast-like fungus and bacteria on the spread of *S. sanguinolentum* may have been due to microbial competition, as observed in earlier microbial competition studies (ÅRSVOLL

1976; BODDY and RAYNER 1983). To eliminate the effect of the inter- and intratree variation more trees should be included in further studies.

When inoculating the microbes it was assumed that the fungi would grow in a narrow vertical zone from the inoculum point (HALLAKSELA 1984a) without much horizontal spread to sampling points of the other inoculation holes. Generally, the situation proved to be so, even though it is known that tracheids in spruce xylem form clockwise spiral-grained conduits (HARRIS 1989). The inoculated bacteria on the other hand, were able to effectively spread in a lateral direction.

The results obtained indicate greater host tree selective defense reactions towards the inoculant decay fungi than towards bacteria. Bacteria may aid decay fungi in the initial steps of infection of the wounded tree. For example bacteria are more successful in surviving in the moist sapwood than the decay fungi (GREAVES 1971), as has also been shown in this study. Hydration increases tha accessibility of wood cell components to the hydrolytic enzymes secreted by the bacteria (GOODMAN et al. 1986). Bacteria isolated from *Picea abies* are able to secrete enzymes capable of degrading cell wall components as pectin (*B. pumilus*), xylan (*B. subtilis, B. pumilus, B. circulans*) and cellulose (*B. subtilis* and *B. circulans*) (HALLAKSELA et al. 1991; HALLAKSELA and SALKINOJA-SALONEN 1992). Bacteria may predominate in the ray cells (GREAVES 1969) of freshly colonized wood and in cases where they are able to hydrolyse wood components they increase wood permeability, e. g. by destroying the margo of the bordered pits (ERIKSSON et al. 1990). The thin pectin-rich cell walls of the ray parenchyma cells, with their high starch and protein content, are attractive to microbes (JOHANSSON and STENLID 1985), and enable the microbes to enter the cells in the inner parts of the tree. Bacteria may contribute in the initial degradation of crystalline cellulose, which is then further broken down by the multicomponent enzyme complex produced by fungi (ERIKSSON et al. 1990). In this study it was found that fluorescent *Pseudomonas* and *Acinetobacter* spp. isolated from living *Picea abies*, were able to grow on antifungal wood resins (HALLAKSELA and SALKINOJA-SALONEN 1992) a bacterial function that could assist sensitive fungi to overcome the natural defense barriers of the tree.

Direct interaction between bacteria and decay fungi can result in either a stimulation or an inhibition of growth of the fungal partner (BLANCHETTE and SHAW 1978; SHORTLE et al. 1978). The bacterial flora was maintained over the whole experimental period of five years. In this study the presence of endophytic fungi *A. cylichnium* or *N. premnophila*, prior to inoculation seems not to have affected invasion by the inoculated decay-fungi (Figs. 1 and 3). The decay fungi, *H. annosum* and *S. sanguinolentum*, were common both in trees largely infected with *A. cylichnium* and/or *N. premnophila* (e. g. trees 14, 15 and 11) and in trees almost devoid of these endophytes (e. g. tree 1 level 1 m and tree 13 level 5 m). The non-decay fungi have no direct interaction with these decay-fungi. The results obtained indicate a different selective host tree defence towards the inoculant fungi than towards the bacteria and endophytic fungi.

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Summary

Wood-decay fungi *Heterobasidion annosum* and *Stereum sanguinolentum*, non-decay fungi *Nectria fuckeliana* and *Ascocoryne cylichnium*, a yeast-like fungus and bacteria *Bacillus pumilus* and *Enterobacter agglomerans* were inoculated into radial bore holes in the stems of healthy

80-year-old *Picea abies* at 1, 3 and 5 m above ground-level. After the trees were felled one, two, three and five growing seasons following inoculation, the microbial flora in the stems was identified by re-isolation of microbes at distances of 5–100 cm above the inoculation planes. All the microbes used in inoculations were of spruce origin.

Most trees were colonized by the endophytes *A. cylichnium* and/or *Neobulgaria premnophila* prior to microbial inoculation. The colonization of wood by the introduced decay-fungi, *H. annosum* and *S. sanguinolentum*, was successful during the first two, respectively three growing seasons regardless of accompanying microbes in the inoculum, but later the decay-fungi rapidly disappeared. The simultaneous inoculation by *N. fuckeliana, A. cylichnium*, the yeast like fungus and the bacteria *B. pumilus* and *E. agglomerans* with *S. sanguinolentum* may suppress invasion by the latter. Of the inoculated microbes, *A. cylichnium* and the bacteria had successfully invaded almost all the trees. The results obtained indicate a different selective host tree defence towards the inoculant fungi than towards the bacteria and endophytic fungi. Invasion of bacteria was more dependent on wounding and persisted over five years. The individual trees differed greatly in their susceptibility towards infection.

Résumé

Interactions précoces de Heterobasidion annosum et Stereum sanguinolentum avec des champignons et bactéries non lignivores, à la suite d'inoculations dans le tronc de Picea abies

Les lignivores *H. annosum* et *S. sanguinolentum*, les non lignivores *Nectria fuckeliana* et *Ascocoryne cylichnium*, un champignon de forme levure et les bactéries *Bacillus pumilus* et *Enterobacter agglomerans*, ont été inoculés dans un trou radial au tronc de *Picea abies* sains âgés de 80 ans, à un, trois et cinq mètres de hauteur. Après abbatage des arbres, 1, 2, 3 et 5 ans plus tard, la flore microbienne a été identifiée par réisolements 5 à 100 cm au-dessus des inoculations. Tous les germes utilisés provenaient d'épicéa.

La plupart des arbres étaient colonisés par les endophytes *A. cylichnium* et/ou *Neobulgaria premnophila* avant l'inoculation. La colonisation du bois par les lignivores introduits, *H. annosum* et *S. sanguinolentum* était effective pendant respectivement les deux et trois premières saisons de végétation, indépendamment des microorganismes accompagnants; mais plus tard, les lignivores ont disparu rapidement. L'inoculation de *S. sanguinolentum* simultanément avec *N. fuckeliana, A. cylichnium*, le champignon levure, *B. pumilus* et *E. agglomerans* peut empêcher l'invasion du lignivore. Parmi les espèces inoculées, *A. cylichnium* et les bactéries ont réellement envahi presque tous les arbres. Les résultats montrent l'existence d'une défense des arbres différente vis-à-vis des champignons inoculés et vis-à-vis des bactéries et champignons endophytes. L'invasion par les bactéries était plus dépendante des blessures et persistait pendant 5 ans. Les arbres différaient beaucoup individuellement pour leur sensibilité à l'infection.

Zusammenfassung

Interaktionen von Heterobasidion annosum und Stereum sanguinolentum mit keine Fäule verursachenden Pilzen und Bakterien nach Inokulation in den Stamm von Picea abies

Die Fäuleerreger *H. annosum* und *S. sanguinolentum*, die keine Fäule verursachenden Pilze *Nectria fuckeliana, Ascocoryne cylichnium*, ein hefeartiger Pilz sowie die Bakterien *Bacillus pumilus* und *Enterobacter agglomerans* wurden in radiale Bohrlöcher im Stamm von gesunden 80jährigen Fichten in Höhen von 1, 3 und 5 m über dem Boden inokuliert. Die Bäume wurden nach 1, 2, 3 und 5 Vegetationsperioden gefällt, und die Mikroflora wurde durch Reisolierungen in Abständen von 5–100 cm über der Inokulationsstelle bestimmt. Alle zu den Inokulationen verwendeten Isolate stammten aus Fichtenholz.

Die meisten Bäume waren bereits vor der Inokulation von den Endophyten *A. cylichnium* und/oder *Neobulgaria premnophila* besiedelt. Die inokulierten Fäuleerreger *H. annosum* und *S. sanguinolentum* konnten den Baum während der ersten zwei bzw. drei Vegetationsperioden besiedeln, ohne daß die Begleitflora im Inokulum einen Einfluß hatte. Später jedoch verschwanden die Fäuleerreger rasch. Die Invasion des Gewebes durch *S. sanguinolentum* konnte durch die gleichzeitige Inokulation von *N. fuckeliana, A. cylichnium*, des hefeartigen Pilzes und der Bakterien *B. pumilus* und *E. agglomerans* unterdrückt werden. *A. cylichnium* und die Bakterien konnten fast alle Bäume erfolgreich besiedeln. Die Ergebnisse lassen auf eine differenzierte Abwehr des Wirtsbaumes gegenüber Fäuleerregern, Bakterien und endophytischen Pilzen schließen. Die Besiedlung durch Bakterien hing eher von Verletzungen ab und blieb über 5 Jahre konstant. Die einzelnen Bäume zeigten große Unterschiede in ihrer Infektionsanfälligkeit.

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Variation in combined fatty acid and sterol profiles of Ascocoryne, Nectria, and Neobulgaria-strains isolated from Norway spruce

By M. M. MÜLLER and ANNA-MAIJA HALLAKSELA

Abstract

The diversity of *Ascocoryne cylichnium, Nectria fuckeliana* and *Neobulgaria premnophila* isolates expressed as variation in their fatty acid and sterol composition was investigated. Despite high within-species variation, distinct differences in the fatty acid and sterol Tcomposition between species were evident.

Key words: *Nectria - Ascocoryne - Neobulgaria* - Norway spruce - fatty acid - sterol.

1 Introduction

Ascocoryne cylichnium (Tul.) Korf. and *Neobulgaria premnophila* (Roll-Hansen & Roll-Hansen) are common inhabitants in sound wood of Norway spruce (*Picea abies* L.). *Nectria fuckeliana* (Booth) is commonly found in wounded Norway spruce stems (ROLL-HANSEN AND ROLL-HANSEN, 1980; HUSE, 1981). *A. cylichnium* and *N. premnophila* are believed to invade the trees through the roots whereas *N. fuckeliana* infects through wounds and dead knots (ETHERIDGE, 1970; ROLL-HANSEN and ROLL-HANSEN, 1980; HUSE, 1981). Reliable identification of *Ascocoryne* and *Neobulgaria* is possible from their sexual fruiting stages but *Ascocoryne* and *Neobulgaria* usually do not produce their teleomorphs on artificial media. On the basis of combined sterol and fatty acid profiles of vegetative mycelia, these two genera can be distinguished from each other (MÜLLER, KANTOLA and KITUNEN, 1994). HALLAKSELA (1993) reported on the effects of bacteria and the above mentioned non-decay fungi on the growth and persistence of *Heterobasidion annosum (Fr.) Bref.* and *Stereum sanguinolentum* (Alb&Schw. ex. Fr.) inoculated in a Norway spruce stand in Ruotsinkylä, southern Finland. A considerable proportion of the trees in HALLAKSELA's investigation contained indigenous strains of *A. cylichnium* and *N. premnophila*. The aim of the present investigation was to confirm identification of the *A. cylichnium* and *N. premnophila* strains isolated by HALLAKSELA (1993), to describe the diversity of the *A. cylichnium, N. premnophila* and *N. fuckeliana* isolates from the trees and to compare them with the inoculated strains.

2 Materials and methods

Inoculation and sampling of the experimental trees is described in detail by HALLAKSELA (1993). Briefly, twenty healthy 80-year old Norway spruce (*Picea abies* (L.) H. Karsten) trees were chosen within an area of 0.5 ha in a spruce stand in southern Finland. Sixteen trees were inoculated at 1, 3 and 5 m above ground. Before inoculation 16 holes (6 cm deep and 0.5 cm wide) were bored at each level and an increment core was taken for isolation of the indigenous microbes. Then fifteen holes were inoculated with the following microbes in various combinations: *H. annosum* (two strains), *S. sanguinolentum* (two strains), *N. fuckeliana*, *A. cylichnium*, a white yeast-like fungus, *Bacillus pumilus (isolate K74A)*, and *Enterobacter agglomerans* (K74B). In total 48 different combinations of the above mentioned microbes were prepared for inoculation. All these strains were originally isolated from wounded Norway spruce. Four replicate trees were inoculated with identical combinations of microbes and a single tree from each experimental group was felled after one, two, three and five growing seasons. Six 5 cm thick discs from 0, 20, 40, 60, 80 and 100 cm above each inoculation level were taken for microbial isolations. From each of the four uninoculated trees, three discs were cut at heights 1, 3 and 5 m above ground.

The fungal strains were isolated as described by HALLAKSELA (1993) and stored on malt agar slopes at +4^{0}C. From the initial 268 *N. fuckeliana*, 2040 *A. cylichnium* and 1560 *N. premnophila* isolates; 17, 66 and 13 isolates of the respective strains were available (several of the isolates had died during storage on the agar slopes) for this study. Additionally, the inoculated strains *A. cylichnium* Ac70 and *N. fuckeliana* Nf96, were analysed for their fatty acids and sterols. The *A. cylichnium* strains were separated into three groups according to growth rate and colony colour on malt extract agar: dark violet slow, pale violet slow and white slow. White fast-growing strains, resembling *A. cylichnium* white slow strains were identified in the first instance as *N. premnophila* based on their colour, growth rate and formation of mycelial coils corresponding to the description given by ROLL-HANSEN and ROLL-HANSEN (1979a). Because of their uniform ovoid conidia, the violet slow, pale violet slow and white slow strains were classified as *A. cylichnium* and not as *A. sarcoides* (Jacq. ex S.F.Gray) Groves & Wilson which have allantoid or rod-shaped conidia (ROLL-HANSEN and ROLL-HANSEN, 1979b). Culture collection strains used for reference included: *A. cylichnium* ATCC44015, ATCC44019; *A. sarcoides* ATCC44014; *N. fuckeliana* 1637/7, 1637ss1, 1637ss2, 1637ss3 and 60-70; *N. premnophila* ATCC44017, ATCC44018 and 68-140a/2. These strains were obtained from Prof. Roll-Hansen, the Norwegian Forest Research Institute, Ås, Norway.

