# Ectomycorrhizal fungi and *Pinus sylvestris*: aluminium toxicity, base cation deficiencies and exudation of organic anions

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# Ectomycorrhizal fungi and *Pinus sylvestris*: aluminium toxicity, base cation deficiencies and exudation of organic anions

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## Abstract

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The finding of microscopic-small tunnels in mineral grains in podzol soils was the incentive for a research programme on rock-eating mycorrhizae. The research described in this thesis was part of that program. The tunnels were thought to be created by ectomycorrhizal (EcM) fungi through exudation of low molecular weight organic anions (LMWOA). LMWOA can detoxify Al by forming stabile non-toxic complexes and can enhance mineral weathering, thereby mobilising base cations for plant and fungus. In this study we demonstrated that a role for EcM-fungi in detoxification of Al and in mobilisation of base cations from mineral grains is likely since both toxic Al levels and low base cation supply induced LMWOA exudation by ectomycorrhizae.

The risk for Al toxicity is often assessed by regarding the ratio of base cations (BC) to Al in the soil solution. Adverse effects on tree growth are expected at BC:Al<1. We demonstrated that Al toxicity is not determined by the BC:Al ratio. Growth reductions in *Pinus sylvestris* and *Picea abies* seedlings were better predicted by the concentration of BC and Al in solution than by the BC:Al ratio. At a constant BC:Al ratio of 1, shoot growth decreased with increasing concentrations of Al. Seedlings of *P. sylvestris* responded to Al by the exudation of LMWOA. Indeed, EcM fungi enhanced this exudation, suggesting a role for EcM-exuded LMWOA in detoxifying Al. The exudation was further enhanced when levels of toxic Al were combined with deficient Mg and P supply. EcM fungi did not physically hinder Al uptake and did not improve uptake of Ca or Mg under Al toxicity.

Ectomycorrhizae affected the exudation of LMWOA and weathering of minerals when plants were deficient in Mg, K or P. The exudation of LMWOA (mainly oxalate and malonate) was nutrient-specific and EcM species-specific. Exudation of LMWOA by the EcM symbiosis could not be predicted from the exudation of LMWOA by seedlings or EcM fungi grown in pure culture. The oxalate exudation by ectomycorrhizae in response to Mg and K deficiencies correlated well with the ectomycorrhizal induced weathering of minerals containing K and Mg .Weathering of the K-containing muscovite was almost doubled if seedlings were colonised by *P. involutus* compared to seedlings colonised by other EcM fungi or without mycorrhizae. There was no effect of EcM fungi on the weathering of Mg-containing hornblende. Localised Mg addition stimulated EcM foraging under Mg deficiency. Hyphal foraging and transport of Mg to the roots was independent of P supply or P transport.

**Keywords**: aluminium (Al), base cations, BC:Al ratio, magnesium (Mg), organic anions, oxalate, malonate, ectomycorrhizal fungi, *Paxillus involutus, Pinus sylvestris* (Scots pine)

## Pictures



*Pictures on pages 73, 101 and 150 taken by R. Landeweert*
*Pictures on pages 1 and 123 courtesy of E. Hoffland*
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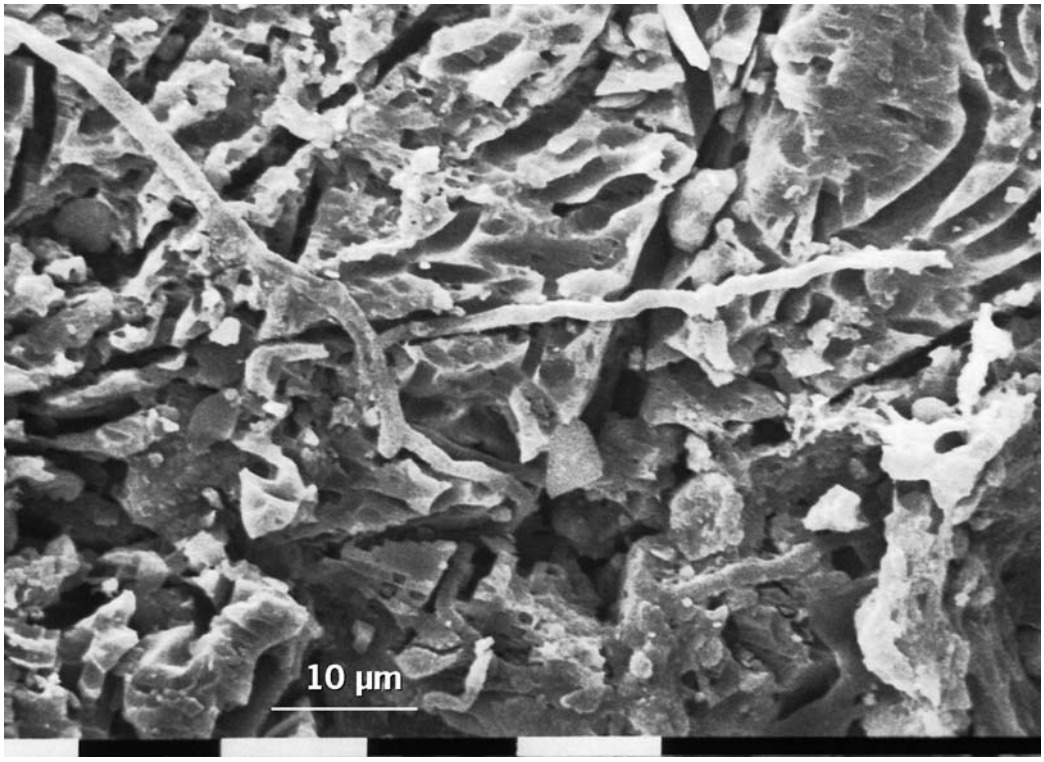
# General introduction



## Ectomycorrhizal fungi

Ectomycorrhizal fungi are a group of soil fungi that form a mutualistic symbiosis with the roots of host plants, almost exclusively trees and shrubs. Under natural conditions, most plants are associated with mycorrhizal fungi. The structure, development and role in ecosystems of the ectomycorrhizal symbiosis have been described in the book on the mycorrhizal symbiosis by Smith and Read (1997), from which the information in this section derives. It has been estimated that 5000-6000 fungal species are ectomycorrhizal, and most of them are able to associate with a broad range of host plant species. Ectomycorrhizal plant species constitute the dominant tree species in the northern boreal (*Pinaceae* family) and temperal (*Fagaceae* family) forests, and of the southern temperal and subtropical forests (*Myrtaceae* family), which occupy a vast land surface, and have economic importance as a major source of timber. Under natural conditions, most plants are associated with mycorrhizal fungi, but only approximately 3% of the seed plants are considered ectomycorrhizal.

Ectomycorrhizal fungi do not penetrate the root cells, a feature that distinguishes them from other mycorrhizal fungi that form intracellular connections in plant roots. Ectomycorrhizae, the association between root tips and ectomycorrhizal fungi, are characterised by three structural components: the *sheath* or *mantle*, formed by a network of hyphae enveloping the root, the *Hartig net,* a labyrinthine network of hyphae occupying the space in between the epidermal and cortical cells, and the *extraradical (or extramatrical) mycelium*, the hyphae radiating out from the sheath.

Ectomycorrhizal fungi, with few exceptions, completely depend on their host for carbohydrates, while the host plants receive water and nutrients taken up by the mycorrhizal fungus. The apoplastic exchange of carbon, water and nutrients takes places in the Hartig net. The extraradical mycelium connects the ectomycorrhizal root tip with the surrounding soil and with the fruit bodies, and plays a key role in the uptake water and nutrients. Because of their extensive proliferation, the ectomycorrhizal hyphae explore the soil more thoroughly than the tree roots alone. With their small diameter, hyphae can access micro sites that are inaccessible to root tips, and due to their high surface to volume ratio, they have a higher adsorbing capacity for water and nutrients. In addition, ectomycorrhizal fungi can mobilize N and P

from organic or mineral sources through the exudation of enzymes and organic anions. In general, ectomycorrhizal fungi improve the nutrient condition and growth of their host plants.

## Rock-eating mycorrhizae

In 1997, tunnel-like pores were discovered in mineral grains originating from the mineral E horizon of podzol soils (Jongmans *et al.*, 1997). These tunnels were distinguished from etch pits and other forms of chemical weathering by their smooth walls, rounded end, and constant diameter. Ectomycorrhizal fungi were hypothesised to be responsible for this mineral tunnelling: Ectomycorrhizal trees dominated the vegetation on the podzol soils in which the tunnels were found, and whereas the tunnels provided a good fit for fungal hyphae, they were too narrow to be accessed by tree roots. Ectomycorrhizal fungi can exude low molecular weight organic anions (LMWAO) (Gadd, 1999) that have high weathering potential (Drever & Stillings, 1997). Because of their association with a host tree, they have a source of carbon available in the mineral soil horizon, in contrast to most saprotrophic fungi or bacteria that derive their carbon from the decomposition of organic matter, which is rare in the mineral E horizon.

These findings have triggered the start of a research programme called "Rock-Eating Mycorrhizae: Where, Why, How?" at Wageningen University. Tunnelled minerals were seen as a result of the weathering ability of ectomycorrhizal fungi and this raised intriguing questions on the role of ectomycorrhizal fungi in ecological processes such as biogeochemical cycles and plant uptake of base cations. The work presented in this dissertation was conducted within the context of this research programme. It focussed on tree uptake of base cations and on the exudation of LMWAO by ectomycorrhizal fungi and tree seedlings under aluminium (Al) toxicity and base cation deficiencies. If ectomycorrhizal fungi could improve plant uptake of base cations and enhance the exudation of LMWOA, this might play an important role in counteracting the negative effect of atmospheric deposition on plant growth.

## Organic anions

Organic acids are organic compounds that contain at least one carboxyl (COOH) group. The function and behaviour of organic acid anions in plant exudates and in soil have been reviewed extensively (Hocking, 2001, Jones, 1998, Ryan *et al.*, 2001, Strobel, 2001). Within plants, organic acids have an important role in cellular me-

tabolism. Typical total concentrations of organic acids in the roots range between 10-20 mM (1-4% of total root dry weight). Within the plant cell cytosol, most organic acids exist as the fully dissociated anions (with $COO^{-1}$), and most likely organic acids are also exuded in the form of organic anions. Organic anions are a common part of root exudates. Exudation of organic anions may be stimulated by nutrient deficiencies and metal toxicities, especially Al. Other sources of organic anions in the soil solution are root degradation and release by soil micro-organisms. Typical concentration in the soil solution range between 0.5-10 μM, but concentration in the rhizosphere may be considerable higher. Due to their negative charge, organic anions can form complexes with metal ions in solution and adsorb to soil particles, thereby reducing metal toxicity and enhancing mineral dissolution.

## Atmospheric deposition

In the last decades of the 20th century there was wide-spread public fear for large scale forest deterioration in Europe and North-America, commonly termed "*Neuartige Waldschäden*", new type or recent forest decline, or more dramatically "*Neuartige Waldsterben*", forest dieback. This forest deterioration was attributed to atmospheric deposition of acidifying pollutants ('acid rain'). Forests are complex ecosystems with many interrelated and interacting processes, and a direct link between cause and effect is therefore difficult to quantify in field observations. There have been many theories about the possible mechanisms leading to forest decline (reviewed by Wellburn, 1994), including bad management, direct harmful effect of atmospheric pollutants on the leaves or needles, pathogen attack, ozone effects, droughts, but the indirect effects of atmospheric deposition through changes in the soil chemistry stand out (Godbold & Hüttermann, 1994, Johnson & Lindberg, 1992a, Ulrich & Pankrath, 1983). Due to atmospheric deposition of acidifying elements, the pH of soil solution declines, resulting in increased leaching of base cations (K, Ca, Mg) and increased concentrations of dissolved Al (Johnson & Lindberg, 1992a, Mulder *et al.*, 1987, Ulrich *et al.*, 1980). Both Al toxicity and base cations deficiencies are considered to attribute to forest decline.

## Aluminium toxicity in forest ecosystems

At pH below 4 the major form of inorganic Al in the soil solution is the phytotoxic $Al^{3+}$ (Kinraide, 1991). The exact mechanisms of Al toxicity in plants are not yet fully clarified despite considerable research (for reviews see: Delhaize & Ryan, 1995, Kochian, 1995, Kochian *et al.*, 2004, Ma *et al.*, 2001, Ryan *et al.*, 2001).

Symptoms of Al toxicity are stunted root growth, which leads to poor uptake of water and nutrients, and deficiencies of P, Ca and Mg. Aluminium blocks ion channels over the root cell wall, and inhibits the uptake of Ca and Mg specifically by kinetic processes in the root cell walls. Increased concentrations of Ca and Mg can -partly- mitigate Al toxicity (Kinraide, 2003). The ratio of the concentration of base cations (BC) to Al in the soil solution is seen as indicator of Al toxicity for forest ecosystems (Cronan & Grigal, 1995, Sverdrup *et al.*, 1992). This BC:Al ratio, with the BC comprising either Ca, Ca+Mg or Ca+Mg+K, is used as a chemical criterion in the calculation of acceptable loads of atmospheric deposition, with a critical threshold value 1 for BC:Al, below which reduced forest growth can be expected (De Vries, 1988, Hettelingh *et al.*, 1995, Sverdrup & De Vries, 1994). The use of the BC:Al ratio to determine the effect of Al has been heavily criticised (Binkley *et al.*, 2000, De Wit, 2000, Falkengren Grerup *et al.*, 1995, Högberg & Jensén, 1994, Løkke *et al.*, 1996). The fact that the BC:Al ratio can be useful to indicate conditions that are unfavourable to plant growth does not necessarily mean that plant growth is directly determined by the BC:Al ratio. Experimentally validated mechanistic explanations of the relation between the BC:Al ratio and tree response are lacking. The predictive value of the BC:Al ratio in the soil solution compared to Al concentration, and several assumptions underlying the use of the BC:Al ratio were tested in Chapter 2.

## Mitigation of Al toxicity by ectomycorrhizal fungi

A factor that is not accounted for in the determination of Al toxicity is that most tree roots are ectomycorrhizal. Ectomycorrhizal fungi may protect their host trees against the negative effects of Al toxicity (Finlay, 1995, Jentschke & Godbold, 2000, Meharg, 2003). Most experiments on Al toxicity in trees have been done with nonmycorrhizal seedlings, but experiments in which nonmycorrhizal and ectomycorrhizal seedlings were compared showed that, in most cases, ectomycorrhizal seedlings maintained higher growth rates than nonmycorrhizal seedlings under Al toxicity (Table 1).

Ectomycorrhizal fungi generally improve the nutrient condition and growth rate of their host plants. Aluminium reduces root growth, thereby reducing exploration of the soil and reducing the uptake of water and nutrients, making the plants more susceptible to drought and other stresses. Under these conditions, the role of the extramatrical mycelium in the uptake of water and nutrients becomes even more important. This was also shown in an experiment by Schier & McQuattie (1996) where either ectomycorrhizal colonisation or increased nutrient supply improved growth rate and reduced Al toxicity symptoms in *Pinus rigida* seedlings.

**Table 1:** Effect of ectomycorrhizal colonisation on growth rate and concentrations of Al, Ca and Mg in seedlings, compared to nonmycorrhizal seedlings.

| Tree species | Ectomycorrhizal species | Al mmol $l^{-1}$ | Effect ectomycorrhizal fungi | Reference |
|---|---|---|---|---|
| *Pinus banksiana* | *Rhizopogon rubescens*<br>*Suillus tomentosus* | 0.037-0.37 | Lower growth rate<br>No effect on Al concentration needles<br>Lower % of root tips colonised by *R. rubescens* with high Al | Jones *et al.*, 1986 |
| *Pinus rigida* | *Pisolithus tinctorius* | 0-0.05 0.2 | Higher growth rate in Al treatments<br>Lower concentrations of K, Ca, Al | Cumming & Weinstein, 1990a |
| *Picea abies* | *Suillus bovinus* | 0-0.2-…-30 | Higher growth rate | Göransson & Eldhuset, 1991 |
| *Picea abies* | *Paxillus involutus* | 0-0.2-0.8 | Lower growth rate in control treatment, higher growth rate in Al treatment<br>Lower Al concentrations shoot | Hentschel *et al.*, 1993 |
| *Eucaliptus rudis* | *Pisolithus tinctorius* | 0-500 mg $AlCl_3$/ml | Lower concentration of Al, higher concentration of Ca and Mg in shoot | Egerton-Warburton *et al.*, 1993 |
| *Pinus rigida* | *Pisolithus tinctorius* | 0-0.46-...-3.71 | Higher growth rate under Al and control treatment<br>Lower concentrations of K, Mg and Al, higher conc. of P | Schier & McQuattie, 1995 |

In pot experiments, it is impossible to separate the effect of improved nutrient conditions through ectomycorrhizal colonisation from other mechanisms by which the fungus protects their host plants (Jentschke & Godbold, 2000, Meharg, 2003) and therefore improved growth rates in pot experiments cannot be used to show that ectomycorrhizal fungi *protect* their host plants.

Ectomycorrhizal fungi may protect the tree root by the exclusion of Al from the root cortex and improved uptake of Ca and Mg (Cumming & Weinstein, 1990a, Egerton-Warburton *et al.*, 1993, Finlay, 1995). Aluminium toxicity mainly affects the root apex, which is the major site of Mg and Ca uptake (Häussling *et al.*, 1988). As this is also the site where the sheath and Hartig net are formed, it seems likely that this hyphal network modifies the entry of Al and other ions. In Chapter 3 we used a semi-hydroponic set up to investigate whether ectomycorrhizal fungi would exclude Al and improve the uptake of Ca and Mg by *Pinus sylvestris* (Scots pine) seedlings. In semi-hydroponic systems, the delivery of nutrients to the roots is ensured, and growth differences due to higher proliferation and nutrient delivery by the ectomycorrhizal fungi are avoided.

Ectomycorrhizal fungi may also protect their tree host from Al by the exudation of LMWOA (Ahonen Jonnarth *et al.*, 2000, Cumming & Weinstein, 1990a, Schier & McQuattie, 1995). The LMWOA form strong non-toxic complexes with Al, which do not bind to the root cell wall and are not taken up. Root exudation of LMWOA in response to Al seems a general response of Al tolerant plant varieties (Ryan *et al.*, 2001). Most research on this mechanism, however, has been carried out with herbaceous plant species, and it is uncertain if woody plants respond similarly. Trees can withstand considerable concentrations of Al (>100 µM) in the rooting environment compared to herbaceous plant, to which a few µM of Al can be toxic (Moyer-Henry *et al.*, 2005). Aluminium toxicity is often accompanied by P and Mg deficiencies, which might also induce the exudation LMWOA. In Chapter 4, we tested if LMWOA exudation under Al toxicity is specific for Al, or for the accompanying P and Mg deficiency. Furthermore, we tested if simultaneous Al toxicity and Mg and P deficiency has a synergistic effect on the exudation of LMWOA.

## Rock-eating mycorrhizae: contribution to tree uptake of base cations

Ectomycorrhizal fungi may improve tree uptake of base cations by enhanced weathering of mineral grains. Most research on the nutrition effects of ectomycorrhizal fungi has dealt with the uptake of N and P (Smith & Read, 1997), which are the elements that are generally growth-limiting for forest growth (Tamm, 1985). Base

cations can become growth-limiting as a result of biomass removal by harvesting and leaching. Atmospheric deposition leads to increased leaching of base cations. It has also been recognised, however, that acidification of the soil solution will also increase the dissolution of base cations, which may partly compensate the increased leaching (Johnson & Lindberg, 1992). Ectomycorrhizal fungi may enhance weathering by the exudation of LMWOA, but so far exudation of LMWOA has not been linked to base cation deficiencies of tree or fungi. The results of experiments on LMWOA exudation of ectomycorrhizal fungi and *P. sylvestris* seedlings, grown in symbiosis or in pure culture, in response to nutrient deficiencies are described in Chapter 5. By comparing the exudation of seedlings and fungi, grown in symbiosis or in isolation, we aimed to answer the question: who is driving this response, tree or fungus?

In Chapter 6, the capacity of ectomycorrhizal fungi to enhance weathering of minerals containing K and Mg and to improve plant uptake of K and Mg was investigated in a pot experiment.

If ectomycorrhizal fungi exude LMWOA with high weathering capacity in response to Mg deficiency, it might also be expected that the hyphae will also forage for Mg and transport Mg. Transport of Mg through ectomycorrhizal fungi has been shown, but hereby it was assumed that this was a passive co-transport with P, driven by P deficiency (Jentschke *et al.*, 2001). The foraging behaviour and transport of Mg through the ectomycorrhizal hyphae in response to Mg deficiencies of their host plant was investigated in Chapter 7.

