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Growth, Nutrient Uptake and Ectomycorrhizal Function in *Pinus sylvestris* Plants Exposed to Aluminium and Heavy Metals

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Abstract

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The present work focused on the effects of elevated concentrations of Al and heavy metals on Scots pine (*Pinus sylvestris* L.) and the potential role of ectomycorrhiza in modifying these effects.

Ectomycorrhizal colonisation enhanced the growth and nutrient uptake by seedlings. To some extent, colonisation also alleviated reduced nutrient uptake which was a feature of seedlings growing in the presence of the metals. This effect was particularly noticeable with respect to P uptake. In general, mycorrhizal seedlings grew better and had an improved P, K, Mg and S status compared with non-mycorrhizal seedlings. Significant differences were also found in nutrient uptake among seedlings colonised by different fungi. One fungus, *Hebeloma* cf. *longicaudum*, was more sensitive to the Al treatment than the pine seedlings. The use of the base cation / Al ratio as an indicator of the potential detrimental effects to trees to acidification and Al is discussed.

The production of oxalic acid was found to increase when mycorrhizal and non-mycorrhizal seedlings were exposed to Al or Cu. Colonisation by *Suillus variegatus* or *Rhizopogon roseolus*, in particular, resulted in a marked increase. These results demonstrate that there is a capacity, especially by certain ectomycorrhizal fungi, for increased production of the metal-chelating oxalic acid when root systems are exposed to increased levels of metals.

In a field experiment, spraying with solutions of Ni/Cu sulphate or acidified water did not affect the growth of small pine trees. As part of the same experiment, defoliation was carried out on the pine trees in order to reduce carbon supply below-ground. Defoliation altered the proportions of different mycorrhizal morphotypes: Tuberculate types decreased and smooth types increased. The treatment did not affect the level of mycorrhizal colonisation of short roots, which was nearly 100%.

Key words: aluminium, nickel, cadmium, copper, *Pinus sylvestris*, ectomycorrhiza, oxalic acid, defoliation

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Contents

Appendix

Papers I-IV

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

I Ahonen-Jonnarth U, Göransson A & Finlay R. Growth and nutrient uptake of ectomycorrhizal *Pinus sylvestris* seedlings treated with elevated Al concentrations and decreased levels of base cations. Manuscript for *Tree Physiology*.

II Ahonen-Jonnarth U & Finlay R. Effects of elevated nickel and cadmium concentrations on growth and nutrient uptake of mycorrhizal and non-mycorrhizal *Pinus sylvestris* seedlings. Manuscript.

III Ahonen-Jonnarth U, van Hees PAW, Lundström U & Finlay R. 2000. Production of organic acids by mycorrhizal and non-mycorrhizal *Pinus sylvestris* seedlings exposed to elevated concentrations of aluminium and heavy metals. *New Phytologist* (accepted).

IV Saikkonen K, Ahonen-Jonnarth U, Markkola AM, Helander M, Tuomi J, Roitto M & Ranta H. 1999. Defoliation and mycorrhizal symbiosis: a functional balance between carbon sources and below-ground sinks. *Ecology Letters* **2**: 19-26.

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Terms and concepts used in the thesis

- *acid soil* – a soil is acid if the pH value of its aqueous-solution phase is < 7.0. Hydrogen and aluminium are largely responsible for soil acidity. Soil acidity is common in regions where precipitation is high enough to leach appreciable quantities of exhangeable base-forming cations (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}) from the surface layers of soil. (Brady 1990).
- *base cations* – non acid cations, such as Ca^{2+}, Mg^{2+}, K^{+} are not, technically speaking, base cations (Brady 1990) which means that in soil solutions with pH < 8, they cannot react as proton acceptors (Ulrich 1995). However, they are referred to as base cations, because when adsorbed by soil colloids, they reduce acidity and increase the soil pH (Brady 1990). The proportion of cation exchange capacity (CEC) that they satisfy is usually termed percentage base saturation (Brady 1990).
- *cation binding site* or *cation exchange site* – a site to which a cation can be bound and where it can be replaced by another cation. On cell walls, these sites can be carboxylic groups. (Marschner 1995).
- *detoxification* – treatment of toxic substances by removal (e.g. into vacuols) or transformation into a lower toxicity.
- *elevated concentration* – a concentration which is higher than that an organism normally experiences in its natural environment.
- *fungal strain* or *fungal isolate* – a fungal pure culture which originates from the vegetative mycelium of one fruitbody, sclerotium or mycorrhizal root tip.
- *growth limiting level of a nutrient element in plant tissue (expressed as N or element / N ratio)* – the species specific N concentration or element/N ratio in plant tissue indicating the value below which a decrease in relative growth rate may occur. Concentrations of N needed for maximal growth rate are first determined for each species investigated. Other nutrients are then compared to N, and element/N ratios, in weight basis, are used for identification of nutrient deficiency. The deficiency levels for N and element/N ratios are based on experimental work. See Ingestad (1979), Ericsson & Kähr (1993), Ericsson et al. (1998). Even if concentrations vary among different tree species from boreal forests, element/N ratios are relatively similar (Göransson, pers. comm.). Normally only one element can be growth limiting at a certain time (Liebig's law).
- *growth under conditions of maximal relative growth rate with free access to nutrients* – genetically limited growth rate (in fixed conditions, for example, of light, temperature and CO_2). This concept is based on hydroponic studies (for example Ingestad 1979). Plants are grown in a system where roots are sprayed with nutrient solution containing low concentrations of nutrients. Plant growth is exponential so nutrient concentrations in the solution are increased exponentially during the growth period. Further experiments have revealed that maximal growth rate can be maintained even if concentrations of some nutrients

are decreased from the original concentrations of free access to nutrients. See Ingestad (1979), Ericsson & Kähr (1993), Ericsson et al. (1998).

- *luxury uptake* – uptake of a nutrient element not resulting in a growth rate increase. This can be defined using the element/N ratio in the plant tissues, or by comparing N to a specific N concentration for a certain species. See Ingestad (1979), Ericsson & Kähr (1993), Ericsson et al. (1998). It can also be defined as storage uptake. A higher uptake may lead to toxic effects.
- *metal tolerance or resistance* – the ability of an organism to cope with toxic concentrations of metals.
- *metal toxicity* – degree of inhibition of any organism function.
- *nutrient status* – relationships of nutrient concentrations in plant tissues. Evaluation of whether nutrients are in balance or imbalance can be made, for example, according to element/N ratios in the plants (see Ingestad 1979, Ericsson and Kähr 1993, Ericsson et al. 1998).
- *sensitivity to metals* – a relative description (low, moderate, high) of an ability of an organism to cope with toxic concentrations of metals.

Abbreviations

- LMW - low molecular weight
- BC – base cations (K, Ca and Mg)
- dw – dry weight

Introduction

Aims of the present investigation

Possible forest die-back has now been a source of concern to forest owners, politicians and scientists for several decades. Acidification of soil, leading to increased solubility of aluminium and heavy metals, was first put forward as a hypothesis to explain forest die-back by Ulrich et al. (1980), stimulating intensive research into Al toxicity to trees. Since then, numerous studies have examined the effects of metals upon nutrient uptake by plants. However most of these investigations have been carried out in the absence of mycorrhizal fungi, which, in most ecosystems, are crucial components involved in nutrient uptake by plants. The short roots of trees in boreal forests are practically all mycorrhizal and function as the main organs of nutrient uptake. Mycorrhizal fungi thus form an important interface between tree roots and the soil, affecting nutrient uptake and responses to potentially toxic levels of different elements. The base cation / Al ratio has been suggested to be a useful tool in models for defining critical loads in order to estimate risks for decreased growth of trees due to acidification and Al toxicity.

This work focuses on the effects of elevated concentrations of aluminium (Al) and heavy metals on Scots pine (*Pinus sylvestris* L.) and the possible role of ectomycorrhiza in modifying the effects of the metals. A possible detoxification mechanism, production of organic acids, was investigated. In another experiment, pine trees were also defoliatiated in order to decrease the amounts of carbon transported below ground. The main questions in these studies were:

- How do different ectomycorrhizal fungal species modify the effects of Al, Ni and Cd on the tree seedlings?
- How do low (Ca+Mg+K)/Al ratios affect tree seedlings?
- Could production of low molecular weight organic acids function as a defence mechanism against elevated concentrations of metals (Al, Cu, Ni, Cd)?
- Are pine trees affected by moderate Ni-Cu treatment in the field?
- Are levels of mycorrhizal colonisation or the species composition of mycorrhizal morphotypes affected by defoliation?

Mycorrhiza – a common symbiosis

Mycorrhizal symbiosis between plants and fungi was described for the first time by Frank (1885). Mycorrhiza play a role in nutrient uptake of plants affecting uptake of P, N and different micronutrients such as Cu, Zn (Smith & Read 1997). The plant host serves as the main carbohydrate source for most types of mycorrhizal fungi, and changes in the amounts of carbon transported below

ground to mycorrhizal roots may thus affect the mycorrhizal fungi. The vast majority of terrestrial plant genera in the world are mycorrhizal (Smith & Read 1997).

Different types of mycorrhiza have developed during evolution. In *endomycorrhiza* the fungus grows inside the plant cells without penetrating the host plasmalemma. Arbuscular mycorrhiza (AM) is a common type of endomycorrhiza occurring in many crop plants. In *ectomycorrhiza*, the fungus does not penetrate the host plant cells but forms a mycelial network *between* the cortical cells of the short roots called the *Hartig net.* The surface of these short roots is often covered with a structure called a *mantle* or *sheath.* This structure may vary in thickness and be more or less developed in different symbiotic associations but forms an important interface between the roots and the soil solution through which nutrients must pass. The ectomycorrhizal mantle is often connected to an *extramatrical mycelium* which extends from the root into the soil, playing an important role in nutrient acquisition (Fig. 1).

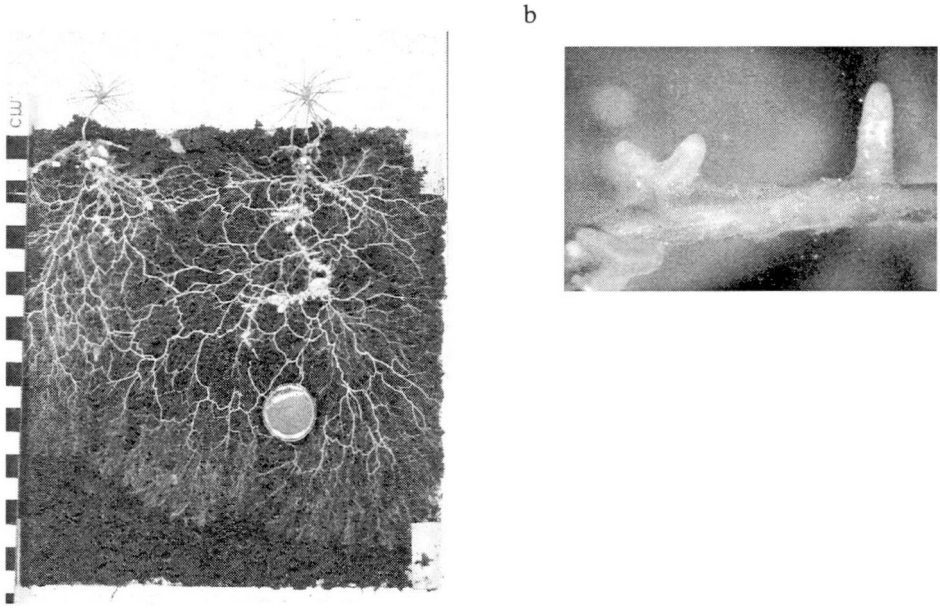

Figure 1. Ectomycorrhizal seedlings with a large extramatrical mycelium of *Suillus bovinus* (a) and mycorrhizal short roots colonised by *Laccaria bicolor* (b). The surface of these short roots is covered with a mantle which forms an important interface between the roots and the soil solution through which nutrients must pass. Inside the short roots, between the cortical cells the fungus forms a mycelial network, Hartig net. Figure (a) from R. Finlay.

This thesis concentrates on ectomycorrhiza which is the dominant type of mycorrhiza in trees of boreal forests. Almost all short roots of the trees are mycorrhizal and non-mycorrhizal tree short roots are seldom found in nature (Termorshuizen & Schaffers 1991, Nylund et al. 1995, Taylor et al. 2000).

Ectomycorrhizal fungi affect nutrient uptake of trees and are thus important to include in investigations of tree nutrient physiology. In addition, ectomycorrhiza have been found to increase metal tolerance of plants (Leyval et al. 1997, Hartley et al. 1997a, Jentschke & Godbold 2000) and should be taken into consideration when for example toxicity of Al or heavy metals is evaluated.

Aluminium

Aluminium is the third most common element in the Earth's crust. It is not classified as an essential element for plants but it has been suggested to have some beneficial effects on plant growth, for example by alleviating effects of other elements such as Cu or H (low pH) in toxic concentrations (Marschner 1995). Aluminium occurs in soil in different forms: solid-phase Al, organic and inorganic Al complexes, exchangeable Al and solution-phase Al (Schaedle et al. 1989).

The highest Al concentrations are found in mineral soil, and concentrations in the organic layers, such as humus, are lower. In Sweden, maximum values of 0.4 mM have been measured for total Al concentrations in soil solution (Bergkvist 1987, Bengtson et al. 1988). In a strongly acidified soil in Solling, Germany, concentrations as high as 1 mM Al have been found (Matzner & Prenzel 1992). In drying soil, Al concentrations may become even higher, as pointed out by Tamm & Andersson (1985). Commonly the values in relatively unpolluted soils are below 0.1 mM. The concentration of quickly reacting Al (Clarke et al 1992), also termed biologically active Al (mainly inorganic Al), is usually lower than the total Al concentration (van Hees et al. 1999). A large proportion of the Al is usually bound to organic material or, depending on pH, converted to a solid phase form such as gibbsite (Delhaize & Ryan 1995). Ionic forms of Al vary depending on pH, and solubilisation of Al increases strongly at pH values under 4.5. Toxicity of different forms of Al is difficult to study, because there are always several forms of Al present (Kinraide 1991). In addition, one form of Al, triskaidekaaluminium referred to as Al_{13}, may be toxic to plants in very low concentrations (Kinraide 1991).