The strains were cultivated in Petri dishes containing potato-tomato-agar: 150 ml of vegetable juice (Eden, Eden-Waren, Bad Soden/TS, Germany), 39 g of Potato-dextrose agar (Difco) supplemented with 5 g of Bacto agar (Difco) and made up to 1 litre in distilled water. After solidification a sterile cellophane membrane (diam. 8 cm, British Cellophane Ltd, UK) was placed on the agar surface to make the mycelium sampling easy at the end of the incubation. After 36 days incubation

at 21°C, hyphal samples of 1.5 g (fresh weight) were taken and lyophilized. The lyophilized samples were homogenized in a ball mill after freezing in liquid nitrogen. The culture collection strains were cultivated as 3-5 replicates and our isolates as 2-3 replicates on separate batches of potato-tomato-agar for extraction of fatty acids and sterols. The fatty acid methyl esters and sterols were analysed by gas chromatography. Details of the procedure are described by MÜLLER, KANTOLA and KITUNEN (1994). The results were subjected to variance analysis, discriminant analysis and principal component analysis using the Systat for Windows, version 5. (Systat Inc., Evanston, Illinois, USA). Two fatty acid and sterol profiles were considered to differ if a significant difference was found between one or more extractives and/or TAE (=total amount of extractives as % of dry weight).

3 Results

3.1 A. cylichnium and N. premnophila

For statistical analysis, the *Ascocoryne spp.*, *N. fuckeliana* and *N. premnophila* culture collection strains were used as *a priori* groups. Figure 1 A shows that these three *a priori* groups could be well separated by a discriminant analysis of their combined fatty acid and sterol contents. When our isolates where subjected to classification by the model obtained from the culture collection strains (Fig. 1A), the white fast strains preliminarily identified as *N. premnophila* clustered with the *N. premnophila* culture collection strains (Fig. 1B). The violet, pale violet and white slow strains clustered with the *Ascocoryne* spp. culture collection strains. The main differences between the *A. cylichnium* strains and the *N. premnophila* strains were in the ranges of cis-9,12-octadecadienoic acid (9,12-18:2), cis-9-octadecenoic acid (c18:1), one unidentified extractive (CB) and ergost-22-en-3-one (ergostenon) (Table 1). No subclustering of the three colony types (violet, pale violet and white slow) could be detected by comparing the principal components of the combined fatty acid and sterol profiles (Fig. 2).

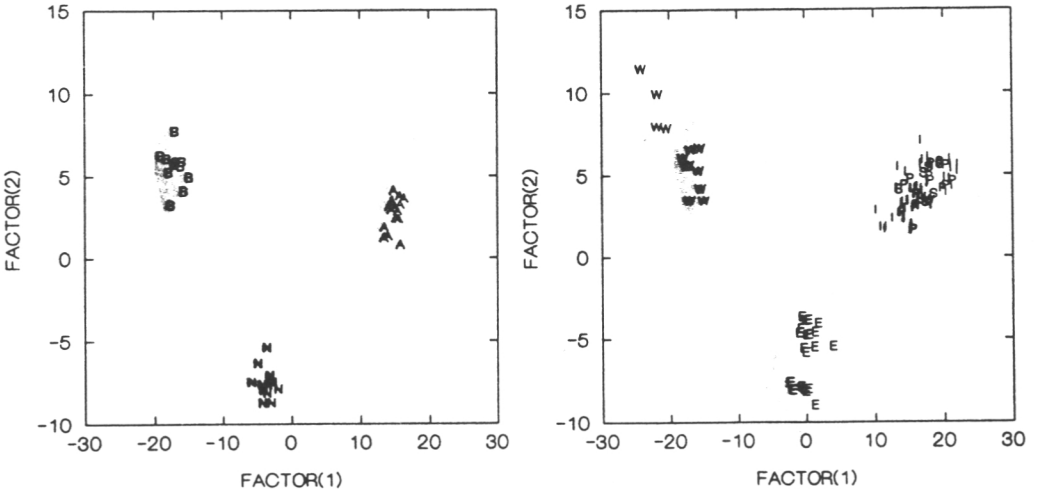

Fig. 1. Canonical variable plot of the combined fatty acid and sterol profiles. A. Culture collection strains *Ascocoryne* spp. (A), *N. premnophila* (B) and *N. fuckeliana* (N). All replicates (3–5) are shown. – B. Our isolates (letters) compared with the culture collection strains (shaded area). Symbols represent average values of 2–3 replicates: E = *N. fuckeliana*; P = *A. cylichnium* subgroup pale violet slow; I = *A. cylichnium*, subgroup violet slow; S = *A. cylichnium*, subgroup white slow; W = *N. premnophila*

Table 1. **Fatty acids and sterols from A. cylichnium, N. fuckeliana and N. premnophila strains. The amounts are given as % of all ~~compounds~~ listed (dash = < 0.1 %)**

Extractive (Ordered according to increasing retention time)	Abbreviation	*Ascocoryne cylichnium*					*Neobulgaria premnophila*				*Nectria fuckeliana*				
		Inoculum	Isolated strains		Culture collection strains*		Isolated strains		Culture collection strains		Inoculum	Isolated strains		Culture collection strains	
		Average (s.d)	(n=66) Average	Range	(n=3) Average	Range	(n=13) Average	Range	(n=3) Average	Range	Average (s.d.)	(n=17) Average	Range	(n=5) Average	Range
AA (Unidentified)	AA	–	–	–	–	–	–	–	0.0	0–0.1	–	0.2	0–1.4	1.4	0.3–2.6
Tetradecanoic acid	14:0	0.3(0.1)	0.4	0.1–0.9	0.5	0.5–0.6	0.6	0.0–1.0	0.9	0.8–1.2	0.1(0.0)	0.2	0.1–0.7	0.3	0.1–0.6
Pentadecanoic acid	15:0	0.2 (0.0)	0.2	0.1–0.4	0.6	0.2–1.1	0.5	0.4–0.7	0.6	0.5–0.9	0.5 (0.0)	0.5	0.2–0.6	0.3	0.2–0.7
Cis-9-hexadecenoic	9–16:1	0.6 (0.0)	0.6	0.4–1.0	1.0	0.9–1.3	0.5	0.1–0.9	0.4	0.40	0.2 (0.0)	0.2	0.2–0.4	0.2	0.1–0.4
Hexadecanoic acid	16:0	11.8 (0.1)	11.8	8.9–19	15.5	13–20	13.2	11–16	15.7	13–20	10.3 (0.1)	11.2	8.7–14	12.6	11–15
13-Heptadecenoic acid T	17:1	0.9 (0.2)	1.0	0.3–2.1	1.1	0.4–1.9	0.5	0.3–1.0	0.5	0.4–0.7	–	–	–	–	–
2-Hydroxy-hexadecanoic acid	OH-16:0	0.3 (0.0)	0.3	0.2–0.5	0.3	0.2–0.5	0.5	0.3–1.0	0.7	0.5–0.9	0.8 (0.0)	1.0	0.6–1.9	1.7	0.8–2.1
Cis-9,12-octadecadienoic acid	9,12–18:2	25.1 (0.5)	28.7	22–35	30.0	21–36	43.7	39–49	42.0	39–44	49.1 (2.2)	46.6	32–51	38.0	29–55
Cis-9-octadecenoic acid**	c18:1	50.6 (1.8)	44.1	34–54	36.9	31–46	17.1	12–20	13.0	11–15	19.6 (0.5)	15.8	5.8–24	10.3	8.5–11
Trans-9-octadecenoic acid	t18:1	0.7 (0.0)	0.7	0.2–1.2	0.5	0.2–0.9	0.7	0.5–1.0	0.7	0.6–0.9	0.1 (0.1)	–	0–0.2	0.2	0.1–0.3
Octadecanoic acid	18:0	0.9 (0.1)	0.7	0.4–1.5	1.4	1.0–1.8	0.7	0.4–1.2	0.8	0.4–1.1	0.6 (0.0)	0.6	0.4–0.7	0.7	0.5–0.9
Cis-6,9-octdecadienoic acid (T	6,9–18:2	0.6 (0.1)	0.6	0.4–1.0	0.5	0.4–0.6	0.7	0.4–1.0	0.8	0.7–0.9	0.9 (0.1)	0.9	0.7–1.2	0.8	0.7–1.0
CA (Unidentified)	CA	0.1 (0.0)	0.1	0.1–0.4	0.0	0.0–0.1	0.8	0.3–1.5	0.8	0.3–1.5	0.7 (0.2)	0.7	0.4–1.3	1.1	0.5–1.7
CB (Unidentified)	CB	–	–	0–0.2	0.1	0.0–0.1	0.8	0.3–1.8	0.8	0.2–1.6	0.3 (0.2)	0.3	0–0.4	0.1	0–0.2
Heneicosanoic acid	21:0	0.1 (0.0)	0.2	0–0.3	0.2	0.1–0.3	0.2	0.2–0.4	0.4	0.3–0.6	–	0.1	0–0.6	0.0	0–0.1
2-Hydroxy-docosanoic acid	OH-22:0	0.1 (0.0)	0.1	0.1–0.4	0.1	0.10	0.2	0.1–0.4	0.4	0.3–0.5	0.7 (0.3)	0.9	0–2.3	1.8	0.9–2.4
DA (Unidentified)	DA	0.1 (0.0)	0.2	0.1–0.4	0.2	0.1–0.2	0.2	0.0–0.3	1.2	0.2–2.9	0.3 (0.1)	0.4	0.2–1.1	0.5	0.3–0.6
2-Hydroxy-tetracosanoic acid (T	OH-24:0	0.4 (0.0)	0.4	0.2–0.6	0.4	0.3–0.5	0.3	0.1–0.6	0.6	0.5–0.9	0.9 (0.1)	1.1	0.7–2.6	2.3	1.0–3.2
Ergosta-x,x,x-trien-3-ol (T	Ergosta-trienol	0.2 (0.2)	0.4	0–0.9	0.4	0.3–0.5	0.1	0.0–0.3	0.4	0.3–0.5	0.5 (0.0)	0.7	0.2–1.4	1.2	0.9–1.4
Ergost-22-en-3-one (T	Ergo-stenon	-	0.2	0–1.2	0.6	0.5–0.8	4.9	4.2–5.9	4.8	4.1–5.8	–	0.1	0–0.3	0.2	0–0.3
Ergosta-5,7,22-trien-3-ol	Ergo-sterol	7.1 (1.1)	9.3	6.1–13	9.6	7–12	13.7	11–18	14.4	11–19	14.4 (1.7)	18.6	12–39	26.3	16–34
Total amount of extractives	TAE	10.3 (1.1)	9.9	4.7–17	11.1	7.0–17	7.6	3.4–14	4.8	3.9–5.8	5.9 (0.3)	5.7	4.3–7.6	4.0	3.7–4.5

* Includes one *A. sarcoides* strain; ** Includes Cis-9,12,15-octadecatrienoic acid. T = tentative identification by mass spectrometry; TAE is given as % of dry weight.