In the last Chapter 8, the outcomes are discussed and put in perspective of the results of the whole Rock-Eating Mycorrhizae research programme in a review.

## *Pinus sylvestris* and ectomycorrhizal fungal species used

The tree species used this thesis research is *Pinus sylvestris* (Scots pine[1]), belonging to the *Pinaceae***.** It is a common and widespread tree species in boreal and temperate forest. *Pinus sylvestris* is native in Europe and North Asia, and introduced into North America. It prefers a sandy, well-drained, acidic soil, and can grow well on infertile and dry soils. Because of it hardiness and low nutrient requirements, it is planted for erosion control, and used to reforest coal mine spoils and burned sites (Sullivan, 1993). In North America, it is a popular Christmas tree. The wood, re-

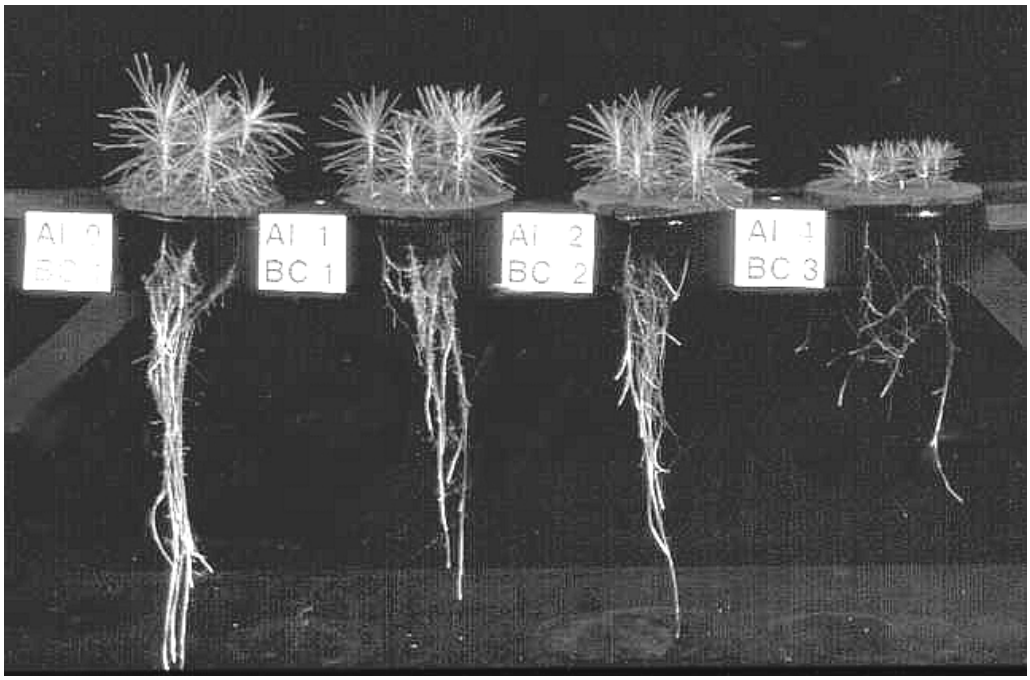
[1] The term 'Scotch' pine, although widely used in North America, is grammatically incorrect, as it does not refer to the famous drink but to Scotland, where the term Scotch is considered offensive

ferred to by the timber trade as "redwood" or "deal", is a general purpose timber, used for fencing, carpentry, building, flooring, box and packing case manufacture, railway sleepers, pit wood, fibreboard, chipboard, and telegraph poles.

The ectomycorrhizal fungi used were taken either from the field (Chapter 3) or from laboratory cultures (Chapters 4-7). To do laboratory experiments under controlled condition, it is necessary to have ectomycorrhizal species that can be cultured in pure culture, have a relatively high growth rate and can form ectomycorrhizae with tree seedlings under artificial conditions. The ectomycorrhizal fungi *Paxillus involutus*, *Hebeloma longicaudum*, *Piloderma croceum*, *Suillus bovinus*, *Rhizopogon roseolus* and *Laccaria bicolor,* used in this study, possess all these characteristics.

# 2

# Aluminium concentration versus the Base Cation to Aluminium ratio as predictors for aluminium toxicity in *Pinus sylvestris* and *Picea abies* seedlings.

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## Summary

Aluminium (Al) toxicity is considered an important factor in forest deterioration caused by soil acidification. A ratio of base cations (BC) to Al in the soil solution lower than 1 is widely used as an indicator for potentially adverse effects on tree health. In our view, the validity of the assumptions underlying the use of the BC:Al ratio as an indicator for Al toxicity in trees has never been evaluated properly. Here we evaluate the importance of the base cations Ca and Mg in counteracting Al toxicity. *P. sylvestris* and *P. abies* seedlings were grown on nutrient solution with a range of Al (0-0.25-0.5-1-2 mM) and base cation (0.25-0.5-2 mM) concentrations, giving BC:Al ratios of 1 at different levels of Al. Increasing concentrations of Al in solution caused growth reductions, which could not be counteracted by increasing concentrations of BC in solution with *P. sylvestris* and only partly counteracted with *P. abies*. Increased concentrations of Al in solution decreased the concentrations in shoot and root of both Ca and Mg, while increased concentrations of BC in solution increased tissue concentrations of BC. Growth reductions were, however, not a result of BC deficiencies, as growth reduction already occurred in tree seedlings that maintained adequate concentrations of Ca and Mg. All growth and uptake variables measured showed a higher or equal correlation with the absolute concentrations of Al or Al+BC in solution than with the BC:Al ratio. We conclude that Al toxicity is determined solely by the concentration of Al in solution. Shoot growth decreased significantly as dissolved Al increased at a constant BC:Al ratio of 1. In *P. abies*, but not in *P. sylvestris*, dissolved BC can positively affect uptake of BC and growth, which might partly alleviate the toxic effects of Al. Our results show that the mechanistic explanation for the effect of the BC:Al ratio is insufficient to describe Al toxicity. Care should be taken when using models based on the BC:Al ratio to predict the effect of Al on tree growth.

## Introduction

Aluminium (Al) toxicity is considered an important factor in forest decline caused by acidification of the soil solution (Ulrich *et al.*, 1980). Soil acidification leads to increasing concentrations of aluminium and to decreasing concentrations of the base cations (BC) K, Ca and Mg in the soil solution (Puhe *et al.*, 2000, Ulrich, 1983). At pH values lower than 4, dissolved Al is mainly present as $Al^{3+}$, which is toxic to plants (Kinraide, 1991). The exact mechanisms of Al toxicity are not fully understood, despite considerable attention to the Al toxicity problem (Kochian, 1995, Matsumoto, 2000, Taylor, 1995). Symptoms of Al toxicity include halted root growth and Ca and Mg deficiencies, resulting in growth reductions. Impaired root growth results from inhibited cell division and cell elongation, for which a disruption of regulatory processes in the cytoplasm and at the plasma membrane are considered key factors (Marschner, 1991). Al reduces the uptake of Ca and Mg by replacing them at the binding sites in the root apoplast, where high concentrations of Ca and Mg are needed for high uptake rates (Marschner, 1991). Increasing the concentration of Ca and Mg in the soil solution can mitigate Al toxicity (Alva *et al.*, 1986, Blamey *et al.*, 1992, Tan *et al.*, 1991).

In model calculations on the impact of soil acidification on forest ecosystem functioning the ratio of BC to Al (BC:Al ratio) in the soil solution is widely used as a chemical indicator for Al toxicity. Adverse effects on tree health are expected below a threshold value of 1 for BC:Al (Hettelingh *et al.*, 1991, Posch *et al.*, 1997, Sverdrup & De Vries, 1994, Sverdrup *et al.*, 1992a, Sverdrup *et al.*, 1992b). This threshold value of BC:Al=1 is used to determine long term acceptable loads for atmospheric deposition, which are used for policy making (e.g. Hettelingh *et al.* (1991), Posch *et al.* (1997). The applicability of the BC:Al ratio (with Ca as BC) was reviewed by Cronan & Grigal (1995) who concluded that -given a set of other criteria- a BC:Al ratio lower than 1 in the soil solution indicates a 50% risk of adverse impacts on tree growth or nutrition. However, in the studies reviewed, aluminium toxicity symptoms occurred at a wide range of BC:Al ratios (0.1-5). Cronan & Grigal (1995) also showed that several studies show good correlation between aluminium toxicity effects and BC:Al ratio, whereas other studies do not show an effect of BC or BC:Al ratio. From the studies reviewed it does not become clear whether the BC:Al ratio is useful because it generally indicates conditions with high Al and low

BC, or because the BC:Al ratio itself has a mechanistic meaning. The use of the BC:Al ratio (with either Ca+Mg or Ca+Mg+K comprising BC) to predict aluminium toxicity was also proposed by Sverdrup & De Vries (1994) and Sverdrup *et al.* (1992b). However, in the studies presented by Sverdrup & De Vries (1994) and Sverdrup *et al.* (1992b), the growth of different types of trees at a BC:Al ratio of 1 varied between 50% and 100% of the growth of control trees.

The use of the BC:Al ratio to calculate aluminium toxicity effects on forest ecosystems has been criticised because several of the underlying assumptions, as stated explicitly in Sverdrup *et al.* (1992b), are not supported theoretically or experimentally (Binkley & Högberg, 1997, Högberg & Jensén, 1994, Løkke *et al.*, 1996). Firstly, tree growth is supposed to be linearly proportional to root growth, which in turn is supposed to be linearly proportional to the uptake of the limiting nutrient. The BC are assumed to be the limiting nutrients. By defining the effect of aluminium as base cation deficiencies, the direct toxic effects of aluminium are ignored. There is no experimental evidence for a linear relationship between uptake of BC, root growth and tree shoot growth under aluminium toxicity. Also, forest tree growth is generally limited by nitrogen availability, not BC (Binkley & Högberg, 1997). Secondly, uptake of BC is supposed to be proportional to the concentrations of adsorbed BC on active root surfaces. This assumption ignores the selectivity of roots in the uptake of nutrients, and is contradicted by experiments involving Ca (Ryan *et al.*, 1994) and Mg (Godbold & Jentschke, 1998). Thirdly, Al and BC adsorption on the root is supposed to be governed by concentrations in the bulk soil solution via by Gapon ion-exchange coefficients originally derived for soil organic matter. In fact the concentrations in the rhizosphere are modified by plants through nutrient uptake and mass flow, and can vary considerably from those in the bulk soil solution (Marschner, 1991). Another point of critique is that the BC:Al ratio is unspecific as it can be based on several elements (K, Ca and Mg) which are all essential for growth (Göransson & Eldhuset, 2001). Furthermore, the predictive power of the threshold value of 1 for the BC:Al ratio is undermined by the interchangeable use of Ca (Cronan & Grigal, 1995, Rost-Siebert, 1983), Ca+Mg (Sverdrup *et al.*, 1992b) or Ca+Mg+K (Sverdrup & De Vries, 1994) for BC.

Direct experimental evidence that the BC:Al ratio in the soil solution determines tree growth is lacking. Rost-Siebert (1983) combined a range of Ca concentrations with a range of Al concentrations at constant pH in a hydroponic experiment. The negative impact of Al on root length was counteracted by Ca when the Ca:Al ratios in nutrient solution were higher than 1. However, the effect of Al concentrations or the Ca:Al ratio on nutrient uptake or shoot dry weight were not measured. In several pot

experiments, BC:Al ratios have been varied by manipulating pH, BC and Al together (Gobran *et al.*, 1993, Sucoff *et al.*, 1989, Wolfe & Joslin, 1989). The pH itself has a negative effect on tree growth and the uptake of BC (Arduini *et al.*, 1998, Godbold *et al.*, 1995). In other experiments either Al (Arovaara & Ilvesniemi, 1990) or base cation concentrations (Ericsson *et al.*, 1998) were varied. From these studies it is not possible to deduct if the toxic effect on growth is the result of a decreased BC:Al ratio, or the direct result of low BC, high Al, or decreased pH.

Clearly, experimental evidence to validate the assumptions underlying the use of the BC:Al ratio and the threshold level of BC:Al=1 to predict Al toxicity is incomplete. In this study we evaluate these very assumptions by determining the importance of base cations in counteracting Al toxicity over a whole range of Al and base cation concentrations, giving BC:Al ratios of 1 at different levels of Al and constant pH. We consider the BC to consist of Ca and Mg. K is not included in the definition of BC because uptake of K is usually unaffected by Al (Marschner, 1991). We hypothesise (1) that Al toxicity is not governed by the BC:Al ratio but by the absolute concentrations of Al and BC and (2) that purported experimental evidence in support for the relevance of BC:Al ratios is due to a coincidence of low BC:Al ratios with either harmful high Al levels or with low levels of base cations causing deficiencies. The BC:Al ratio will be compared with the concentrations of Al and BC in soil solution as a predictor for growth and BC uptake using regression analysis. To test the validity of the threshold level of 1 for the BC:Al ratio, we will compare shoot growth of seedlings grown at a BC:Al ration of 1 but at different concentrations of both Al and BC.

## Material and methods

*Seedling growth and treatment:*

Seeds of *Pinus sylvestris* (NLZT, Ommen, The Netherlands) and *Picea abies* (German 84008) were soaked overnight in demineralised water, surface-sterilised in 15% $H_2O_2$ for 30 min and germinated in moist coarse quartz sand, previously treated at 120°C for 24 hours. The experiment was conducted in the period February-April 1999 in Wageningen in a greenhouse at 60% relative humidity. The plants received direct daylight and were supplementary illuminated for 16 h $day^{-1}$ with HPI lamps at 400W.

Twenty-eight days after being sown, the seedlings were transplanted to 50-liter containers filled with continuously aerated nutrient solution (pH 4.0). The concentra-

tions in the basal nutrient solution were (in mM): 0.6 $NO_3$; 0.6 $NH_4$; 0.06 $H_2PO_4$; 0.5 K; 0.062 Mg; 0.187 Ca; 0.06 $SO_4$, and (in µM): 95 Fe (as Fe-EDTA), 46 B, 0.3 Cu, 0.1 Mo, 9.2 Mn, 0.8 Zn. The concentrations were chosen so that they resemble those found in forest soils (adapted from (Jentschke *et al.*, 1991). Eleven days after the seedlings had been transplanted, experimental treatments were applied. Treatments included 5 Al levels (0-0.25-0.5-1-2 mM) and 3 Base Cations (BC) levels (0.25-0.5-2 mM). Ca and Mg were supplied in a constant 3:1 molar ratio as in the basal solution above. Other nutrient concentrations were as given above. Each level of Al was combined with each BC level, resulting in 15 treatments. Because a BC:Al ratio of 1 is often considered as the threshold value for Al toxicity we choose concentrations of Al and BC resulting in ratios covering a range from below to above 1. The pH in all treatments was adjusted to 4.0 and subsequently checked and adjusted again if necessary. Concentrations of Al, Ca, Mg, P, K and Fe in the solution were measured weekly and replenished when needed (i.e. P).

Nine weeks after the start of the treatments all seedlings were harvested. Four sets of 5 seedlings each were used to determine dry weight and chemical analysis, and one set was used for root length measurements. Seedlings were separated into shoot and root and dried at 70°C for 48 hours. For chemical analysis of the root samples, the 4 sets within each treatment were pooled to obtain the minimally required sample mass.

*Chemical analysis:*

Nitrogen and phosphorus concentrations in roots and shoots were determined colorimetrically by automated flow analysis after digestion with $H_2SO_4$-salicyclic acid-$H_2O_2$ and selenium. Tissue concentrations of Al, Ca, Mg, K were measured by ICP-AES after digestion with $HNO_3$-$H_2O_2$-HF.

*Root length:*

Root length was measured using the cross section method (Newman, 1966).

*Model description and statistical analysis:*

All data on shoot and root growth, root length and concentrations of Ca, Mg and Al in shoot and root were analysed with linear regression analysis (stepwise procedure in SPSS 10.0). Data on shoot and root dry weight were log-transformed to increase homogeneity of variance. The regression model used was $Y=\beta_0+\beta_1\times A+\beta_2\times B$, with Y = dependent variable ($^{10}$log dry weight shoot, $^{10}$log dry weight root, root length, shoot and root concentrations of Ca, Mg and Al), $\beta_{0\text{-}2}$ = regression constants, and

A = Al or $Al^{1/2}$ and B = BC or $BC^{1/2}$. The square root of Al and BC were used where necessary to obtain a linear relationship. The effect of the BC:Al ratio on growth and uptake of BC was assumed to be determined via the adsorption of BC to the root cell wall. The regression model used was $Y=\beta_0+\beta_1*C$, with C = the reduction factor for adsorption to the root cell wall $BC^{1/2}/(BC^{1/2} + k_G*Al^{1/3})$, derived from Sverdrup *et al.* (1992b), with $k_G$ = 0.0125 the Gapon exchange coefficient for Al and BC. Model performance will be compared with the $R^2_{adj}$, which is the coefficient of determination adjusted for the effect of a different number of independent variables in the models.

Shoot growth at BC:Al in the soil solution of 1 but with different concentrations of both Al and BC were compared by a one-way ANOVA (SPSS 10.0).

## Results

*Plant growth:*

Shoot dry weight of both *P. sylvestris* and *P. abies* significantly decreased with increasing Al concentrations in solution. Only with *P. abies* did shoot dry weight increase with increasing BC in solution (Fig. 1. a,d; Table 1). Shoot dry weight correlated better with Al for *P. sylvestris* ($R^2$=0.83) and Al+BC for *P. abies* ($R^2$=0.79) than with BC:Al ratio ($R^2$=0.51 and 0.62 for *P. sylvestris* and *P. abies* resp.) (Table 1). Root dry weight of both *P. sylvestris* and *P. abies* showed the same trend as shoot dry weight (Fig. 1b,e), but the responses and correlation with Al and BC as well as the BC:Al ratio were weaker (Table 1) than for shoot dry weight.

Shoot dry weights at BC:Al in the soil solution of 1 but with different concentrations of both Al and BC (Fig. 2) were significantly different for both *P. sylvestris* ($P$<0.001) and *Picea abies* ($P$<0.001).

*Root length:*

Root length of both *P. sylvestris* and *P. abies* decreased with increasing Al levels in the nutrient solution (Fig. 1c,f; Table 1). There was no correlation with the BC concentration in solution (Table 1). Root length was weakly correlated with both the Al concentration ($R^2$=0.45 and 0.43) and the BC:Al ratio ($R^2$=0.15 and 0.35).