Aluminium concentrations in unpolluted sites in *current year needles* of 170 and 320 ppm have been reported (Helmisaari 1990, Reich et al. 1994) and concentrations in polluted sites of up to 900 ppm (Reich et al. 1994). Values in the polluted site studied by Reich et al. (1994) were assumed to contain mainly Al taken up from soil.

Effects of Al on plants

Trees in boreal forests are generally better adapted to Al than crop plants because they grow in acid soils with higher Al concentrations. Al toxicity was suggested to be a possible cause of forest die-back by Ulrich et al. (1980) stimulating intensive research into Al toxicity to trees. It has been suggested that Al does not

affect plant growth directly but may be a factor causing nutrient imbalances (Arovaara & Ilvesniemi 1990, Ilvesniemi 1992, Janhunen et al. 1995).

In a review describing Al toxicity to plants Rengel (1996) describes the first symptoms of Al toxicity as decrease of net Ca^{2+} uptake, blockage of Ca^{2+}-channels in the plasma membrane, a decrease in net uptake of Mg^{2+} and NO^{-}_{3}, reduction in K^{+} efflux, callose accumulation and malate extrusion. Hexokinase activity, ATPase activity, DNA synthesis, tubulin assembly, calmodulin function and mitosis have all been found to be inhibited by Al (Schaedle et al. 1989). The phytotoxicity of Al, even in low concentrations, arises from its strong affinity for oxygen donor compounds such as inorganic phosphate, ATP, RNA, DNA, proteins, carboxylic acids and phospholipids (reviewed by Martin 1988). Aluminium can also induce expression of genes which have been found to be induced during oxidative stress (Richards et al. 1998).

There is some evidence which indicates that Al causes premature cell maturation and senescence in roots: vascular tissue differentiation, emergence of lateral roots, vacuolisation of root cortical cells closer to the meristem, and vacuolisation of meristematic cells also accompany exposure to toxic levels of Al (Schaedle et al. 1989). A major difficulty in the study of Al localization in roots and its relation to Al phytotoxicity is the presence of large amounts of bound Al which are presumably non-toxic and mask the toxic Al fraction in the tissues (Schaedle et al. 1989).

Al has been found to affect nutrient uptake of plants, especially uptake of base cations and P. One possible explanation is replacement of divalent cations by Al at cation exhange sites in the plant cell apoplast and further decreased uptake of cations (Keltjens 1995). One explanation for decreased uptake of Ca and Mg may be the effect of Al on the regulator protein calmodulin and further on membrane ATPase activity possibly involved in cation uptake (Siegel & Haug 1983). Another explanation could be the observed replacement of Ca and Mg from binding sites on root surfaces by electropositive Al (Godbold et al. 1988), but binding of Mg on binding sites on roots does not appear to control uptake of Mg into shoots (Godbold & Jentschke 1998). Al may decrease P availability by complexing P in the root apoplast which may cause P deficiency in the shoots (Schaedle et al. 1989, Cumming & Weinstein 1990a).

Non-mycorrhizal tree seedlings have been used in many studies of toxicity of different elements to trees. In studies by Göransson & Eldhuset (1991), non-mycorrhizal *Pinus sylvestris* seedlings grew at a similar growth rate to non-exposed control seedlings at Al concentrations as high as 6 mM under conditions of free access to nutrients whereas *Picea abies* seedlings showed decreased growth rate at 0.3 mM Al. In another study growth reduction of *P. abies* was pH dependent: at pH 3.2 root growth was decreased by 0.4 mM Al, but at higher pH growth was already decreased at 0.1 mM Al (Godbold et al. 1995). Birch (*Betula*

pendula) shows a decreased growth rate at 3 mM Al under conditions of free access to nutrients (Göransson & Eldhuset 1987). However growth of non-mycorrhizal birch seedlings has been shown to decrease with 0.2 mM Al treatment when P is the growth limiting element (Clegg & Gobran 1995). Toxic Al concentrations for different tree species have been presented by Schaedle et al. (1989). *P. sylvestris* is generally defined as a Al tolerant species, more tolerant than for example *P. abies*.

Pure culture studies of ectomycorrhizal fungi exposed to Al

Many ectomycorrhizal fungi have been found to tolerate high concentrations of Al, but there are big differences both between different species and between strains of single species exposed to Al. For example, a *P. tinctorius* strain originating from a site of old coal mining waste grew at much higher Al concentrations than strains from rehabilitated or forest sites (Egerton-Warburtin & Griffin 1995). On the other hand, Marschner et al. (1999) found a *P. involutus* strain from a less contaminated site to be more tolerant to Al than a strain from a heavily acidified site. In the study of these authors, a three compartment Petri dish system was used which enabled exclusion of P from compartments containing Al thus hindering Al-P precipitation. Differences in Al tolerance between strains of *P. tinctorius* were also found by Thompson & Medve (1984).

Effects of Al on growth and nutrient uptake vary remarkably between different ectomycorrhizal fungi. The biomass yield of *Suillus variegatus* was decreased to about half that of controls at 2 mM Al (Žel & Gogala 1989). This fungus, as well as *Paxillus involutus*, could grow on nutrient solutions containing 370 mM Al (10 g/l) (Hintikka 1988). In another study, growth of *P. involutus* isolates was decreased more than 50% in substrate containing 2.0 mM Al (Marschner et al. 1999). *Hebeloma crustuliniforme* and *Rhizopogon rubescens* showed decreased growth in solutions containing 0.37 mM Al, but they still grew at 0.74 mM Al (Browning & Hutchinson 1991). *Hebeloma mesophacus* did not grow at 13 mM Al, but growth decreased about 50 % at 3.7 mM (Kong et al. 1997). Growth of *Laccaria bicolor* was unaffected on a substrate containing 1.0 mM Al but the growth was decreased at pH values below 3.0 (Jongbloed & Borst-Pauwels 1992). Growth of different fungi in metal containing substrate has been reviewed by Hartley et al. (1997a). Exposure to Al can cause both decreased and increased uptake of Ca, Mg, K or P by ectomycorrhizal fungi depending on the growth conditions used and fungal species investigated (Browning & Hutchinson 1991, Jonbloed & Borst-Pauwels 1992, Žel & Bevc 1993).

The membrane fluidity of Al tolerant *Lactarius piperatus* has been found to increase due to Al treatment (Žel et al. 1993a). This was shown as a relative increase in amounts of less ordered membrane domains. The opposite was found with Al sensitive *Amanita muscaria* (Žel et al. 1993b). The hormonal balance of ectomycorrhizal fungi may also be affected by Al. For example cytokinin activity

in Al tolerant *L. piperatus* was increased due to a treatment of 10 mM Al (Kovač & Žel 1994).

Ca has been found to ameliorate growth retarding effects of Al on *H. mesophacus* (Kong et al. 1997) and *H. crustuliniforme* (Browning & Hutchinson 1991). However, Ca acted synergistically at high Al concentration decreasing the growth of *S. tomentosus* whereas it did not have any effect on *R. roseolus* (Browning & Hutchinson 1991), *Lactarius rufus* and *L. hepatica* (Jongbloed & Borst-Pauwels 1992). Furthermore, Ca could alleviate decreased nitrate reductase activity in *H. mesophacus* due to the Al treatments at the two pH values used, 6.8 and 4.3. Ca could also alleviate decreased acid phosphatase activity due to Al at the higher concentration at pH 6.8 (Kong et al. 1997). In plant roots, the reason for Ca alleviation of Al toxicity has been suggested to be a) Ca induced reduction in cell-surface negativity leading to Al replacement or b) restoration of Ca at the cell surface (if it has been reduced to a growth limiting level due to Al) (Kinraide 1998). Mg has been found to alleviate Al toxicity in *L. rufus* and P in *L. rufus* and *L. hepatica* (Jongbloed & Borst-Pauwels 1992).

Mechanisms of Al tolerance

Tolerance of Al has been studied in many experiments and different possible mechanisms have been suggested. However, it should be kept in mind that Al tolerance may involve simultaneous operation of a number of mechanisms and it is probably a mistake to seek simple one-mechanism responses (Taylor 1991). To decrease the toxic effects of Al, plants may exclude Al from roots or detoxify Al ions inside the plant tissues (Taylor 1991). Al may be excluded from plant cells by immobilisation at the cell wall, by inhibited uptake because of selective permeability of the plasma membrane, or by active Al efflux from the cells (Taylor 1995). Al may also be detoxified with the aid of exudates which chelate Al. Exudation of organic acids that bind Al near the sensitive root apex has been suggested as an Al tolerance mechanism (Kochian 1995). The role of organic acids is discussed further in this thesis (III). Phosphate exudation and complexing of Al is one possible mechanism with which to detoxify Al (Taylor 1991). Mucilage, gelatinous material consisting mainly of polysaccharides and polygalacturonic acid, offers some protection for roots against Al toxicity (Marschner 1995, Rengel 1996). Although mucilage is not a common product of tree roots (Schaedle et al. 1989), slime production by ectomycorrhizal fungi may play a role in metal tolerance (Denny & Ridge 1995). Raised pH has been suggested as a way of reducing Al toxicity (Bennet & Breen 1991). Direct evidence for this mechanism was obtained by Degenhardt et al. (1998) who observed an increase in rhizosphere pH due to Al in an Al-resistant *Arabidopsis* mutant in contrast to wild type plants. Low pH in soil solution may also decrease Al toxicity by reducing cell-surface potential and decrease Al binding (Kinraide et al. 1992, Delhaize & Ryan 1995, Godbold et al. 1995). However, this phenomenon is not, likely to be important in plants grown in acid soils where lower pH increases Al solubility, and proton uptake competes with Ca and Mg

uptake (Marschner 1995). Inside the plant cells, Al may be chelated in the cytosol or compartmented into vacuoles (Taylor 1995). Al-tolerant enzymes may be evolved, or enzyme activity may be increased in order to tolerate Al (Taylor 1995). Inside certain plants, Al may be bound to oxalate and thus be detoxified (Ma et al. 1997a). Al has been shown to induce expression of several genes, including genes for metallothionein-like proteins (Snowden et al. 1995), but these genes have still not been linked to an Al-tolerance mechanism (Delhaize & Ryan 1995) .

Formation of Al-phosphate granules on the mycelium of *Suillus variegatus* has been suggested to detoxify Al (Väre 1990). This may not reflect an active process but a passive Al-P precipitation when relatively high concentrations of P and Al are present in the growth substrate. Al polyphosphate complexes in vacuoles of *Laccaria bicolor* have also been shown with aid of ^{27}Al-NMR (Martin et al. 1994) when high P concentrations were used in growth media.

Ectomycorrhizal symbiosis and Al

Ectomycorrhizal colonisation has been shown to ameliorate growth reductions or decreased uptake of nutrients in tree seedlings exposed to Al. In some cases the better growth of mycorrhizal Al-exposed seedlings compared to non-mycorrhizal ones appears to be due to better nutrient uptake.

Growth decreases of *Pinus rigida*, *Pinus strobus* and *Picea abies* seedlings occurring in response to Al have been found to be alleviated by ectomycorrhizal colonisation by *Pisolithus tinctorius* or *Paxillus involutus* (Cumming & Weinstein 1990ab, Hentschel et al. 1993, Schier & McQuattie 1995, 1996). Decreased relative growth rate of *Pinus sylvestris* has been shown to occur at lower Al concentrations in non-mycorrhizal seedlings than in seedlings colonised by *Suillus bovinus* (Göransson & Eldhuset 1991). The root endodermis has been found to be the primary barrier to radial Al transport both in mycorrhizal and non-mycorrhizal spruce seedlings (Jentschke et al. 1991a).

Decreased uptake of Ca and Mg in both mycorrhizal and non-mycorrhizal tree seedlings has commonly been found due to Al exposure (Schaedle et al. 1989, Cumming & Weinstein 1990a, Schier et al. 1990, Jentschke et al. 1991ab, Schier & McQuattie 1995, 1996, Jentschke & Godbold 2000). In polluted experimental field sites in Poland, measurements of nutrient concentrations in needles indicated that Mg and possibly K were the main growth limiting nutrients (Reich et al. 1994). In experiments by Jentschke et al. (1991a), mycorrhizal colonisation of *Picea abies* by *Lactarius rufus* or *L. theiogalus* did not prevent Al from reaching the root cortex cells and displacing Mg and Ca. Not all base cation concentrations are generally decreased due to elevated concentrations of Al: concentrations of K have been found to increase with increasing Al concentrations in many studies (Schaedle et al. 1989).

Ectomycorrhizal symbiosis has been found to have an effect on P uptake of Al exposed tree seedlings. In experiments by Cumming & Weinstein (1990a), uptake of P was reduced due to Al treatment in non-mycorrhizal *Pinus rigida* but not in mycorrhizal plants. Enhanced nutrient uptake, especially of P, was concluded to be the reason for reduced Al toxicity in mycorrhizal *Pinus strobus* seedlings compared to non-mycorrhizal ones in a study by Schier & McQuattie (1995).

Effects of different nitrogen sources on Al toxicity have been found in different investigations. In *Pinus rigida* seedlings, exacerbation of Al toxicity by NO_3^- was explained by reduction in P uptake in non-mycorrhizal but not in mycorrhizal seedlings (Cumming & Weinstein 1990b). However, in other studies elevated NO_3^- did not affect Al toxicity in *P. rigida* seedlings (Schier & McQuattie 1999). Al toxicity in non-mycorrhizal and mycorrhizal *Pinus rigida* has also been found to be ameliorated by elevated NH_4^+ concentrations (Schier & McQuattie 1999).