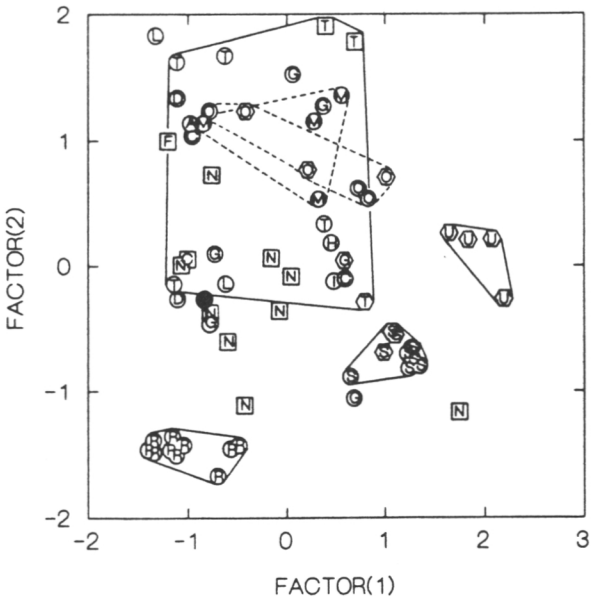

Fig. 2. Principal component analysis of the *A. cylichnium* isolates, I, P and S in Fig. 1B. The strains isolated from the uninoculated reference trees are encircled with solid lines and those from trees M (12, sterile tree prior to inoculation) and O (14, strong indigenous *Ascocoryne* infection prior to inoculation) are encircled with broken lines. Each symbol represents the average of 2–3 replicates: ● = inoculum, ○ = violet slow, □ = pale violet slow, ⬡ = white slow, and the letters correspond to tree number in HALLAKSELA (1993): A = 1, C = 3, D = 4, F = 6, G = 7, H = 8, I = 9, L = 11, M = 12, N = 13, O = 14, R = 17, S = 18, T = 19 and U = 20

The initially inoculated *A. cylichnium* strain and the 66 strains which clustered with the *Ascocoryne* spp. group in Figure 1B were subjected to principal component analysis to determine whether they fell into subgroups and how related the isolated strains were compared to the initially inoculated *A. cylichnium* strain Ac70 (Figure 2). The analysis revealed that strains from three of the four reference trees cluster in distinct groups (R,S and U, Fig. 2). Trees R and U may have each been infected by one single strain, as all isolates from each tree clustered together and represented the same colony type: violet slow and white slow, respectively. Different colony types were isolated from the reference trees S and T. The seven isolates from tree T varied considerably in their combined sterol and fatty acid contents. Also, the isolates from the inoculated trees N, G and D (9, 5 and 2 isolates, respectively) varied considerably on the basis of combined fatty acid and sterol profiles (Fig. 2). It is notable that the cluster of tree M differs from the inoculated strain despite the fact that it was initially devoid of *A. cylichnium*. Cluster M largely overlaps that of tree O which was infected at all three sampling levels (1, 3 and 5 m) by indigenous *A. cylichnium* strain(s) prior to inoculation (HALLAKSELA 1993). The combined fatty acid and sterol profiles of the strains isolated from the reference trees differed statistically significantly ($p<0.05$) from that of the inoculated strain except for two strains isolated from tree T (a result not shown in Fig. 2). The fatty acid and sterol profiles of 21 of the 36 *A. cylichnium* strains isolated from the inoculated trees (and 23 of all the 66 investigated strains) did not differ significantly from that of the inoculum.

3.2 N. fuckeliana

The strains identified as *N. fuckeliana* clustered into two groups close to the culture collection strains (E in Fig. 1B). This subclustering was also revealed in principal component analysis (Fig. 3). The seven *N. fuckeliana* strains of the lower cluster in Figure 1B are separated in terms of factor 1 from the others in Figure 3. The combined sterol and fatty acid profiles of the left and right groups in Figure 3 differed considerably from each other as reflected by the wide range of 9,12-18:2, c18:1 and ergosterol in the *Nectria* isolates in Table 1. Morphologically, however, all 268 strains isolated during this experiment were uniform and none of the so-called atypical *Nectria* strains forming long conidia chains (ROLL-HANSEN and ROLL-HANSEN, 1980) were observed. *N. fuckeliana* strains similar to Nf96 were isolated both from sites (= position on sampling disc immediately above the inoculation hole) inoculated with Nf96 as well as from sites not inoculated as indicated by circular symbols in both the left and right groups of Figure 3. Surprisingly, two isolates distinctly different from Nf96 where isolated from sites were Nf96 was initially inoculated (triangular symbols outside shaded area in Fig. 3).

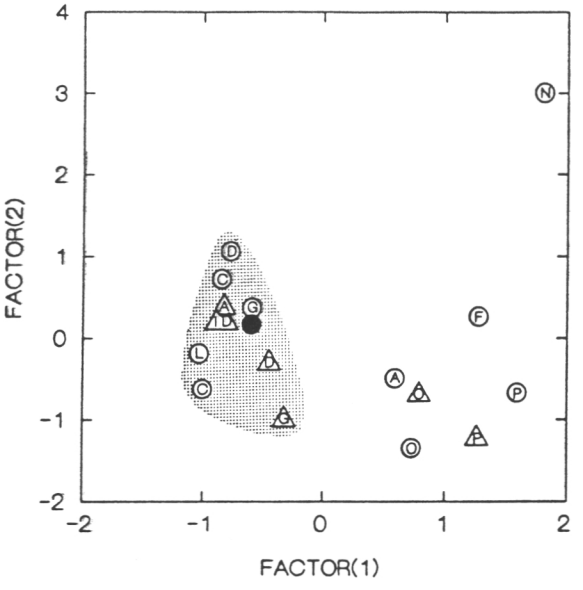

Fig. 3. Principal component analysis of the *N. fuckeliana* isolates. The shaded area covers those fatty acid-sterol results not significantly different ($p < 0.001$) from that of the inoculum. Each symbol represents the average of 2–3 replicates: ● = inoculum, ○ = isolated from a site not inoculated with *N. fuckeliana*, △ = isolated from a site inoculated with *N. fuckeliana*. Letters refer to tree number as in Fig. 2

Table 2. Five variables giving the highest loading for the factors in Figures 1, 2, and 3. Abbreviations used are those listed in Table 1

Figure 1 (*Ascocoryne*, *Neobulgaria*, and *Nectria* culture collection strains)

Factor 1		Factor 2	
Extractive	Loading	Extractive	Loading
OH-16:0	0.924	c18:1	–0.891
6,9-18,2	0.893	16:0	0.725
OH-24:0	0.825	18:2	0.697
TAE	–0.824	14:0	0.674
Ergosterol	0.670	15:0	0.574

Figure 2 (*A. cylichnium* strains)

Factor 1		Factor 2	
Extractive	Loading	Extractive	Loading
Ergostenon	–0.210	Ergostenon	0.468
c18:1	0.160	OH -16:0	–0.240
16:1	0.090	OH-24:0	–0.216
TAE	0.070	c18:1	0.205
CA	–0.067	Ergosterol	–0.199

Figure 3 (*N. premnophila* strains)

Factor 1		Factor 2	
Extractive	Loading	Extractive	Loading
Ergosterol	0.984	15:0	–0.719
OH-24:0	0.979	16:0	–0.565
OH-16:0	0.959	t18:1	0.559
Ergostatrienol	0.937	21:0	0.492
OH-22:0	0.929	DA	–0.487

The loadings given in Table 2 are correlations between variables and the factor in question. A high loading of a fatty acid or sterol corresponds to a strong effect in separation along the factor axis. Both extractives appearing abundantly as well as those appearing in low quantities proved to be valuable for the multivariate analysis as inferred from their high loadings (Table 2). Three extractives, OH-16:0, OH-24:0 and ergosterol, gave high loadings in all three multivariate analyses applied here.

4. Discussion

The preliminary identification of *N. premnophila* strains according to growth rate, formation of mycelial coils and colony pigmentation could be confirmed by a discriminant analysis of their combined sterol and fatty acid profiles (Fig. 1B). The clusters of our isolates do not completely overlap with the area of the culture collection strains (shaded area) which may be a reflection of the restricted number of culture collection strains investigated.

The fatty acid and sterol contents of the culture collection strains given in Table 1 are within or close to the range obtained earlier on orange serum agar (MÜLLER, KANTOLA and KITUNEN, 1994). The largest deviation was obtained with *N. premnophila* which contained 4.1-5.8 % and 0.6-2.0 % of ergostenon when cultivated on potato-tomato-agar (this study) and orange-serum-agar (earlier work), respectively.

The combined fatty acid and sterol profiles of the two *A. cylichnium* culture collection strains, ATCC44015 and ATCC44019, did not differ significantly from those of the *A. sarcoides* strain ATCC44014. This is in accordance with an earlier suggestion of ROLL-HANSEN and ROLL-HANSEN (1979b) that these organisms should be considered the same species. Thus we treated *A. sarcoides* together with *A. cylichnium* as one group in the statistical analysis for Fig. 1B.

According to their combined fatty acid and sterol profiles, the indigenous endophytic *A. cylichnium* population is rather diverse even within a tree stand covering a small area as in this case where the trees were at most 100 m from each other. Some of the trees apparently harboured a single *A. cylichnium* strain. It is possible that the proportion of reisolations of the inoculated strain Ac70 was actually lower than 23/66 since the combined fatty acid and sterol profiles are not strain specific. It is surprising to find even in tree M, strains of *A. cylichnium* different from that inoculated though the stem of this tree was, prior to inoculation, totally devoid of culturable microbes at the sampling levels of 1, 3 and 5 m at the start of the experiment (HALLAKSELA, 1993). Perhaps this tree harboured indigenous but weak sporadic *A. cylichnium* infections. The tree was sampled five years after inoculation, a time period sufficiently long for considerable changes in the degree of infection to have taken place. The overall microbial flora varied considerably among individual trees (HALLAKSELA, 1993), suggesting large variations in the defence mechanisms of spruce trees which may explain the diversity of the indigenous *A. cylichnium* strains found here.

The inoculated *N. fuckeliana* strain Nf96 was obviously a weak competitor as only 54 % of the 240 inoculation sites (= position on sampling disc immediately above the inoculation hole) were infected by *N. fuckeliana* compared to 26 % infection of sites which had remained uninoculated (unpublished data). Also the fatty acid and sterol profiles suggest that natural infections by *N. fuckeliana* took place in addition to those started from artificial inoculation. The number of *N. fuckeliana* strains available for this investigation (17) was unfortunately low, compared to the high numbers initially isolated. Hence, it is possible that there were, in fact, more than the two types of *N. fuckeliana* distinguished in this work.

The lists of extractives giving high loadings, presented in Table 2, support earlier results on the importance of sterols, in addition to fatty acids, for the characterization of fungi (MÜLLER, KANTOLA and KITUNEN, 1994). For this particular type of fungi, the hydroxy acids seem to be valuable extractives, both for principal component as well as for discrimination analysis.

To date fatty acid profiles have often been used for the characterization of fungal taxa (JABAJI-HARE, 1988; LÖSEL, 1988; JOHNK and JONES, 1992). Inclusion of sterols and the application of discriminant analysis to the data increases the resolution of this method considerably (MÜLLER, KANTOLA and KITUNEN, 1994). The method can be applied for identification of fungal species and demonstration of within-species variation.

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Summary

Sixty-six *A. cylichnium*, 17 *N. fuckeliana* and 13 *N. premnophila* strains obtained from sixteen preinoculated and four reference trees of Norway spruce were characterized by analysing their combined fatty acid and sterol profiles. The preliminary identification based on growth rate, colour and hyphal and conidial morphology could be confirmed using discriminant analysis of their combined fatty acid and sterol profiles. The within-species variation of the fatty acid and sterol profiles was high. The within-tree variation among *A. cylichnium* isolates was low in three reference trees but high in the inoculated trees. Fifteen out of 36 *A. cylichnium* strains isolated from the inoculated trees differed from the inoculated strain probably because most of the trees were infected by indigenous *A. cylichnium* strains prior to inoculation. Seven out of seventeen isolated *N. fuckeliana* strains investigated differed from that inoculated despite the fact that no indigenous infections by *N. fuckeliana* were found prior to inoculation.

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III

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Identification of *Bacillus* Species Isolated from *Picea abies* by Physiological Tests, Phage Typing and Fatty Acid Analysis

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The bacterial species inhabiting Norway spruce (*Picea abies* (L.) H. Karsten) were investigated, with special attention paid to *Bacillus* species. The trees had been inoculated one or five growing seasons earlier with a mixture of fungi and bacteria to observe the interaction of externally introduced microbes with a living tree.

After both one and five growing seasons, the most frequent *Bacillus* species was *B. pumilus*. The second most frequent species were *B. subtilis* and *B. cereus*. *B. pumilus* strains were pectolytic and *B. subtilis* were cellulolytic, which probably enabled them to attack the bordered pits of xylem cells. The *Bacillus* strains collected from the trees were identified by classical physiological tests, by determination of whole-cell fatty acid composition, and by phage typing. Results from these three analytical techniques were in good agreement. Whole-cell fatty acid analysis was found to be sufficiently accurate to be used alone as a routine method for the identification of *Bacillus* species inhabiting living spruce. *Key words: Bacillus, Norway spruce, chemotaxonomical methods, whole-cell fatty acids, phage typing.*

INTRODUCTION

There are several reports on the presence of bacteria in the woody interior of Norway spruce (*Picea abies* (L.) H. Karsten) soon after wounding sound trees (Kallio, 1973; Bonnemann, 1979; Roll-Hansen & Roll-Hansen, 1980; Hallaksela, 1984; Solheim & Selås, 1986; Ali El Atta & Hayes, 1987). Bacteria were found not only close to the wound, but also in healthy looking wood xylem above the region affected by fungi. This may indicate that the bacteria pioneer the infection process (Kallio, 1976; Hallaksela, 1984). Kallio (1974) showed that bacteria are antagonistic to decay fungi *in vitro*. However, the inoculation of antagonistic bacteria into the wounds of spruce was not able to prevent the invasion of decay fungi, and cannot be used as a biological control method (Hallaksela, 1984).

In order to gain better understanding of the effect of microbes in the decay of living wood it is necessary to identify the microbes infecting the tree. The most common bacterial genera isolated from the xylem of living conifer tree, with or without wounds, were reported to belong to the family of Enterobacteriaceae (Bacon & Mead, 1971; Aho et al., 1974; Bagley et al., 1978; Mireku, 1981; Schmidt & Kebernik, 1984). The occurrence of gram-positives in live tree xylem is less well documented.