*Tissue concentrations of Ca, Mg and Al:*

The concentrations of Ca and Mg in shoot and root of *P. sylvestris* and *P. abies* decreased significantly with increasing Al in solution and increased significantly with

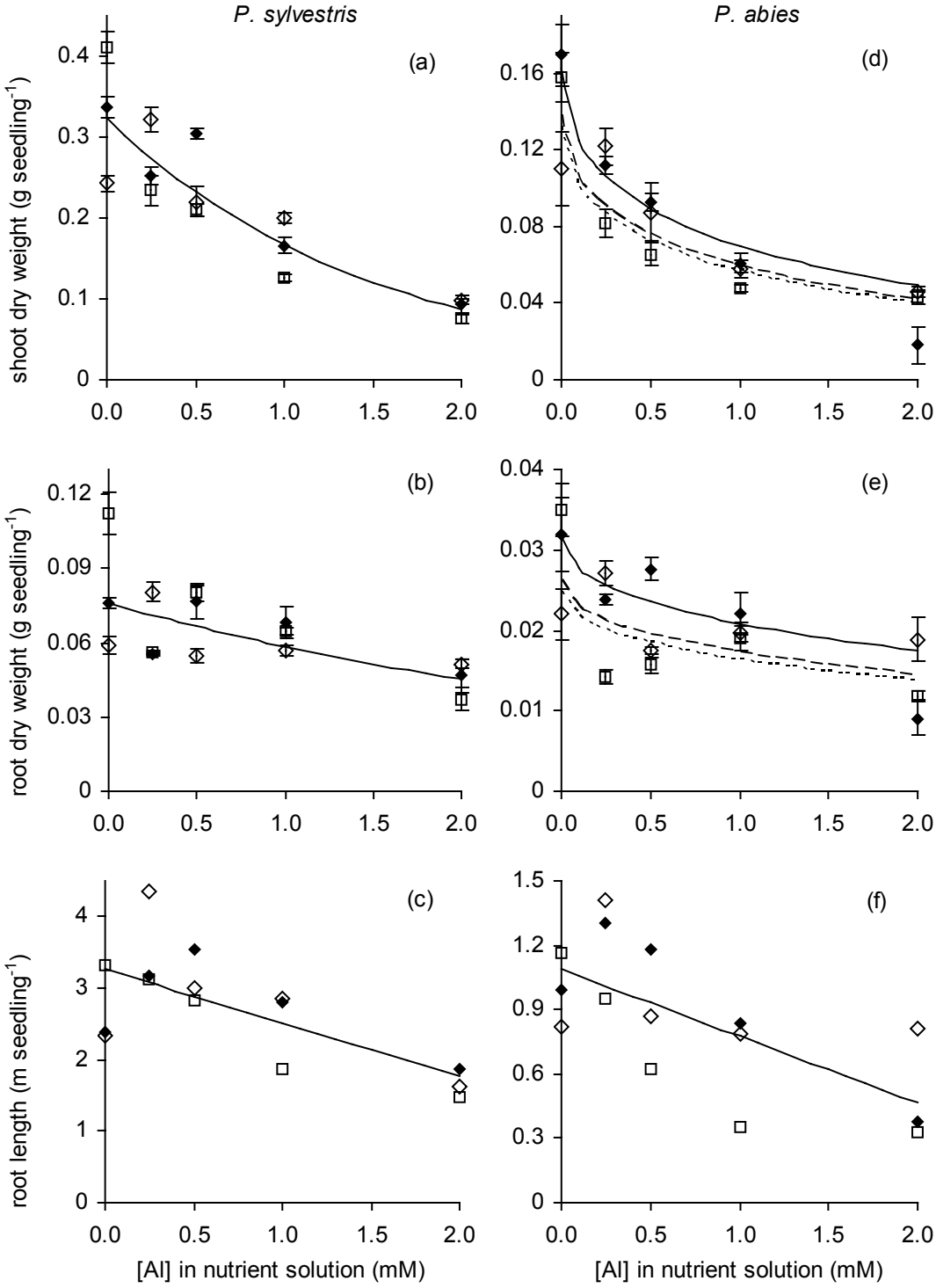



**Figure 1** Shoot and root dry weight (means of 4 ± s.e.) and root length (n =1) of *P. sylvestris* and *P. abies* grown for 9 weeks on nutrient solutions with 5 levels of Al and 3 levels of base cations (BC). Lines are the results of the regression model. Dotted line □ 0.25 mM BC; Dashed line ◊ 0.5 mM BC; Solid line ♦2 mM BC (in Fig. 1a-b-c-f lines coincide: no significant effect of BC in the regression model).

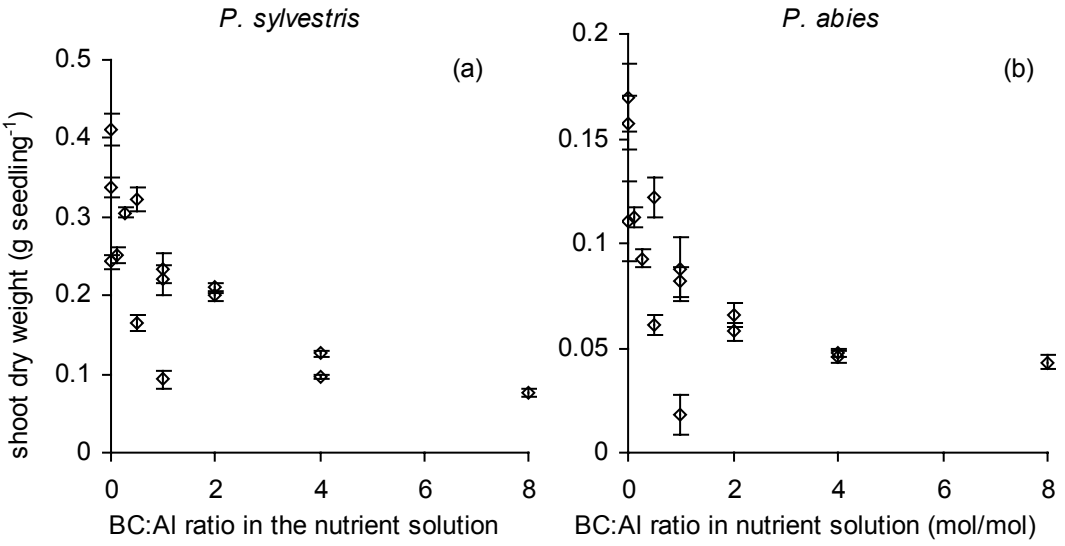



**Figure 2** Shoot dry weight of *P. sylvestris* and *P. abies* grown for 9 weeks on nutrient solution with different ratios of BC:Al (means of 4 ±s.e.).

increasing BC in the solution (Fig. 3a,b,d,e; Table 1). The Al concentration in the shoot of *P. sylvestris* strongly increased with 0.25 mM Al in the nutrient solution, but at higher Al levels did not increase further (Fig. 3c). There was no correlation with levels of BC in the nutrient solution (Table 1). The Al concentrations in the shoot of *P. abies* increased more gradually with increasing Al levels in the nutrient solution and were lower with 2 mM BC in solution than with 0.25 and 0.5 mM BC in solution (Fig. 3f). For each of the shoot and root concentrations of Ca, Mg or Al, the regression model with the additional effect of Al and BC in solution gave correlations higher than or equal to the correlations for the regression model with BC:Al ratio, for both *P. sylvestris* and *P. abies* (Table 1).

## Discussion

Our results do not support the assumptions underlying the use of the BC:Al ratio to indicate aluminium toxicity. Firstly, growth reductions are not determined by the BC:Al ratio. Increasing Al in solution reduced growth. Increasing BC in solution did not have an effect on this growth reduction in *P. sylvestris*. Therefore, the BC:Al ratio does not seem a meaningful predictor for Al toxicity in *P. sylvestris*. In *P. abies* the growth reductions could partly be counteracted by increasing BC in solution (Fig. 1a-e, Table 1). The additional effects of Al and BC, however, better explained growth reduction in *P. abies* than the BC:Al ratio did. The relatively high $R^2_{adj}$ for

**Table 1** Coefficients of determination ($R^2_{adj}$) of the regression between growth response or tissue nutrient concentrations of *P. sylvestris* and *P. abies* seedlings, and either the concentrations of Al and Base Cations (Al+BC) in the nutrient solution, or the ratio of BC to Al (BC:Al). The effect of BC:Al ratio was modelled via BC adsorption to the root cell walls, calculated with Gapon ion exchange reaction. The $R^2_{adj}$ is given instead of the $R^2$ to account for a different number of independent variables in the regression models. Shoot and root dry weight were log-transformed to homogenise variance.

| Dependent variable | *P. sylvestris* | | *P. abies* | |
|---|---|---|---|---|
| | Independent variable | $R^2_{adj}$ | Independent variable | $R^2_{adj}$ |
| $^{10}$lg Shoot dry weight | Al [a] | $0.83^{***}$ | √Al+√BC | $0.79^{***}$ |
| | BC:Al | $0.51^{***}$ | BC:Al | $0.62^{***}$ |
| $^{10}$lg Root dry weight | Al [a] | $0.40^{***}$ | √Al+√BC | $0.44^{***}$ |
| | BC:Al | $0.24^{***}$ | BC:Al | $0.47^{***}$ |
| Root length | Al [a] | $0.45^{**}$ | Al [a] | $0.43^{**}$ |
| | BC:Al | 0.15 | BC:Al | $0.35^{**}$ |
| Shoot Ca conc. | √Al+√BC | $0.62^{***}$ | √Al+√BC | $0.86^{***}$ |
| | BC:Al | $0.57^{***}$ | BC:Al | $0.66^{***}$ |
| Shoot Mg conc. | √Al+√BC | $0.83^{***}$ | √Al+√BC | $0.82^{***}$ |
| | BC:Al | $0.80^{***}$ | BC:Al | $0.76^{***}$ |
| Shoot Al conc. | √Al [a] | $0.57^{***}$ | √Al+BC | $0.69^{***}$ |
| | BC:Al | $0.43^{***}$ | BC:Al | $0.61^{***}$ |
| Root Ca conc. | √Al+√BC | $0.73^{**}$ | √Al+BC | $0.60^{**}$ |
| | BC:Al | $0.67^{***}$ | BC:Al | $0.43^{**}$ |
| Root Mg conc. | √Al+√BC | $0.91^{***}$ | √Al+√BC | $0.77^{***}$ |
| | BC:Al | $0.84^{***}$ | BC:Al | $0.69^{***}$ |
| Root Al conc. | Al [a] | $0.92^{***}$ | √Al [a] | $0.90^{***}$ |
| | BC:Al | $0.52^{***}$ | BC:Al | $0.50^{**}$ |

[a] = effect of BC not significant. ($P$>0.05); $^{**}P$<0.01; $^{***}P$<0.001.

the BC:Al ratio can probably be explained by the fact that low BC:Al ratios partly overlap with high Al concentrations.

Secondly, growth reductions are not (solely) the result of BC deficiencies, as in our experiment growth reduction occurred in tree seedlings that maintained adequate tissue concentrations of Ca and Mg. In all treatments the Ca concentrations in the shoot were above the critical level of 20 mmol $kg^{-1}$ d.w. for *Pinus spp*. (Reuter *et al.*, 1997) (Fig. 3a,d). The Mg concentrations in the shoot decreased with increasing Al concentrations in solution below the critical level of 30 mmol $kg^{-1}$ for *Pinus spp.* (Reuter *et al.*, 1997) (Fig. 3b,e). With increasing BC concentrations in solution the shoot Mg concentrations were increased. At the BC level of 2 mM the shoot Mg concentrations were increased even to above the critical level, but this was not enough to counteract the dry weight reduction caused by increased Al concentrations in solution (Fig. 1a,d). This indicates that growth reduction has to have other causes than Ca and Mg deficiency.

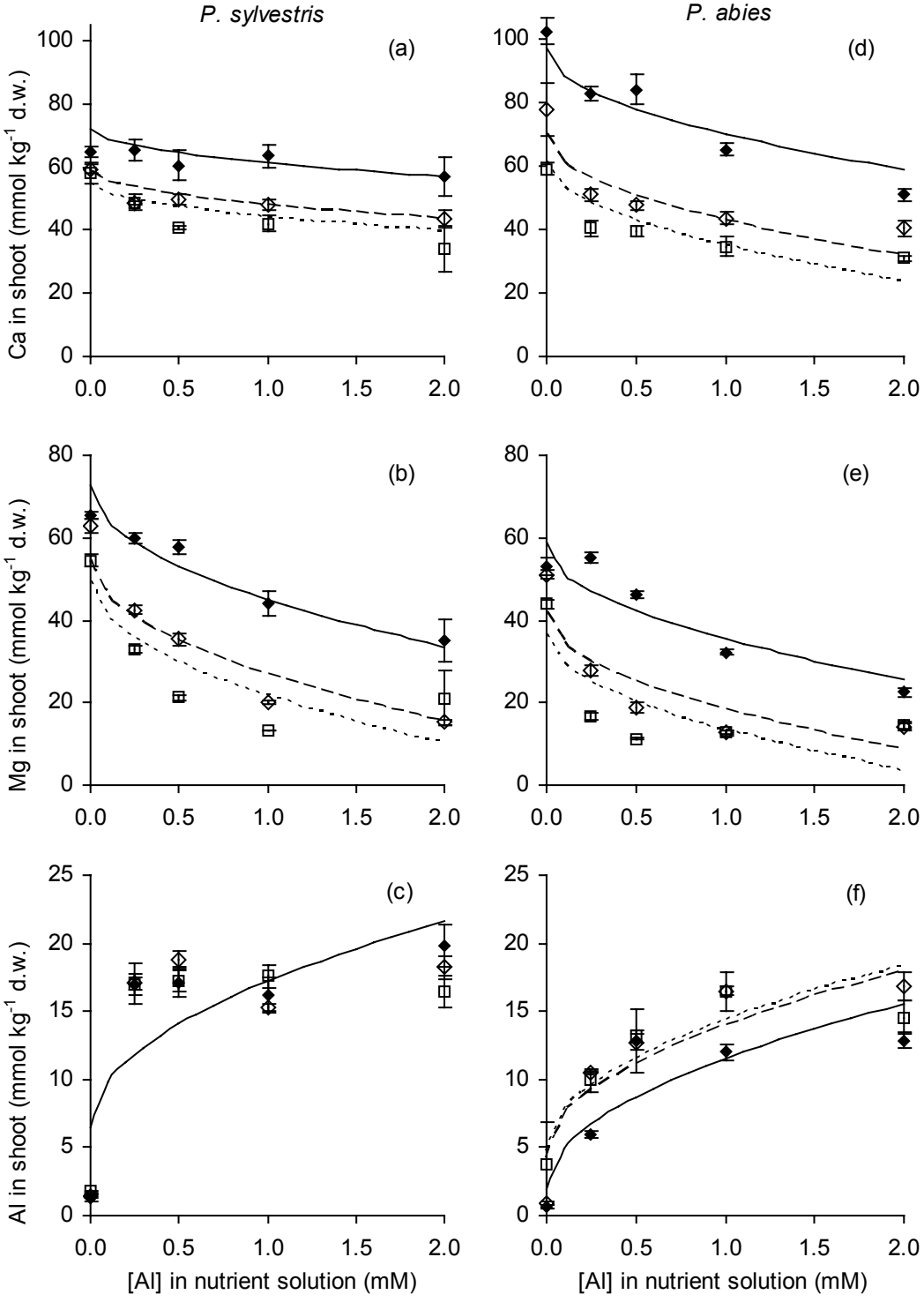



**Figure 3** Concentrations of Ca, Mg and Al in shoot of *P. sylvestris* and *P. abies* (means of n = 4 ± s.e.), grown for 9 weeks on nutrient solutions with 5 levels of Al and 3 levels of base cations (BC). Lines are the result of the regression model. Dotted line □ 0.25 mM BC; Dashed line ◊ 0.5 mM BC;Solid line ♦ 2 mM BC.

Thirdly, Al in solution decreased the concentrations in shoot and root of both Ca and Mg. Increased concentrations of BC in solution increased tissue concentrations of BC. The negative effect of Al, however, was not neutralised by higher BC in solution (Fig. 3a-d, Table 1), as expected if the ratio of BC:Al in solution determined uptake. Additional effects of Al and BC in solution better described the Ca and Mg in shoot and root than the BC:Al ratio.

By using ranges of both Al and BC in solution we could indicate that Al toxicity, seen as growth reduction and reduced uptake of BC, is not determined by the BC:Al ratio, but rather by the concentration of dissolved Al alone. Shoot growth significantly decreased at BC:Al ratios of 1 but with increasing Al in solution. The concentration of BC in solution can have a positive effect on the uptake of BC and -for *P. abies*- growth, which might partly alleviate the effects of Al. This was however better described by the absolute concentrations of Al and BC in solution than by the BC:Al ratio. Consequently, even if a the threshold value of 1 for BC:Al ratio might be practical, e.g. in calculating critical loads of acid atmospheric deposition, it is theoretically misleading. Moreover, the erroneous BC:Al concept is used to advocate the widespread application of liming in Scandinavian forests, with potentially dire ecological consequences (Binkley & Högberg, 1997).

Our results may provide some information on the mechanics of Al toxicity. Growth reduction at high Al concentrations in solution does not seem to be the direct result of too high Al concentrations in the shoot (Fig. 3c,f). The shoot Al concentrations were raised at an Al concentration in solution of 0.25 mM, but remained more or less constant with further increasing Al concentrations in solution while shoot dry weight decreased gradually. It is not likely that the reduction in root length with increasing Al concentrations in solution affected the uptake of other nutrients, as the tree seedlings were grown on a constantly stirred nutrient solution. The concentrations of nutrients others than Ca and Mg remained adequate (N and K) or only became deficient at the highest Al levels in the nutrient solution (P) (results not shown), and can therefore not explain the pattern of growth reduction as found. Growth reductions must therefore be the result of elevated Al concentrations in the rooting medium, leading to a disruption of cell functioning as proposed by Kochian (1995), Taylor (1995) and Matsumoto (2000).

The growth conditions under which the experiment was conducted and the concentration ranges of both Al and BC are different from those encountered in the field. In the forest soil solution Al and BC interact with other components, creating conditions that are more complex than the situation in our controlled experimental set up. The response curves can therefore not be extrapolated to the field. By keeping all

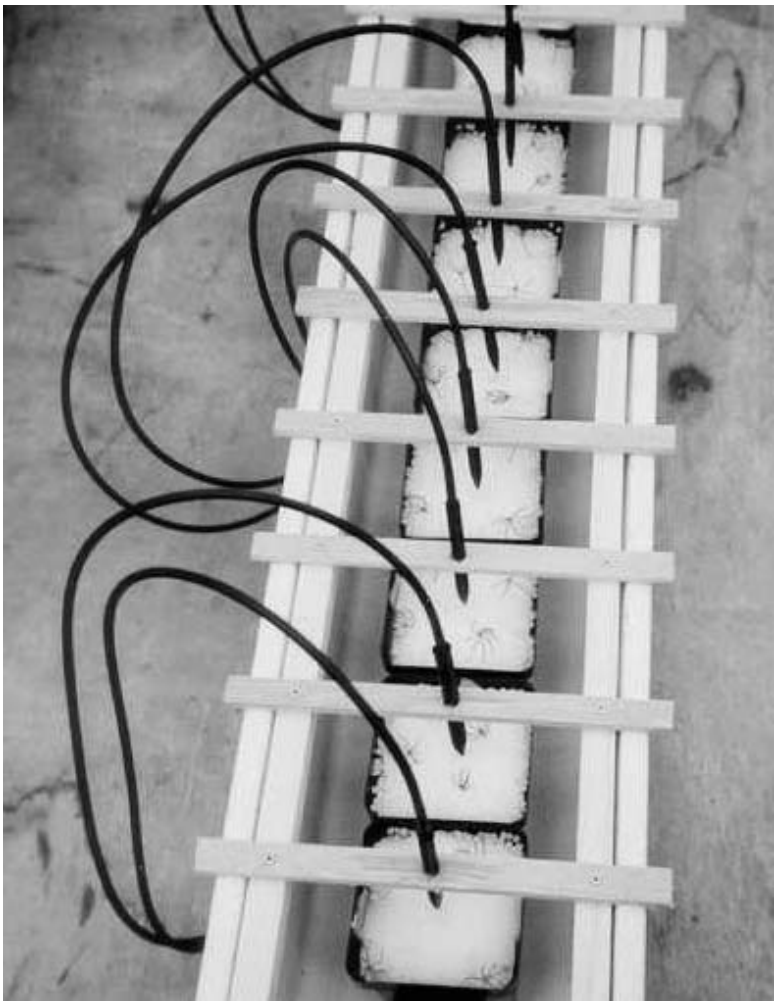
factors apart from Al and BC constant, we could however show that the mechanistic explanation for the effect of the BC:Al ratio is insufficient to describe the response of tree seedlings to a change in Al and BC concentrations. Care should be taken when using models based the BC:Al ratio to predict the effect of Al on tree growth.

## Acknowledgements

The authors like to thank Willeke van Tintelen and Jaap Nelemans for technical assistance, Gerdine Gaikhorst, Arie van de Berg and Monique Driessen for chemical analysis. This study was funded by the NWO (Netherlands Organisation for Scientific Research).

# 3

# Effect of ectomycorrhizal colonisation on the uptake of Ca, Mg and Al by *Pinus sylvestris* under aluminium toxicity



Laura van Schöll, Willem G. Keltjens, Ellis Hoffland and Nico van Breemen
*Forest Ecology and Management (2005) 215 352-360*

## Summary

Aluminium toxicity has been considered an important factor in forest decline. In earlier pot experiments, ectomycorrhizal tree seedlings were reported to have higher growth rates than nonmycorrhizal seedlings under aluminium toxicity. In this paper we test if this is caused by exclusion of Al and higher uptake of Ca and Mg by the ectomycorrhizal roots. *Pinus sylvestris* seedlings, grown for 3 months on a semi-hydroponic system, were continuously drip-irrigated with nutrient solution, containing 0 or 1.5 mM Al. The seedlings were nonmycorrhizal or colonised by ectomycorrhizal fungal species from a podzol soil. The presence of 1.5 mM Al in solution significantly decreased the dry weights of needles and roots compared to the control, and increased mycorrhizal colonisation. Yet growth was not affected by mycorrhizal colonisation. Concentrations of Al in the needles were significantly higher at 1.5 mM Al in solution than at 0 mM Al, and significantly higher in ectomycorrhizal seedlings than in nonmycorrhizal seedlings. Concentrations of Ca and Mg in the needles were significantly lower at 1.5 mM Al in solution than at 0 mM Al, but were not affected by ectomycorrhizal colonisation. In conclusion, ectomycorrhizal colonisation did not mitigate aluminium toxicity in our semi-hydroponic system. We suggest that better growth of soil-grown ectomycorrhizal tree seedlings compared to nonmycorrhizal tree seedlings should be explained by improved uptake of immobile nutrients such as P through a better soil exploration by the external mycelium, or by detoxification of Al by organic anions exuded by the fungi.