Use of the base cation (K + Ca + Mg) / Al ratio as an indicator of Al toxicity

The Ca/Al ratio has been used to estimate toxic effects of Al on plant growth, because it has been found to correlate better with decreased growth of plants than Al or Ca concentrations themselves (Rost-Siebert 1983, Cronan & Grigal 1995). Mg was also included in the ratio as a base cation by Sverdrup et al. (1992) because uptake of both Ca and Mg was found to be affected by Al and competition between Al, Ca and Mg was suggested to occur at root surface binding sites (Godbold et al. 1988). Potassium (K) was also later included in the base cation (BC)/Al ratio (Ca+Mg+K)/Al (Sverdrup & Warfvinge 1993, Sverdrup et al. 1994). The BC/Al model assumes a rather passive uptake of base cations based on concentrations in the apoplast, which in turn depend upon concentrations in the soil solution. A BC/Al ratio of 1 has been used as a general threshold value under which damage to tree growth is plausible. For *P. sylvestris* the intermediate risk ratio has been defined as 0.6 and for *P. abies* the ratio is 0.9 (Warfvinge & Sverdrup 1995). However, this model has not been accepted by all scientists because it does not take into account biological complexity (for example mycorrhiza and production of Al chelating substances such as low molecular weight organic acids) or soil heterogeneity (concentration gradients and spatial heterogeneity) affecting nutrient uptake of trees (Högberg & Jensén 1994, Falkengren-Grerup et al. 1995, Løkke et al. 1996, Binkley & Högberg 1997).

Heavy metals

The term "heavy metals" is conventionally applied to metals having a density greater than 5 g/cm^3. Some of the heavy metals, such as Fe, Zn, Cu and Ni, are essential for plants as microelements, some are classified as beneficial (for example Co) and some others, such as Cd, are not essential, (Marschner 1995). Average concentrations in a range of plants are 100 μg Fe g^{-1} dw, 20 μg Zn g^{-1} dw, 6 μg Cu g^{-1} dw and 1-10 μg Ni g^{-1} dw (Marschner 1995). At high

concentrations heavy metals are toxic to living organisms. Critical leaf concentrations (μg g^{-1}dw) above which the growth of many species is affected are 500 for Fe, 200-300 for Zn, 15-20 for Cu, 10-50 for Ni, and 8-12 for Cd (Balsberg-Påhlsson 1989, Marschner 1995). Hyper-accumulator species, however, may contain much higher concentrations, for example 1000 μg Cu g^{-1} dw or 30 000 μg Ni g^{-1} dw (Marschner 1995).

Total heavy metal concentrations in soils are generally much higher than concentrations of so called "bioavailable" heavy metals. Bioavailable heavy metals are soluble or exhangeable metals which it is possible for organisms to take up and which are measured in soil solution after extraction, for example $CaNO_3$ or $BaCl_2$ + EDTA (Weissenhorn et al. 1995, Derome 2000). On the Kola Peninsula in Russia, 8 km from a Cu-Ni smelter, the closest sites where conifers still survive, the mean Ni and Cu concentrations in soil solution were 692 and 347 μg l^{-1}, respectively (Lindroos et al. 1996). In soil solutions in uncontaminated spruce and beech forests in one study in Sweden, the highest mean concentrations of Fe, Zn, Cu, Ni and Cd were 5000, 150, 7, 10 and 2.5 μg l^{-1}, respectively (Bergkvist 1987). Heavy metals are not distributed evenly in soils, but their distribution is patchy (Berthelsen et al. 1995). Cu and Pb are commonly accumulated in soils, whereas Zn, Cd and Ni are more mobile, especially in acidified soils (Bergkvist et al. 1989).

Effects of heavy metals on plants

General symptoms of heavy metal toxicity to plants are chlorosis and decreased growth (Foy et al. 1978). Toxic effects include disturbance of enzymes, for example those involving photosynthesis (Clijsters & van Assche 1985). Enzyme inactivation may occur via metal sensitive groups of enzymes such as SH or histidyl groups (Prasad 1995). Activity of other enzymes may increase, especially those involving stress metabolism (van Assche & Clijsters 1990). The transport of photosynthetic assimilates to sinks is affected in some cases (Balsberg Påhlsson 1989). Plasmalemma integrity may be disturbed in several ways. For example, Cu may disturb the plasmalemma via: a) oxidation and cross-linking of protein thiols, b) inhibition of proton influx because of inhibition of plasmalemma ATPase or c) production of free radicals which may cause peroxidation of unsaturated fatty acids in biomembranes (reviewed in Meharg 1993). In some cases, nutrient leakage occurs through damaged membranes (Balsberg Påhlsson 1989). Heavy metals have been found to cause deficiency of essential nutrients and they generally cause water stress, reduced CO_2 uptake and disturbances in gas exchange (Schlegel et al. 1987, Balsberg Påhlsson 1989, Barceló & Poschenrieder 1990). Hormonal effects may affect uptake of heavy metals: exposure of *Picea abies* roots to cytokinin decreased uptake of Pb to shoots (Vodnik et al. 1999).

Pure culture studies of ectomycorrhizal fungi exposed to heavy metals

Growth of ectomycorrhizal fungi on substrates containing heavy metals has been tested in several studies. Large variation in the effects of heavy metals on growth has been found between species and isolates (Hartley et al. 1997a, Blaudez et al. 2000). Variation in sensitivity to heavy metals between different fungal species has also been observed as reduced amounts of extramatrical mycelium of certain species following heavy metal treatment (Marschner et al. 1996, van Tichelen et al. 1999). This affects both the heavy metal binding capacity of the extramatrical mycelium and capacity for colonisation of new short roots. Ectomycorrhizal species differ in their capacity to bind heavy metals on the cation exhange sites on the mycelium (Marschner et al. 1998). In pure culture studies, no clear relationship has been found between sensitivity of fungal strains to heavy metals and levels of contamination of their sites of origin (Denny & Wilkins 1987a, Colpaert & van Assche 1992a, Howe et al. 1997, Blaudez et al. 2000). A possible reason for the large variability in heavy metal sensitivity could be tolerance against Mn^{2+} ions, as suggested by Hartley et al. (1997a). Mn concentrations in acid soils may increase to levels where lower uptake (as a tolerance mechanism) is advantageous. Tolerance of this divalent ion may confer tolerance of other divalent cations, for example Cd^{2+}, Zn^{2+}, Pb^{2+} and Cu^{2+}, because these ions are thought to be taken up by the same transporter as Mn^{2+}.

Tolerance in pure culture does not necessarily mean that a specific isolate is also tolerant in symbiosis. *Scleroderma flavidum* was the most sensitive fungus of four fungi tested in agar culture (Jones & Hutchinson 1986) but in symbiosis with *Betula papyrifera* it was the fungus best alleviating growth reduction due to Ni and Cu (Jones & Hutchinson 1986). On the other hand, in a study by Colpaert & van Assche (1993), although the ectomycorrhizal fungus *Thelephora terrestris* had the best growth of many fungi tested on a Cd containing substrate, it proved to be the most sensitive when growing in symbiosis with *P. sylvestris*. One factor which may affect interpretation is, that generally in pure culture studies, growth retardation due to heavy metals in liquid substrate is higher than on agar substrates. The reason may be metal complexation to particles in the agar substrate and thus higher metal exposure of the fungi grown in liquid growth medium (Hartley et al. 1997a).

Growth responses of ectomycorrhizal fungi in the presence of more than one heavy metal have seldom been investigated. Heavy metals may, in certain circumstances, ameliorate growth reduction of ectomycorrhizal fungi caused by other heavy metals. For example Pb or Sb can ameliorate growth reduction by Cd or Zn under certain circumstances and Zn can ameliorate Cd toxicity (Colpaert & van Assche 1992b, Hartley et al. 1997b).

Mechanisms of heavy metal tolerance

Different processes have been linked to heavy metal tolerance in plants. Binding heavy metals to polygalacturonic acids in cell walls has been suggested as one

possible tolerance mechanism (Ernst et al. 1992). Ion efflux and modified uptake systems at the plasmalemma, decreasing excess heavy metal uptake may also contribute to tolerance (Meharg 1993). Tolerance in plants may be gained by maintenance of membrane integrity, or protection of protein function associated with the plasmalemma (Meharg 1993). Heavy metals may also be sequestered in vacuoles as a complex with organic acids (Ernst et al. 1992, Ross & Kaye 1994). An increased ability to transport metals to vacuoles has also been suggested as a tolerance mechanism (Ernst et al. 1992). Metallothioneins may play a role by binding heavy metals (Meharg 1993). Different detoxification mechanisms occur with different heavy metals. For example, phytochelatins are effective in chelating Cu and Cd, whereas regulation of Zn levels in the cytoplasm may be through chelation to malate and compartmentalization within the vacuole (Meharg 1993, Prasad 1995). Histidine has been suggested to be the Ni chelator in Ni accumulator plants (Krämer et al. 1996). Faster fine root turnover has been suggested as an exclusion mechanism (Kahle 1993).

As an "avoidance" mechanism, fungi may reduce uptake or increase efflux of heavy metals (Leyval et al. 1997). In fungal cells, heavy metals may be bound to cell wall components such as chitin and pigments such as melanin (Gadd 1993). Tyrosinase activity, which enhances melanin pigmentation, has been found to increase in ectomycorrhizal fungi exposed to Cu (Gruhn & Miller 1991). One possible detoxification mechanism is binding heavy metals to organic acids, either outside or inside the cell. This is further discussed in this thesis. Heavy metal binding to polyphosphate granules has been discussed as a detoxification mechanism but has not yet been clearly demonstrated (Leyval et al. 1997, Hartley et al. 1997a). Difficulties arise because both the form of polyphosphate and the localisation of heavy metals may be affected by specimen preparation, in particular chemical fixation, which may cause membrane leakage and allow redistribution of chemical elements (Orlovich & Ashford 1993, Jentschke & Godbold 2000). Heavy metals may also be detoxified by binding to metallothinein-like proteins in fungi (Morselt et al. 1986, Howe et al. 1997).

Ectomycorrhizal symbiosis and heavy metals

Ectomycorrhizal symbiosis has been found to ameliorate the toxic effects of heavy metals on host plants in a number of cases (Brown & Wilkins 1985, Denny & Wilkins 1987b, Jones & Hutchinson 1986, 1988ab, Colpaert & van Assche 1993, Jentschke et al. 1999a). The capacity to alleviate toxic effects depends on the ectomycorrhizal species and heavy metals involved (Wilkins 1991, Hartley et al. 1997a, Godbold et al. 1998). *Scleroderma flavidum* decreased the growth reduction of *Betula papyrifera* exposed to 34 μM or 85 μM Ni nickel whereas *Lactarius rufus* could only provide some initial protection against Ni toxicity (Jones & Hutchinson 1986, 1988a). It is not surprising that there is large variation in this capacity, because there is a large interspecific and intraspecific variation in heavy metal sensitivity in fungi (Hartley et al. 1997a). Ectomycorrhizal fungal species themselves may be affected by heavy metals more strongly than their host

plants. In certain species, ectomycorrhizal colonisation has been observed to be decreased due to heavy metal treatment (Jones & Hutchinson 1986, Dixon 1988, Dixon & Buschena 1988), and in some cases ectomycorrhizal colonisation appears to be more sensitive to heavy metals than growth of the host plant (Hartley et al. 1999a).

There has been much discussion about the role of ectomycorrhiza in preventing excess uptake of heavy metals (see Jentschke & Godbold 2000). In some cases decreased uptake of heavy metals has been found together with heavy metal tolerance (Dixon 1988, Bücking & Heyser 1994, van Tichelen et al. 1999). Fungi producing dense extramatrical mycelium have been suggested to be able to bind and retain metals and hinder their uptake by plants (Colpaert & van Assche 1993). In experiments by van Tichelen et al. (1999), even though the biomass of *S. luteus* was reduced by 50% due to Cu treatment, the fungus prevented accumulation of Cu in the needles. Binding of metals to extrahyphal polysaccharide slime has been proposed as a mechanism of excluding Zn from plants (Denny & Wilkins 1987b, Denny & Ridge 1995). Ni precipitation by P was suggested as a detoxification mechanism against Ni (Jones & Hutchinson 1988b). In addition, the seedlings colonised by *Scleroderma flavidum* did not require metabolic energy to prevent N translocation from roots to shoots (Jones et al. 1988). Pregrowth conditions may influence the ectomycorrhizal effect on heavy metal uptake. For example, Zn uptake to shoots of *P. sylvestris* seedlings exposed to elevated Zn concentrations was lower when the seedlings were colonised by *Suillus bovinus* which was, before inoculation, grown on a Zn containing medium (Bücking & Heyser 1994). Cu or Zn uptake of *P. sylvestris* seedlings has also been shown to be dependent on their mycorrhizal status and fungal symbionts (Colpaert & van Assche 1993, van Tichelen et al. 1999). Pb have been shown to reach the cortex in a similar manner both in mycorrhizal and non-mycorrhizal short roots, and the endodermis was found to act as a barrier to radial transport in both cases (Jentschke et al. 1991c). However, inhibited transport suggesting a filtering capacity of the fungal mantle, has also been reported (Turnau et al. 1996). Localisation studies have often been performed using chemically fixed material, in which the position of heavy metals may have been changed during the process of fixation, embedding and cutting of sections (Jentschke & Godbold 2000).

One mechanism by which ectomycorrhizal fungi may ameliorate heavy metal toxicity is through improved nutrient uptake (Jentschke et al. 1999a). Cadmium toxicity (defined as decreased shoot and root growth and chlorophyll content of old needles) to Norway spruce has been shown to be alleviated by colonisation with *Paxillus involutus*, probably through improved P nutrition (Jentschke et al. 1999a). In the same experiment, *Laccaria bicolor* did not have the same kind of ameliorating effect even though both species reduced Cd concentrations in young needles compared with those from non-mycorrhizal seedlings (Jentschke et al. 1999a). Cu exposed mycorrhizal plants have also been shown to have higher P

and ammonium uptake capacities than non-mycorrhizal plants (van Tichelen et al. 1999).

Hartley et al. (1999b) performed a series of experiments with several heavy metals (Cd, Pb, Zn, Sb, Cu) in the growth substrate as single pollutants or in combination. These authors found lower relative toxicity, defined as decreased growth, when mixtures of heavy metals were used than when single metals were used. These results indicate that soils contaminated by a mixture of heavy metals might not be as toxic as results from individual investigations predict (Hartley et al. 1999b). On the other hand, indications of additive effects of Cd and Cu on non-mycorrhizal *Picea sitchensis* have also been reported (Burton et al. 1986).

Low molecular weight (LMW) organic acids

Low molecular weight organic acids are common substances in nature, playing a part in many metabolic reactions. In soils, they are released by mycorrhizal plant roots and bacteria and are produced during microbial decomposition of organic material (Fox & Comerford 1990). They are also used as nutrients by microorganisms (Lundström 1994, Jones & Darrah 1994). Organic acids can bind elements such as metals, and their role as detoxification agents has been widely discussed; they also play a role in weathering processes (Lundström 1994).