The development of rapid diagnostic methodology in microbiology permits the characterization of versatile microbial communities. Investigation of microbial communities such as those present in the tree rhizosphere, on the needle surface or in the woody interior, involves large numbers of isolates that would be extremely laborious to examine with the classic

methodology based on morphology and physiological tests developed for clinical microbes.

In order to find suitable methods for identifying the *Bacillus* species associated with trees, we isolated *Bacillus* species from Norway spruce, and studied them using whole-cell fatty acid analysis and phage typing, cell morphology and physiological properties.

This study is part of a larger investigation of the role of microbes in Norway spruces earlier in the decay process.

MATERIALS AND METHODS

Experimental design

Eight healthy, 80 year old Norway spruce trees, from a stand in Ruotsinkylä (60°22′ N, 20°0′ E) were inoculated with mixtures of bacteria and fungi (*Heterobasidion annosum* (Fr.) Bref. 70-84 or 61-84, *Stereum sanguinolentum* (Alb. & Schw. ex Fr.) Fr. 96-84 or L50-84, *Nectria fuckeliana* (Booth) 96-84, *Ascocoryne cylichnium* (Tul.) Korf 70-84, a white yeast like fungus 70-84, *Bacillus pumilus* K74A and *Enterobacter agglomerans* K74B). The inoculated fungi (Hallaksela, 1984) and bacteria (Kallio, 1974) all originated from Norway spruce trees. Trees were inoculated either with decay fungi *S. sanguinolentum* or *H. annosum*, but other microbes were the same in each tree.

Water suspensions of the cultures were injected into bore holes of 0.5 cm × 6 cm (48 holes/tree) at three different heights above ground. Holes were sealed with grafting wax (Oy Åström, Finland).

The trees, inoculated May 1981 and May 1986, were felled in winter 1985 and 1986 respectively, when the air temperature was below 0°C. 5-cm-thick discs were sawn directly above the inoculation whorl and then at 20 cm intervals until 150 cm. Discs were sealed in plastic bags, and stored at −20°C until microbes were isolated from them.

Isolation of bacteria

Samples of wood chips (0.5 g fresh weight, 48 samples/disc) were aseptically cut and homogenized (Ultra Turrax T18/10) in 20 ml of sterile water. The chip slurries were plated on Bacto nutrient agar (Difco), Taylor's medium modified for bacteria of spruce (Kallio, 1973), Pseudomonas agar F (Difco) with 0.001% crystal violet, and Bacto MacConkey agar (Difco). Nutrient agar plates were inoculated with pasteurized slurry (5 min at 90°C in a water bath) in order to select for endosporeforming bacteria. Taylor's medium was used to count the total viable bacteria, and Pseudomonas F and MacConkey media were used to isolate gram-negative strains. Plates were incubated aerobically at +21°C for four days and bacterial colonies were restreaked until pure.

Morphological and physiological characterization

Size, spore formation and possible rhizoid growth were recorded in all isolated strains. The following physiological characteristics were determined: 1. Gram reaction, 2. Utilization of and gas production from glucose (Hugh & Leifson, 1953), 3. Hydrolysis of starch and casein, 4. Growth in anaerobic agar, 5. Growth at 50°C and 60°C, 6. Growth in 7% sodium chloride (Norris et al., 1981), 7. Hydrolysis of cellulose-azure (Couts & Smith, 1976), and 8. Pectate gel pitting (Cupples & Kelman, 1974), with sodium polypectate (M. Burger Enterprise, Madison, U.S.A.). All strains were also identified with API 20B strips (API Systems S.A., La Balme les Grottes, Montalieu Vercieu, France).

Phage typing

B. cereus (31 strains) were tested with phages of *B. cereus*, *B. thuringiensis* and *B. mycoides* (12 phages). *B. pumilus* (38 strains) and *B. subtilis* (22 strains) were tested using phages of

B. pumilus, *B. subtilis* and *B. licheniformis* (19 phages). Phage typing was carried out as described by Väisänen & Salkinoja-Salonen (1989).

Whole-cell fatty acid analysis

Fatty acids were analysed with a Hewlett Packard HP 5898A microbial identification system based on gas chromatography of the fatty acid methyl esters (Miller & Berger, 1985). The results were processed using database software (version 3.0) of Microbial ID Inc. (Newark, DE USA). The dendrograms showing Euclidian distances of the bacterial strains were prepared by cluster analysis (unweighted pair-group method using arithmetic averages; Romesburg, 1984).

Abbreviations used in this study

BCe = Biotype of *B. cereus*, BMy = Biotypes of *B. mycoides*, BPu = Biotypes of *B. pumilus*, BSu = Biotypes of *B. subtilis*. PhCe = Phage type of *B. cereus*, PhMy = Phage type of *B. mycoides*, PhPu = Phage type of *B. pumilus*, PhSu = Phage types of *B. subtilis*. FCe = Fatty acid type of *B. cereus*, FPu = Fatty acid types of *B. pumilus*, FSu = Fatty acid types of *B. subtilis*, see Table 5.

RESULTS

Bacteria in inoculated Norway spruces

The bacterial flora was diverse both one and five growing seasons after inoculation periods (Table 1). *Bacillus* species were the most common bacteria. After morphological and physiological inspection, 5% or more of the *Bacillus* strains were chosen from every tree for testing with API 50CHB and API 20E identification, phage typing and whole-cell fatty acid analysis.

Table 1. *Number of Bacillus strains isolated from Norway spruce inoculated one and five growing seasons earlier*

		Bacillus strains				
Tree code	Total no. of bacteria	*B. cereus*/ *B. mycoides*	*B. pumilus*	*B. subtilis*	Total	% of Total
One growing season						
13	350	95(11)*	153(7)	50(4)	298	85
14	256	35(4)	140(6)	17(2)	192	75
15	250	35(3)	157(4)	38(2)	230	92
16	89	13(3)	40(4)	18(2)	71	80
Five growing seasons						
3	112	–	14(2)	7(4)	21	20
6	220	–	185(7)	2(2)	187	85
9	91	–	48(3)	17(3)	66	73
12	214	104(10)	96(5)	8(3)	208	97
Total	1582				1273	

* Number of *Bacillus* strains characterized in detail by API 50CHB and API 20E strips, phage typing, and fatty acid analyses, are indicated within parentheses.

Bacillus species and biotypes

Although only one *Bacillus* species was present in the inoculate, four species were isolated from the trees (Tables 1, 2). Table 2 shows the biotypes of the 91 *Bacillus* strains selected for

Table 2. *Morphological and physiological characteristics of Bacillus strains isolated from inoculated Norway spruce*

Positive reactions with all strains: production of acetoin, utilization of D-glucose, D-fructose, sucrose, trehalose. Negative reactions with all strains: arginine dihydrolase, lysine and ornithine decarboxylase, urease, tryptophane deaminase, production of H_2S and indole, utilization of erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, L-sorbose, L-rhamnose, dulcitol, lactose, melibiose, melezitose, raffinose, xylitol, D-furanose, D-lyxose, D- and L-fucose, D- and L-arabitol

Bacterial strains:	*B. cereus*	*B. mycoides*	*B. mycoides*	*B. pumilus*	*B. subtilis*	*B. subtilis*
Biotype:	BCe1	BMy1	BMy2	BPu1	BSu1	BSu2
Number of strains:	27	2	2	38	9	13
Cell diameter >1 μm	+	+	+	−	−	−
Rhizoid growth	−	+	+	−	−	−
NO_3^- reduced to NO_2^-	+	+	+	−	+	(to N_2)
β-galactosidase	−	−	−	+	+	+
Cytochrome oxidase	+	+	+	−	−	−
Gelatinase	+	+	+	v	+	+
Citrate utilization	−	−	−	v	v	v
Hydrolysis of:						
starch	−	−	+	−	+	+
casein	+	+	+	+	+	+
cellulose	−	−	−	−	+	+
Pectate gel pitting	−	−	−	+	−	−
Utilization of:						
Glycerol	−	−	−	+	+	+
L-arabinose	−	−	+	+	+	+
Ribose	+	+	−	+	+	+
D-xylose	−	−	+	+	−	−
D-galactose	−	−	−	+	−	−
D-mannose	−	−	+	+	+	+
Inositol	−	−	−	−	+	+
D-mannitol	−	−	+	+	+	+
D-sorbitol	−	−	−	−	+	+
α-methyl-D-mannoside	−	−	−	v	−	−
α-methyl-D-glucoside	−	−	−	−	+	+
N-acetyl-glucosamine	+	+	+	+	−	−
Amygdalin	−	−	−	+	+	+
Arbutin	−	−	+	+	+	+
Esculin	−	−	+	+	+	+
Salicin	−	−	+	+	+	+
D-cellobiose	−	−	+	+	+	+
D-maltose	+	+	+	−	+	+
Inulin	−	−	−	−	+	+
Glycogen	−	−	+	−	+	+
Gentiobiose	−	−	−	v	v	−
D-tagatose	−	−	−	+	−	−

Reactions: +, positive; − negative; v, variable

a closer study. Twenty-seven strains were identified as *B. cereus*, a homogeneous group sharing one identical biotype (BCe1) with regard to all 64 physiological characteristics determined. Four strains showed rhizoid growth. Two of these were in other respects identical to BCe1 (BMy1), but two other rhizoid strains utilized a wider range of sugars (BMy2). These four rhizoid strains were identified as *B. mycoides*.

Thirty-eight strains were identified as *B. pumilus*. These formed a homogeneous group of one single biotype (BPu1). Twenty-two strains, identified as *B. subtilis*, were divided into two biotypes (BSu1 and BSu2) on the basis of differences in the reduction of nitrate and in gentiobiose utilization.

Phage types

Thirty one phages were used for typing the 91 *Bacillus* strains and the results are presented in Table 3. All 27 *B. cereus* strains shared one phage type (PhCe2), being sensitive to only one (7064-B1) of the three tested *B. cereus* phages. Two more *Bacillus* strains also shared the same phage type (PhCe2), but they were designated here as *B. mycoides* because of their rhizoid colony growth configuration. Two *B. mycoides* strains were found to be sensitive to *B. mycoides* phage (11986-B1), as well as to one *B. cereus* phage (7064-B1), PhMy1.

All 38 *B. pumilus* strains were sensitive to only one phage, *B. pumilus* phage EP1. This phage type was designated as PhPu5. *B. pumilus* K74A, which had been inoculated into spruce trees one or five years before isolation of bacteria, was also of this phage type.

The 22 *B. subtilis* strains fell into four different phage types. Phage types PhSu1 (7 strains), PhSu4 (6 strains) and PhSu5 (4 strains) were closely related. The difference between PhSu1 and PhSu4 was based on sensitivity to one morphotype B1 phage (ø105) only, while both were sensitive to another B1-phage (SPP1). PhSu5 differed from PhSu1 also in the sensitivity to one phage, (ø29), which is of the C2 morphotype. Five *B. subtilis* isolates formed a separate phage type (PhSu24), being sensitive to a *B. subtilis* morphotype C2 phage (ø29) only.

Fatty acid composition

The four *Bacillus* species studied fell into five different fatty acid groups (Fig. 1). *B. cereus* (27 strains) and *B. mycoides* (4 strains) could not be separated on the basis of their fatty acid profiles. *B. pumilus* fell into two fatty acid groups, designated FPu2 (32 strains and the inoculated strain K74A) and FPu3 (6 strains). The 22 *B. subtilis* strains fell into two fatty acid groups, designated FSu2 (10 strains) and FSu3 (12 strains).

Fig. 2 shows a dendrogram of Euclidian distances between whole-cell fatty acid compositions of the *Bacillus* strains isolated from Norway spruce trees. The strains fell into three clusters, corresponding to *B. cereus/mycoides*, *B. pumilus* and *B. subtilis*. Group M corresponding to *B. pumilus* (FPu3, 6 strains) was located within the *B. subtilis* cluster of the dendrogram. However, additional physiological tests for growth in anaerobic agar, at 50°C and 60°C, and in 7% sodium chloride in addition to those listed in Table 2, showed that all 6 strains belonged to *B. pumilus* species.

Comparison of physiological tests, phage typing and fatty acid analysis of Bacillus strains

Results obtained with the different methods of classification are summarized in Table 4. *B. cereus* (27 strains) and *B. mycoides* (4 strains) shared one fatty acid type, but could be subdivided into two phage types and three different biotypes.