## Introduction

Aluminium toxicity has been considered an important factor in the forest decline in Europe over the past decades. This forest decline has been attributed to atmospheric deposition, causing soil acidification resulting in increased concentrations of Al in the soil solution (Ulrich *et al.*, 1980). At pH values below 4, dissolved Al is mainly present in the form of $Al^{3+}$, which is toxic to plants (Kinraide, 1991). Al inhibits root growth, which induces poor uptake of water and nutrients, disrupts metabolic processes and impairs the uptake of Ca and Mg (Kochian, 1995, Matsumoto, 2000). Al interferes with the uptake of Mg and Ca by replacing them at the binding sites of the cell wall and plasma membrane of the root cortex cells, by blocking ion channels over the plasma membrane and by reducing the electrostatic attraction of the plasma membrane and cell wall for Ca and Mg (Kinraide, 2001, Kinraide *et al.*, 2004, Kochian, 1995). Because of the interactive effect of Al and Ca and Mg at the cell wall and plasma lemma, an increased supply of Ca and Mg can improve their uptake under Al toxicity (Kinraide, 2003, Van Schöll *et al.*, 2004).

In calculations on the impact of atmospheric deposition on forest ecosystems it is generally assumed that the reduced growth under Al toxicity is the result of reduced uptake of Ca and Mg, which is calculated from the BC:Al ratio of the soil solution (Cronan & Grigal, 1995, Sverdrup & De Vries, 1994, Sverdrup *et al.*, 1992b). However, in this mechanistic approach the influence of ectomycorrhizal colonisation of the tree roots on the uptake of Ca, Mg and Al is ignored (Högberg & Jensén, 1994, Løkke *et al.*, 1996). The effects of Al on tree growth and uptake of Ca and Mg have been investigated mostly with nonmycorrhizal (NM) tree seedlings, whereas in temperate and boreal forests most tree roots are colonised by ectomycorrhizal (EcM) fungi. In ectomycorrhizal roots, the hyphae form a dense network around the root tips (the sheath or mantle) and in the apoplast of the root cortex (the Hartig net), with an extensive mycelium radiating out into the soil. The ectomycorrhizal fungi receive carbon from their host tree that they provide with N and P taken up by the extraradical hyphae. It has been postulated that ectomycorrhizal fungi mediate the uptake of Ca and Mg in tree roots and thereby mitigate the negative effect of Al on the uptake of Ca and Mg and tree growth (Finlay, 1995, Løkke *et al.*, 1996, Van Breemen *et al.*, 2000a).

Knowledge on the effect of ectomycorrhizal fungi on the uptake of Ca and Mg by their host tree is limited and inconsistent (Bücking *et al.*, 2002). In nonmycorrhizal roots, the highest uptake rates of Ca and Mg occur at the root apex, or at sites where lateral roots pass through the Casparian band (Häussling *et al.*, 1988). As these are also the sites of ectomycorrhiza formation, and ectomycorrhizal hyphae have been found to transport Ca (Melin & Nilsson, 1955) and Mg (Jentschke *et al.*, 2000) to the tree roots, it seems likely that uptake of Ca and Mg in ectomycorrhizal roots is mediated by the ectomycorrhizal fungi (Bücking *et al.*, 2002, Häussling *et al.*, 1988). Under aluminium toxicity, the uptake capacity for Ca and Mg at the root surface is reduced, and the extraradical mycelium may enhance uptake by providing a large surface area for uptake.

Uptake of Ca and Mg and tree growth under Al toxicity may be further improved by the exclusion of Al from the root cortex. Binding of Al to the fungal cell walls or sequestration of Al into fungal cell vacuoles (Kottke *et al.*, 1994, Schier & McQuattie, 1996, Turnau *et al.*, 1993, Väre, 1990, Väre & Markkola, 1990) may reduce the flux of Al into the root cortex. Furthermore, Al can be detoxified by complexation with low molecular weight organic anions (Ma *et al.*, 2001, Ryan *et al.*, 2001). Ectomycorrhizal tree seedlings have been found to exude more oxalate than nonmycorrhizal tree seedlings in response to Al in axenic conditions (Ahonen Jonnarth *et al.*, 2000), but this has not been linked to growth responses of the tree seedling.

In pot experiments, ectomycorrhizal tree seedlings maintained higher growth rates than nonmycorrhizal tree seedlings at non-toxic and toxic concentrations of Al in the soil solution (Ahonen Jonnarth *et al.*, 2003, Cumming & Weinstein, 1990a, b, Hentschel *et al.*, 1993, Schier & McQuattie, 1995, 1996). Concentrations of Ca and Mg in ectomycorrhizal tree seedlings were higher (Egerton-Warburton *et al.*, 1993), equal (Ahonen Jonnarth *et al.*, 2003) as well as lower (Hentschel *et al.*, 1993, Schier & McQuattie, 1996) then those of nonmycorrhizal seedlings under Al toxicity. Lower concentrations of Al in the shoots and/or roots of ectomycorrhizal tree seedlings compared to nonmycorrhizal seedlings are seen as indirect evidence for the exclusion or detoxification of Al by the ectomycorrhizal hyphal network (Cumming & Weinstein, 1990a, b, Egerton-Warburton *et al.*, 1993, Kieliszewska-Rokicka *et al.*, 1998). Differences in tissue concentration of Ca, Mg and Al between nonmycorrhizal and ectomycorrhizal seedlings can result from exclusion or complexation of Al and improved uptake of Ca and Mg. However, lower concentrations may also result from dilution due to better growth of the ectomycorrhizal seedlings compared to the nonmycorrhizal seedlings (Jentschke & Godbold, 2000). Generally, ectomycorrhizal fungi improve the nutrient condition of their host plant because the external ectomy-

corrhizal mycelium increases the total absorbing surface, and because the hyphae are able to mobilize N and P from organic and inorganic sources unavailable to the plant roots (Chalot et al. 2002; Smith and Read 1997). Under Al toxicity, the positive effect of ectomycorrhizal colonisation on P uptake by the plant may be even more pronounced as the uptake of P -which is highly immobile in soil- is limited by the reduced root growth. In most experiments on metal toxicity, no distinction can be made between the direct effect of the ectomycorrhizal fungi on the toxicity factors and the indirect effect of better nutrient supply (Jentschke & Godbold, 2000, Meharg, 2003).

In this study, we evaluate the direct effects of ectomycorrhizal colonisation on the Al toxicity effects on *Pinus sylvestris* seedlings. We used a controlled semi-hydroponic growth system in which the ectomycorrhizal external mycelium could develop relatively naturally in the pores of the rooting medium, containing both air and nutrient solution. In semi-hydroponic systems, nutrient delivery to the root is ensured, and a better exploration of the rooting medium by the extrametrical mycelium is generally of small or even no importance (Eltrop & Marschner, 1996). However, under aluminium toxicity, enlargement of the total surface area for uptake by the extraradical mycelium may be important by compensating the reduced uptake capacity for Ca and Mg at the cell walls and plasma lemma. Therefore, in this system, differences between ectomycorrhizal and nonmycorrhizal seedlings can be attributed to direct effects of the ectomycorrhizal fungi on Al toxicity, i.e. exclusion of Al and/or improved uptake of Ca and Mg.

Another advantage of this system is that it is possible to study the pure Al effects at controlled pH and nutrient concentrations. In experiments with soil as the rooting medium, the addition of Al to the soil solution causes exchange reactions between the soil particles and the soil solution, which changes the pH and concentrations Ca and Mg between the Al and the control treatments (Ahonen Jonnarth *et al.*, 2003). We expected the ectomycorrhizal tree seedlings to have higher growth rates due to higher uptake of Ca and Mg and lower uptake of Al than the nonmycorrhizal tree seedlings.

## Material and methods

*Seedling growth and treatment:*

Seeds of *Pinus sylvestris* (NLZT, Ommen, The Netherlands) were soaked overnight in demineralised water and subsequently surface-sterilised in 15% $H_2O_2$ for 30 min.

For the nonmycorrhizal treatment, seeds were germinated in coarse quartz sand, which was previously incubated at 120°C for 24 hours. For the ectomycorrhizal treatment, soil that had been collected from a podzol under a boreal forest (Nyänget, Sweden) (Ilvesniemi *et al.*, 2000) was sieved (0.5 cm). Roots were collected from the sieve, cut into pieces of 2 centimeter and mixed through the original soil again as an inoculum. Seeds were germinated on a mixture (1:3 v:v) of this soil with coarse quartz sand, previously incubated at 120°C for 24 hours. Fourteen weeks after being sown, seedlings were transplanted to pots (8 seedlings per pot), filled with a mixture (3:2 v:v) of PVC pellets (4 mm) and fine gravel (4 mm). Dry weight of shoot and root were approximately 0.03 and 0.02 g. Differences between nonmycorrhizal and ectomycorrhizal seedlings were negligible. Pots were placed in broad containers (140×80×15 cm) loosely covered with paper for an acclimatization period of 3 days and watered daily with nutrient solution. The concentrations in the nutrient solution were (in µM): 250 $NH_4NO_3$ 60 $KH_2PO_4$, 220 $K_2SO_4$, 188 $CaCl_2$, 62 $MgSO_4$ and 95 Fe-EDTA, 46 $H_3BO_3$, 0.3 $CuSO_4$, 0.1 $(NH_4)_6Mo_7O_{24}$, 9.2 $MnSO_4$, 0.8 $ZnSO_4$.

Experimental treatments started 3 days after transplanting. An experimental unit consisted of 20 pots (with seedlings all either nonmycorrhizal or ectomycorrhizal) placed on a longitudinal container (200×18×14 cm) with a drain. The drain fed back into an 80-liter container filled with nutrient solution, from which the pots were continuously drip-irrigated (see picture on page 25). The nutrient solution (composition as above) contained either 0 or 1.5 mM $AlCl_3$. We used 2 experimental units with each 20 pots per treatment. The pH of the nutrient solution was adjusted to 4.0 and checked and adjusted again 3 times a week. Nutrient solutions were refreshed every 2 weeks. Concentrations of Al, Ca, Mg, P, K and Fe in the solution were measured weekly and replenished when needed.

The experiment ended 14 weeks after transplanting of the seedlings. For each experimental unit, the 20 pots were randomly divided into 3 subsamples. The seedlings of 8 pots were pooled and used for dry weight measurements and chemical analysis. The pooled seedlings of another 8 pots were used for root length measurements. The 4 remaining pots per experimental unit were used for determination of mycorrhizal colonisation.

*Root length and mycorrhizal colonisation:*

Root length was measured using the cross section method (Newman, 1966). Mycorrhizal and nonmycorrhizal root tips were distinguished under a dissecting microscope. Ectomycorrhizal morphotypes were described on the basis of macroscopical and microscopical characteristics.

**Table 1** Morphological features of ectomycorrhizas of *P. sylvestris*, obtained from soil inoculum taken from a podzol in Nyänget, Sweden.

| Morphotype | Morphological features |
|---|---|
| L1 | White v-shaped or clustered tips. Loose thin mantle of medium to thick hyphae with trapped air, swollen at the septae, no clamps. External white mycelium surrounding tips, with thick to very thick rhizomorphs consisting of loose hyphae. |
| L2 | Black v-shaped or clustered tips with white top. Loose thin mantle of medium to thick hyphae with trapped air, swollen at the septae, no clamps. External white mycelium surrounding tips, with thick to very thick rhizomorphs consisting of loose hyphae. |
| L3 | Brown v-shaped and single tips. Mantle of smooth broad hyphae loosely packed on the outside, more densely packed underneath, with very obvious clamps. |
| L4 | White 'balloon-like' tips. Mantle formed by thick hyphae, with clamps and emanating cystides. No rhizomorphs. |
| L5 | Brown/blackish, tigerprint tips. Thin mantle of densely packed medium-thick hyphae with clamps. No rhizomorphs. |
| L6 | White/yellow tips v-shaped tips. Thin mantle of very thick hyphae, surrounded by thin brown/golden external hyphae, no clamps. |

*Chemical analysis:*

Phosphorus concentrations in roots and needles were determined colorimetrically by segmented automated flow analysis after digestion with $H_2SO_4$-salicylic acid-$H_2O_2$ and selenium. Root and needle concentrations of Al, Ca and Mg were measured by ICP-AES after digestion with $HNO_3$-$H_2O_2$-HF.

*Statistical analysis:*

The experiment was set up as a complete two-factorial design, with the factors Mycorrhiza (Non Mycorrhizal seedlings; EctoMycorrhizal seedlings) and the factor Aluminium (0-1.5 mM Al in the nutrient solution) and the experimental units as replicates. The significance of treatment effects was determined by analysis of variance (ANOVA), using the GLM procedure of SPSS 10.0.

## Results

*Mycorrhizal colonisation:*

In the nonmycorrhizal treatments, with either 0 or 1.5 mM Al, mycorrhizal colonisation was negligible (<5% of total root tips). In the mycorrhizal treatments, ectomycorrhizas were distinguished into 6 morphologically different types (Table 1) that

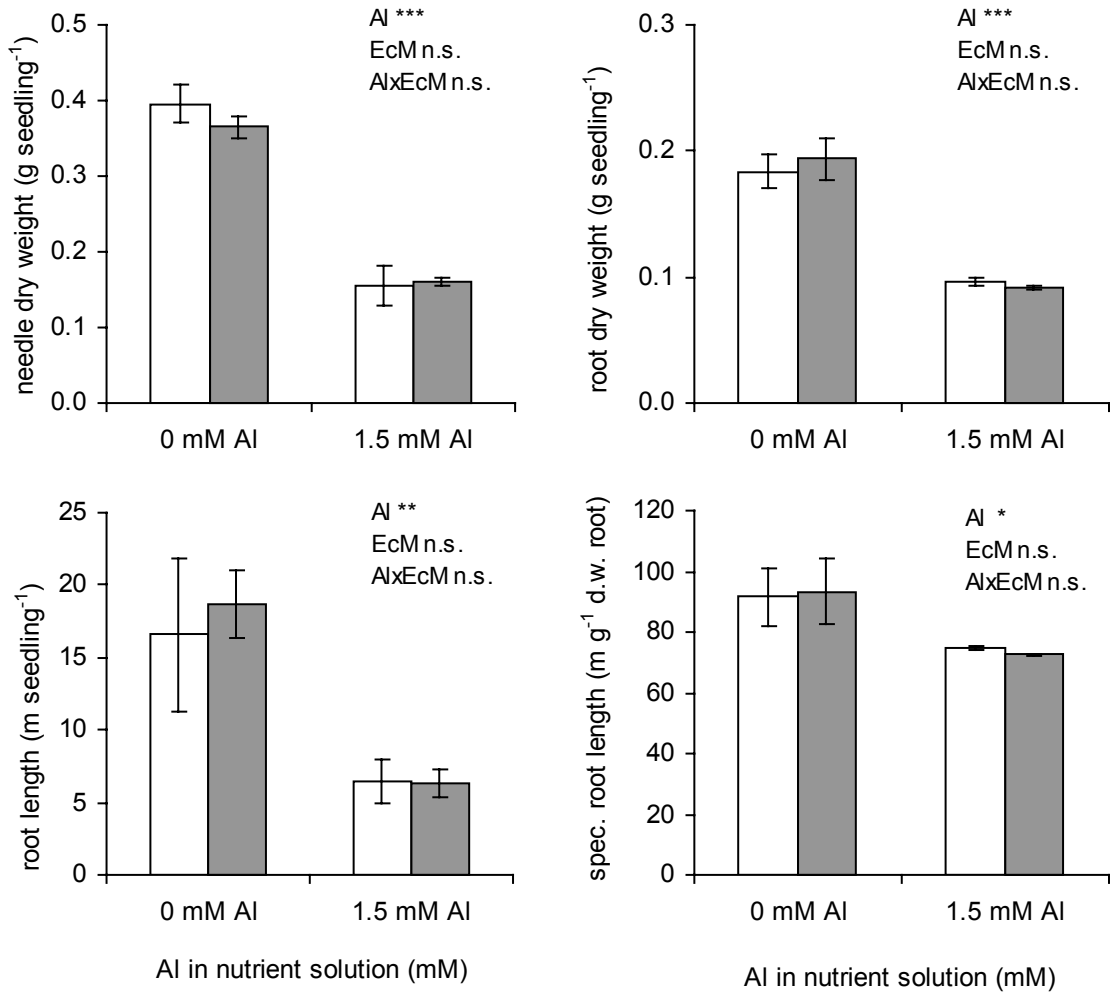



**Figure 1** Needle and root dry weight, root length and specific root length of *P. sylvestris*, grown for 14 weeks on a semi-hydroponic system with 0 or 1.5 mM Al in the nutrient solution. Mean of two replicates ± standard deviation. **P*<0.05, ***P*<0.01, ****P*<0.001, n.s.=not significant. White bars=nonmycorrhizal; Shaded bars=ectomycorrhizal seedlings

were not further identified. In the mycorrhizal treatment with 1.5 mM Al in solution the mycorrhizal colonisation was high (>80% of total root tips). In these pots an abundant white extraradical mycelium had developed. Types L1 to L5 were found, with L1 being most abundant. In the mycorrhizal treatment with 0 mM Al only types L5 and L6 were found, but mycorrhizal colonisation was low (50-80% of total root tips) compared to the mycorrhizal 1.5 mM Al treatment.

*Plant growth:*

Dry weights of needles and roots, root length and specific root length were significantly lower at 1.5 mM Al than at 0 Al (Fig. 1). Mycorrhizal colonisation did not significantly affect these 4 growth parameters. Interaction effects between aluminium in the nutrient solution and mycorrhizal colonisation were not significant.

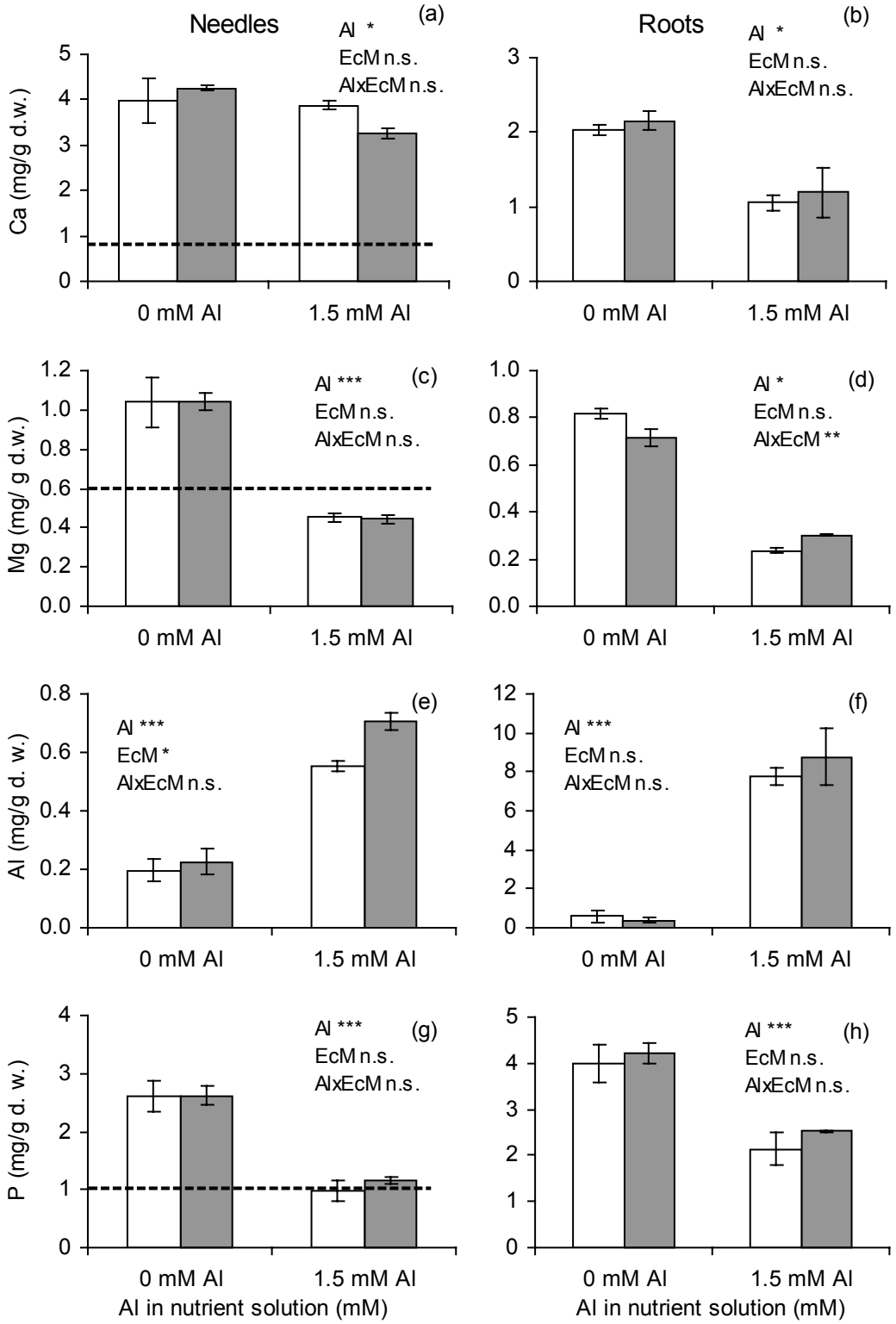



**Figure 2** Concentrations of Ca, Mg, Al and P in the needles and roots of *P. sylvestris,* grown for 14 weeks on a semi-hydroponic system with 0 or 1.5 mM Al in the nutrient solution. Dashed lines indicate concentrations were deficiency occurs (Reuter *et al.*, 1997). Mean of two replicates ± standard deviation. **P*<0.05, ***P*<0.01, ****P*<0.001, n.s.=not significant. White bars=nonmycorrhizal; Shaded bars=ectomycorrhizal seedlings

*Tissue concentrations of Ca, Mg and Al:*

Needle concentrations of Ca, Mg and P were significantly lower and the needle concentration of Al was significantly higher with Al in the nutrient solution than without Al (Fig. 2a,c,e,g). Mycorrhizal colonisation did not affect needle concentrations of Ca, Mg or P, but increased the needle concentration of Al. There was no significant interaction between Al in nutrient solution and mycorrhizal colonisation on needle contents of Ca, Mg, P or Al.