Production of organic acids, especially oxalate, is a well known phenomenon in ectomycorrhizal fungi (Cromack et al. 1979, Malajczuk & Cromack 1982 Lapeyrie et al. 1990, Griffiths et al. 1994, Sun et al. 1999). In hydroponic systems, calcium oxalate crystals have been found on non-mycorrhizal fine roots of *Picea abies* (Fink 1992).

Oxalate production has been suggested to have an important role in P solubilisation (Cromack et al. 1979, Knight et al. 1992, Griffiths et al. 1994, Cannon et al. 1995) and citric acid may play a role in K mobilisation (Wallander & Wickman 1999). Oxalate retained in hyphal mats of mycorrhizal species has been proposed to increase sulphate availability and calcium oxalate crystals may function as a reservoir of calcium (Dutton & Evans 1996). Christiansen-Weniger et al. (1992) speculated that the higher N_2 fixation they found in Al-tolerant, organic acid producing wheat, was possibly due to feeding by bacteria on the organic acids. Jones & Darrah (1994) suggested that organic acids in acid soils may have been developed as a general mechanism for micronutrient uptake and potential Al detoxification whereas these roles are less important in soils with high pH. Oxalic acid is thought to act in pathogenesis leading to weakening of cell walls through acidification of host tissue and sequestering of calcium from host cell walls (Dutton & Evans 1996). On the other hand, production of oxalic acid has also been connected to disease suppression by ectomycorrhizal fungi growing in symbiosis with plants (Duchesne et al. 1989).

Detoxification of Al and heavy metals by LMW organic acids

Aluminium has been found to increase production of organic acids by roots, especially oxalic acid (Ma et al. 1997a), malic acid (e.g. Pellet et al. 1997) and citric acid (Pellet et al. 1995, Ma et al. 1997b). The role of malate in Al tolerance may be to inhibit the blocking of the root cell plasma membrane Ca^{2+} channel (Huang et al. 1996). Citric acid has been shown to be an effective Al-chelator and has been suggested to decrease Al toxicity (Jones & Darrah 1994). On the other hand, both malate (Jones et al. 1996) and citrate (Jones & Darrah 1994) may be readily broken down by microorganisms. Elevated production of organic acids, either inside or outside the plants, has often been found in Al tolerant but not in sensitive cultivars, but the role of organic acids in Al tolerance has still not been thoroughly clarified (Jones 1998, Parker & Pedler 1998). The main role of organic acids in Al tolerance in plants may be to exclude Al from the apoplasm and symplasm of Al sensitive root apexes (Kochian 1995, Jones et al. 1996).

Higher concentrations of organic acids have been found in heavy metal tolerant plants than in sensitive plants (Thurman & Rankin 1982; Godbold et al. 1984; Harmens et al. 1994, Yang et al. 1997), but a major role of organic acids in detoxification of heavy metals has been questioned (Thurman & Rankin 1982, Harmens et al. 1994). Citrate and malate have been found in Ni hyperaccumulators (Homer et al. 1991, Sagner et al. 1998). Some copper-tolerant (fungicide tolerant) wood-rotting fungi are able to produce large amounts of oxalic acid which forms copper oxalates, but no direct correlation between oxalic acid production and copper tolerance has been shown (see Dutton & Evans 1996).

Ectomycorrhiza and decreased carbon supply

In the field experiment described in this thesis involving Cu-Ni and acid rain exposure of *P. sylvestris*, defoliation was performed on experimental trees in order to study the effects of decreased photosynthetic capacity on mycorrhizal colonisation, the composition of mycorrhizal morphotypes and growth of the trees.

Observations of a variety of plant species suggest that above ground herbivory affects mycorrhizal associations negatively by decreasing fungal colonisation of short roots (Gehring and Whitham 1994a). One explanation is that the ability of plants to support mycorrhiza decreases if herbivores remove significant amounts of photosynthetic tissues (Gehring and Whitham 1994a). Decreased carbon supply via defoliation has been found to inhibit sporophore production of ectomycorrhizal fungi (Last et al. 1979) and there are indications that current photosynthate is allocated to ectomycorrhizal fruitbodies (Lamhamedi et al. 1994). About 10 to 30% of the photosynthetically fixed carbon is estimated to be used by the ectomycorrhizal fungal partner (Fogel and Hunt 1979, Vogt et al. 1982, Finlay and Söderström 1992, Rygiewicz and Andersen 1994, Markkola et al. 1995, Smith & Read 1997). Herbivory or defoliation has been found to decrease mycorrhizal colonisation in tree roots (Gehring and Whitham 1991,

1994b, Del Vecchio et al. 1993). However Markkola (1996) did not find any decrease in mycorrhizal colonisation in a pot experiment with defoliated *Pinus sylvestris* seedlings.

Methodological considerations

Growth systems used in experiments – advantages and disadvantages

Ingestad-type hydroponic studies in which nutrients are added by frequent spraying onto root systems hanging from the lid of growth boxes (eg. Ingestad 1979) give good information on the nutrients needed by plants and effects of imbalances in nutrient availability. Toxic effects of different elements on plant roots have also been investigated using this system in order to find toxic concentrations at which growth and nutrient uptake are affected under different conditions of nutrient supply (Göransson & Eldhuset 1991, Ericsson et al. 1998). Because nutrients are added frequently to the root surfaces, some phenomena occurring naturally in soil, such as depletion gradients due to uptake, and accumulation gradients because of drying, are strongly reduced (even though they still occur to some extent). Other approaches are thus also needed. Under natural conditions, nutrient solutions do not circulate freely around the root systems but can stand and gradually dry out during dry periods. The role of the ectomycorrhizal mycelium is difficult to study in Ingestad type systems, even with the aid of sloping plate systems (Kähr & Arveby 1986) since there is no solid substrate to allow three dimensional growth of a full extramatrical mycelium. In addition, hydrophobic fungi grow poorly when the substrate is too wet (Stenström 1991, Unestam 1991).

Semi-hydroponic systems have been developed in which a solid substrate is flushed frequently by nutrient solution (eg. Nylund & Wallander 1989). Growth of ectomycorrhizal fungi in these systems is better than in the Ingestad type systems, because the fungi have a solid substrate in which they can grow in a more natural, three-dimensional way. In semi-hydroponic systems, ectomycorrhizal seedlings often grow less well than non-mycorrhizal seedlings (Colpaert & Verstuyft 1999). The beneficial effect of the extramatrical mycelium on nutrient uptake, compared to non-mycorrhizal roots, is decreased by frequent nutrient addition. Both mycorrhizal and non-mycorrhizal roots are given an opportunity to take up nutrients effectively without the same necessity for a well developed extramatrical mycelium. Lower growth of mycorrhizal seedlings in these systems has been explained in terms of competition for carbon between the fungus and the plant. Another explanation may be retention of N in the mycobiont at least with some ectomycorrhizal fungal species (Colpaert et al. 1996). Recently Colpaert & Verstuyft (1999) showed that competition for P may be an important

reason for lower growth of ectomycorrhizal seedlings in the semi-hydroponic systems using modified Ingestad nutrient solutions, possibly because these solutions were created for plants only, and the needs of the fungi have thus not been taken into account.

Sand culture systems have been used for studies of the influence of toxic metals on mycorrhizal and non-mycorrhizal seedlings (Jentschke et al. 1991b, Schier & McQuattie 1995, 1996 Jentschke et al. 1999b). Perlite (Cumming & Weinstein 1990ab) or mixtures of vermiculite, sphagnum peat moss, perlite and pumice have also been used as growth substrate in pot experiments (Entry et al. 1987). Most experiments using the systems described above contain frequent changes of nutrient solutions around the root systems. This increases the degree of control in the experiments, and reduces the potential effects of accumulation of nutrients and changes of pH. These systems are thus suitable for physiological studies of the effects of toxic elements. However, use of these controlled systems decreases the ecological relevance of the studies (Fig. 2). Under natural conditions soil solutions do not circulate around the root systems and gradients of nutrients and Al may sometimes occur, for example when soil dries. Soil solutions are diluted with rain water, and some of the nutrients may be leached to deeper layers (some fungi may even have specialised to catch these percolated nutrients in B- and even C-horizons). The role of ectomycorrhizal fungi as soil exploiting organisms is different in natural soils from that in experiments with frequent flushing of substrate.

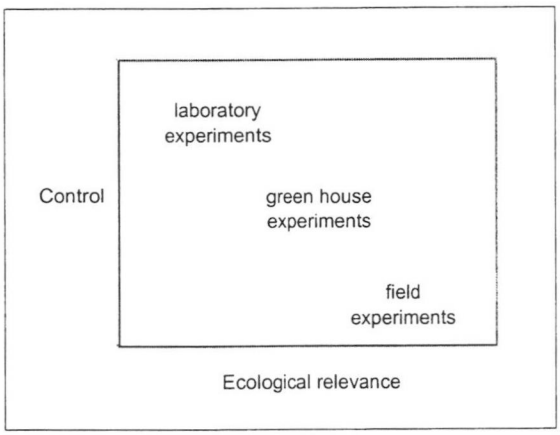

Figure 2. Relationship between control and ecological relevance in biological experiments.

Experiments with sand as a growth substrate have also been performed in greenhouse and field experiments, for example Al pot experiments (Ilvesniemi 1992, Janhunen et al. 1995). Results from pot experiments at field sites depend on how much nutrients have been added to the system, and the ratios in which they are added. At field sites seedlings become mycorrhizal quickly and if Ingestad type nutrient solutions (Ingestad 1979) have been used any P deficiences found may partly arise from use of the P by the ectomycorrhizal fungi which is not taken into consideration when the nutrient solution is prepared for plant needs only (Colpaert & Verstuyft 1999). More complicated systems (having hopefully more

ecological relevance) suffer from loss of control making the results more difficult to interpret (Fig 2).

Hydroponic systems do not use a solid-phase substrate, which may react with nutrient solutions and toxic elements in it (Jentschke & Godbold 2000). All other systems have a solid-phase substrate, and in studies including ectomycorrhizal fungi are chosen because they provide physical support and a solid-liquid phase interface in which the fungal mycelium can grow. None of the solid materials used (vermiculite, perlite, peat, sand) is biologically inert, and elements may be released from the solid material. Thus ions may be released and nutritional effects changed in a complicated way (see Jentschke & Godbold 2000). Ingestad nutrient solution (Ingestad 1979), or modifications thereof, have been used as a nutrient source in many experiments. Another approach has been the use of nutrient solutions with compositions corresponding to those of soil solution measured in field samples (Jentschke et al. 1991ab).

Sand from the B-horizon of a local forest, containing both Al and base cations, was used in the laboratory experiments in this thesis. The goal was to use a natural substrate and investigate how ectomycorrhizal fungi affect seedling growth and nutrient uptake under different conditions in which nutrient capture from sites unavailable to roots might play a role. The system used in these experiments has some disadvantages: the system is not so controlled, and there are difficulties when comparing effects of metals on large and small plants. The latter problem may be decreased by comparing the seedlings inside one mycorrhizal group, for example comparing percentages of biomass compared to respective mycorrhizal or non-mycorrhizal control.

Pot experiments with Al, Ni or Cd

In the metal experiments *Pinus sylvestris* L. seedlings were grown in pots containing B-horizon sand and small quartz stones (I) or with small quartz stones and quartz sand (II). This approach was chosen because it was considered important to allow full development of an extramatrical mycelium and to allow development of nutrient gradients in the substrate. Nutrients were supplied in the form of modified Ingestad solutions (Ingestad 1979, for details, see I, II) using peristaltic pumps. The pots were supplied with nutrients 1-2 times per day allowing nutrient gradients to be created in the pots (Fig. 3).

Effects of Al were studied in two experiments (I). In the first experiment, 0 mM or 2.5 mM Al was added to the pots together with a strongly diluted 1/10 Ingestad (1979) nutrient solution. The seedlings were either non-mycorrhizal or they were inoculated with *Laccaria bicolor* (Maire) Orton strain S238, *Hebeloma crustuliniforme* (Bull.) Quél. strain 89.001 or *Hebeloma* cf. *longicaudum* (Pers.: Fr.) Kumm. ss. Lange strain BL 97.05. In the second Al experiment, 0 mM Al (Al-) or 0.7 mM Al (Al+) was added to the pots together with nutrient solution

with or without base cations. The seedlings were either non-mycorrhizal or they were inoculated with *L. bicolor* S238 (I). During the second Al experiment the pots were watered liberally every second to every third week in order to hinder accumulation of exchanged cations in solution, and the water draining from the pots was gathered for analysis. In the heavy metal experiment the seedlings were left non-mycorrhizal or inoculated with *L. bicolor*. The pots were watered with nutrient solution containing 0, 85 µM Ni, 170 µM Ni or 8.9 µM Cd. The experiments lasted one growth period, about 5 months. After harvest the root systems were investigated for mycorrhizal colonisation with aid of dissection and and compound microscopes.

Figure 3. Seedlings from the first Al experiment (I). Nutrient solution was supplied to the pots by a peristaltic pump.

The dry weights of roots and shoots were recorded. Nutrient concentrations in roots and shoots were analysed as well as concentrations of Al or Ni and Cd. Soil solutions were analysed for nutrient elements and Al or Ni and Cd.

Interpretation of nutrient deficiencies

Ratios of different elements relative to nitrogen are commonly used to identify growth limiting nutrients. The use of these ratios is based on Liebigs "law of the minimum" published by van Liebig in 1840 (Salisbury & Ross 1985). According to this law, growth of plants is limited by a single factor at a specific moment. When this factor becomes non-limiting, some other factor becomes limiting instead. A particular nutrient may be a limiting factor at a particular time. The role of different nutrients as growth limiting factors has been investigated using nutrient/N ratios based on laboratory studies by Ingestad (1979), Ericsson & Kähr (1993, 1995) and Ericsson et al. (1998). The estimated N needed for maximal growth of *Pinus sylvestris* and *Picea abies* in controlled laboratory experiments is 18-20 mg/g dw (Ingestad 1979, Ingestad & Kähr 1985) and N concentrations up to 20 mg/g have been measured in current-year needles after balanced nutrient addition (Linder 1995).