This *B. cereus/mycoides* fatty acid type was analysed in more detail by comparison to culture collection strains. The dendrogram in Fig. 3 shows that the *B. cereus/mycoides* fatty acid type, compiled of 26 reference strains and 31 Norway spruce strains, formed two major

Table 3. *Phage typing of Bacillus strains isolated from inoculated Norway spruces*

		Sensitivity[a] to *Bacillus* phages[b]															
Morphotype:		A1	B1		B1	A1	A1	A1	B2	B2	B2	C2	C2	A1			
Bacillus species	Total no. of isolates	7064-B1	*B. cereus* 12826-B1	27877-B1	*B. mycoides* 11986-B1	*B. thuringiensis* HER211 (Bastille)	HER230 (P400)	HER231 (Bat1)	HER78 (morl)	HER234 (Bat10)	HER235 (Bat11)	HER232 (Bat5)	HER236 (Bat18)	*B. pumilus* 6631-B1	EP1	FP2	FP7
B. cereus	27	+	–	–	–	–	–	–	–	–	–	–	–	NT	NT	NT	NT
B. mycoides	2	+	–	–	–	–	–	–	–	–	–	–	–	NT	NT	NT	NT
	2	+	–	–	+	–	–	–	–	–	–	–	–	NT	NT	NT	NT
B. pumilus	38	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	–	+	–	–
B. subtilis	7	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	–	–	–	–
	6	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	–	–	–	–
	4	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	–	–	–	–
	5	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	–	–	–	–

[a] +: sensitive, –: resistant, NT: not tested.
[b] The phages are shown with their host species and morphotypes (according to Akermann & DuBow, 1987, H.-W.
[c] Phage type coding is as described by Väisänen & Salkinoja-Salonen (1989).

branches, with Euclidian distance of 15 units. The *B. cereus* from Norway spruce located in three subgroups (C, H, I) of which two were close to each other (subgroups H, I). Strains with rhizoid colony morphology were separated in three subgroups (J, L, O) of which subgroup J closely resembled *B. cereus* subgroups H and I but differed greater from other rhizoid subgroups L and O.

B. pumilus (38 strains) belonged to one biotype and phage type, but fell into two separate fatty acid types FPu2 (32 strains) and FPu3 (6 strains, see Table 4).

Biotypes BSu1 and BSu2 of *B. subtilis* did not coincide with the fatty acid groups (FSu2, FSu3), nor with the phage types (Table 4). It looks as if phage types were independent of both fatty acid groups and biotypes.

Bacillus in infections of Norway spruce trees after one or five growing seasons

Table 5 shows the distribution of various *Bacillus* types among trees inoculated one or five growing seasons earlier. The *B. cereus/mycoides* group decreased in frequency during the five-growing-season period. *B. pumilus* (FPu2), identical to the one inoculated, became established and remained unchanged throughout the study period in each of the eight trees studied. On the other hand, many different biotypes, phage types, and fatty acid types of *B.*

C2	B3	B3	B3	A1	A1	A1	A1	A1	A1	A1	B1	B1	B1	C2	
HER182 (GA-1) *Bacillus* sp.	12139-B1	HER190 (Θ1) *B. licheniformis*	HER191 (BLE)	6051-B1 (ø e)	27370-B1 (SP01)	HER97 (PBS1)	HER147 (SP8)	HER148 (SP10)	HER174 (SP50) *B. subtilis*	HER183 (H1)	15841-B1 (S-a)	HER46 (ø 105)	HER149 (SPP1)	HER243 (ø 29)	Phage[c] type
NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	PhCe2
NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	PhCe2
NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	PhMy1
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	PhPu5
–	–	–	–	+	+	+	–	–	+	+	–	+	+	+	PhSu1
–	–	–	–	+	+	+	–	–	+	+	–	–	+	+	PhSu4
–	–	–	–	+	+	+	–	–	+	+	–	+	NT	–	PhSu5
–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	PhSu24

Akermann, pers. comm., and E.-L. Nurmiaho-Lassila &Väisänen, unpublished results).

subtilis were found. One year after inoculation only biotype 2 of *B. subtilis* was found, after five years both biotypes 1 and 2, each of them combined to many different phage types and fatty acid types. In fact only one combination BSu2, PhSu5, FSu3 was repeatedly isolated. The most persistent *Bacillus* strains were the *B. pumilus* strains, which did not change over five years.

DISCUSSION

As far as we know, no study has been published on the identification of *Bacillus* species in living conifers. We found the genus *Bacillus* to be an important component in the microbial flora.

Bacillus strains isolated from the inoculated trees were either pectolytic (*B. pumilus*) or cellulolytic (*B. subtilis*). This suggests that these bacteria were capable of moving within the spruce xylem from cell to cell by degrading the margo of bordered pits, formed of cellulose, and the pit membrane (torus) rich in pectic material. *Bacillus* cells are too thick to move freely from cell to cell (Liese & Schmidt, 1986).

Three independent methods were used in this study to characterize *Bacillus* strains in Norway spruce: biotyping by physiological tests (Norris et al., 1981), phage typing (Väisänen & Salkinoja-Salonen, 1989) and fatty acid typing (Miller & Berger, 1985). These combined

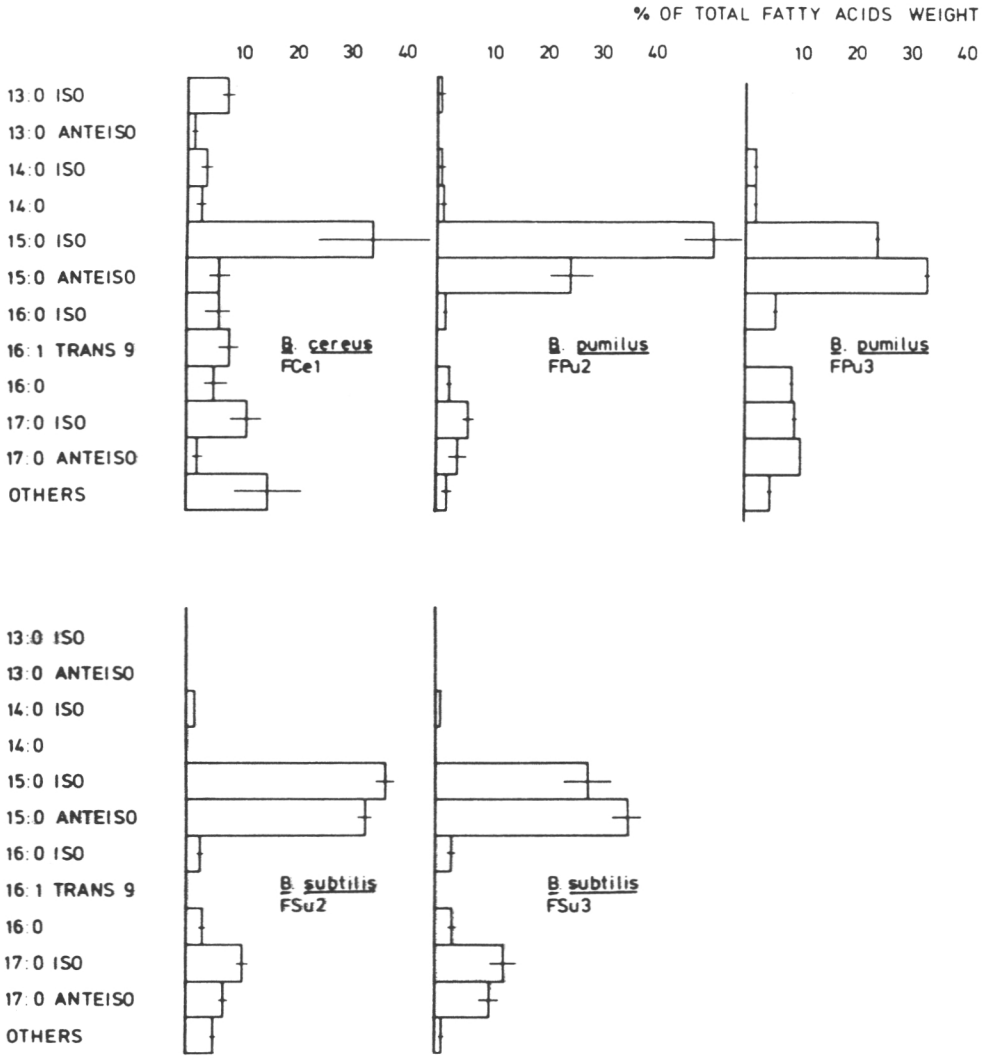

Fig. 1. Whole-cell fatty acid profiles of *Bacillus* strains isolated from inoculated Norway spruce. Fatty acid types were coded as described by Väisänen & Salkinoja-Salonen (1989), whose designation FCe is identical to FCe1 used here. Fatty acids: 13 : 0 iso, 11-methyldodecanoic acid; 13 : 0 anteiso, 10-methyldodecanoic acid; 14 : 0 iso, 12-methyl-tridecanoic acid; 14 : 0, tetradecanoic acid; 15 : 0 iso, 13-methyltetradecanoic acid; 15 : 0 anteiso, 12-methyltetradecanoic acid; 16 : 0 iso, 14-methylpentadecanoic acid; 16 : 1 trans 9, trans-9-hexadecanoic acid; 16 : 0, hexadecanoic acid; 17 : 0 iso, 15-methylhexadecanoic acid; 17 : 0 anteiso, 14-methylhexadecanoic acid.

techniques show that the four *Bacillus* species found in the Norway spruces fall into 15 different types: one of *B. cereus*, two of *B. mycoides*, two of *B. pumilus* and ten of *B. subtilis* (Table 4).

Only the rhizoid growth of *B. mycoides* distinguishes it from *B. cereus* (Sneath, 1986). In the present study, two different biotypes (BMy1 and BMy2) with rhizoid colony morphology were found, with biotype BMy1 being identical to *B. cereus* biotype BCe1. In the dendro-

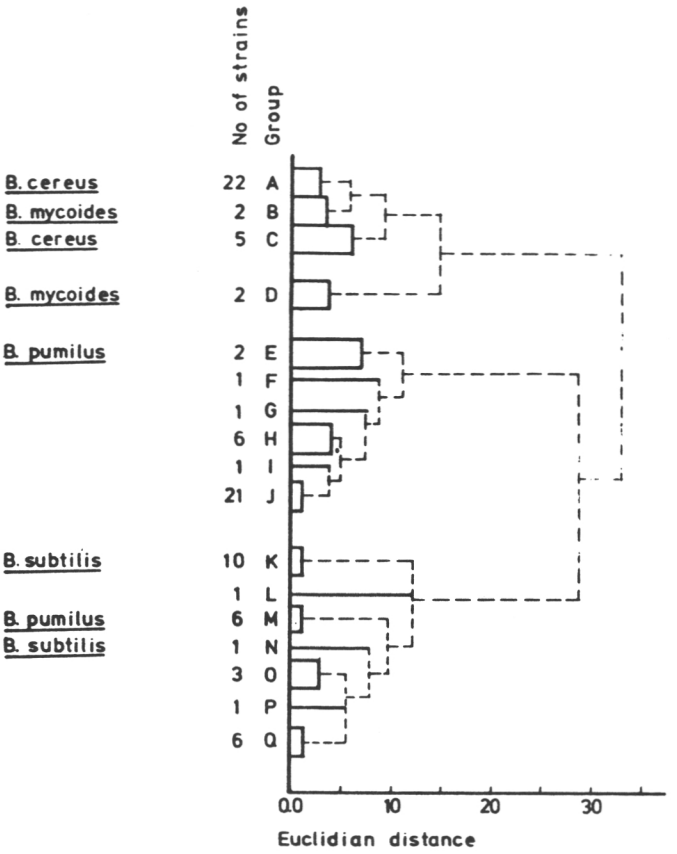

Fig. 2. Dendrogram of whole-cell fatty acid compositions of *Bacillus* strains isolated from inoculated Norway spruce. Group A: *B. cereus*; BCe1, PhCe2, FCe1. Group B: *B. mycoides*; BMy1, PhCe2, FCe1. Group C: *B. cereus*; BCe1, PhCe2, FCe1. Group D: *B. mycoides*; BMy2, PhMy1, FCe1. Groups E, F, G, H, I, J: *B. pumilus*; BPu1, PhPu5, FPu2. Group K: *B. subtilis*; BSu1, PhSu1, FSu2., BSu1, PhSu4, FSu2., BSu1, PhSu24, FSu2., BSu2, PhSu1, FSu2., BSu2, PhSu24, FSu2. Group L: *B. subtilis*; BSu2, PhSu5, FSu3. Group M: *B. pumilus*; BPu1, PhPu5, FPu3. Group N: *B. subtilis*; BSu2, PhSu5, FSu3. Group O: *B. subtilis*; BSu1, PhSu4, FSu3., BSu2, PhSu1, FSu3. Group P: *B. subtilis*; BSu2, PhSu1, FSu3. Group Q: *B. subtilis*; BSu1, PhSu1, FSu3., BSu2, PhSu1, FSu3., BSu2, PhSu4, FSu3., BSu2, PhSu5, FSu3. Physiological, phage and fatty acid types given in Table 4.

gram (Fig. 3) strains of biotype BMy2 (groups L and O) were located in a different branch than the strains of biotypes BMy1 (group J) and BCe1 (groups C, H and I). This shows that the rhizoid growth pattern does not coincide with the other characteristics that distinguish strains of *B. mycoides/cereus* group. *B. cereus* strains of phage type *PhCe*2, and *B. mycoides* strains of phage type PhMy1 shared by ATCC strains 7064 (*B. cereus*) and 11986 (*B. mycoides*) respectively, were identical to those found on the surface of Norway spruce needles in Finland (Väisänen & Salkinoja-Salonen, unpublished results), but differed from those found in paper and board machines (Väisänen & Salkinoja-Salonen, 1989).