Root concentrations of Ca, Mg and P were significantly lower and the root concentration of Al was significantly higher with 1.5 mM Al in the nutrient solution than with 0 Al (Fig. 2b,d,f,h). Mycorrhizal colonisation did not affect root concentrations of Ca, Mg, P or Al. There was a small but significant interaction effect between Al in nutrient solution and mycorrhizal colonisation for Mg concentration in the root, but no significant effect for P, Ca and Al.

## Discussion

The semi-hydroponic system used in this study appears very suitable to study the direct effect of Ectomycorrhizal fungi on the Al toxicity systems of their host plant. In the treatments without Al added to the nutrient solution, nonmycorrhizal and Ectomycorrhizal tree seedlings had similar dry weight and nutrient uptake (Fig. 1). Because nutrient delivery to the roots is ensured in semi-hydroponics experimental systems, this result indicates that under these conditions mycorrhizal colonisation has little if any effect on the nutrient condition and growth of the host plant, as also shown by (Eltrop & Marschner, 1996)). So, the nonmycorrhizal and ectomycorrhizal seedlings of the 0 mM Al treatments can be regarded as relevant controls for the effect of Ectomycorrhizal colonisation in the 1.5 mM Al treatments. By contrast, in pot experiments, ectomycorrhizal colonisation improved seedling growth even in the controls without Al in solution (Ahonen Jonnarth *et al.*, 2003, Cumming & Weinstein, 1990a, Hentschel *et al.*, 1993, Schier & McQuattie, 1995), making it difficult to evaluate the mechanisms of improved growth at higher Al levels.

Our results do not support the hypothesis that ectomycorrhizal fungi mediate the uptake of Ca and Mg under Al toxicity as suggested by Finlay (1995), Løkke *et al.* (1996) and Van Breemen *et al.* (2000). Ectomycorrhizal colonisation did not affect the uptake of Ca and Mg with either 0 or 1.5 mM Al in solution. With 0 mM Al in solution, the concentrations of Ca and Mg in the needles were adequate for *Pinus spp.* (Reuter *et al.*, 1997)(Fig. 2). With 1.5 mM Al in solution, the Ca concentration in the needles remained adequate, but the Mg concentration was below the defi-

ciency level for *Pinus spp.* (Reuter *et al.*, 1997)(Fig. 2). As this reduction in root uptake of Ca and Mg is the result of specific impairment by Al at the root surface (Kinraide, 2001), we expect the effect of ectomycorrhizal colonisation on the uptake of Ca and Mg will be similar in soil and in semi-hydroponics.

Even at the high percentage of mycorrhizal root tips, ectomycorrhizal colonisation did not exclude Al from uptake. The concentration of Al in the roots of ectomycorrhizal and nonmycorrhizal seedlings did not differ, but ectomycorrhizal seedlings had higher concentrations of Al in their needles than nonmycorrhizal seedlings (Fig. 2). As the dry weight of nonmycorrhizal and ectomycorrhizal seedlings did not differ within the Al treatments (Fig. 1), this must have been the result of higher translocation of Al to the needles of the ectomycorrhizal seedlings. These results are not in agreement with earlier pot experiments where ectomycorrhizal colonisation mitigated Al induced growth reductions in soil grown tree seedlings (Ahonen Jonnarth *et al.*, 2003, Cumming & Weinstein, 1990a, Hentschel *et al.*, 1993, Schier & McQuattie, 1995) and where lower concentrations of Al in the needles of Ectomycorrhizal seedlings compared to nonmycorrhizal seedlings were found (Cumming & Weinstein, 1990a, b, Egerton-Warburton *et al.*, 1993, Kieliszewska-Rokicka *et al.*, 1998). These lower concentrations of Al in the needles may have been the result of dilution due to better growth of ectomycorrhizal tree seedlings and not the result of exclusion of Al by the ectomycorrhizal fungi. Accumulation of Al in the fungal sheath was found in several studies (Egerton-Warburton & Griffin, 1995, Schier & McQuattie, 1995, 1996, Turnau *et al.*, 1996), but not in others (Jentschke *et al.*, 1991, Kuhn *et al.*, 1995) (Kottke *et al.*, 1994), and may be dependent on plant and fungal species. In the field, trees are colonised by a variety of ectomycorrhizal fungal species. For exclusion to be a general mechanism by which ectomycorrhizal fungi protect their host tree, the ability to exclude Al would be expected to be a characteristic of ectomycorrhizal species originating from sites with high Al in the soil solution. Adaptation or lower sensitivity to Al or heavy metals in ectomycorrhizal fungal species from contaminated sites has been shown *in vitro* in several studies (Colpaert *et al.*, 2004, Egerton-Warburton & Griffin, 1995, Leski *et al.*, 1995). The tree seedlings in our experiment were colonised by fungi from a podzol soil, which is typically characterized by low pH and high concentrations of Al in the soil solution.

Lower concentrations of Al in the needles of soil grown tree seedlings may also be the result of detoxification by complexation of Al with organic acids, exuded by the Ectomycorrhizal fungi into the rhizosphere. Complexes of Al with organic acids are not taken up by the root (Ma & Hiradate, 2000). In soil, organic acid exudation will

be more effective than in our semi-hydroponic system, where rhizosphere effects are small due to the continuous through-flow.

Addition of Al influenced the mycorrhizal colonisation of the tree seedlings. Both the percentage of root tips colonised and the number of ectomycorrhizal fungal types found were higher in the treatment with 1.5 mM Al than in the treatment without Al. At 1.5 mM Al an abundant white mycelium was present in all pots of the ectomycorrhizal treatment, which was not observed in the pots without Al. In other studies, contrasting effects of Al on ectomycorrhiza formation were found. Entry *et al.* (1987) and Hentschel *et al.* (1993) found that the percentage of root tips colonised was decreased by addition of Al, but no effect of Al addition was found by Kieliszewska-Rokicka *et al.* (1998) and Schier & McQuattie (1995, 1996). Kasuya *et al.* (1990) found that the mycorrhizal potential could increase or decrease with increasing Al concentrations, depending on the ectomycorrhizal fungal species. In the control treatment without Al root growth may have been to fast for ectomycorrhizal colonisation (Kottke & Oberwinkler, 1986). In addition, the ectomycorrhizal fungi may have thrived better in the Al treatment because of their adaptation to high Al concentrations. Improved growth in response to non-toxic Al concentrations (hormesis) is frequently observed in plants (Barceló & Poschenrieder, 2002), and has been found in an Al tolerant ectomycorrhizal fungus when grown *in vitro* (Zel & Bevc, 1993).

In conclusion, our results do not support the hypothesis that ectomycorrhizal colonisation mitigates aluminium toxicity in *Pinus sylvestris* seedlings by improved uptake of Ca and Mg or exclusion of Al. In soil-grown plants, root growth and rhizosphere effects are more important than in our semi-hydroponic system (Eltrop & Marschner, 1996, Marschner, 1991). An alternative explanation for better growth of soil grown ectomycorrhizal tree seedlings could be improved uptake of immobile nutrients such as P through a better soil exploration by the external ectomycorrhizal mycelium, or detoxification of Al by organic anions exuded by the fungi.

## Acknowledgements

The authors like to thank Willeke van Tintelen and Jaap Nelemans, Willem Menkveld and Rijndert de Fluiter for their help in performing the experiment, Gerdine Gaikhorst, Arie van de Berg and Frank Bakker for chemical analysis, Renske Landeweert for morphotyping of the ectomycorrhizas, and Thomas Kuyper and an anonymous reviewer for useful comments on the manuscript. LvS was funded by the NWO (Netherlands Organisation for Scientific Research).

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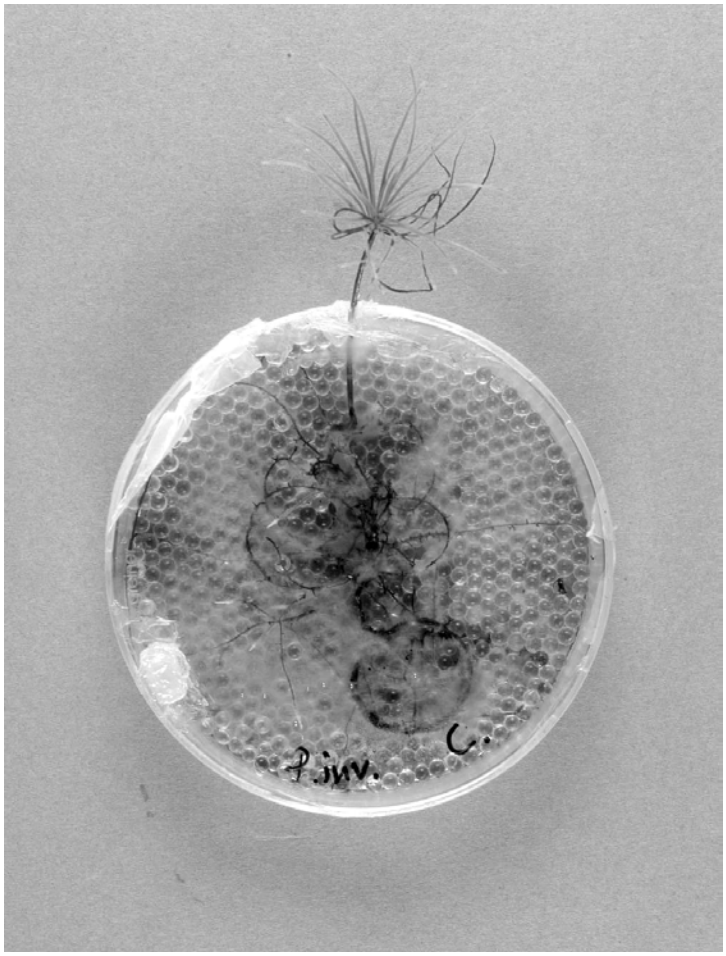
# Effect of phosphorus and magnesium deficiency on aluminium-induced oxalate exudation by nonmycorrhizal and ectomycorrhizal *Pinus sylvestris* (Scots pine)



Laura van Schöll and Ellis Hoffland
*Submitted for publication.*

## Summary

Most trees in boreal forests form a symbiotic association with ectomycorrhizal fungi on their roots. Ectomycorrhizal fungi can exude low molecular weight organic anions (LMWOA) that have been hypothesized to play a role in the detoxification of Al in the soil solution and in the mobilisation of P and Mg from mineral grains. The goal of this study was to separate the effect of Al toxicity from the effect of P and/or Mg deficiency on the exudation of LMWOA. We also tested whether Al toxicity and Mg and P deficiency have a synergistic effect on LMWOA exudation. Seedlings of *Pinus sylvestris* L. (Scots pine), nonmycorrhizal or colonised by ectomycorrhizal fungi (*Paxillus involutus, Rhizopogon roseolus, Piloderma croceum, Laccaria bicolor, Suillus bovinus*) were cultured on a layer of glass beads with nutrient solution. Aluminium induced the exudation of oxalate in both nonmycorrhizal and ectomycorrhizal seedlings. Simultaneous Al toxicity with Mg and P deficiency significantly increased the oxalate exudation compared to Al toxicity alone, which signifies that exudation is a response to combined effects. The increase was most notably in nonmycorrhizal seedlings, which only exuded low amounts of oxalate under Al toxicity with complete nutrient supply. Without Al, Mg or P deficiency did not induce the exudation of oxalate. Ectomycorrhizal fungal species affected the amount and type of organic anion exuded. Differences among seedlings colonised by different ectomycorrhizal species were as big or bigger than between nonmycorrhizal and ectomycorrhizal seedlings.

## Introduction

Aluminium (Al) toxicity is a major restriction for plant growth on acid soils. With decreasing pH in the soil solution the concentration of $Al^{3+}$, which is toxic to plants, increases. The mechanisms of Al toxicity and tolerance have not yet been fully elucidated (Kochian *et al.*, 2004, Zheng & Yang, 2005). Symptoms include reduced root growth and deficiencies of P, Mg and Ca. A general mechanism of Al tolerant plants seems to be the exudation of low molecular weight organic anions (LMWOA) such as citrate, oxalate and malate in response to toxic concentrations of $Al^{3+}$ (Kochian *et al.*, 2004, Ma *et al.*, 2001, Ryan *et al.*, 2001). These LMWOA can form strong non toxic complexes with Al, which do not bind to the cell wall (Postma, 2003) and are not taken up by the root (Ma & Hiradate, 2000).

Most work on LMWOA exudation and tolerance to $Al^{3+}$ has dealt with crop plants, to which a few μM of $Al^{3+}$ in the soil solution is toxic (Ma *et al.*, 2001). Trees, however, can tolerate far higher concentrations of Al (>150 μM) than crop plants (Moyer-Henry *et al.*, 2005).

Most trees form a symbiotic association with ectomycorrhizal (EcM) fungi on their roots. The hyphae of the ectomycorrhizal fungi form a sheath around the root tips and a dense network in the apoplast of the root cortex, the Hartig net. Hyphae radiating out from this ectomycorrhizal root tip play an important role in the acquisition of N and P (Smith & Read, 1997). Ectomycorrhizal fungi may also protect the roots from dissolved Al (Meharg, 2003) and enhance the dissolution of nutrients from mineral grains by the exudation of LMWOA (Landeweert *et al.*, 2001, Van Breemen *et al.*, 2000a).

Tree seedlings colonised by ectomycorrhizal fungi have yielded higher biomass than nonmycorrhizal seedlings under Al toxicity in pot experiments (Ahonen Jonnarth *et al.*, 2003, Cumming & Weinstein, 1990a, Hentschel *et al.*, 1993, Schier & McQuattie, 1995, 1996). It is unclear, however, if this growth improvement results from a better nutrient status of the ectomycorrhizal than the nonmycorrhizal seedlings or if the ectomycorrhizal fungi protect the seedlings from Al toxicity by exclusion of Al or exudation of LMWOA (Jentschke & Godbold, 2000, Meharg, 2003). Earlier we showed that ectomycorrhizal colonisation of the root system did not physically prevent uptake of Al by the tree when the role of root exudates was minimised by con-

tinuous through-flow of the rooting medium (Chapter 5: Van Schöll *et al.*, 2005).

Results of the effect of Al on the exudation of LMWOA by ectomycorrhizal fungi and tree species are variable. Al induced the oxalate production by the ectomycorrhizal fungus *Pisolithus tinctorius,* but not by *Laccaria bicolour,* in pure culture (Cumming *et al.*, 2001). Ahonen Jonnarth *et al.* (2000) found that ectomycorrhizal colonisation of *Pinus sylvestris* (Scots pine) increased the exudation of oxalate and in some cases Al significantly enhanced this exudation. No LMWOA exudation in response to Al toxicity was found with nonmycorrhizal *Picea abies* (Norway spruce) (Heim *et al.*, 1999). However, Al increased oxalate exudation by *Picea rubens* (red spruce) cells in suspension culture (Minocha & Long, 2004).

Cumming *et al.* (2001, 1990c) have suggested that LMWOA exudation by ectomycorrhizal tree roots is not a response to Al toxicity, but a scavenger response to Al-induced deficiency of P. Al depresses P uptake by plants by reducing root growth, leading to reduced uptake of immobile nutrients such as P, and by forming insoluble Al-phosphates. Exudation of LMWOA in response to P deficiency has been found for several herbaceous plants (Kochian *et al.*, 2004, Ryan *et al.*, 2001) and nonmycorrhizal and ectomycorrhizal *Pinus sylvestris* (Chapter 5:Van Schöll *et al.*, 2006). When grown in symbiosis, dissolution of P containing minerals by ectomycorrhizal fungi is stimulated by P deficiency, presumably through increased LMWOA exudation (Wallander *et al.*, 2003). Phosphorus deficiency significantly increased the exudation of malonate by the ectomycorrhizal fungus *Paxillus involutus* in pure culture (Chapter 5: Van Schöll *et al.*, 2006).

Exudation of LMWOA by ectomycorrhizal fungi may also play a role in the mobilisation of base cations from mineral grains (Landeweert *et al.*, 2001). Al inhibits the uptake of Ca and Mg, and this may be counteracted by increasing the concentrations of dissolved Ca and Mg (Kinraide, 2003, Chapter 2:Van Schöll *et al.*, 2004). Nonmycorrhizal and ectomycorrhizal *P. sylvestris* significantly increased the exudation of oxalate when Mg was omitted from the nutrient supply (Chapter 5: Van Schöll *et al.*, 2006). The ectomycorrhizal fungus *Paxillus involutus* increased oxalate production in the absence of Mg and K from the growth medium (Paris *et al.*, 1996), but not when either Mg or K were omitted (Chapter 5:Van Schöll *et al.*, 2006).

This study aimed at separating the effect of Al toxicity from the effect of P or Mg deficiency on the exudation of LMWOA by tree seedlings and ectomycorrhizal fungi. We also tested whether Al toxicity and Mg and P deficiency have an additive effect on LMWOA exudation. In forest ecosystems, Al toxicity generally coincides with low P and Mg availability. In most experiments on Al toxicity, however, either an optimal nutrient composition has been used to avoid confounding effects of nutri-

ent deficiencies on plant growth, or a pure Al solution with a 0.5 mM $CaCl_2$ solution as a control to avoid confounding effects of complexation or interaction of Al with other elements. We expected higher exudation rates from the ectomycorrhizal seedlings compared to the nonmycorrhizal seedlings because of the known capacity of ectomycorrhizal fungi to exude LMWOA.

## Material and methods

The EcM fungal species used were *Paxillus sylvestris* (Batch: Fr.) Fr. 'nr 17', *Piloderma croceum* J. Erikss. & Hjorst. 'BL97-01', *Suillus bovinus* (L.: Fr.) Roussel and *Laccaria bicolor* (Maire) P.D. Orton (all kindly provided by Roger Finlay, SLU, Uppsala, Sweden) and *Rhizopogon roseolus* (Corda) Th.M. Fries var. intermedius Svrcek (kindly provided by C. Plassard, INRA, Montpellier, France). Seeds of Scots pine (*Pinus sylvestris* L.) were soaked overnight in demineralised water, surface-sterilised in 30% $H_2O_2$ for 30 min. and rinsed with autoclaved demineralised water. Seeds were germinated on agar (8 g $l^{-1}$) with glucose (10 g $l^{-1}$). After 6 days, individual seedlings were transferred to growth tubes (2 cm diam. 15 cm length) together with 3 plugs of MMN agar (Marx, 1969), which were sterile (NM treatment) or taken from the front of an actively growing mycelium (EcM treatments) for inoculation. Growth tubes contained 20 gram of a mixture of fine quartz sand:perlite:nutrient solution (ratio 2 kg: 0.13 kg: 0.685 l) and had been autoclaved for 30 minutes at 120°C. Composition of the nutrient solution (µM): 1540 $NH_4NO_3$; 100 $NH_4H_2PO_4$; 362 $KNO_3$; 72 $(NH_4)_2SO_4$; 77 $CaCl_2$; 101 $K_2SO_4$ (modified from Ingestad 1979). The growth tubes were closed with cotton wool and a plastic cap, and the bottom was covered with aluminium foil. The tubes were placed in a climate chamber (16 hours photoperiod at 70 Watts/$m^2$, day/night temperatures of 20/16°C and 80% humidity).