Element/N ratios were first obtained from experiments in which pine seedlings were grown at their maximal growth rate under conditions of free access to nutrients (Ingestad 1979) (Table 1). The elemental composition of the plant tissues was then measured. These experiments have given information about nutrient uptake at maximal growth rate under free access to nutrients. As pointed out by Göransson & Eldhuset (1995), decreases in element ratios below the values observed during maximal growth rates under conditions of free access to nutrients

do not necessarily result in decreased growth, because tree seedlings may take up nutrients in excess of requirements (luxury uptake) when they are freely available. When interpreting apparent nutrient deficiency, one must therefore consider this possibility. Further experiments have revealed that maximal growth rate can be maintained even if concentrations of some nutrients are decreased from the original free access concentrations (Ericsson & Kähr 1993, Ericsson et al. 1998), and growth limiting element/N levels may be obtained from these studies and from the original studies of Ingestad (1979) (Table 1).

Table 1. Estimated deficiency levels for N concentrations and element/N ratios (on a weight basis) in birch seedlings, and the values in pine seedlings grown under conditions of free access to nutrients.

	N conc.	N / N *100	K / N *100	P / N *100	Ca / N *100	Mg / N *100	S / N *100
Deficiency birch*	16-18	100	ca. 23	10	1-2	ca. 3	ca. 5-6
Free access pine**	22.0	100	43	14	6	6	9

*limit for deficiency for birch (Ericsson and Kähr 1993, Ericsson et al. 1998)
**contents in pine seedlings grown with free access (FA) to nutrients (Ingestad 1979)

These element ratios compare uptake of an element with that of N, because N is needed in relatively large amounts by plants and it is commonly the growth limiting element, at least in boreal forests. Even if concentrations differ between tree species, element/N ratios are supposed to be similar between them (Göransson, pers. comm.). In some cases, however, element/N ratios are difficult to interpret, for example when N contents are different in different treatments. It is not very well known whether other nutrients are needed by the plant in the same ratios when availability of N varies greatly. However the ratios are informative, suggesting which nutrients are near or below the growth limiting level. On the other hand the ratios help to identify differences which are statistically significant but may not be very important ecologically (or at least are not growth limiting). Decreases in luxury uptake may thus be found, but the decreased uptake may still be enough for adequate growth. One complicating factor in interpreting element concentrations of needles is that needle dry weight varies seasonally because carbohydrates form a major part of needle dry weight and the content of carbohydrates may vary greatly during the year (see Helmisaari 1990, Linder 1995).

Production of LMW organic acids by mycorrhizal and non-mycorrhizal seedlings

A cultivation method was developed to enable exposure of ectomycorrhizal plants with intact extramatrical mycelium to solutions containing different metals, in this case Al (0, 0.1, 0.74 and 3.7 mM), Cu (0.157 mM), Ni (17 µM) or Cd (0.44 µM). *Pinus sylvestris* seedlings were inoculated with *Suillus variegatus* (two isolates), *Rhizopogon roseolus* or *Paxillus involutus* (two isolates) in a peat-vermiculite system (Finlay 1989). Inoculated seedlings were transferred to Petri dishes

containing glass beads and exposed to elevated metal concentrations in two ways: a) immediately following transfer, b) after allowing mycorrhizal seedlings to develop an extraradical mycelium which colonised the interface between the upper surface of the beads and the metal solution (Fig. 4). Production of organic acids in mycorrhizal and non-mycorrhizal systems was measured by withdrawing samples from the solution and analysing by HPLC.

Advantages and disadvantages of the Petri dish method

The method appeared to be suitable for studying the production of a wide range of substances by the intact, extra-radical, ectomycorrhizal mycelium under axenic conditions. Pre-inoculated ectomycorrhizal seedlings were able to grow in the glass bead-liquid-air interface and hydrophobic species (*S. variegatus*, *P. involutus* and *R. roseolus)* produced relatively large amounts of extramatrical mycelium. Many new mycorrhizal roots were formed during the 2-3 weeks incubation when 1/10 strength Ingestad solution was used in the Petri dishes during the initial growth period of extraradical mycelium. In one pilot experiment, a hydrophilic fungus *Laccaria bicolor* did not produce as much mycelium as the other fungi used. The axenic conditions enable the examination of compounds, such as organic acids, which would otherwise be subject to microbial degradation. and have a relatively short half-life. This is particularly true of acids such as malate which has a relatively short half life in non-sterile soil (Jones *et al.* 1996). One potential problem with the system is binding of substances to the glass beads, but this was not found for the organic acids measured in this study. Another problem is, that root tips grow away from the fungi they are inoculated with so that mycorrhizal seedlings often include small parts of young, elongated non-mycorrhizal roots. In this study, the length of these pieces was measured, but there was no correlation between production of acids and the length of the non-infected root parts. These pieces were not cut before exposure, because cut root ends could then have leaked organic acids into the solution.

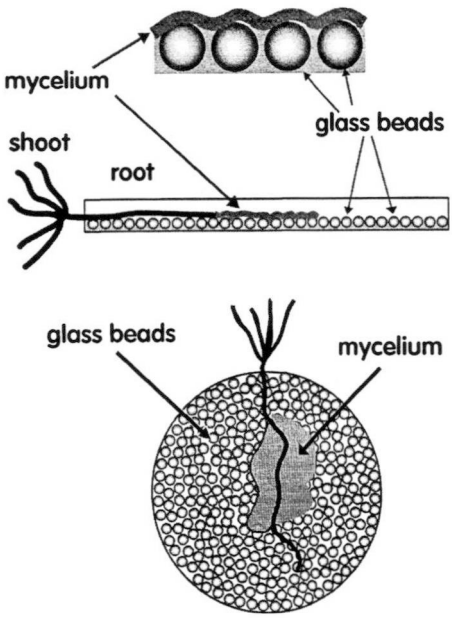

Figure 4. The Petri dish system used in the experiments with organic acids (III). Axenically synthesised ectomycorrhizal seedlings were transferred to Petri dishes containing glass beads where exposure to different metals was performed, either directly, or after an extramatrical mycelium had developed.

Field experiment with defoliation, Cu-Ni and acid rain treatments

The Ni-Cu field study was conducted at Kevo Subarctic Research Station (69°45N, 27°E) in northern Finland during 1991-1993. In spring 1991, sixty Scots pines from a natural stand which were at least 15 years old were transplanted into pots on an experimental field site. The trees were exposed to four pollution treatments consisting of factorial combinations of two factors, simulated acid rain (pH 3) and heavy metal (Ni-Cu) pollution: irrigated control, Ni-Cu treatment, pH 3 treatment, and Ni-Cu + pH 3 treatment. Total amounts (mg/m^2) of Cu, Ni and S, respectively, added during three growth periods were: control – 0, 0, 172; acid rain – 0, 0, 4663; metal – 39, 24, 202, metal+acid rain – 39, 24, 4577. The irrigation treatments were applied by sprinklers over the canopy and ground area of each plot twice a week during three summers. Three levels of artificial defoliation were performed cumulatively during two seasons: (a) unmanipulated controls (0% defoliation), (b) moderately defoliated: 30 % defoliation in 1992 and 30% in 1993 resulting in 60 % total needle loss; and (c) severely defoliated: 75 % defoliation in 1992 and total defoliation in 1993 resulting in 100 % needle removal, excluding current-year needles. Trees were defoliated before the current-year shoot elongation had ceased and when needle growth had just started at the beginning of July. The intention was to mimic the natural damage pattern caused by the European pine sawfly (*Neodiprion sertifer*). The trees were harvested in autumn 1993. The biomass of different needle age classes was recorded as well as that of fine roots (<1mm diameter), intermediate roots (1–2 mm diameter) and coarse roots (>2mm diameter). The proportion of living fungi in the fine roots was estimated by measuring ergosterol content (Nylund and Wallander 1992). Ergosterol content in the roots is supposed to correlate with a metobolically active fungal biomass (Nylund & Wallander 1992) and by using a certain constant this value can be transformed to estimate the correnspondingfungal biomass. The total biomass of living fungi in the fine roots was estimated by multiplying the proportion of living fungi by the total fine root biomass. The fine roots were examined for mycorrhizal colonisation and morphotypes.

Results and discussion

The effect of ectomycorrhizal colonisation on growth and nutrient uptake of pine seedlings

Well developed ectomycorrhiza had a generally positive effect on growth and nutrient uptake of seedlings in the pot experiments. In the first Al experiment (I), a low level of nutrient supply was chosen and the growth of all seedlings was relatively low. However seedlings with well colonised ectomycorrhizal root systems grew better than non-mycorrhizal or poorly colonised ones. Qualitative descriptions of the colonisation of root systems by the different fungi are presented in table 2. Growth of the seedlings with poorly colonised root systems was similar to that of the non-mycorrhizal ones.

Table 2. Colonisation by *Laccaria bicolor, Hebeloma* cf. *longicaudum* and *Hebeloma crustuliniforme* in the first Al experiment.

	Al 1 treatment	Al 2 treatment
L. bicolor	intensive	intensive
H. crustuliniforme	poor	poor
*H.*cf. *longicaudum*	intensive	poor

In two other experiments, the second Al experiment (I) and the Ni-Cd experiment (II), higher concentrations of nutrients were added to the pots but only small amounts of nutrient solution were added each day. A classical mycorrhizal effect was found in these experiments and ectomycorrhizal plants were much bigger than the non-mycorrhizal ones.

The growth of non-mycorrhizal and mycorrhizal seedlings was limited by different nutrients. Nitrogen levels in the mycorrhizal seedlings were below the level found in seedlings grown at maximal growth rate under free access of nutrients, and N was the growth limiting element in these seedlings. In the non-mycorrhizal seedlings, N concentrations were near or above the level found in seedlings grown at maximal growth rate, and for those seedlings P was the growth limiting element. Mg and S concentrations also sometimes approached growth limiting levels, as interpreted using Mg/N and S/N ratios.

In both Al experiments, the non-mycorrhizal seedlings had dark purple needles symptomatic of P-deficiency which in some cases is caused by Al toxicity (Hutchinson 1984, Schaedle et al. 1989). Element analysis confirmed that the non-mycorrhizal seedlings were P-deficient. The most plausible reason for the lower P concentration in the non-mycorrhizal seedlings is the lack of extramatrical mycelium in the growth substrate, and thus a much smaller surface area available for nutrient uptake. In the first Al experiment, the P concentrations were decreased in the seedlings inoculated with *H.* cf. *longicaudum* and exposed to 2.5 mM additional Al. *H.* cf. *longicaudum* did not tolerate the Al treatment and

hardly grew in the Al 2 treatment. An additional factor possibly explaining the higher P concentrations in well colonised seedlings could be solubilisation of precipitated P by the mycorrhizal fungi, which has been demonstrated in *Pisolithus tinctorius* (Cumming & Weinstein 1990c), but this capability was not measured in the fungi used in the present experiments.

In the first experiment, there were differences in the nutrient uptake of seedlings depending on whether they were non-mycorrhizal or colonised by *L. bicolor* or *H.* cf. *longicaudum* (those inoculated by *H. crustuliniforme* were not analysed). The seedlings colonised by *L. bicolor* had lower concentrations of N than the non-mycorrhizal seedlings or the seedlings colonised by *H.* cf. *longicaudum*. However the seedlings colonised by *L. bicolor* had higher P concentrations than the other seedlings. It is possible that *L. bicolor* had a large extramatrical mycelium with a higher N demand than *H.* cf. *longicaudum*, and with the help of this large mycelium the fungus could supply greater amounts of P to the seedlings. Sequestration of N or P by ectomycorrhizal mycelium has been put forward as a possible reason for P deficiency in mycorrhizal plants grown in semi-hydroponic systems (Colpaert et al. 1996, Colpaert & Verstuyft 1999). There was no sign of P deficiency in mycorrhizal plants in the present experiment but sequestration of N might have occurred in the mycelium of *L. bicolor*. A related species, *Laccaria laccata*, occurs in plant nurseries with high N availability and moisture (Dahlberg, pers. comm.) and it was the only species which increased in occurrence in a Cu and Zn contaminated site at Gusum, Sweden (Rühling et al. 1984, Tyler 1984). It is possible that *L. bicolor* is a weak competitor but can tolerate high metal concentrations (Rühling et al. 1984, I, II) and moisture (Unestam & Sun 1995) and takes advantage when competition is low.

Improved nutrient status due to ectomycorrhizal colonisation may reduce metal toxicity. Decreased metal toxicity to plants has been observed to occur via improved nutrient status rather than amelioration of direct toxic effects (Schier & McQuattie 1995, Jentschke et al. 1999a). The results obtained suggest that when nutrient solutions around roots are not changed frequently the role of the extramatrical mycorrhizal mycelium is larger than in the experiments with frequent nutrient replacement. Thus if ectomycorrhizal function is under investigation, it should be taken into consideration that frequent replacement of the nutrient solution surrounding the roots will reduce the relative importance of the mycorrhizal mycelium. On the other hand, adding low amounts of nutrient solutions to the pots gives some other side effects such as accumulation of nutrients and changes in pH and thus a larger loss of control.

Responses to Al treatment by different species of ectomycorrhizal fungi

In the first Al experiment (I), one of the three fungi used to inoculate the seedlings, *H.* cf. *longicaudum*, appeared to be sensitive to Al and hardly grew in the Al2 treatment. In the Al 2 treatment, there were hardly any attached sand

particles around the root systems when these were removed from the pots in contrast to the Al 1 treatment and to the well colonised root systems inoculated with *L. bicolor*. Growth of the seedlings inoculated by *H.* cf. *longicaudum* was strongly decreased by the Al 2 treatment and similar to that of non-mycorrhizal plants. In contrast, the seedlings colonised by this fungus had the highest biomass yields in the absence of added Al and abundant mycelium attached to their root surface. The nutrient uptake of these Al treated seedlings was also quite similar to that of the non-mycorrhizal seedlings. In pure culture studies, differences have been found in growth of different ectomycorrhizal fungi on Al containing substrates (Hintikka 1988, Thompson and Medve 1984, Marschner et al. 1999). The growth of *Hebeloma mesophacus* decreased about 50% on medium containing 3.7 mM Al (Kong et al. 1997) and *H. crustuliniforme* showed decreased growth at 0.37 mM Al but still grew at 0.74 mM Al (Browning and Hutchinsson 1991). In the present study, the Al concentration in soil solution in the Al 2 treatment was about 3.34 mM at the end of the experiment. *Laccaria bicolor* was tolerant to Al in the present experiment as well as in an experiment by Jongbloed and Borst-Pauwels (1992). Jongbloed and Borst-Pauwels (1992) found *L. bicolor* to be sensitive to pH values below 3, a value lower than the pH of 3.8-4.8 measured in soil solution at the end of the experiment in the present study.