The inoculated *B. pumilus* (K74A, Kallio, 1974) and the reisolated *B. pumilus* strains shared the same phage type (PhPu5), but fell into two clusters according to fatty acid analysis (FPu2 and FPu3, Fig. 1). *Bacillus* species are dominant also at paper and board

Table 4. *Summary of the results from the three different methods for typing Bacillus strains isolated from inoculated Norway spruces*

Bacillus species	Number of strains	Bio-type	Phagetype	Fatty acid type
B. cereus	27	BCe1	PhCe2	FCe1
B. mycoides	2	BMy1	PhCe2	FCe1
	2	BMy2	PhMy1	FCe1
B. pumilus	32	BPu1	PhPu5	FPu2
	6	BPu1	PhPu5	FPu3
B. subtilis	1	BSu1	PhSu1	FSu2
	2	BSu1	PhSu1	FSu3
	3	BSu1	PhSu4	FSu2
	2	BSu1	PhSu4	FSu3
	1	BSu1	PhSu24	FSu2
	1	BSu2	PhSu1	FSu2
	3	BSu2	PhSu1	FSu3
	1	BSu2	PhSu4	FSu3
	4	BSu2	PhSu5	FSu3
	4	BSu2	PhSu24	FSu2

machines, the dominant species being *B. licheniformis*, *B. cereus* and *B. polymyxa* group. *B. pumilus* was found sporadically and was of the same fatty acid type but different phage type than in living tree (Väisänen & Salkinoja-Salonen, 1989).

The *B. subtilis* variety PhSu1, FSu3, found in this study, was similar to those found in needles of Norway spruce (Väisänen & Salkinoja-Salonen, unpublished results). Five other *B. subtilis* strains forming a biovar PhSu24, FSu2, have not been reported elsewhere.

The present work thus shows that while species of the genus *Bacillus* were the most dominant gram positives in living Norway spruce, only a few species, and of these species only few phage types were present. *B. pumilus* was dominant remaining unchanged for years in the spruce xylem and occurring only in one biotype, phage type, and two fatty acid types. *B. subtilis* was seen in many phage types. It thus seems that the living tree is a highly selective environment, suggesting specific interaction between the host tree and its endophyte bacteria. Other environments, where *Bacillus* species are dominant, such as paper and board machine, where cellulose and the other wood polymers are available, display a much wider spectrum of *Bacillus* (Väisänen & Salkinoja-Salonen, 1989).

The identification based on the whole-cell fatty acid analyses correlated well with that based on physiological tests and cell morphology. Of the 91 strains of Norway spruce studied here, all but 6 *B. pumilus* strains could be assigned to the correct species on basis of fatty acid analysis alone. Whole-cell fatty acid analysis thus proved a suitable routine method for determining *Bacillus* inhabiting living spruce.

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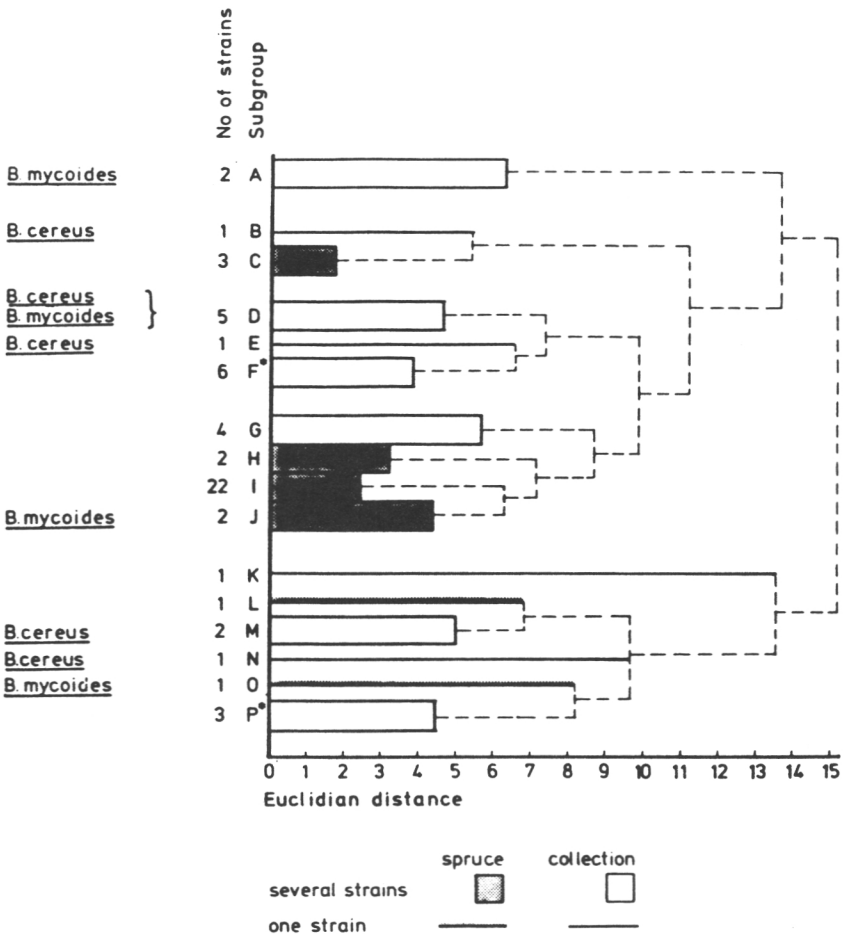

Fig. 3. Comparison of whole-cell fatty acid compositions of *B. cereus* and *B. mycoides* strains isolated from inoculated Norway spruce and culture collection strains. Fatty acid subgroups: A: *B. mycoides*; NCFB-682, NCFB-718. B: *B. cereus*; NCFB-634. C: *B. cereus*; NS-114, NS-116, NS-117. D: *B. cereus*; ATCC-27877, NCFB-719, NCFB-721, NCFB-827. *B. mycoides*; NCFB-1136. E: *B. cereus*; NCFB-1938. F: *B. cereus*; ATCC-7064, DSM-31 (type strain)*, NCFB-719, NCFB-720, NCFB-722, NCFB-723. G: *B. cereus*; ATCC-12826, NCFB-577, NCFB-579, NCFB-836. H: *B. cereus*; NS-115, NS-118. I: *B. cereus*; NS-20a, NS-55, NS-56, NS-57, NS-58, NS-59, NS-60, NS-61, NS-62, NS-77, NS-78, NS-79, NS-80, NS-81, NS-84, NS-85, NS-86, NS-87, NS-88, NS-89, NS-90, NS-91. J: *B. mycoides*; NS-54, NS-83. K: *B. mycoides*; NCFB-826. L: *B. mycoides*; NS-76. M: *B. cereus*; NCFB-578, Wageningen-B1. N: *B. cereus*; NCFB-826. O: *B. mycoides*; NS-113. P: *B. mycoides*; ATCC-6462 (type strain)*, NCFB-1152, Wageningen-B2. Strain numbers from NS-54 to NS-118 were isolated from inoculated Norway spruce. NCFB = National Collection of Food Bacteria (AFRC Institute of Food Research, Reading Laboratory, England); Wageningen = Culture collection of the Agricultural University of Wageningen, The Netherlands; DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG); ATCC = American Type Culture Collection (Rockville Md., USA).

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Table 5. *Distribution of Bacillus types isolated from Norway spruce inoculated one and five growing seasons earlier*

Combinations* of biotypes (B), phage types (Ph), and fatty acid types (F)

Tree code	*B. cereus* B Ph F	*B. mycoides* B Ph F	*B. pumilus* B Ph F	*B. subtilis* B Ph F
One growing season				
13	1 2 1 (10)	2 1 1 (1)	1 5 2 (5)	2 1 3 (3)
			1 5 3 (2)	2 5 3 (1)
14	1 2 1 (3)	1 2 1 (1)	1 5 2 (4)	2 4 3 (1)
			1 5 3 (2)	2 5 3 (1)
15	1 2 1 (3)	2 1 1 (1)	1 5 2 (4)	2 24 2 (2)
16	1 2 1 (3)	–	1 5 2 (3)	2 24 2 (2)
Five growing seasons				
3	–	–	1 5 2 (2)	1 4 3 (2)
				1 4 2 (2)
6	–	–	1 5 2 (4)	1 1 3 (1)
			1 5 3 (2)	1 24 2 (1)
9	–	–	1 5 2 (3)	1 1 3 (1)
				1 1 2 (1)
				1 4 2 (1)
12	1 2 1 (9)	1 2 1 (1)	1 5 2 (5)	2 1 2 (1)
				2 5 3 (2)

* Type numbers are as in Table 4.

Numbers of strains with each combination are given in parentheses.

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IV

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Bacteria Inhabiting Artificially Inoculated Xylem of *Picea abies*

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Bacteria inhabiting the xylem of Norway spruce (*Picea abies* (L.) H. Karsten) were investigated. The trees had been wounded and artificially inoculated with fungi and bacteria obtained from wounds of naturally infected spruce. One and five growing seasons after inoculation the Gram-negative bacterial population present in the stem of inoculated trees were analysed.

The Gram-negative bacteria isolated from the trees were identified on the basis of morphological, biochemical and physiological tests and whole-cell fatty acid composition. The predominant strains were *Enterobacteriaceae* fermenter strains (*E. agglomerans* or *E. sakazakii*), fluorescent and yellow pigmented *Pseudomonas*, *Acinetobacter* and *Moraxella* spp. All Gram-positive bacteria were *Bacillus* species.

The Gram-negative bacteria of Norway spruce differed from the Gram-positive species in possessing stronger lipolytic activity and in their ability to utilize pine resins for growth. Gram-positive bacteria were generally able to utilise cellulose and hemicellulose, whereas among the Gram-negative bacteria only one xylanolytic (yellow *Pseudomonas*) strain was found. *Key words: chemotaxonomic methods, whole-cell fatty acids, xylan, pectin, cellulose, pine resin, pine oil, Enterobacter, Pseudomonas, Acinetobacter, Moraxella, Enterobacteriaceae, Bacillus.*

INTRODUCTION

Wounding is known to promote the invasion of bacteria into the woody interior of trees (Kallio, 1973; Bonnemann, 1979; Roll-Hansen & Roll-Hansen,1980; Hallaksela, 1984; Solheim & Selås, 1986; Ali El Atta & Hayes, 1987). However, little is known about the subsequent interaction between bacteria and the woody tissues within a living tree, in particular as to whether or not they initiate decay in the wounded tree. The limited number of bacterial species known to be able to gain access to the xylem of living trees include members of the family *Enterobacteriaceae* e.g. *Erwina* spp. (Bacon & Mead, 1971) and *Enterobacter* spp. (Bagley et al., 1978) in addition to *Pseudomonas* (Mireku, 1981; Schmidt & Kebernik, 1984).

In order to gain a better understanding of occurrence and degradation abilities of bacteria in a wounded living tree, we isolated and identified bacteria from Norway spruce (*Picea abies* (L.) H. Karsten). Identification was based on cell morphology, physiological and biochemical properties, whole-cell fatty acid analysis and the ability of the isolated bacteria to hydrolyse wood components. The present paper deals with the Gram-negative species and follows a recent report characterizing the Gram-positive bacterial population in this conifer species (Hallaksela et al., 1991).

MATERIAL AND METHODS

Eight healthy, 80-year-old Norway spruce trees, from a stand in Ruotsinkylä (60°22′ N, 20°0′ E) were inoculated with mixtures of fungi and bacteria (*Bacillus pumilus* K74A and *Enterobacter agglomerans* K74B). Experimental design, inoculation and isolation of bacteria were as described by Hallaksela et al. (1991). The inoculated fungi (Hallaksela, 1984) and bacteria (Kallio, 1974) were all originally isolated from Norway spruce trees.