Seventeen weeks after inoculation the seedlings (dry weight ±0.05 g) were transferred to Petri dishes (diam. 9 cm), containing 25 gram of glass beads (diam. 3 mm) and 9 ml of nutrient solution, the composition of which varied as noted below (system adapted from Ahonen Jonnarth *et al.* (2000)). The roots were washed in autoclaved demineralised water and spread out over the beads. The shoot protruded from a notch cut out along the side of the dishes. The dishes were sealed with TESA painters tape and anhydrous lanolin around the base of the stem, placed horizontally in a growth box and covered with aluminium foil. They were left in the laboratory for one week to accustom the seedlings to the new rooting medium, and then placed in a climate chamber (specifications as above). Every 2 weeks the Petri dishes were

**Table 1** Composition of the nutrient solution (µmol $l^{-1}$), adapted from (Ingestad, 1979): optimal nutrient composition for growth of *Pinus sylvestris* seedlings.

| | Complete | -Mg | - P | -Mg -P |
|---|---|---|---|---|
| $NH_4NO_3$ | 770 | 770 | 780 | 780 |
| $KH_2PO_4$ | 67 | 67 | | |
| $NH_4H_2PO_4$ | 50 | 50 | 30 | 30 |
| $KNO_3$ | 181 | 181 | 181 | 181 |
| $(NH_4)_2SO_4$ | 36 | 36 | 36 | 36 |
| $CaCl_2$ | 39 | 39 | 39 | 39 |
| $MgSO_4$ | 60 | | 60 | |
| KCl | 50 | | 117 | |
| $KSO_4$ | | 25 | | 60 |
| $Na_2SO_4$ | | 34 | | |

**Note** In addition, all nutrient solutions contained microelements in concentration (mg $l^{-1}$): 2.65 Fe; 0.25 B; 0.01 Cu; 0.005 Mo; 0.25 Mn; 0.03 Zn

brought back to their original weight by adding sterile demineralised water. After 8 weeks, 3 ml of fresh nutrient solution was added to each dish.

Eleven weeks after transferring the seedlings to the glass beads, 4.6 ml of the nutrient solution was replaced with fresh nutrient solution (pH 4). Five days later, nutrient solution was removed from the dishes and frozen at -22˚C till determination of organic anions. Shoots and roots were separated, dried at 70˚ for 48 hrs and weighted.

The nutrient supplies in the Petri dishes were: complete nutrient solution (C), nutrient solution without Mg (–Mg); nutrient solution with 25% P (–P); complete nutrient solution with 1 mM Al (+Al); nutrient solution without Mg and 25% P plus 1 mM Al (+Al–Mg–P). Composition of the nutrient solutions are given in Table 1. For the +Al and +Al–Mg–P treatments, seedlings initially received either the complete or the –Mg–P solution. The nutrient solution added after eleven weeks contained 2 mM Al ($AlCl_3$) to reach a final Al concentration in the dishes of 1 mM. The concentration of 1 mM Al was chosen because earlier experiments showed that this concentration was toxic, but not lethal for our tree and fungal species.

Initially there were 5 replicates for each nutrient composition-ectomycorrhizal treatment combination, but because samples of the mycorrhizal treatments without visible ectomycorrhizas were discarded, the number of replicates at the end of the

experiment varied among treatments (n=3 or 4). Colonisation by *S. bovinus* was variable, and only the Complete and the +Al complete treatments were analysed.

*Determination of Low Molecular Weight Organic Anions*

The LMWOA oxalate, malonate, fumarate, tartrate, malate, succinate, and maleate were analysed by capillary zone electrophoresis (Waters Corp. Milford, MA, USA) following the method of Westergaard *et al.* (1998). The detection limit for each LMWOA is around 1 µM.

*Statistical analysis*

Statistical analysis was performed with SPSS 12.0.1 for WINDOWS (SPSS Inc., Chicago, IL, USA). The significance of main effects of the independent variables "Ectomycorrhizal species" (nonmycorrhizal, *Paxillus involutus, Rhizopogon roseolus, Piloderma croceum, Laccaria bicolor, Suillus bovinus)* or "Nutrient supply" (Complete; –Mg; –P; +Al complete; +Al–Mg–P) on the dependent variables dry weight of shoot and root, and concentrations of total LMWOA, oxalate and malonate was determined by a robust one-way analysis of variance (ANOVA) with the Brown-Forsythe test statistic followed by the Games-Howell *post hoc* test (equal variances not required). Within the Al treatments, the interactive effect of ectomycorrhizal species (nonmycorrhizal, *Paxillus involutus, Rhizopogon roseolus, Piloderma croceum, Laccaria bicolor)* and nutrient supply (+Al complete; +Al-Mg-P) was determined by a two-way ANOVA for oxalate and total LMWOA. Significance of differences between all pair wise comparisons of treatment means (incl. *S. Bovinus* +Al) was determined with the Tukey's honestly significant difference (HSD) *post hoc* test. Data on oxalate were transformed (square root) to homogenise variance (Levene's test). For the Complete, –Mg or low P treatments the interaction effect with ectomycorrhizal species could not be tested as there were several treatment combinations without detectable LMWOA and hence no variation.

## Results

*Ectomycorrhizal colonisation and plant growth*

Root systems of the inoculated tree seedlings were colonised well in most dishes. The surface of the dishes with tree seedlings colonised by *P. involutus* were covered for 30-100% with mycelium. With *R. roseolus* and *P. croceum*, the mycelium did not extend further than 1 cm from the roots, covering at most 50% of the dishes surface. Most root tips of seedlings inoculated with *L. bicolor* were ectomycorrhizal,

**Table 2** Dry weight of shoot and root of *P. sylvestris* seedlings, nonmycorrhizal or colonised by different ectomycorrhizal fungal species.

| EcM species | shoot dry weight (gram) | | root dry weight (gram) | | |
|---|---|---|---|---|---|
| Nonmycorrhizal | 0.068 | *0.002* | 0.058 | *0.002* | a |
| *L. bicolor* | 0.062 | *0.001* | 0.077 | *0.002* | b |
| *P. croceum* | 0.064 | *0.002* | 0.072 | *0.003* | b |
| *R. roseolus* | 0.068 | *0.002* | 0.068 | *0.003* | ab |
| *S. bovinus* | 0.064 | *0.002* | 0.063 | *0.004* | ab |
| *P. involutus* | 0.068 | *0.003* | 0.069 | *0.003* | b |

**Note:** Mean of all nutrient composition treatments with standard error in italics. Values followed by the same letter are not significantly different.

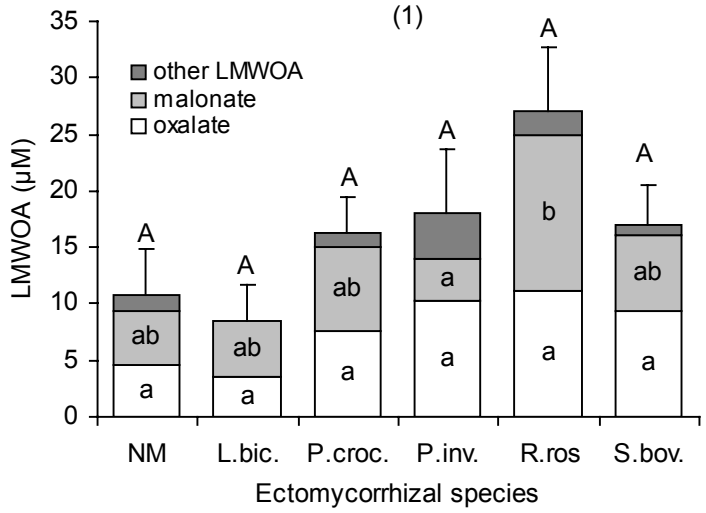
but there was no outgrow of hyphae to the glass beads or nutrient solution. Shoot dry weights (Table 2) were not affected by either ectomycorrhizal colonisation ($P$=0.3) or composition of the nutrient solution ($P$=0.3). Root dry weights (Table 2) were affected by ectomycorrhizal colonisation ($P$<0.001) but not by composition of the nutrient solution ($P$=0.8). Nonmycorrhizal seedlings had significantly lower root dry weight than seedlings colonised by *L. bicolor*, *P. croceum* or *P. involutus*, but root dry weights among seedlings colonised by different ectomycorrhizal fungal species were not significantly different (Table 2).

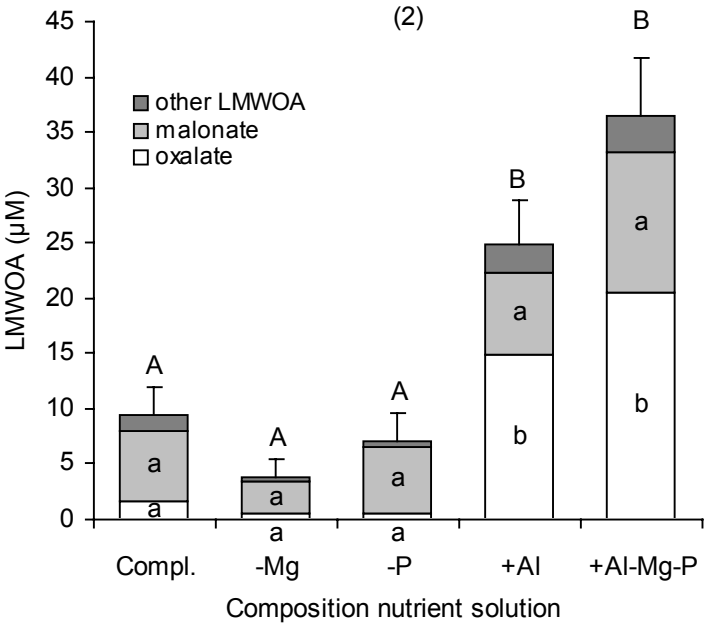
*LMWOA exudation*

The dominant LMWOA exuded were oxalate and malonate (Fig. 1 and 2). Fumarate, tartrate, malate, succinate and maleate were detected occasionally but without a clear trend over the nutrient treatments or species. The total amount of LMWOA exuded was significantly affected by ectomycorrhizal species ($P$=0.032) and by nutrient supply ($P$<0.001). Adding Al to the nutrient solution, either complete or lacking Mg and P, significantly increased the total amount of LMWOA exuded, mainly in the form of oxalate, but absence of Mg or low P supply had no effect (Fig. 2). In the presence of Al, there was a significant effect of nutrient supply ($P$=0.001) and of ectomycorrhizal species ($P$=0.055), but no significant interactions effect ($P$=0.365) (Fig. 3). Seedlings colonised by *R. roseolus* exuded significantly more LMWOA than nonmycorrhizal seedlings or seedlings colonised by *L. bicolor* or *P. croceum* (Fig. 3).

The overall oxalate exudation was significantly affected by nutrient supply ($P$<0.001), but not by ectomycorrhizal species ($P$=0.237) (Fig. 1). Oxalate was mainly detected in the dishes with Al added to the nutrient solution (Fig. 2). In the presence of Al, there was a significant effect of nutrient supply ($P$<0.001) and ecto-

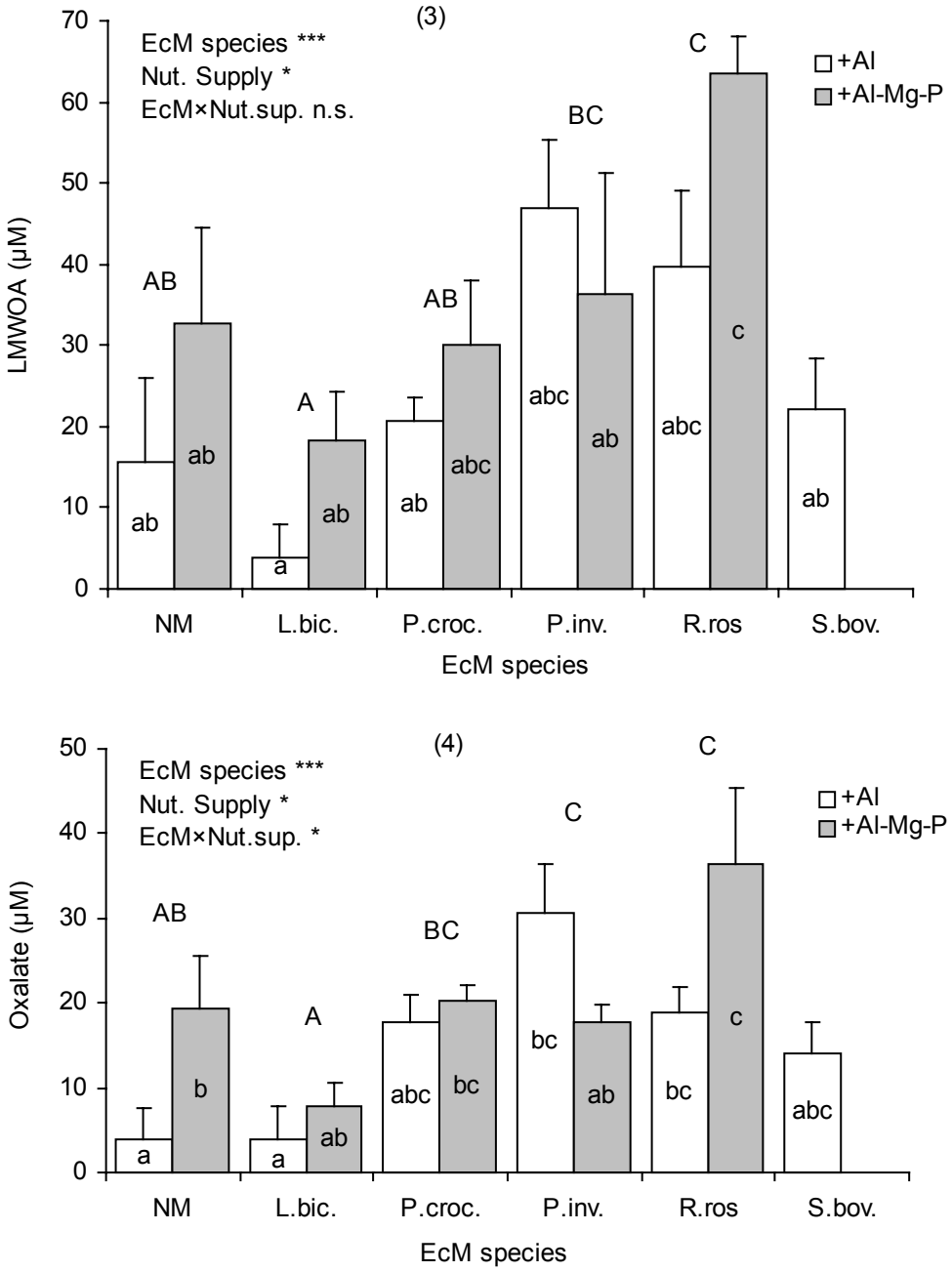
mycorrhizal species ($P$=0.021) and a significant interaction effect ($P$=0.044). Seedlings colonised by *R. roseolus* and *P. involutus* had higher concentrations of oxalate exudation than nonmycorrhizal seedlings or seedlings colonised by *L. bicolor* (Fig. 4). For the nonmycorrhizal seedlings, oxalate exudation in the +Al treatment was lower than in the +Al–P–Mg treatment (Fig. 4).




Figures 1 and 2 Main effect of ectomycorrhizal species (Fig. 1) or nutrient and Al supply (Fig. 2) on exudation of LMWOA (total), oxalate and malonate by *P. sylvestris* seedlings (NM=nonmycorrhizal). Error bars give standard error for totals. For each type of organic anion, bars with the same letter are not significantly different (P<0.05); capital letters above bars refer to totals.

Malonate concentrations varied strongly, with concentration below the detection limit in 2/3 of the dishes. The malonate concentration was significantly affected by ectomycorrhizal species ($P$=0.037), but not by composition of the solution ($P$=0.084). Seedlings colonised by *R. roseolus* exuded more malonate than those colonised by *P. involutus* (Fig 1).




**Figures 3 and 4** Main effect of nutrient supply and ectomycorrhizal species on exudation of LMWOA (Fig.3) or oxalate (Fig. 4) by *P. sylvestris* seedlings (NM=nonmycorrhizal). Error bars give standard error. Bars with the same letter are not significantly different ($P$<0.05); capital letters above bars refer to main effect of inoculation. n.s.= not significant, * =$P$<0.05, ***=$P$<0.001

## Discussion

Aluminium induced the exudation of oxalate in *P. sylvestris* seedlings and ectomycorrhizal fungi (Fig. 2) which confirms the results of Ahonen Jonnarth *et al.* (2000). Oxalate is a strong detoxifier of Al due to its high affinity for Al (stability constant $\log K_{Al}$=6.5) (Hue *et al.*, 1986). A similar response has been previously found for *Picea rubens* (red spruce) (Minocha & Long, 2004) and several herbaceous plants (Ma *et al.*, 1997, Ma & Miyasaka, 1998, Yang *et al.*, 2005). Exudation of malonate was not affected by addition of Al (Fig 2). Malonate is a moderate detoxifier of Al (stability constant $\log K_{Al}$=5.7) (Hue *et al.*, 1986). It is commonly found in the root exudates of leguminous plants (Wouterlood *et al.*, 2005) and nonmycorrhizal and ectomycorrhizal *P. sylvestris* (Chapter 5:Van Schöll *et al.*, 2006), but there are no reports on Al-induced malonate exudation.

Simultaneous Al toxicity with Mg and P deficiency significantly increased the oxalate exudation compared to Al toxicity alone (Fig. 4), which signifies that exudation is a response to combined effects. The increase was most notably in nonmycorrhizal seedlings, which only exuded low amounts of oxalate under Al toxicity with complete nutrient supply. For the ectomycorrhizal species, the increase was not significant, and in seedlings colonised by *P. involutus* the simultaneous Al toxicity and Mg and P deficiency decreased the oxalate exudation. In studies dealing with crop plants, P deficiency under Al toxicity either decreased the organic anion exudation compared to Al toxicity with sufficient P (Dong *et al.*, 2004, Ligaba *et al.*, 2004, Nian *et al.*, 2003) or did not affect the organic anion exudation under Al toxicity (Delhaize *et al.*, 1993, Ma & Miyasaka, 1998, Yang *et al.*, 2000) which is similar to what we found for the ectomycorrhizal seedlings. It is, however, uncertain if the mechanisms triggering the exudation of LMWOA in herbaceous plants, which are already sensitive to a few μM Al, are similar to those in trees, which can tolerate far higher concentrations of Al (>150 μM). The synergistic effect may also have been caused by the concomitant Mg deficiency. As far as we know, comparison of the exudation of organic acids in response to Al toxicity and Mg deficiency has not been previously assessed. The effect of nutrient supply on fungal and plant response to Al underlines the importance of considering the complexity of the natural situation in comparison to the experimental system.

The increase in oxalate exudation was not a response to Mg omission or low P in itself, because omission of Mg or low P in the nutrient solution without Al did not induce oxalate exudation (Fig. 2). This is in contrast to Van Schöll et al. (2006: Chapter 5) where Mg omission from the nutrient supply, in a comparable experimental set up, increased the oxalate exudation of nonmycorrhizal and ectomycorrhi-

zal *P. sylvestris* and where the total amount of LMWOA exuded by sand-grown nonmycorrhizal and ectomycorrhizal *P. sylvestris* increased in response to P deficiency. Differences in timing and experimental set up presumably influenced the physiological activity and response of the seedlings and fungi.