H. cf. *longicaudum* was more sensitive to the Al treatment than its *Pinus sylvestris* host seedlings. It is not clear whether the apparent sensitivity of *H.* cf. *longicaudum* in the first experiment would play an important role under natural conditions because the Al concentration used was rather high. Other side effects as such as Cl, Mn, or Fe toxicity and the lower pH may also have played a role. However, concentrations of Al may become high in drying soil (Tamm & Andersson 1985) and the differences between the different mycorrhizal isolates clearly demonstrate that the potential effects of mycorrhizal symbionts should be taken into account when modelling responses of plants to changed soil chemistry arising from acidification.

Effects of Al on growth and element uptake of pine seedlings

Growth

In the first experiment, seedling growth was not affected by the Al treatment directly but mycorrhizal colonisation of root systems by *H.* cf. *longicaudum* appeared to be negatively affected by Al treatment, resulting in a decreased mycorrhizal stimulation of growth. In the second experiment, growth of non-mycorrhizal seedlings was lower in the Al+ treatment, especially when no extra base cations were added. A similar, non-significant (P= 0.07), trend was observed for shoot growth of mycorrhizal seedlings but root growth of mycorrhizal seedlings was not affected by the treatments. The maximal reductions in root and shoot weight between the Al-BC+ and Al+BC- treatments were 23 and 25%, respectively, for non-mycorrhizal seedlings and 15% and 17% for mycorrhizal seedlings. These reductions occurred when the maximum concentrations of Al in

the soil solution at the end of the experiment were 0.22 mM and concentrations in the leachate following watering were 0.49 mM. These values are much lower than in the study of Göransson and Eldhuset (1991) where decreased growth of non-mycorrhizal pine seedlings was shown in the 3 mM Al treatment with high nutrient availability, and in experiment 1 where N and P limited seedlings were not affected by 2.5 mM Al treatment.

Base cations (Ca, Mg and K)

Concentrations of Ca and Mg were decreased in the roots of both non-mycorrhizal and mycorrhizal seedlings in the first experiment with a high Al treatment. In the second experiment with lower Al treatment Ca and Mg uptake were only decreased in the non-mycorrhizal roots due to the Al treatment. However, no shortage of Ca or Mg was found. Shoot concentrations of Ca or Mg were not affected by the Al treatments in either of the experiments. Decreased Ca and Mg uptake have been found in a number of other studies with trees and uptake by mycorrhizal and non-mycorrhizal seedlings is generally affected in the same way (see Jentschke and Godbold 2000). Nutrient imbalances have been suggested to be the main reason for negative effects of Al on growth rather than direct toxicity (Janhunen et al. 1995). Al has been found to reduce uptake of Mg and Ca in mycorrhizal spruce seedlings (Jentschke et al. 1991ab) and mycorrhizal *Pinus strobus* and *P. rigida* seedlings (Cumming and Weinstein 1990b, Schier et al. 1990, Schier and McQuattie 1995, 1996). In *Pinus nigra* exposed to 100 µM Al decreased uptake of Ca and Mg (Boxman et al. 1991). Göransson and Eldhuset (1991) found reduced uptake rates of Ca and Mg when non-mycorrhizal pine seedlings were exposed to 1 mM Al and mycorrhizal seedlings (colonised by *Suillus bovinus*) were exposed to 3 mM Al. In *Picea abies*, Al was found to reduce Mg concentrations to a similar level of deficiency in both mycorrhizal and non-mycorrhizal seedlings but needle chlorosis was found to be delayed in the mycorrhizal seedlings (Hentschel et al. 1993). It has been speculated that mycorrhiza might increase Mg efficiency of the seedlings or delay leaf senescence via hormonal effects, for example by enhancing cytokinin export from the roots (Jentschke and Godbold 2000).

The results of the present study indicate that Ca and Mg were taken up in excess amounts when available and stored in the seedlings. In other studies Mg uptake by Norway spruce was found to be affected by Al at low pH (3.2) but not at high pH (5.0) (Godbold and Jentschke 1998). In the roots, Al displaced Mg and Ca from cation exchange sites on the root cortical cell walls (Godbold and Jentschke 1998) but the authors concluded that the amount of Mg bound to cation exchange sites on the roots does not control the Mg concentration of the needles. In another study by Ericsson et al. (1998) of non-mycorrhizal birch seedlings Mg uptake was decreased due to 0.5 mM Al treatment. In *Pinus sylvestris* (nursery) seedlings, decreased Mg and Ca uptake has also been found after $Al(NO_3)_3$ exposure (Oleksyn et al 1996).

These experiments indicate that of K, Ca and Mg, Mg is the primary growth limiting element in these conditions, using this specific substrate. Ratios of Mg/N, especially in the non-mycorrhizal seedlings, were near the growth limiting level and decreased due to Al treatments. Ratios of K/N or Ca/N were not near the growth limiting level in any of the treatments.

Effects of the Al treatments on the K uptake differed from the effects on Ca and Mg uptake. In the first Al experiment, K uptake was generally *increased* due to the Al treatment. Similar effects on *P. sylvestris* have been found in pot experiments in the field (Ilvesniemi 1992, Janhunen et al. 1995). In the second Al experiment, K uptake was unaffected by any of the treatments.

Phosphorus

Mycorrhiza generally increased the P concentration of seedlings in the Al experiments. Non-mycorrhizal seedlings were P deficient and this was exacerbated due to Al in the shoots of the non-mycorrhizal seedlings in the second Al experiment. A similar reduction of P uptake in response to Al treatment in non-mycorrhizal *Pinus rigida* seedlings, but not in mycorrhizal plants, has also been found by Cumming and Weinstein (1990a). In the first Al experiment, Al did not directly affect P concentrations of the seedlings (only via apparent inhibition of the mycelial growth of *H.* cf. *longicaudum*). This was somewhat surprising because the high Al concentration in soil solution could have lead to P-Al precipitation thus lowering the P available to the plants. An alleviating effect of higher concentrations of base cations could be one explanation of this result, for example by hindering Al precipitation or by protecting P transporters on the plasma membrane. In a pot experiment in the field, Al treatment was found to decrease P concentration of *P. sylvestris* (Janhunen 1995). In the second Al experiment, P concentration was kept at the same level in the mycorrhizal seedlings in all treatments (1.3-1.6 mg/g dw) whereas in the shoots of the non-mycorrhizal seedlings the P concentration was only 0.7–0.9 mg/g dw in the Al treatments.

In some Al experiments, P has been found to accumulate in roots due to Al treatment (Entry et al. 1987, Cumming et al. 1986) and Al has been found to be complexed with P on the surface of mycelium (Väre 1990). Aluminium polyphosphate has also been found in vacuoles of *L. bicolor* (Martin et al. 1994). These results indicate that Al may precipitate together with P at least when P is available in relatively high concentrations. It is likely that excess P was not available for extensive Al precipitation in the present experiment since no elevated P concentrations were found in the roots, and in most of the treatments P was under the level found in seedlings growing at maximal growth rate. Furthermore in the first Al experiment, P concentrations were similar in both Al treatments in non-mycorrhizal seedlings as well as in the seedlings colonised by *L. bicolor*, even though the Al concentration was 0.015 mM in the Al 1 treatment and 3.34 mM in the Al 2 treatment. If much P precipitation had occurred,

decreased shoot P concentrations in the first Al experiment would have been expected.

Aluminium uptake

Al concentrations in the roots and shoots were not well correlated with growth. Aluminium treatment elevated Al concentrations of the shoots in the first experiment, but in the second experiment Al concentrations of shoots were at the same level irrespective of added Al. Aluminium concentrations of 170 and 320 ppm have been found in unpolluted sites in *current year needles* (Helmisaari 1990, Reich et al. 1994) and values up to 900 ppm have been found in polluted sites (Reich et al. 1994). Values in polluted site have been assumed to contain mainly Al taken up from soil (Reich et al. 1994). Mean Al concentrations of the shoots in experiment 1 were 440-590 ppm in the treatment with no added Al and 590-840 ppm in the Al 2 treatment. Although the latter values are larger the former ones they do not reflect the large difference in measured values in the soil solution of the two treatments, 0.015 mM in control pots and 3.34 mM in the pots with added Al. The results from experiment one thus indicate that 1) high Al concentration (3.34 mM) in the soil solution could not decrease the growth of nutrient limited *Pinus sylvestris* seedlings directly (but only via the effects on the mycorrhizal fungus *H.* cf. *longicaudum*) and that 2) Al uptake is controlled and excess uptake can partly be inhibited. In the second experiment in the present study, Al decreased the growth of the seedlings, especially when no base cations were added to the nutrient solutions. However, this decrease could not be explained by higher shoot uptake of Al, because no differences were found between Al contents in the shoots. Mean Al concentrations of the shoots in the experiment 2 were between 330-490 ppm.

Investigation of BC/Al ratios in laboratory conditions

One of the original goals of the Al experiments was to investigate the effects of low BC/Al ratios (preferably below 0.5) on the growth and nutrient uptake of ectomycorrhizal seedlings, because ratios below 1 in soil solution have been suggested to indicate risks of detrimental effects of acidity when evaluating critical loads for acidic deposition (Sverdrup & Warfvinge 1993, Barkman 1998). However, testing this BC/Al model under laboratory conditions by creating stabile BC/Al ratios in solution while also using natural, solid growth media is difficult. In the first Al experiment, using B-horizon sand as a growth substrate, a large change from BC/Al=0.04 in nutrient solution to 1.35 in soil solution at the end of the experiment was found. Even in nutrient solution studies, for example in hydroponic systems without any solid-phase interactions, these ratios are changed (although less dramatically than in the present experiments) due to different uptake rates of different cations (Göransson, pers. comm.).

The large increase in the BC/Al ratio in the first experiment from 0.04 in the added nutrient solution to 1.35 in soil solution at the end of the experiment was due to much higher Ca, Mg and K concentrations in soil solution in the pots

treated with additional Al. Base cation concentrations (mM) in the soil solution of the Al 1 and Al 2 treatments at harvest were as follows (values for the added nutrient solution shown in parenthesis): K - 0.069 & 0.23 (0.083), Ca - 0.324 & 3.49 (0.0075), Mg – 0.128 & 0.658 (0.012). The aluminium concentration in the Al 2 treatment was 2.5 mM in the nutrient solution and 3.3 mM in the soil solution in the end of the experiment (0.015 mM in the Al 1 treatment at the end of the experiment). It is possible that high concentrations of base cations in the soil solution had ameliorated Al toxicity. In the second Al experiment it was hypothesised that Al could have a decreasing effect on growth at a lower exposure concentration (0.74 mM) than in the first Al experiment since 1) the pots were flushed at 2-3 week intervals in order to hinder cation accumulation and 2) as an additional treatment base cations were removed from the nutrient solution. By collecting and analysing the leachates the BC/Al ratio could be followed during the experiment. A growth decrease was found in the second Al experiment even though BC/Al ratios in soil solution were still relatively high, over 3 even in the most extreme Al treatments. It is thus tempting to conclude that the experiment should have continued for a longer time, or that possibly a higher Al concentration should have been chosen.

One consequence of using a system in which low amounts of nutrient solution are added to the pots without frequent (several times per day) flushing is possible changes in pH. The pH may be changed during the experiment, as in the present experiments, especially if the substrate used is not inert. In the first experiment in the pots treated with additional Al, the pH of the soil solution was 3.8 at the end of the experiment - the same as the nutrient solution added at the beginning of the experiment In the control pots, the pH of the soil solution was 4.8. These effects were the same in all mycorrhizal treatments. Added Al presumably replaced H^+ and base cations from the sand used. In the second experiment, mycorrhizal colonisation was found to increase the pH of the soil solution (about 0.5 units) and Al+ treatment was found to produce an equivalent pH decrease. Increased pH in response to mycorrhizal colonisation may arise from changed patterns of utilisation of different N sources. In addition, mycorrhizal roots have been found to release fewer hydrogen ions per ammonium ion taken up than non-mycorrhizal roots (Rygiewicz 1984 a,b). The acidifying effect of Al may arise from Al^{3+} replacing H^+ from the substrate since the pH was the same in the nutrient solutions added. However, adjusting pH to the same level in the pots of all treatments is not without complications, either. H^+ stress becomes higher with control solution not containing Al because Al^{3+} displaces H^+ from plant membrane surfaces and thus decrease H^+ stress (Kinraide 1998).

One important question with all laboratory experiments is how well the results obtained mirror natural conditions. Direct toxic effects of Al may not be the most important general problem affecting boreal forest trees, because total concentrations measured in field samples seldom exceed concentrations found to be toxic to trees species from (European) boreal forests. Other acidification-

related aspects such as cation leaching are probably more important, as well as combined effects of base cation depletion and Al. What would be the next step for laboratory investigations concerning acidification and Al? In the present study mycorrhizal mycelium appeared to both increase pH of the substrate and reduce leaching of cations from the pots. If the amount of extramatrical mycelium in soil decreases due to pollution as in a study by Wallander & Nylund (1992) with high N concentration, both soil pH and base cation leakage may be affected. The stimulating effect of ectomycorrhiza on growth and nutrient uptake of pine seedlings was very clear, as shown in many other studies (Smith & Read 1997). In future, effort should be put into investigating differences between seedlings colonised by different ectomycorrhizal fungi. This could be done by inoculating with one fungus at a time or by inoculating several fungi on one seedling in order to understand the response of communities to Al or acidification stress. An important question is whether cation leaching and removal when harvesting trees is a real threat, affecting cation depletion. The possible role of ectomycorrhizal fungi in base cation weathering (Jongmans et al. 1997) should also be investigated.

The base cation (K + Ca + Mg) / Al ratio - a tool for estimating Al toxicity?