Morphological, physiological and biochemical characterization

Morphological, physiological and biochemical characterization of the bacterial strains was performed using the following tests: 1. Reaction to the Gram stain, 2. Glucose utilization and gas production (Hugh & Leifson, 1953), 3. Fluorescence on Pseudomonas agar F (Difco 0448-01), 4. API 20NE (nonfermenters), or API 20E and API 50CHE (fermenters) strip tests (API Systems S.A., La Balme les Grottes, Montalieu Vercieu, France), 5. Depolymerization of DNA on DNA test agar w/methyl green (Difco 0220-01-3), 6. Hydrolysis of corn oil (mixed triglycerides of myristic, palmitic, stearic, hexadecanoic, oleic, and linoleic acids) on Lipase medium (Ewing, 1986, p. 522), 7. Hydrolysis of Tween 20 (sorbitol ester of lauric acid), and Tween 80 (sorbitol ester of oleic acid) (Bergan, 1981, p. 678), 8. Hydrolysis of extracted pine resin; monitored as clear zones under growing colonies on agar plates containing 1.5% water agar with an overlay of 5 ml bacterial medium amended with aqueous spruce wood extract (Kallio, 1973) supplemented with 0.5% pine resin, pine oil or pine fatty acid fractions (pine resin fractions were a gift from Kari Honkanen (Veitsiluoto Oy, Oulu),

Table 1. *The sources of the reference strains and other microbes used*
ATCC = American Type Culture Collection (Rockville Md., USA), CDC = Center for Disease Control (Atlanta, Georgia, USA), DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG), NS = isolated from Norway spruce xylem (Hallaksela et al., 1991), MV = isolated from Norway spruce needles (M. Salkinoja-Salonen and O. Väisänen, unpub. results).
T = type strain

Bacterial species	Strain
Acinetobacter calcoaceticus	ATCC 19606
Bacillus cereus	NS-88
Bacillus circulans	NS-119
Bacillus pumilus	NS-34, -66
Bacillus subtilis	NS-75
Enterobacter agglomerans	CDC 1461–67 (T)
Flavobacterium capsulatum	ATCC 1466 (T), DSM 30196
Flavobacterium devorans	DSM 30198
Hafnia alvei	ATCC 1164
Pseudomonas fluorescens	ATCC 13525 (T)
Pseudomonas paucimobilis	ATCC 29837 (T)
Pseudomonas putida	PAW 1; Dr. D. A. Williams, University of North Wales, Bangor, UK
Pseudomonas syringae	1; Dr. D. Sands, Montana State University, Bozeman
Pseudomonas vesicularis	ATCC 11426 (T)
Pseudomonas, yellow	MV-43A, -43B, -44A, -45A, -46B, -47, -60
Serratia liquefaciens	ATCC 27592 (T)
Steureum sanquinolentum	NS-L50, NS-96
Yersinia kristensenii	IHI 40938; National Public Health Institute, Finland

9. Hydrolysis of starch, 10. Hydrolysis of cellulose using cellulose-azure (Couts & Smith, 1976, Sigma C-7391), 11. Hydrolysis of pectin on the medium of Cuppels & Kelman, 1974 at pH 6.8, sodium polypectate (M. Burger Enterprise, Madison, U.S.A), 12. Hydrolysis of xylan by using remazol brilliant blue R-D-xylan (Biley et al., 1985; Sigma M-5019), 13. Hypersensitivity test on tobacco (*Nicotiana tabacum* cv. White Barley) for some *Pseudomonas* strains (NS-202, NS-212, NS-223 and NS-261) as described by Klement et al., 1964.

The microbes used in wood component hydrolysis tests included the Gram-positive bacteria as *Bacillus pumilus*, *B. subtilis*, *B. circulans*, *B. cereus* and the decay fungus *Stereum sanguinolentum* (Table 1), isolated from Norway spruce (NS strains) as described earlier by Hallaksela et al. (1991), and the Gram-negative bacteria described in this paper.

Whole-cell fatty acid analysis

Whole-cell fatty acids were analysed from 24 h bacterial cultures as described in Väisänen et al. (1989) using the Microbial Identification System, MIS (Microbial ID, Newark, Delaware, versions 3.0 and 3.2, and CLIN Library).

For comparative proposes the fatty acid profiles of yellow *Pseudomonas* strains, similarly isolated and identified, from needles (M. S. Salkinoja-Salonen & O. Väisänen, unpublished results) and xylem of Norway spruce are also described. Two slowly growing yellow pigmented *Pseudomonas* strains (NS strains 230 and 232) isolated from the xylem of Norway spruce and most of the strains from spruce needles were analysed after 96 h and 168 h in culture, respectively.

RESULTS

Bacterial population isolated from wounded and inoculated Norway spruce after one and five growing seasons

A total of 1582 bacterial strains were isolated from eight individual Norway spruce trees of which 296 (19%) were Gram-negative (Table 2). Gram-negative bacteria were less dominant as compared to the Gram-positives in all but one tree (tree no. 3 in Table 2). This individual tree had been felled in October, five growing seasons after wounding and inoculation with microbes, before permanent frost while the others were felled after the onset of frost (Table 2).

The 296 Gram-negative strains were subjected to API 20E (fermenters) or API 20NE (nonfermenters) strip tests and for fluorescence. Further characterization of ca. 20% of the strains from each tree was made using whole cell fatty acid analysis and API 50CHE (fermenter strains) and the DNAse test.

Fermenter strains

In addition to 41 fermenter bacteria a strain similar to *E. agglomerans* K74B, originally inoculated into the wounded trees, was recovered from all the trees. These NS strains were identified from the physiological and biochemical tests as *Enterobacter agglomerans* (25 NS strains, and K74B), or *E. sakazakii* (16 NS strains). Both species were very similar, differing only in two important characters; production of arginine dihyrolase and lysine decarboxylase (Table 3). All the *Enterobacteriaceae* strains were found in two fatty acid clusters; H (38 NS strains, K74B, and 4 reference strains) and K (3 NS strains) which did not coincide with the above mentioned species as identified by API (Fig. 1).

The fatty acid cluster H contained strains with similarities to both *E. agglomerans* and *E. sakazakii*. The type strains of *E. agglomerans* (CDC 1461–67) and *Serratia liquefaciens* (ATCC 27592) and the reference strains of *Hafnia alvei* (ATCC 11604) and *Yersinia*

Table 2. *Numbers of Gram-negative bacteria isolated from Norway spruce one and five growing seasons after wounding and inoculation*

Tree code	Month of tree felling	Total no. of bacteria	Number of Gram-negative strains			% of all strains
			Fermenters	Nonfermenters	Total	
One growing season post wounding:						
13	January	350	54(12)*		54	15
14	January	256	50(8)	19(4)	69	27
15	January	250	4(2)	11(2)	15	6
16	January	89	8(3)	12(4)	20	22
Five growing seasons post wounding:						
3	October	112	42(7)	39(10)	81	72
6	January	220	10(4)	11(3)	21	10
9	January	91	17(4)	8(2)	25	27
12	January	214	7(1)	2(1)	9	4
Total		1582	192(41)	80(26)	296	19

* The number of Gram-negative strains chosen for closer characterization are indicated within parentheses.

kristensenii (IHI 40928) were also found within this cluster too. The cluster K contained 3 strains with the physiological characteristics of *E. agglomerans*.

Sixteen NS strains in the H cluster were biochemically similar to *E. sakazakii* and differed from *S. liquefaciens* (ATCC 27592) and *H. alvei* (ATCC 11604) by producing arginine dihydrolase and not producing ornitine decarboxylase. As the reference strain of *Y. kristensenii* was found in the fatty acid cluster H, the NS-strains were further tested for growth on blood agar. The one positive NS strain was found in the fatty acid cluster K and differed from *Y. kristensenii* by not producing urease, nor acid from inositol or sorbitol, and by its ability to produce acid from melibiose and raffinose.

The K cluster fermenters differed from those within H, in the fatty acid dendrogram, by more than 20 units. No reference strain was available to cross match them. Thus there was no evidence for new species designations other than *E. agglomerans* or *E. sakazakii* which were the species identified on basis of their physiological traits (Fig. 1 and Table 3).

Nonfermenter strains

The nonfermenter spruce strains (26 NS strains in total) were distributed among twelve fatty acid clusters (Fig. 1).

Fatty acid clusters A, B, C, and D contained strains with low physiological activity and were identified as *Acinetobacter* (2 NS strains) and *Moraxella* (4 NS strains) (Table 3).

The reference strain of *A. calcoaceticus* (ATCC 19606) together with the *Acinetobacter* NS strains clustered in the fatty acid cluster A, but the latter differed from the reference strain in showing fewer positive assimilation reactions. The four *Moroxella* NS strains each had distinct fatty acid compositions which confirmed the four different fatty acid clusters.

The remaining nonfermenting strains (fatty acid clusters E, F, G, I, J, L, M, and N) were assigned to the genus *Pseudomonas* on the basis of their physiological and biochemical properties. The clusters E and F were composed of single strains (Table 3 and Fig. 1). The strain NS-223 (cluster F) was identified as *Pseudomonas facilis* using the MIS.

Table 3. *Physiological characteristics of strains of Gram-negative bacteria isolated from wounded and inoculated Norway spruce*

Reactions: +, positive; −, negative; v = variable; nt = not tested, Positive reactions with all strains tested: Production of acetoin, Acid from: Glycerol, L-arabinose, Ribose, D-xylose, Galactose, Glucose, Fructose, Mannose, Mannitol N-acetyl-glucosamine, Arbutin, Maltose, Trehalose, Gluconate, 2-ketogluconate. Negative reactions with all strains tested: Production of indole, Acid from: Erythritol, D-arabinose, L-xylose, Adonitol, Dulcitol, α-methyl-D-mannoside, Amygdalin, Inulin, Glycogen, Xylitol, D-lyxose, D-tagatose, L-arabitol.

	Enterobacter:			*Pseudomonas:*					
Bacterial strains:	*sakazakii*	*agglomerans*	*agglomerans***	*fluorescent*	yellow	*P. vesicularis****	spp.	*Acinetobacter* sp.	*Moraxella* sp.
Number of strains:	16	25	1	9	8	1	2	2	4
Fluorescence	nt	nt	nt	+	−	−	−	nt	nt
β-galactosidase	+	+	+	−	+	+	−	−	−
Lysine decarboxylase	+	−	−	nt	nt	nt	nt	nt	nt
Ornithine decarboxylase	−	−	−	nt	nt	nt	nt	nt	nt
Citrate utilization	+	+	+	nt	nt	nt	nt	nt	nt
Urease	−	−	−	−	−	−	−	−	−
Gelatinase	−	−	−	V	−	−	V	−	−
DNAse	+	+	+	nt	nt	nt	nt	nt	nt
Nitrate reduction	+	+	−	V	V	−	V	+	−
Cytochrome oxidase	−	−	−	+	V	+	+	−	+
Acid from:									
Glucose (ferm.)	+	+	+	−	−	−	−	−	−
Sorbose	−	−	−	nt	nt	nt	nt	nt	nt
Rhamnose	+	+	+	nt	nt	nt	nt	nt	nt
Inositol	−	−	−	nt	nt	nt	nt	nt	nt
Sorbitol	−	−	−	nt	nt	nt	nt	nt	nt
α-methyl-D-glucoside	+	+	+	nt	nt	nt	nt	nt	nt
Esculin	+	+	+	V	+	+	−	−	−
Salicin	+	+	+	nt	nt	nt	nt	nt	nt
Cellobiose	+	+	+	nt	nt	nt	nt	nt	nt
Lactose	+	+	+	nt	nt	nt	nt	nt	nt
Melibiose	+	+	+	nt	nt	nt	nt	nt	nt
Sucrose	+	+	+	nt	nt	nt	nt	nt	nt
Melezitose	−	−	−	nt	nt	nt	nt	nt	nt
Raffinose	+	+	+	nt	nt	nt	nt	nt	nt
Gentiobiose	+	+	−	nt	nt	nt	nt	nt	nt
D-turanose	+	+	+	nt	nt	nt	nt	nt	nt
D-fucose	−	−	−	nt	nt	nt	nt	nt	nt
L-fucose	−	−	−	nt	nt	nt	nt	nt	nt
D-arabitol	+	+	+	nt	nt	nt	nt	nt	nt
5-ketogluconate	−	−	+	nt	nt	nt	nt	nt	nt
Assimilation of:									
Glucose	nt	nt	nt	+	+	+	+	−	−
Arabinose	nt	nt	nt	+	+	−	+	−	+
Mannose	nt	nt	nt	+	+	−	V	−	−
Mannitol	nt	nt	nt	+	V	−	−	−	−
N-acetyl-glucosamine	nt	nt	nt	+	+	−	V	−	−
Maltose	nt	nt	nt	−	+	+	V	−	−
Gluconate	nt	nt	nt	+	V	−	+	−	−
Caprate	nt	nt	nt	+	−	−	−	+	−
Adipate	nt	nt	nt	−	−	−	V	−	−
Malate	nt	nt	nt	+	V	−	+	+	−
Citrate	nt	nt	nt	+	+	−	V	−	−
Phenylacetate	nt	nt	nt	−	−	−	V	−	−
Phytopathogenity	nt	nt	nt	−*	nt	nt	nt	nt	nt

* Fluroescent NS strains (202, 212, 223, 261) were tested, ** = the inoculated strain K74B, *** = NS strain 268