Ectomycorrhizal fungal species significantly affected the amount and type of organic anions exuded. *R. roseolus* caused significantly more malonate exudation than *P. involutus* (Fig. 1). Seedlings colonised by *P. involutus* or *R. roseolus* exuded significantly higher amounts of oxalate than nonmycorrhizal seedlings or seedlings colonised by *L. bicolor* under Al toxicity (Fig. 4). This pattern correlates well with the oxalate production of these fungi in pure culture: *L. bicolor* is a poor oxalate producer (Cumming *et al.*, 2001) whereas *R. roseolus* and *P. involutus* have been found to be active oxalate producers compared to other species (Arvieu *et al.*, 2003, Casarin *et al.*, 2003, Gharieb & Gadd, 1999, Lapeyrie, 1988, Paris *et al.*, 1996). Also, seedlings colonised by *R. roseolus* exuded significantly higher amounts of oxalate than nonmycorrhizal seedlings in the study by Ahonen *et al.* (2000). In contrast to our results and Van Schöll et al. (2006: Chapter 5), however, they did not find any difference in oxalate exudation between nonmycorrhizal seedlings and seedlings colonised by *P. involutus.*

The concentrations of organic anions were an order of magnitude lower than the concentration of Al, which raises the question of the quantitative importance of detoxification by complexation. Zheng *et al.* (1998) found that an externally applied oxalate:Al ratio of 1:1 alleviated Al toxicity symptoms, but no effect was found with an molar ratio of 1:2. The oxalate:Al ratios observed here were still considerable lower. Model calculations, however, suggest that the concentrations of organic anions in the apoplast of the root cortex are more relevant that the concentrations at the root surface or in the external solution (Kinraide *et al.*, 2005, Mariano, 2003). Continuous production and resistance to diffusion out of the roots can cause a build up of organic anions in the root cortex that is considerably higher than the concentration at the root outer surface or bulk solution, and this might be sufficient to reduce the concentration of $Al^{3+}$ in the root cortex. In this respect, the ectomycorrhizal roots might have an advantage over the nonmycorrhizal roots; not only can the hyphae in the Hartig net increase the exudation of oxalate in the root cortex, the Hartig net and the ectomycorrhizal sheath may also act as a diffusion barrier to organic molecules (Behrmann & Heyser, 1992, Vesk *et al.*, 2000), thereby effectively increasing the build up of organic anions in the root cortex. The permeability of the fungal sheath is species dependent (Bücking *et al.*, 2002)

The present set up of the experiment did not allow us to link the exudation of

LMWOA to Al resistance of the seedlings. Shoot or root dry weights were not affected by the composition of the nutrient solution. Root dry weight was significantly affected by ectomycorrhizal colonisation (Table 2), but this was not correlated to LMWOA exudation. In experiments with herbaceous plant species, root elongation is a good indicator of Al resistance of plants (Mariano & Keltjens, 2004) and can be measured within days or even hours. With *P. sylvestris* (and most woody plant species) root elongation rates are too low to detect significant differences in such time frame. Additional experiments, linking LMWOA exudation to plant resistance and growth rates under Al toxicity are required to elucidate whether ectomycorrhizal species with high LMWOA exudation are more effective in protecting their host plant.

Differences in Al-induced organic acid exudation among the seedlings colonised by various ectomycorrhizal species are as big or bigger than those between nonmycorrhizal and ectomycorrhizal seedlings. This high variability among ectomycorrhizal fungal species shows again that generalisation of the effect of ectomycorrhizal colonisation is not possible. In general, more knowledge is needed on the functional roles of the different species, and how this is related to their occurrence within and among different ecosystems with different natural conditions.

## Acknowledgements

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# 5

# Organic anion exudation by ectomycorrhizal fungi and *Pinus sylvestris* in response to nutrient deficiencies

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## Summary

Low Molecular Weight Organic Anions (LMWOA) can enhance weathering of mineral grains. We tested the hypothesis that ectomycorrhizal fungi and tree seedlings increase their exudation of LMWOA when supply of Mg, K and P is low to enhance the mobilisation of MG, K and P from mineral grains. Ectomycorrhizal fungi and *Pinus sylvestris* seedlings were cultured in symbiosis and in isolation on glass beads with nutrient solution or with sand as a rooting medium, with a complete nutrient supply or with Mg, K, P or N in low supply. Concentrations of all di-carboxylic LMWOA in the rooting medium were measured. Nonmycorrhizal seedlings released predominantly malonate. Colonisation with *H. longicaudum* decreased the amount of organic anions exuded, whereas *P. involutus* and *P. croceum* increased the concentration of oxalate but not the total amount of LMWOA. P deficiency increased the concentration of LMWOA by nonmycorrhizal and ectomycorrhizal seedlings. Mg deficiency increased the concentration of oxalate by nonmycorrhizal and ectomycorrhizal seedlings but not the concentration of total LMWOA. *P. involutus* grown in pure culture responded differently to low nutrient supply compared to symbiotic growth. Ectomycorrhizal fungi did not increase the total concentration of LMWOA compared to nonmycorrhizal seedlings but, depending on the fungal species, affected the type of LMWOA found.

## Introduction

Most trees in boreal forests are associated with ectomycorrhizal fungi (Smith & Read, 1997). Ectomycorrhizal (EcM) fungi can improve the uptake of N and P by their host plant through a better exploration of the soil and higher total absorbing surface of the roots and hyphae compared to roots alone. In addition, ectomycorrhizal fungi can mobilise N and P from organic sources largely unavailable to plant roots (Chalot & Brun, 1998, Smith & Read, 1997). In most forest ecosystems, N is considered the limiting element for tree growth (Binkley & Högberg, 1997, Tamm, 1991). For trees growing on strongly weathered soils with high loads of atmospheric N deposition, Mg and K can become limited (Landman *et al.*, 1997, Uebel & Heinsdorf, 1997). Currently, ectomycorrhizal fungi were suggested to play a role in the acquisition of P, K and Mg from mineral grains (for reviews: Landeweert *et al.*, 2001, Wallander & Hagerberg, 2004). Ectomycorrhizal fungi can transport K and Mg (Jentschke *et al.*, 2001), but knowledge on the influence of ectomycorrhizal fungi on the uptake of base cations by their host tree is limited and inconsistent (Bücking *et al.*, 2002, Hoffland *et al.*, 2004).

An important source of Mg and K for plant growth is the dissolution of mineral grains such as feldspars and hornblendes. Mineral weathering can be enhanced by low molecular weight organic anions (LMWOA) (Drever & Stillings, 1997). LMWOA in soil solution are the product of decaying plant material, root exudation and activity of micro-organisms (Jones, 1998). Several plants increase the exudation of LWMOA when grown under P deficiency (Ryan *et al.*, 2001). Wouterlood *et al.*(2005) suggest that LMWOA exudation has become a constitutive trait of plants that are adapted to low P environments. Similarly, ectomycorrhizal fungi might increase mineral weathering by exuding LMWOA in response to P, Mg or K deficiencies (Hoffland *et al.*, 2004).

There have been no reports on the effects of cation deficiencies on LMWOA exudation by ectomycorrhizal fungi or tree seedlings. Both trees and ectomycorrhizal fungi, however, have been found to exude LMWOA and to enhance mineral weathering. When grown *in vitro* without their plant symbiont, oxalate exudation by several ectomycorrhizal fungi has been found in several studies (Arvieu *et al.*, 2003, Casarin *et al.*, 2003, Cumming *et al.*, 2001, Gharieb & Gadd, 1999, Lapeyrie, 1988,

Lapeyrie *et al.*, 1987, Paris *et al.*, 1996). Tree seedlings colonised by ectomycorrhizal fungi exuded more oxalate than nonmycorrhizal tree seedlings under axenic conditions (Ahonen Jonnarth *et al.*, 2000, Casarin *et al.*, 2003). In the field, higher levels of oxalate and dissolved P were found in soil solution under mats of ectomycorrhizal fungi than in soil solution without fungal mat (Griffiths *et al.*, 1994).

Ectomycorrhizal fungi differ in their capacity to exude LMWOA (Ahonen Jonnarth *et al.*, 2000, Arvieu *et al.*, 2003, Casarin *et al.*, 2003, Wallander & Wickman, 1999) but no effort has been made so far to relate this capacity to their origin or ecological function. If ectomycorrhizal fungi increase the exudation of LMWOA to enhance weathering under cation deficiencies it can be expected that ectomycorrhizal fungi occurring in the mineral soil layer exude more organic anions in response to cation deficiencies than those that occur in the organic soil layer. Recently, it was shown that up to 60% of the total ectomycorrhizal root tips was found in the mineral soil layer, and that there was a distinct distribution pattern of ectomycorrhizal species over the soil profile, with some species occurring in either the mineral or the organic layer (Landeweert *et al.*, 2003, Rosling *et al.*, 2003). Heinonsalo *et al.* (2004) found a high carbon allocation to ectomycorrhizal fungi in mineral soil layers. Also, more carbon allocation to hyphae growing over K containing-minerals than over quartz was shown (Rosling *et al.*, 2004). These findings indicate a functional role of ectomycorrhizal fungi present in the mineral soil horizons in the weathering of mineral grains.

In pot experiments, ectomycorrhizal seedlings were able to mobilise and take up P, K, Mg and Fe from rock phosphate, phlogopite (mica), biotite or apatite (Glowa *et al.*, 2003, Leyval & Berthelin, 1989, Wallander *et al.*, 1997), but this did not always improve growth or uptake compared to nonmycorrhizal seedlings (Wallander & Wickman, 1999). Ectomycorrhizal fungi were able to weather the minerals vermiculite (Paris *et al.*, 1995b) and phlogopite (Paris *et al.*, 1995a), and to solubilise calcium and iron phosphates (Lapeyrie *et al.*, 1991, Leyval & Berthelin, 1986). Simultaneous omission of Mg and K from the growth medium increased weathering of phlogopite by ectomycorrhizal fungi (Paris *et al.*, 1996). Correlations between the concentration of organic anions in the soil solution, weathering and uptake of dissolved elements and ectomycorrhizal colonisation are however not clear (Leyval & Berthelin, 1993, 1991, Wallander, 2000). This can be explained by high spatial variability and the high turnover of organic anions under non-sterile conditions (Jones, 1998).

In this study, we tested the hypothesis that ectomycorrhizal fungi enhance the exudation of LMWOA when base cations are in low supply. The response of tree seed-

lings and ectomycorrhizal fungi grown in pure culture can be different from the response when grown in symbiosis. Therefore, the same experimental set up was used to compare the response of the ectomycorrhizal fungus *Paxillus involutus* with the response of *Pinus sylvestris* tree seedlings, either nonmycorrhizal or colonised by *P. involutus*, to omission of Mg or K in the nutrient solution. The fungal species used were *P. involutus,* which is commonly associated with organic soil horizons, *Piloderma croceum* (also known as *P. fallax*), which has been found in both the organic and mineral horizon, and *Hebeloma longicaudum* which has been found in the mineral horizon (Laiho, 1970, Landeweert *et al.*, 2003).

## Material and methods

*Plant and fungal material*

The ectomycorrhizal (EcM) fungi *Paxillus involutus* (Batch: Fr.) Fr. 'nr 17', *Piloderma croceum* J. Erikss. Z. Hjorst 'BL97-01'and *Hebeloma longicaudum* ()Pers.:Fr.) Kumm (all kindly provided by Roger Finlay, SLU, Uppsala, Sweden) were maintained on MMN medium (Marx, 1969). Seeds of *Pinus sylvestris* L. (Scots pine) were soaked overnight in demineralised water, surface-sterilised in 30% $H_2O_2$ for 30 min. and rinsed with autoclaved demineralised water. To check for eventual microbial contamination they were transferred to water agar plates (8 g $l^{-1}$) with glucose (10 g $l^{-1}$) for germination. The experiments were set up under sterile conditions in a continuous flow hood, with sterile materials and all growth media autoclaved before use. Nutrient solutions in all experiments were based upon Ingestad's (1979): mineral nutrient requirements of *P. sylvestris,* aimed at optimal growth. These concentrations may deviate from the forest soil solutions, where concentrations of certain nutrients are low but replenished continuously, and N is the element generally limiting growth.

Three axenic experimental systems were used:

1. Petri dishes where the fungus grows in pure culture on a layer of glass beads and nutrient solution

2. Petri dishes where the roots of a tree seedling and associated fungus grow on a layer of glass beads and nutrient solution, with the shoots protruding from the Petri dish (adapted from Ahonen Jonnarth *et al.* (2000), see picture at page 39).

3. Serum incubation flasks, in which the tree seedlings grow on quartz sand mixed with nutrient solution (see picture at page 53).

*1.* Paxillus involutus *on glass beads*

*P. involutus* was cultured for 2 weeks on agar (15 g $l^{-1}$) with glucose (10 g $l^{-1}$) and nutrients added according to experimental treatment (composition given in Table 1). The experimental treatments were: (1) Complete nutrient solution; nutrient solution with either (2) 25% of N, (3) 25% of P, (4) 25% of K or (5) 0% of Mg. After 2 weeks, plugs were cut from an actively growing mycelial front and transferred to Petri dishes (diam. 9 cm., one plug per Petri dish) containing 25 gram glass beads (diam. 3mm) (Fisher Scientific, Netherlands) and 8.5 ml of nutrient solution (composition as above) with glucose (10 g $l^{-1}$). The pH of the solutions was 5.6. The glass beads had been immersed in 0.1 M HCl overnight followed by rinsing in nutrient solution and demineralised water. There were 4 replicates for each treatment.

After 4 weeks, 4 ml of the nutrient solution was replaced with fresh nutrient solution. One week later, part of the nutrient solution was collected for determination of LMWOA and frozen immediately (-22°C) till analysis. The remaining nutrient solution was used for pH measurement. The concentrations of LMWOA measured were not corrected for the dry weight increment of the fungi during the experiment, which was considered negligible compared to the amount of solution (maximal 11 mg dry

**Table 1** Composition of the nutrient solution (µmol $l^{-1}$), adapted from (Ingestad, 1979): optimal nutrient composition for growth of *Pinus sylvestris* seedlings. In addition, all nutrient solutions contained microelements in concentration (mg $l^{-1}$): 2.65 Fe; 0.25 B; 0.01 Cu; 0.005 Mo; 0.25 Mn; 0.03 Zn.

| | $NH_4NO_3$ | $KH_2PO_4$ | $NH_4H_2PO_4$ | $KNO_3$ | $(NH_4)_2SO_4$ | $CaCl_2$ | $MgSO_4$ | KCl | $KSO_4$ | $Na_2SO_4$ | $NaNO_3$ | $CaNO_3$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Experiment 1* | | | | | | | | | | | | |
| Complete | 770 | 67 | 50 | 181 | 36 | 39 | 60 | 50 | | | | |
| 0% Mg | 770 | 67 | 50 | 181 | 36 | 39 | | | 25 | 34 | | |
| 25% K | 738 | | 117 | 75 | 36 | 39 | 60 | | | | 29 | 39 |
| 25% N | 77 | 67 | 50 | 181 | 36 | 39 | 60 | 117 | | | | |
| 25% P | 780 | | 30 | 181 | 36 | 39 | 60 | 117 | | | | |
| *Experiment 2* | | | | | | | | | | | | |
| Complete | 770 | 67 | 50 | 181 | 36 | 39 | 60 | 50 | | | | |
| 0% Mg | 770 | 67 | 50 | 181 | 36 | 39 | | | 25 | 34 | | |
| 0% K | 738 | | 117 | | 36 | 39 | 60 | | | | 104 | |
| *Experiment 3* | | | | | | | | | | | | |
| Complete | 1540 | 133 | 100 | 362 | 72 | 78 | 120 | 100 | | | | |
| 0% Mg | 1540 | 133 | 100 | 362 | 72 | 78 | | 50 | | 68 | | |
| 40% P | 1540 | 100 | | 362 | 62 | 78 | 120 | 230 | | | | |

weight fungus vs 8.5 ml solution added). Evaporation from the dishes was assumed to be negligible because the dishes were sealed off, and this was checked by comparing the weight of the dishes at the start and end of the experiment. Surface area covered by the mycelium was outlined on the upper lid of the Petri dish and measured. The mycelium was then removed from the glass beads, separated from the agar plug, dried at 70°C and weighted.

*2. Nonmycorrhizal or ectomycorrhizal P. sylvestris seedlings on glass beads*

After 6 days germination on water agar (see paragraph 'plant and fungal material' above), individual seedlings of *P. sylvestris* (± 0.006 g dry weight) were transferred to growth tubes together with 3 plugs of agar, which were sterile (nonmycorrhizal treatment) or taken from the front of an active growing mycelium (EcM species: *P. involutus*; *H. longicaudum*; *P. croceum*). Growth tubes contained 20 gram of a mixture of fine quartz sand:perlite:nutrient solution (ratio 2 kg: 0.13 kg: 0.685 l) and had been autoclaved for 30 minutes at 120°C. Composition of the nutrient solution was (in µM) 1540 $NH_4NO_3$; 100 $NH_4H_2PO_4$; 362 $KNO_3$; 72 $(NH_4)_2SO_4$; 77 $CaCl_2$; 101 $K_2SO_4$ (modified from Ingestad (1979)). The growth tubes were closed with cotton wool and the outside bottom was covered with aluminium foil. They were placed in a climate chamber (16 hours photoperiod at 70 Watts/$m^2$, day/night temperatures of 20/16°C and 80% humidity). During the incubation period, each tube received 3 ml of demineralised water twice.

At 14 weeks after inoculation the seedlings (± 0.07 g dry weight) were transferred to Petri dishes (diam. 9 cm), containing 25 gram of glass beads (diam. 3mm) (as above) and 9 ml of nutrient solution. The roots were washed in autoclaved demineralised water and spread out over the beads. The shoot protruded from a notch cut out along the side of the dishes. The dishes were sealed with anhydrous lanolin around the base of the stem and parafilm, covered with aluminium foil and placed horizontally in a growth box. They were left in the laboratory so that the seedlings could acclimatise for 1 week, and then placed in a climate chamber (specifications as above). The experimental treatments were: complete nutrient solution (Compl.), nutrient solution without Mg (–Mg) and nutrient solution without K (–K) (composition given in Table 1). There were 6 replicates for every treatment. After 2 and 4 weeks the Petri dishes were brought back to their original weight by addition of sterile demineralised water to correct for transpiration losses. Water was added with a sterile pipette tip through a small hole burned into the upper lid of the Petri dish, that was sealed off again with sterile lanoline.

After 5 weeks following transfer to the glass beads, all Petri dishes were brought

back to their original weight by the addition of demineralised water, where after 4.6 ml of the nutrient solution was replaced with fresh nutrient solution. Five days later, the weight loss of each Petri dish was noted and nutrient solution was removed from the dishes for determination of organic anions and frozen at -22°C till analysis. The concentrations of LMWOA measured were corrected for the transpiration of the tree seedlings as calculated from the weight loss of the Petri dishes. No correction was made for the weight increment of the tree seedlings and fungi during growth on the glass beads (± 0.09 g dry weight increment), resulting in a slight overestimation of the exudation (± 3% assuming a fresh weight/dry weight ratio of 3). Surface area covered by the hyphae was outlined on the upper lid of the Petri dish and measured. A contamination check was done in triplicate for each dish by plating 0.1 ml of the solution on King's medium B (King *et al.*, 1954) and checking for bacterial or fungal growth after 48 hours. Contaminated dishes were discarded.

*3. Nonmycorrhizal or ectomycorrhizal P. sylvestris seedlings grown on sand in incubation flasks*

After 2 weeks germination on water agar (see paragraph 'plant and fungal material' above), individual seedlings (± 0.006 g dry weight) were transferred to incubation flasks (250 ml) together with six plugs of agar, which were sterile (nonmycorrhizal treatment) or taken from the front of an active growing mycelium (EcM species: *P. involutus* or *H. longicaudum*). The flasks contained 150 g of moist fine quartz sand and had been autoclaved at 120°C for 30 min. The sand had been flushed with demineralised water till the effluent reached the pH of the demineralised water to remove easily dissolvable Mg, dried at 70°C and mixed with nutrient solution (170 ml $kg^{-1}$ sand). The flasks were closed with cotton wool and the outside bottom was covered with aluminium foil. The flasks were left for 3 days in the lab to allow the seedlings to acclimatise before being placed in a climate chamber (specifications as above).

Experimental treatments were: (1) Complete nutrient solution (adapted from Ingestad (1979)), nutrient solution (2) without Mg or (3) with 42% P (composition of the nutrient solution in Table 1). At three times during the experiment, flask were weighted and brought back to their original weight by the addition of nutrient solution (in total 15 ml) and demineralised water.

After 6 months, all flasks were brought back to original weight to correct for transpiration losses. The sand and seedlings were removed from the flasks in non-sterile conditions. The soil solution was extracted immediately by centrifugation of the sand at 5500 RCF and partly directly frozen at -22°C till organic anion analysis. The

concentrations of LMWOA were not corrected for the weight increment of the seedlings (± 0.38-0.51 g dry weight) resulting in a slight overestimation of the exudation (± 4-6% assuming a fresh weight/dry weight ratio of 3). The remaining soil solution was used for pH measurements. Mycorrhizal colonisation of the roots was checked under a dissecting microscope, after which shoots were dried at 70°C (48 hours) and weighed. Root dry weights could not be determined because of sand attached to the roots that could not be removed.