The base cation / Al ratio has been suggested to be a suitable tool for estimating the risk of acidification related tree damage and need for remediative treatments such as liming (Sverdrup and Warfvinge 1993). The BC/Al model assumes passive uptake of base cations based on concentrations in the apoplast which in turn depend upon concentrations in the soil solution. However, uptake of some nutrients may occur partly via specific carriers and it is questionable how much Al in ecologically relevant concentrations can affect this kind of uptake. In addition, it has been shown in many studies that mycorrhiza affect nutrient uptake of trees (Smith & Read 1997) including uptake of base cations (Finlay 1995). Binding of Mg to cation exchange sites on *Picea abies* roots was not found to control uptake of Mg by shoots (Godbold & Jentschke 1998). Uptake of nitrate increases pH which in turn decreases Al solubility. In some cases higher pH in acidified sites has been explained to be dependent on increased nitrate uptake (see Högberg and Jensén 1994). In addition it is questionable whether uptake of K should be regarded as similar to that of Ca and Mg. Potassium is a monovalent cation which (unlike Ca and Mg) only forms weak complexes and does not compete strongly for binding sites requiring divalent cations (Marscher 1995). Uptake of K has also often been found to be increased in Al treated plants in contrast to uptake of Ca and Mg (Ilvesniemi 1992, Janhunen 1995) as also demonstrated in the first Al experiment (I). The BC/Al model has been criticised because it does not take into consideration the complexity of the rhizophere, including gradients of pH, nutrients and Al (Högberg & Jensén 1994, Falkengren-Grerup et al. 1995, Løkke et al. 1996). Another possibility would be to measure tree nutrient status by foliar analysis, and evaluate the need for remediative actions such as liming or fertilisation (Linder 1995). Using needle data, seasonal

variation should be taken into consideration (Helmisaari 1990, Linder 1995). Soil conditions must also be taken into consideration to evaluate possible negative effects of liming.

Effects of Ni and Cd on growth

In the heavy metal pot experiment, a general trend of decreased growth due to Ni was found for both mycorrhizal and non-mycorrhizal seedlings. Decreased root growth has been found to be one of the first signs of heavy metal toxicity (Foy 1978, Godbold & Hüttermann 1985), but in the present study it was shoot growth rather than root growth which was sensitive to heavy metal treatments. Shoot growth was decreased by up to 46 % in the non-mycorrhizal seedlings and 36 % in the mycorrhizal Ni 2 treated seedlings compared to the respective controls without metal exposure, but the decrease was statistically significant for the shoots of the mycorrhizal seedlings only. The relative differences between the shoots of the mycorrhizal and non-mycorrhizal seedlings were even larger in the Cd and Ni 1 treated seedlings than in the Ni 2 treated seedlings. The capacity of ectomycorrhizal fungi to ameliorate growth reductions in their host plant has been shown to depend upon the fungal species involved and the Ni concentration that the symbionts are exposed to. *Scleroderma flavidum*, *Laccaria proxima* and *Lactarius hibbardae* but not *Lactarius rufus* were found to alleviate toxic effects of Ni on growth of *Betula papyrifera* seedlings during four months exposure to 35 µM Ni, but at concentrations of 85 µM Ni only seedlings colonised by *S. flavidum* grew better than non-mycorrhizal controls (Jones & Hutchinson 1986). In the present study, *Laccaria bicolor* could not clearly alleviate toxic effects of Ni on growth but it did generally increase the growth of the seedlings.

No significant effect on growth of the seedlings was found with 8.9 µM Cd although the mean dry weight of the non-mycorrhizal Cd treated shoots was 32 % lower than that of respective control. In the shoots of the mycorrhizal seedlings this decrease was 6 %. One explanation for the lack of a statistically significant difference between the shoots of the mycorrhizal and non-mycorrhizal seedlings may be the large variation in non-mycorrhizal control seedlings. In another study with constant exposure to 0.5 and 5.0 µM Cd, *Laccaria bicolor* was found not to alleviate toxic effects of Cd on *Picea abies* seedlings whereas *Paxillus involutus* did at the lower Cd concentration (Jentschke et al. 1999a). These authors found that colonisation by *L. bicolor* was decreased due to Cd treatments, whereas colonisation by *Paxillus involutus* was not. In the present study almost all short root tips were colonised by *L. bicolor* but both the method of exposure and the growth substrate was different in the two experiments: pure quartz sand in Jentschke et al. (1999a) and a mixture of B-horizon soil and quartz sand in the present study.

The plants and ectomycorrhizal fungi in the present study were thus given an opportunity to take up nutrients, not only from the added nutrient solution, but also from the substrate. Heavy metal exposure was performed by adding Ni or Cd

together with low amounts of nutrients in the watering solution. The exposure was thus cumulative and heavy metal gradients were formed in the pots. Heavy metals in contaminated soils are also heterogeneously distributed (Berthelsen et al. 1995). Metals negatively affected the nutrient contents of some seedlings, but the exposed seedlings had a root biomass similar to control plants. Non-mycorrhizal seedlings appeared to invest relatively more carbon on root than shoot growth, as suggested by the changed R/S ratio in the non-mycorrhizal seedlings. Both shortage of P and N are known to increase R:S ratio (Ericsson 1995), and lower P uptake was associated with a higher R:S ratio in the present study. It is possible that the roots could grow away from the gradient of high heavy metal concentrations, however, despite the high root weights, the shoot uptake of P and Mg was decreased.

Effects on nutrient uptake due to Ni, Cd and mycorrhizal colonisation

Nitrogen was interpreted to be the main limiting factor for the growth of the mycorrhizal seedlings in the control and Cd treated seedlings with aid of element/N ratios, but in the Ni treated seedlings P was under the growth limiting level. For the non-mycorrhizal seedlings, the P/N ratio was under the growth limiting level in all treatments, and this ratio was decreased after heavy metal treatments, especially the Ni treatments. The decreasing P concentration after Ni treatment was similar in both mycorrhizal and non-mycorrhizal seedlings, but mycorrhizal seedlings had higher P concentrations than non-mycorrhizal ones. In a study of Ni treated *Betula papyrifera* seedlings (Jones & Hutchinson 1988b) mycorrhizal colonisation was not found to have a great effect on P content. These authors found a correlation between P and Ni and suggested that high P values were correlated with Ni-binding sites which could take part in detoxification of Ni. In the present study, mycorrhizal seedlings improved P uptake, probably due to the larger surface area for nutrient uptake provided by the external mycelium of *L. bicolor*. In contaminated sites, just as in unpolluted soil, mycorrhiza play an important role in tree nutrient capture in exploiting new sites for nutrient uptake at a lower net carbon cost than building much thicker non-mycorrhizal roots. If there are no ectomycorrhizal fungi in a heavy metal contaminated site, nutrient uptake of seedlings will be impaired. Heavy metals can be precipitated with P which may decrease the availability of P (Gadd 1993). Some fungi are able to dissolve P precipitates, for example *Pisolithus tinctorius* has been found to use $AlPO_4$ as a P source (Cumming & Weinstein 1990c), but it is not known whether *L. bicolor* is able to do so.

The phosphorus concentration in the Cd treated roots of the non-mycorrhizal seedlings was decreased by 22% compared to the non-mycorrhizal control. Thus with low Cd exposure, P concentration was decreased in the roots of the non-mycorrhizal seedlings whereas seedlings colonised by *Laccaria bicolor* could maintain the same root P content as the non-exposed control seedlings. In contrast, no positive effect of *L. bicolor* on seedling P status in Cd exposed *Picea*

abies seedlings was found by Jentschke et al. (1999a), but these authors found a positive effect on P status of the seedlings by another ectomycorrhizal fungus, *Paxillus involutus*. It is possible that the exposure of Jentschke et al. (1999a) was higher that that in the present study as indicated by higher Cd concentrations in the roots. Mycorrhizal fungi may generally help to maintain adequate P concentrations in seedlings exposed to low Cd concentrations.

Ectomycorrhizal colonisation did not affect Ni concentration of the seedlings but total uptake was higher in the larger mycorrhizal seedlings. In a study by Jones & Hutchinsson (1986) *Scleroderma flavidum* could alleviate the growth reduction of *Betula papyrifera* due to Ni, but did not decrease transport of Ni into the needles. The seedlings colonised by this fungus had higher Ni levels in the roots (Jones & Hutchinsson 1986). In the present study, no clear ameliorating effect of mycorrhizal colonisation by *L. bicolor* was found even though the percentage decrease in the shoot growth was lower in the mycorrhizal seedlings in the Ni 1 treatment. There appear to be differences in how different ectomycorrhizal fungi react to Ni. In a study by Jones et al. (1988), Ni uptake by paper birch was found to depend on the ectomycorrhizal fungus used: non-mycorrhizal seedlings or seedlings colonised by *Lactarius rufus* needed metabolic energy in order to reduce Ni translocation from roots to shoots, whereas seedlings colonised by *Scleroderma flavidum* did not, which was suggested to contribute to the increased Ni tolerance of the seedlings colonised by *Scleroderma flavidum*.

Both mycorrhizal and non-mycorrhizal pine seedlings had same the Cd concentrations in the roots and shoots, but the total Cd content was double in the roots and almost three times higher in the shoots of the bigger mycorrhizal seedlings. In the study by Jentscke et al (1999a), using *Picea abies*, Cd concentrations were around 2 ppm in 0.5 μM Cd exposed old needles and around 1 ppm in 5 μM Cd exposed old needles. In the present study, with *Pinus sylvestris*, the shoot Cd concentrations were around 10 ppm in the shoots of both mycorrhizal and non-mycorrhizal seedlings exposed to Cd (needles were not analysed separately). Lower Cd concentrations, 0.32-1.23 ppm, were found in *Pinus sylvestris* needles exposed to 44.5 μM Cd for two months of a six month growth period (Colpaert & Van Assche 1993). These authors found the highest concentrations in non-mycorrhizal needles compared to needles from the seedlings colonised with any of 9 different mycorrhizal fungi used. Root concentrations were lower in the present study with pine (almost 130 ppm) than in spruce seedlings where the root concentrations were around 350 ppm in 5 μM Cd exposed seedlings (80 ppm in 0.5 μM Cd exposed seedlings) (Jentschke et al 1999a) but higher than in *P. sylvestris* roots, 5.5-24.3 ppm, in the study of Colpaert & Van Assche (1993). In the present study, *L. bicolor* seemed to have taken up or bound Cd rapidy, because in soil solutions at the end of the experiment, a lowered Cd concentration was found in the mycorrhizal pots whereas the Cd concentration found in the soil solution in the non-mycorrhizal pots was about the same as in the added nutrient solution. In the Ni treatments, no

such differences were found. This might be explained by the higher concentrations used leading to a situation where cation binding sites were already relatively saturated with Ni but there were still were plenty of sites left for Cd.

Field experiment

The pollution treatments, Ni-Cu and/or acid spraying, did not have any effects on any of the tree growth parameters or on mycorrhizal colonisation. In this same experiment, peroxidase activities of the needles showed a increasing trend due to heavy metal treatment ($p=0.098$), especially in the current year needles of the defoliated trees (Roitto et al. 1998). A major problem in experimental approaches is generally to optimise realistic and biologically sensitive manipulations. Instead of high loads reported only for sites close to emission sources, moderate and realistic pollution levels were simulated, which did not cause drastic or visible injuries to forest ecosystems. The sulphur load was about eight-fold and the metal load nearly 50-fold compared with the background deposition in the Kevo district (Laurila et al. 1991). However, loads were low compared to, for example, the annual deposition near the Severonickel smelter complex in Monchegorsk in the Kola Peninsula, North-Western Russia, corresponding to deposition found in an area extending about 40-50 km from the complex (Jevtjugina 1991). It is possible that most of the heavy metals in soil become immobilized by humic substances (Ephraim & Marinsky 1986, Bergkvist et al. 1989). Thus, moderately high Ni-Cu spraying during three growth periods did not affect growth of pines or mycorrhizal colonisation or composition of morphotypes.

Production of LMW organic acids by Al and heavy metal exposed mycorrhizal and non-mycorrhizal *Pinus sylvestris* seedlings

Several different organic acids, principally oxalic, citric and malic acids have been found to be produced in increased amounts by roots of non-mycorrhizal buckwheat (*Fagopyrum esculentum*), *Cassia tora*, maize (*Zea mays*) and wheat (*Triticum aestivum*) exposed to aluminium (Pellet et al. 1995, Ma et al. 1997ab, Pellet et al. 1997). In the present study, a trend for increased exudation of oxalic acid due to Al exposure was found in Petri dishes containing pine seedlings inoculated with *S. variegatus* or *R. roseolus* but not *P. involutus* (only 0.1 mM Al exposure was performed with *P. involutus*) (III). Due to high variability in the data, this increase was significant in only two of four Al experiments where Al also increased oxalic acid production of non-mycorrhizal seedlings to intermediate values, lower than those of Al exposed mycorrhizal plants. In buckwheat, oxalic acid was shown to be a specific detoxification substance against Al (Ma et al. 1997a), and Al induced exudation of oxalic acid has been demonstrated from root tips (Zheng et al. 1998). The root apex has been shown to be a site for Al toxicity (see Kochian 1995, Delhaize & Ryan 1995). It is possible that in the present study higher production of oxalic acid by ectomycorrhizal compared to non-mycorrhizal roots depends partly on a large total hyphal tip

surface area compared to the total surface area of the root apices in non-mycorrhizal seedlings. Zheng et al. (1998) found that buckwheat secreted oxalic acid in the region 0-10 mm from the root tip but similar data are not available for *Pinus sylvestris* and the vast majority of its short roots are colonised by ectomycorrhizal fungi under natural conditions. In the system used, white, long roots up to several cm in length were present in the dishes providing a potential surface area for production.

Copper (0.157 mM) significantly increased the exudation of oxalic acid in two experiments using non-mycorrhizal seedlings or seedlings colonised by *S. variegatus*, *P. involutus* or *R. roseolus*. Oxalic acid may detoxify copper by forming copper oxalate, as suggested for some root-rotting fungi (Dutton & Evans 1996). Nickel (17 μM) and cadmium (0.44 μM) did not affect the exudation of low molecular weight organic acids, but the possibility of organic acid production at higher metal concentrations cannot be excluded. Organic acids have been suggested to play a role in heavy metal tolerance, for example citrate in Zn tolerance (Godbold et al. 1984) and malate for Ni tolerance (Yang et al. 1997).