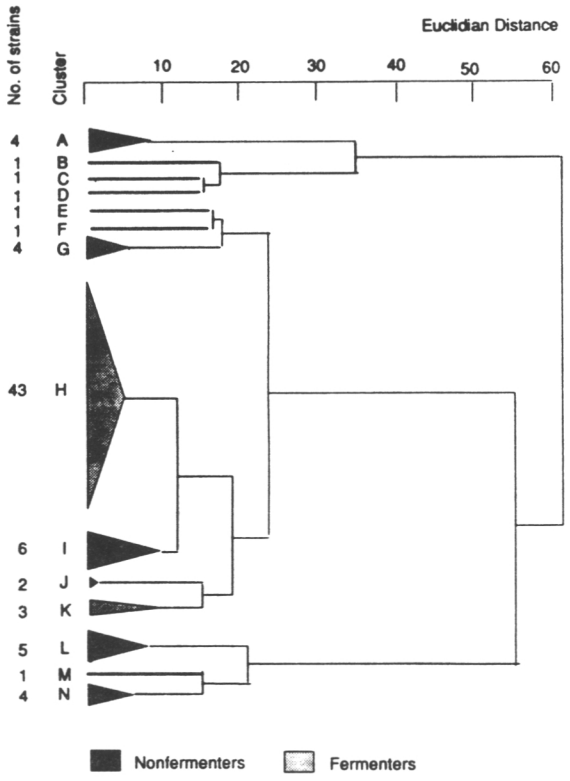

Fig. 1. Whole-cell fatty acid dendrogram of Gram-negative bacteria isolated from inoculated Norway spruce and selected reference strains. Cluster A: *Acinetobacter calcoaceticus* ATCC 19606 *Acinetobacter* sp. NS-201, -203, *Moraxella* sp. NS-206; Cluster B: *Moraxella* sp. NS-264: Cluster C: *Moraxella* sp. NS-207; Cluster D: *Moraxella* sp. NS-210; Cluster E: *Pseudomonas* sp. NS-235; Cluster F: *Pseudomonas* sp. NS-223; Cluster G: *Pseudomonas syringae* 1, fluorescent *Pseudomonas* NS-212, -202, -205; Cluster H: *Enterobacter agglomerans* CDC 1461-67 (T), *Hafnia alvei* ATCC 11604, *Serratia liquefaciens* ATCC 27592 (T), *Yersinia kristensenii* IHI 40928, *Enterobacter agglomerans* NS-K74B (Inoculated strain), NS-204, -208, -209, -211, -214, -217, -218, -226, -231, -234, -236, -238, -239, -240, -241, -246, -247, -248, -251, -254, -265, -267, *E. sakazakii* NS-227, -228, -229, -233, -242, -243, -244, -245, -249, -250, -253, -255, -256, -257, -260, -266; Cluster I: *Pseudomonas fluorescens* ATCC 13525 (T), *P. putida* PAW 1, fluorescent *Pseudomonas* NS-252, -258, -262, -269: Cluster J: fluorescent *Pseudomonas* NS-259, -261; Cluster K: *E. agglomerans* NS-220, -271, -272; Cluster L: *Pseudomonas paucimobilis* ATCC 29837 (T), yellow *Pseudomonas* NS-215, -225, -270, -273; Cluster M: yellow *Pseudomonas* NS-224; Cluster N: yellow *Pseudomonas* NS-213, -230, -232, *P. vesicularis* NS-268. Strains coded as NS were isolated from Norway spruce. Reference strains in Table 1.

Clusters G, I, and J contained fluorescent *Pseudomonas* (9 NS strains) strains (Fig. 1) which were physiologically homogeneous with differences being restricted to the hydrolysis of esculin or gelatin and nitrate reduction (Table 3). Of the fluorescent *Pseudomonas* reference strains, *P. syringae* 1 was detected in cluster G and the type strain *P. fluorescens* (ATCC 13525) together with the reference strain *P. putida* PAW 1 in cluster I. The Norway spruce strains physiologically resembled *P. syringae* 1 and type strain *P. fluorescens* but differed from *P. putida* (PAW 1) in exhibiting more positive assimilation reactions. Moreover the lack of detectable tissue hypersensitivity, hydrolysis of pectate and the positive oxidase reaction indicated that none of these strains were clearly related to the true plant pathogen *P. syringae* (Table 3).

Clusters L, M, N consisted of yellow pigmented *Pseudomonas* species (9 NS strains). The species were quite similar, but varied with respect to the reduction of nitrate, the oxidase reaction, the assimilation of mannitol, gluconate or malate (Table 3) and were identical or closely related to *P. paucimobilis*. Most of the NS strains strongly resembled the *P. paucimobilis* type strain (ATCC 29837) within cluster L (Fig. 1).

One strain in cluster N (NS-268) differed from the others (Table 2 and Fig. 1) and except for the assimilation of malate resembled the *P. vesicularis* type strain (ATCC 11426). The MIS version 3.0 also identified the strain as *P. vesicularis*.

Comparison of the yellow pigmented Pseudomonas strains in the needles and the xylem of Norway spruce

The *P. paucimobilis* fatty acid types in cluster L and N were compared with yellow pigmented *Pseudomons* strains isolated from needles of Norway spruce (M. Salkinoja-Salonen & O.

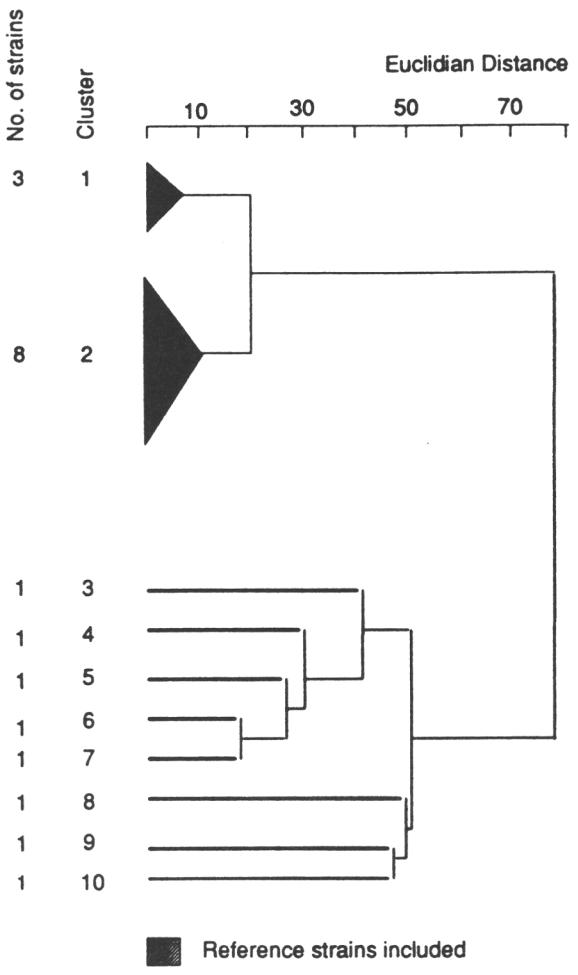

Fig. 2. Comparison of yellow *Pseudomonas* strains isolated from xylem (NS) and neeldes (MV) of Norway spruce and reference strains of *Pseudomonas paucimobilis*, *Flavobacterium devorans* and *F. capsulatum*. Cluster 1. NS-213, -230, -232; Cluster 2. *P. paucimobilis* ATCC 29837, *F. devorans* DSM 30198, *F. capsulatum* ATCC 1466, DSM 30196, NS-215, -225, -270, -273; Cluster 3. MV-56; Cluster 4. MV-44A; Cluster 5. MV-43A; Cluster 6. MV-43B; Cluster 7. MV-46B; Cluster 8. MV-45A; Cluster 9. MV-47; Cluster 10. MV-60.

Väisänen, unpublished results) and the *P. paucimobilis*, *Flavobacterium devorans* and *F. capsulatum* reference strains (Table 1). In the fatty acid dendrogram (Fig. 2) the yellow pigmented strains, originated from needles (MV strains) and from the tree xylem (NS strains), clearly formed two seperate clusters with the reference strains falling into the cluster containing the xylem strains.

Hydrolysis of wood components by microbes isolated from Norway spruce

The ability of the microbes isolated from Norway spruce to hydrolyse different wood components is shown in Table 4. Positive lipolytic activity was found among the nonfermenting Gram-negative bacteria of both the fluorescent *Pseudomonas* and *Acinetobacter* spp. (Table 4). The hydrolysis of short (Tween 20) or long (Tween 80) chain fatty acid esters as well and the pine resin fractions were typical characteristics displayed by the fluorescent *Pseudomonas* strains (Table 4). The abilities to hydrolyse cellulose and/or xylan (hemicellulose component) found in Gram-positive NS strains of *Bacillus subtilis*, *B. pumilus*, and *B. circulans*, were generally absent in Gram-negative bacteria. Xylanolytic activity was greater around colonies of Gram-positive bacteria than around the wood decaying fungus *Stereum*

Table 4. *Utility of wood components by Gram-negative bacteria, Bacillus strains, and decaying fungus isolated from Norway spruce.*
Reactions: +, positive; −, negative; nt = not tested

	Gram-negatives Fermenters:			Nonfermenters:					Gram-positives					
	Enterobacter			*Pseudomonas*					*Bacillus*					
Bacterial strains:	*sakazakii*	*agglomerans*	*agglomerans*	fluorescent	yellow	*vesicularis*	*Acinetobacter sp.*	*Moraxella sp.*	*pumilus*	*pumilus*	*subtilis*	*circulans*	*cereus*	*Stereum sanguinolentum*
Strains code:			K74B			268	201	210	34	66	75	119	88	L50,96
Number of strains:	3	4	1	5	2	1	1	1	1	1	1	1	1	2
Hydrolysis of:														
Corn oil	−	−	−	+	−	−	+	−	−	−	−	−	−	−
Tween 20	−	−	−	+	−	−	+	−	+	+	+	+	−	−
Tween 80	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Utilization of:	−													
Pine resin*	−	−	−	+	+	−	+	−	+	+	−	−	−	+
Pine oil*	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Pine fatty acid*	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Starch	−	−	−	−	+	+	−	−	−	+	+	+	−	−
Cellulose	−	−	−	−	−	−	−	−	−	+	+	+	−	+
Pectate	−	−	−	−	−	−	−	−	+	−	−	+	−	−
Xylan	−	−	−	−	V	−	−	−	+	+	+	+	−	+

* Represent different fractions of pine resin

sanguinolentum (Table 4) also isolated from Norway spruce. The Gram-negative fermenters were able to utilize the hydrolysed products of xylan and cellulose e.g. as cellobiose (Table 2).

DISCUSSION

In addition to our earlier work the present study has shown that Gram-negative bacteria were less dominant in the xylem of Norway spruce as compared to *Bacillus* species (Hallaksela et al., 1991). The Gram-negative bacteria were only detected in significant numbers after the tree had been felled before the onset of frost (Table 2). Gram-negative bacteria may thus disappear from the trees during the winter (Henis, 1987, pp. 13–18) and subsequently recolonize the tree during the warm season. Liese and Schmidt (1986) have shown that although the dimensions of Gram-negative bacteria are sufficiently small to enable movement in the water stream of spruce xylem, they are only able to travel the length of a few cells.

Conditions of long term frost as seen in the Nordic countries seems to block the movement of Gram-negative bacteria in trees and thus may counteract spread of bacterial disease, mainly produced by plant pathogenic Gram-negative bacteria. Diseases linked to *Pseudomonas* spp. such as tip dieback have been reported in tree nurseries in USA (Canfield et

al., 1986), as dieback or canker of apricot trees in France and Hungary (Arnoux, 1986; Klement et al., 1984), as dieback combined with frost in poplars in the Netherlands (de Kam, 1982) and as stem canker of pine in New Zealand (Dick, 1985).

Our results indicate that Gram-positive bacteria may be contributing to the wood decay process in Norway spruce through degradation of the cell wall components pectin (*B. pumilus*), xylan (*B. subtilis*, *B. pumilus* and *B. circulans*) and cellulose (*B. subtilis* and *B. circulans*). These bacteria may be related to bacteria known to cause degradation of cell walls in wood (Greaves, 1968, 1971; Nilsson and Daniel, 1983; Singh et al., 1987). Gram-negative bacteria may also help decaying fungi overcome the natural defense barriers of the tree during their penetration into the cell lumen through decomposition of resins (fluorescent *Pseudomans*, *Acinetobacter*) which are known to protect wood against fungal invasion.

Whole cell fatty acid analysis was used in this study as a tool to group bacteria isolated from Norway spruce. It was found that the currently available library software enabled relatively poor identification of the spruce Gram-negative population which contrasted with the case of the *Bacillus* where high fidelity was found (Hallaksela et al., 1991). This may suggest that the Gram-negative representatives of the respective species were less related to those isolated from other sources and used as the basis of the computer libraries.

The fermenters isolated from Norway spruce were identified as *E. agglomerans* or *E. sakazakii* following biochemical testing. Arginine dihydrolase and lysine and ornitine decarboxylase production were the most important distinguishing characters among strains of these species (Krieg & Holt, 1984, pp. 409–506). *Enterobacter* spp. have been reported in both chlorotic and healthy *Picea glauca* (Mireku, 1981). *Enterobacter* spp. have also been reported in wetwood of Populus stem (Scott, 1984) or elm (Murdock & Campana, 1983). Unfortunately, we could not conclude from their limited data if those strains were *E. agglomerans* or *E. sakazakii*.

A major part of the nonfermenters isolated from Norway spruce appeared to belong to the genus *Pseudomonas*. These isolates formed heterogeneous fatty acid clusters, but were more conserved with regard to physiological characters in forming two basic clusters containing fluorescent *Pseudomonas* and yellow pigmented *Pseudomonas*. Yellow pigmented *Pseudomonas* strains from spruce needles, possessing similar colony morphology, were found after fatty acid analysis to be quite unrelated to the xylem strains. No plant pathogenic species were found. *Pseudomonas* spp. have been identified in both chlorotic and healthy *Picea glauca* (Mireku, 1981) as well in decaying Norway spruce (Schmidt & Kebernik, 1984).

In conclusion, Gram-negative bacteria present in Norway spruce seemed to be less related to the described genera, classified on the basis of presently valid systematics (Krieg & Holt, 1984). *Bacillus* species, in contrast, represented the majority of bacteria in the spruce stem and closely resembled similar species isolated from many other sources (Hallaksela et al., 1991).

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