With the exception of the experiment with Cu, Ni and Cd, mycorrhiza always had an overall significant effect, elevating production of oxalic acid. Colonisation of the seedlings by *S. variegatus* or *R. roseolus* generally increased the concentration of oxalic acid found in the dishes. In those dishes, it was mainly the fungal tissue which was in contact with glass beads or solution. Seedlings colonised by *P. involutus* produced oxalate at the same level as non-mycorrhizal roots. Wallander & Wickman (1999) also found higher soil solution concentrations of oxalic acid (as well as citric and malic acid) with *S. variegatus* than with *P. involutus* in a pot experiment using biotite as a K source. In the present experiments, the basic level of oxalic acid production by non-exposed seedlings was much higher for plants colonised by *S. variegatus* strain BL than for plants colonised by *S. variegatus* strain I 59.

In addition to oxalic acid, other low molecular weight organic acids were also found. These did not show any consistent pattern for all experiments, although concentrations of some of the acids differed significantly between treatments in some experiments. Pellet et al. (1997) found that malic acid bound Al in an Al-tolerant wheat cultivar but no malic acid was found in our study. Citric acid, which can bind Al effectively and has been found to be produced by roots of Al tolerant plants (Pellet et al. 1995, Ma et al. 1997b), was actually decreased in two experiments and increased in one in Al treated dishes. Wallander & Wickman (1999) found large amounts of citric acid in soil solution in experiments with biotite as a potassium source for *Pinus sylvestris* seedlings inoculated with *Suillus variegatus*, but the variability of their data was high. In our study shikimic acid was found in almost all samples from non-mycorrhizal plants and *P. involutus* and *R. roseolus* infected systems, but only occasionally in the systems colonised by *S. variegatus* BL or I 59.

LMW organic acids – for detoxification and weathering?

Negative effects of Al on plant growth have been connected to decreased uptake of Mg and Ca (Godbold et al. 1988, Göransson & Eldhuset 1991, Hentschel et al. 1993, Schier & McQuattie 1996). It has been hypothesised that under natural conditions in podzols under coniferous forest, ectomycorrhizal fungi may alleviate the negative effects of Al on base cation uptake by capturing base cations during weathering in small tubular pores in rocks and small mineral particles (Jongmans et al. 1997, Van Breemen et al. 2000b). Organic acids play a role in weathering (Lundström 1994, Paris et al. 1995) and it has been suggested that ectomycorrhizal fungi may help to drive podzolisation (Van Breemen et al. 2000a). In addition to the general quantitative effect that the mycelium may have on base cation uptake by virtue of an increased surface area for nutrient uptake, plant growth may also benefit from the production of organic acids which are able to chelate and inactivate potentially toxic inorganic Al. Studies by Hue et al. (1986) classified dicarboxylic acids, oxalic, citric and tartaric acids as potentially strong detoxifiers of aluminium compared with malic, malonic and salicylic acids which were classified as intermediate, while monocarboxylic acids, succinic, lactic, formic and acetic acids had only a weak detoxifying capacity. In two acid subsoils these authors estimated that Al-organic acid complexes accounted for 76% and 93% of the total solution Al.

Many weatherable minerals contain a lot of Al and during weathering significant amounts of Al are probably thus released and must be transported away from weathering sites (Van Breemen et al. 2000b, Lundström et al. 2000). Fungi producing LMW organic acids could complex Al and transport it from sites of weathering. Complexing and transporting Al could thus be a detoxification mechanism for fungi to lower Al concentration in weathering sites such as the small tubular holes discussed by Jongmans et al. (1997). The exact mechanisms involved in such a process have not yet been clearly demonstrated. However, it is evident that plants in boreal forests with acid soils are adapted to Al and control Al uptake to the shoot maintaining Al in non-toxic levels.

In this study differences were found in production of organic acids between isolates of same species (*S. variegatus*) and between different species which may affect detoxification or weathering capability of these fungi. Low molecular weight organic acids can be readily degraded in soil systems (Jones et al. 1996, Lundström 1994, Jones & Darrah 1994). There may be little microbial proliferation at the root apex (Jones et al. 1996) but less is know about the microbial environment of the hyphal tips. Sun et al. (1999) speculated that the microbial interface between mycorrhizal hyphal tips and the soil might contain bacteria consuming specific compounds such as mannitol and demonstrated that exuded oxalate was not reabsorbed. It may be enough to create a local increased organic acid production in a small area near the fungal tips in order to hinder for

example Al toxicity, as suggested for plants (Kochian 1995), or to enhance weathering processes.

No changes in mycorrhizal colonisation percentages due to defoliation

In the field experiment (IV), no effects of Cu-Ni exposure were found on the growth parameters measured, fungal colonisation or morphotype composition. Another question investigated was, how defoliaton affects ectomycorrhizal colonisation and growth of pines. Almost all short roots, over 98 %, were colonized by mycorrhizal fungi irrespective of defoliation treatment. Defoliation slightly decreased both the shoot and fine root growth. Defoliation also reduced the total biomass of living fungi on the fine roots estimated using ergosterol analysis, but only because of the loss of the fine roots. Thus, defoliation did not alter the ratios of current-year needle biomass and the fine root or mycorrhizal associate biomass. The small decrease in biomass may be due to the ability of the plants to reallocate nutrients from different parts (both shoots and roots) to new shoot growth in situations when part of foliage is lost.

Herbivory is generally assumed to negatively influence mycorrhizal fungi because of reduced supply of photosynthate to support mycorrhizal fungi following defoliation (see Gehring & Whitham 1994a). Decreased colonisation of short roots by ectomycorrhizal fungi has been found in many studies (Gehring & Whitham 1991, 1994a, b, 1995, Del Vecchio et al. 1993, Gehring et al. 1997, Rossow et al. 1997) but not always (Markkola 1996). In contrast to nearly 100 % ectomycorrhizal colonization in the present study, overall levels of mycorrhizal colonization of unclipped control trees were only about 50 % in the study sites of Gehring & Whitman (1991, 1995) and Gehring et al. (1997). In other studies, high fungal colonization levels of short roots have also been found (Persson 1980, Termorshuizen & Schaffers 1991, Termorshuizen 1993, Nylund et al. 1995, Taylor et al. 2000) as in the present study. Differences between environmental factors may, in part, explain the differences in mycorrhizal colonisation percentages found. Metabolically active fungi are relatively sensitive to dry conditions in general, and thus, seasonality and differences in the ability of trees and fungi to tolerate environmental factors may explain differences in the results. If herbivory delays regeneration of short roots, and if, in unfavourable soil moisture conditions, mycorrhizal fungi are unable to colonize regenerating root tips immediately, the timing of root sampling may thus also explain some of the results.

Defoliation changed ectomycorrhiza morphotype composition of *Pinus sylvestris*

In the field defoliation experiment, crude morphotyping was used. The proportion of roots colonised by the tuberculate type, including *Suillus* was diminished and proportions of smooth types were increased. The decrease after defoliation may

mean that tips colonised by the tuberculate morphotype are strong carbon sinks with strong rhizomorphs and thick mantles, and that the growth rate of this morphotype decreased due to the decreased amount of carbon transported to the roots. The changed composition of mycorrhizal morphotypes suggests competition among different mycorrhizal growth forms owing to their carbon demands and/or intensity of mutualistic association with the host tree. These outcomes may be a consequence of the ability of the plant meristems and fungal symbionts to use resources, thus optimizing proportions of shoot, root and mycorrhizal mutualists. Elevated CO_2 can be seen as an opposite treatment to defoliation because it increases capacity for photosynthesis. In contrast to the effects of defoliation in the present study, elevated CO_2 has been found to increase ectomycorrhizal morhoptypes resulting in a higher incidence of hyphae emanating from the roots of *Betula papyrifera* seedlings (Godbold & Berntson 1997).

Conclusions and future perspectives

Production of organic acids by mycorrhizal and non-mycorrhizal root systems exposed to elevated concentrations of Al, Cu, Ni and Cd was investigated because this process may play a role in metal detoxification. A Petri dish method was developed in order to expose root systems to elevated concentrations of metals either before or after the development of an extramatrical mycelium (III). Stimulation of oxalic acid production by Al and Cu by mycorrhizal and non-mycorrhizal roots was found, but the possible role of oxalic acid in alleviating the toxicity of these metals needs to be tested in further studies. *Suillus variegatus* and *Rhizopogon roseolus* induced higher oxalic acid production compared to non-mycorrhizal roots, whereas the *Paxillus involutus* isolates used did not. There were differences in the basic level (no metal exposure) of oxalic acid produced between two different *S. variegatus* strains investigated. Localised sampling at different distances from the edge of hyphal mats and root tips would give information about sites where oxalate or other LMW organic acids are exuded. The role of bacteria in production and consumption of compounds such as organic acids should be studied, because bacteria are an important part of rhizophere (Garbaye 1994, Perotto & Bonfante 1997). The role of organic acids as metal chelators has received much attention (Jones 1998) but their possible role in weathering interactions still requires further investigation, as does the question of whether and how aluminium is mobilised, taken up and translocated through fungal hyphae.

A growth system with a low supply of nutrient solution, permitting development of nutrient gradients was used in the pot experiments (I, II). The results obtained suggest that when nutrient solutions around roots are not changed frequently the role of the extramatrical mycelium is larger than in the experiments with frequent

nutrient replacement. The choice of growth system will affect interpretation of results. If the goal is to produce seedlings with similar nutrient concentrations in well controlled experiments, the function of the extramatrical mycorrhizal mycelium is partly compensated for in non-mycorrhizal seedlings if a growth system with frequent application of nutrient solution to the root systems is used. This gives non-mycorrhizal plants an opportunity to take up nutrients effectively without a well developed external mycelium. At the same time the ecological relevance of the system decreases. In the system used in the present thesis (I, II) mycorrhizal colonisation affected growth and nutrient uptake of Scots pine seedlings, and a classical mycorrhiza effect was found: mycorrhizal colonisation led to bigger plants, and nutrient uptake also differed between well and poorly colonised (including non-mycorrhizal) seedlings. Concentration of P was higher in well colonised seedlings, whereas N concentration was higher in non-mycorrhizal seedlings than in mycorrhizal seedlings, which indicates that different factors are limiting for the growth of mycorrhizal and non-mycorrhizal seedlings.

Differences in sensitivity to the Al treatment between different ectomycorrhizal fungi were found (I). The *Hebeloma* cf. *longicaudum* strain used, which caused the highest stimulation of growth of the seedlings without additional Al exposure, barely survived the 2.5 mM Al treatment in contrast to *Laccaria bicolor* which was unaffected by the Al treatment. Some ectomycorrhizal fungi may thus be more sensitive to Al than their plant symbionts. It is not clear whether the apparent sensitivity of *H.* cf. *longicaudum* would play an important role under natural conditions because the Al concentration used was rather high. Other side effects as such as Cl, Mn, or Fe toxicity and the lower pH may also have played a role. However, the differences between the different mycorrhizal isolates clearly demonstrate that the potential effects of mycorrhizal symbionts should be taken into account when modelling responses of plants to changed soil chemistry arising from acidification.

It is difficult to devise a good method to test the usefulness of the BC/Al ratio as an estimator of Al toxicity under laboratory conditions. If controlled systems are used with a frequent solution change around the root systems, many important factors are excluded, for example nutrient and Al gradients which occur naturally in soil during wetting and drying and the possible role of ectomycorrhiza in stabilising pH and hindering nutrient leakage from soil. Some indication of pH stabilization was found in the present studies when Al exposure was lower (0.74 mM) and the pH was about 0.5 units higher at the end of the experiment in the pots containing seedlings colonised by *L. bicolor* than in the pots containing non-mycorrhizal seedlings (I). Leakage of base cations was also lower from the mycorrhizal pots. If the amount of extramatrical mycelium in soil decreases due to pollution, as in a study by Wallander & Nylund (1992) with high N concentration, both soil pH and base cation leakage may be affected. The best approach is to perform different experiments trying to simulate different natural

phenomena, and to include results from field observations and experiments in interpretations. In the second Al experiment the Al treatment (0.74 mM) was combined with periodic watering of the pots with excess water during the experiment to simulate rainfall (I). Statistically significant growth decreases were found for the non-mycorrhizal seedlings (22 % for the shoots and 23 % for the roots) but the reductions for the mycorrhizal seedlings were not statistically significant (15 % for the shoots and 5 % for the roots). The results indicate that *L. bicolor* may delay or reduce negative effects on growth due to moderately elevated Al concentrations. Even though BC/Al ratios below 1 were not reached in these experiments, the results gained suggest that the role of mycorrhiza should be taken into consideration when modelling Al effects on trees as has been stressed by Högberg & Jensen (1994), Falkengren-Grerup et al. (1995) and Løkke et al. (1996).

No clear ameliorating effects of ectomycorrhizal fungi were shown against Ni or Cd toxicity even if some trends were found (II). However, well colonised mycorrhizal seedlings were bigger and had higher P, Mg, Ca and K uptake than non-mycorrhizal ones. This study shows a mycorrhizal improvement in nutrient status of the seedlings which may be a key factor for survival of seedlings in metal contaminated sites. Non-mycorrhizal seedlings were able to maintain their root growth when nutrients were added to the system, but functioning of the roots was probably disturbed because nutrient uptake was decreased in response to the heavy metal treatments. Mycorrhizal inoculation of seedlings may be worth performing in heavily contaminated sites where many ectomycorrhizal fungi have disappeared from soil and only can be found in less contaminated areas.

Mycorrhizal colonisation was found to remain at the same level after defoliation, whereas proportions of mycorrhizal types were different after a harsh defoliation: The proportion of tuberculate type was decreased and the proportion of smooth types increased due to defoliation (IV). The decrease after defoliation may mean that the tuberculate morphotype with its well developed rhizomorphs and thick mantles, is a relatively strong carbon sink and that the growth rate of this morphotype decreased due to decreased amounts of carbon transported to the roots. The changed composition of mycorrhizal morphotypes suggests competition among different mycorrhizal growth forms owing to their carbon demands and/or intensity of mutualistic association with the host tree. It would be interesting to investigate carbon allocation to different mycorrhizal types and species under laboratory conditions after decreasing the supply of photo-assimilates due to defoliation or shading.

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