For each treatment, shoots of three replicates were randomly selected for determination of N, P, and Mg. Shoot material was digested with $H_2SO_4$-salicylic acid-$H_2O_2$ and selenium. P and N concentrations in the needles were determined colorimetrically by segmented automated flow analysis and Mg was determined by AAS.

*Determination of di-carboxylic Low Molecular Weight Organic Anions*

The di-carboxylic LMWOA oxalate, malonate, fumarate, tartrate, malate, succinate, and maleate were analysed in all tree experiments by capillary zone electrophoresis (Waters Corp. Milford, MA, USA) following the method of Westergaard *et al.* (1998). The detection limit for each LMWOA is around 1 µM. Citrate could not be measured with sufficient accuracy. High molecular weight organic anions and mono-carboxylic LMWOA were not determined as they have only weak weathering capacity.

*Statistical analysis*

Statistical analysis was performed with SPSS 12.0.1 for WINDOWS (SPSS Inc. Chicago, IL, USA). Independent variables were nutrient supply and -in exp. 2 and 3 only- ectomycorrhizal species. Dependant variables were concentrations of Total LMWOA, oxalate, malonate or fumarate, shoot or fungal dry weight and -in exp. 3 only- shoot concentrations of N, P and Mg. Data were transformed (square root or $^{10}\log(x)$ or $^{10}\log(x+1)$) where necessary to homogenise variance. Because samples of the mycorrhizal treatments without visible ectomycorrhizas or with contaminations were discarded, the number of replicates in experiment 2 and 3 varied among treatments.

In experiment 1, the significance of main effects was determined by one-way ANOVA (analysis of variance), followed by Tukey's honestly significant difference (HSD) *post hoc* test to determine the significance of differences between all pairwise comparisons. For the variable Total LMWOA per mg dry weight, variances remained heterogeneous (Levene's test) and the robust ANOVA with Brown-Forsythe test (equal variances not assumed) was used, followed by the Dunnet T3 *post hoc*

test.

In experiment 2 and 3, the significance of main effects was determined by two-way ANOVA, followed by the Tukey's HSD (equal samples sizes) or Tukey-Kramer (unequal sample sizes) *post hoc* tests to test the significance of differences among all pairwise comparisons. In some cases the variance could not be homogenised (Levene's test), and the Games-Howell *post hoc* test (exp.3: total LMWOA, malonate) or the Dunnet T3 *post hoc* test (exp.3: N conc. in shoot) were used to determine significance of differences among all pairwise comparisons.

## Results

*1.* P. involutus *in pure culture on glass beads*

*P. involutus* was able to grow well in pure culture on a layer of glass beads and nutrient solution. At harvest, the mean surface area of the mycelium was 51% (low P), 62% (low N), 79% (no Mg) 85% (low K) and 90% (complete supply) of the total surface area of the dishes. Dry weight (Table 2) was significantly affected by nutrient supply ($P$=0.002). Reduction of N supply, but not of P, K or Mg, significantly reduced mycelium dry weight compared to the control with complete nutrient solution. There was also a significant effect of nutrient supply on the final pH of the nutrient solution ($P$<0.001) (Table 2). The pH of the nutrient solution was lowered in all treatments at the end of the experiment. At low supply of P it was significantly lower than in the other treatments.

The concentration of total LMWOA was significantly affected by nutrient supply, expressed either as concentration in the solution ($P$=0.026) (Fig. 1a) or per dry weight fungal mycelium ($P$=0.005)(Fig. 1b). The concentration of total LMWOA was significantly higher in the treatment with low N than in the treatment without Mg when expressed as absolute concentration in the solution and significantly hig-

**Table 2** Mycelial dry weight of *Paxillus involutus* and pH of culture solutions with different composition after 4 weeks. Mean of 4 replicates followed by standard error in italics. Values followed by the same letter in a column are not significantly different ($P$<0.05).

| Nutrient supply | Dry weight (mg) | | | pH | | |
|---|---|---|---|---|---|---|
| Complete | 9.0 | *2.4* | bc | 3.33 | *0.09* | a |
| 25% K | 11.0 | *1.4* | c | 3.42 | *0.08* | a |
| 0% Mg | 7.5 | *1.0* | bc | 3.43 | *0.11* | a |
| 25% N | 2.4 | *0.2* | a | 3.54 | *0.09* | a |
| 25% P | 4.5 | *1.7* | ab | 2.82 | *0.10* | b |

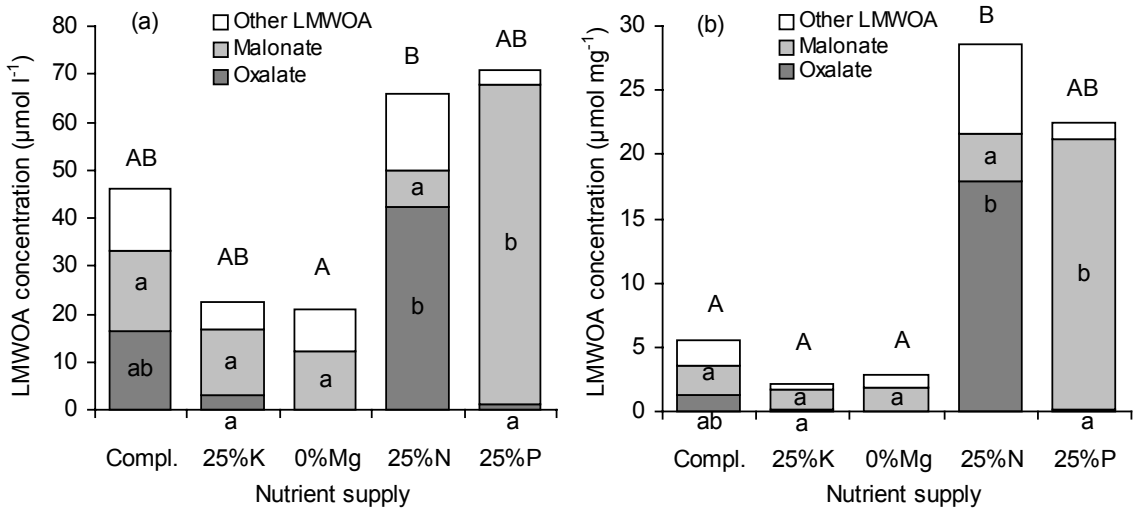


**Figure 1a and b** Main effect of nutrient supply on LMWOA concentration by *P. involutus* cultured on glass beads. Concentrations expressed either as µmol $l^{-1}$ (a) or as µmol $g^{-1}$ DW mycelium (b) (note different scale on y axis). Mean of four replicates. For each type of organic anion, bars with the same letter are not significantly different ($P$<0.05); Capital letters above bars denote Total LMWOA.

her than in the treatments with complete nutrient supply, with low K or without Mg when expressed per fungal dry weight. Oxalate and malonate were the major LMWOA found. Fumarate, tartrate, malate, succiniate and maleate were found occasionally, but there was no consistent treatments effect.

Oxalate concentrations were significantly affected by nutrient supply, either expressed as concentration in solution ($P$=0.008) (Fig. 1a) or amount per dryweight mycelium ($P$<0.001) (Fig. 1b). Oxalate in the low N treatment was significantly higher than in treatments with low P or K (both concentration in solution or when expressed per dry weight) and significantly higher than in the control treatment when expressed per dry weight. No oxalate was found in the treatment with Mg omitted.

Malonate was significantly affected by nutrient supply when expressed as concentration in solution ($P$=0.01) (Fig. 1a) but not when expressed per dry weight mycelium ($P$=0.053) (Fig. 1b). Malonate concentration was significantly higher in treatment with low P compared to all other treatments when expressed as concentration but not when expressed per dry weight.

*2. Nonmycorrhizal or ectomycorrhizal* P. sylvestris *seedlings on glass beads*

Root systems of the inoculated tree seedlings were colonised well by all three fungi, with most root tips ectomycorrhizal. At the end of the experiment, the external mycelium covered on average 34% (*P. involutus*), 19% (*P. croceum*) or 9% (*H.* longi-

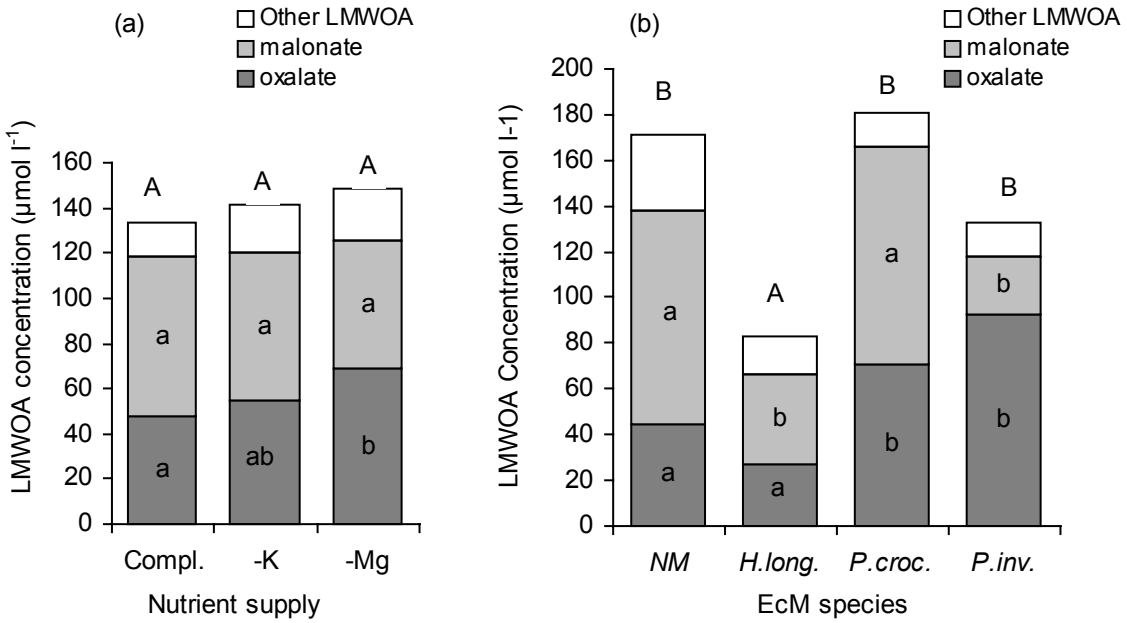


**Figure 2a and b**. Main effect of nutrient supply (a) and mycorrhizal colonisation (b) on concentration of LMWOA by *P. sylvestris* seedlings cultured on glass beads. For each type of organic anion, bars with the same letter are not significantly different ($P$<0.05); Capital letters above bars denote Total LMWOA.

*caudum*) of the total surface area of the dishes. There was no effect of nutrient supply on the mycelium surface area. Dry weight of the shoot and root did not differ significantly between the treatments (data not shown).

The concentration of total LMWOA (Fig. 2, Table 3) was significantly affected by the ectomycorrhizal fungal species ($P$<0.001) but not by nutrient supply ($P$=0.072) There was no interaction effect ($P$=0.45). With tree seedlings colonised by *H. longicaudum* significantly lower concentrations of LMWOA were found than with seedlings colonised by *P. involutus*, *P. croceum* or without mycorrhiza. Oxalate, malonate and fumarate were found in most dishes. Tartrate, malate, succinate and maleate were found occasionally, but there was no consistent pattern over treatment or species.

Oxalate concentrations (Fig. 2, Table 3) were significantly affected by fungal species ($P$<0.001) and nutrient supply ($P$=0.001) but there was no significant interaction effect ($P$=0.073). Concentrations of oxalate were significantly higher with seedlings colonised by *P. involutus* and *P. croceum* than with seedlings colonised by *H. longicaudum* or without mycorrhiza. Dishes with nutrient solution minus Mg had significantly higher concentrations of oxalate than dishes containing complete nutrient solution. Highest concentrations of oxalate were found in dishes with seedlings colonised by *P. involutus* and nutrient solution minus Mg or minus K and in the dis-

**Table 3** Total LMWOA, oxalate and malonate concentrations of culture solution of *P. sylvestris* seedlings, either nonmycorrhizal or colonised by *P. involutus*, *H. longicaudum* or *P. croceum,* cultured for 6 weeks on glass beads and solution with either complete or limited nutrient composition.

| Species | Nutrient supply | n | LMWOA µmol $l^{-1}$ | | | oxalate µmol $l^{-1}$ | | | malonate µmol $l^{-1}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Nonmycorrhizal | Complete | 3 | 132 | *18* | ab | 35 | *3* | a | 70 | *8* | abc |
| | -K | 5 | 183 | *38* | b | 37 | *9* | a | 108 | *31* | bc |
| | -Mg | 3 | 190 | *36* | b | 66 | *5* | ab | 95 | *31* | abc |
| *H. longicaudum* | Complete | 2 | 47 | *20* | a | 17 | *17* | a | 25 | *2* | abc |
| | -K | 5 | 84 | *18* | ab | 19 | *7* | a | 50 | *13* | abc |
| | -Mg | 5 | 97 | *8* | ab | 39 | *3* | a | 35 | *7* | abc |
| *P. croceum* | Complete | 4 | 201 | *60* | b | 56 | *8* | ab | 131 | *51* | bc |
| | -K | 5 | 143 | *22* | ab | 69 | *10* | ab | 61 | *12* | abc |
| | -Mg | 2 | 238 | *28* | b | 105 | *2* | bc | 112 | *22* | c |
| *P. involutus* | Complete | 4 | 111 | *27* | ab | 64 | *15* | ab | 33 | *7* | abc |
| | -K | 3 | 163 | *10* | ab | 125 | *20* | c | 24 | *11* | ab |
| | -Mg | 3 | 131 | *11* | ab | 98 | *7* | bc | 16 | *2* | a |

Number of replicates given by n. Mean followed by standard error in italics. Values followed by the same letter in a column are not significantly different ($P$<0.05).

hes with seedlings colonised by *P. croceum* and nutrient solution minus Mg (Table 3). Malonate concentrations (Fig. 2, Table 3) were significantly affected by species ($P$<0.001) but not by nutrient supply ($P$=0.93) or interaction ($P$=0.44). Concentrations of malonate were significantly higher with seedlings colonised by *P. croceum* or without mycorrhiza than with seedlings colonised by *P. involutus* or *H. longicaudum.* There was no significant overall effect of nutrient supply on malonate concentration.

Fumarate concentrations did not differ significantly between species ($P$=0.614) or nutrient supply ($P$=0.056), nor was there an interaction effect ($P$=0.43).

*3. Nonmycorrhizal or ectomycorrhizal* P. sylvestris *seedlings grown on sand in incubation flasks*

The roots of the seedlings colonised by *P. involutus* were almost completely mycorrhizal, with obvious ectomycorrhizas and abundant hyphae and rhizomorphs. With *H. longicaudum*, growth of extraradical mycelium was less vigorous. The root system contained many ectomycorrhizas with adhering white hyphae, but up to half of the root tips had not formed ectomycorrhizas, although root hairs were lacking.

Ectomycorrhizal colonisation significantly affected shoot dry weight ($P$<0.001) (Table 4). Shoot dry weights of the nonmycorrhizal seedlings were significantly higher

**Table 4** Shoot dry weight (DW) and tissue concentration of N, P and Mg of *Pinus sylvestris* seedlings, either nonmycorrhizal (NM) or colonised by *P. involutus* or *H. longicaudum,* cultured for 6 months on quartz sand and nutrient solution with either complete or deficient nutrient composition. Mean followed by standard error in italics. Values followed by the same letter in a column are not significantly different ($P$<0.05).

| Species | Nutrient supply | Shoot DW (g) | | | N (mg $g^{-1}$ DW) | | | P (mg $g^{-1}$ DW) | | | Mg (mg $g^{-1}$ DW) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NM | | 0.18 | *0.004* | a | 9.53 | *0.09* | a | 1.07 | *0.08* | a | 0.73 | *0.08* | a |
| *H.longicaudum* | | 0.15 | *0.004* | b | 8.97 | *0.21* | a | 0.83 | *0.09* | b | 0.72 | *0.09* | a |
| *P.involutus* | | 0.17 | *0.004* | b | 7.27 | *0.14* | b | 0.99 | *0.06* | a | 0.59 | *0.08* | b |
| | Complete | 0.17 | *0.005* | a | 8.49 | *0.41* | a | 1.09 | *0.06* | a | 0.83 | *0.05* | a |
| | 0% Mg | 0.17 | *0.004* | a | 8.46 | *0.35* | a | 1.11 | *0.04* | a | 0.37 | *0.02* | b |
| | 42% P | 0.17 | *0.005* | a | 8.83 | *0.34* | a | 0.69 | *0.05* | b | 0.84 | *0.03* | a |

than the shoot dry weights of seedlings colonised by *P. involutus* or *H. longicaudum* (Table 3). There was no significant effect of nutrient supply ($P$=0.99) and neither any interaction effect ($P$=0.27).

Nutrient concentrations in the shoot were affected by ectomycorrhizal species and nutrient supply (Table 4). Ectomycorrhizal colonisation significantly affected the shoot concentrations of P ($P$<0.001), Mg ($P$<0.001) and N ($P$<0.001). Nutrient supply significantly affected shoot concentrations of P ($P$<0.001) and Mg ($P$<0.001) but not N ($P$=0.17). The interaction effect was significant only for P ($P$=0.046).

In the minus Mg treatments, all seedlings were Mg deficient (<0.6 mg Mg $g^{-1}$ d.w.for *Pinus spp.* (Reuter *et al.*, 1997) ). In the low P treatment, all seedlings were P deficient (< 1 mg P $g^{-1}$ d.w.for *Pinus spp.* (Reuter *et al.*, 1997). In addition, all seedlings were N deficient (< 10 mg N $g^{-1}$ d.w. for *Pinus spp.* (Reuter *et al.*, 1997)). Seedlings colonised by *P. involutus* had significantly lower concentrations of Mg and N than the nonmycorrhizal seedlings or seedlings colonised by *H. longicaudum.* Seedlings colonised by *H. longicaudum* had significantly lower concentrations of P than nonmycorrhizal seedlings or seedlings colonised by *P. involutus*.

The pH of the soil solution was significantly affected by ectomycorrhizal species ($P$<0.001) and nutrient supply ($P$<0.001) without a significant interaction effect ($P$=0.26). With the nonmycorrhizal seedlings the pH was significantly lower than with the ectomycorrhizal seedlings, and it was lower with the complete or low P nutrient supply than with the minus Mg supply.

The concentrations of total LMWOA (Fig. 3, Table 5) were significantly affected by species ($P$=0.011) and nutrient supply ($P$=0.003), but the interaction effect was not

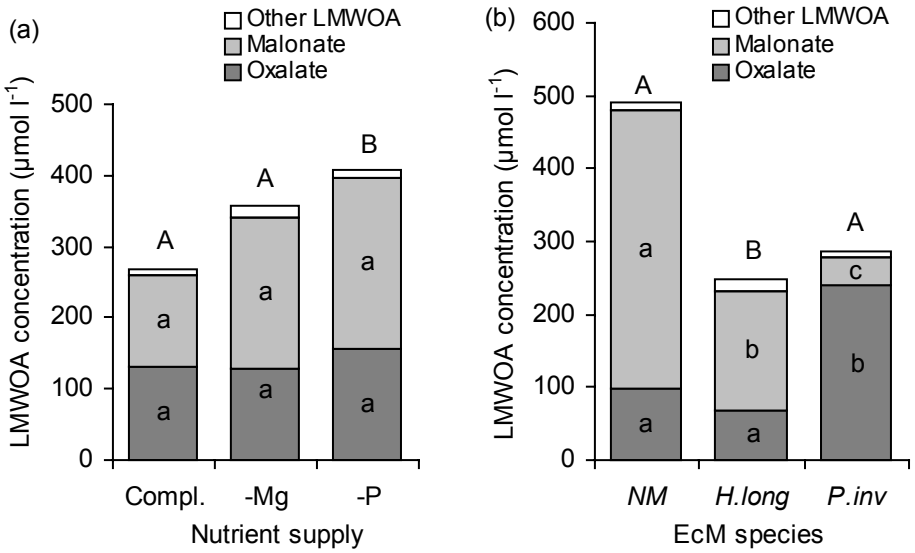


**Figure 3 a and b** Main effect of nutrient supply (A) and mycorrhizal colonisation (B) on concentration of LMWOA by *P. sylvestris* seedlings cultured on quartz sand and nutrient solution. For each type of organic anion, bars with the same letter are not significantly different ($P$<0.05); Capital letters above bars denote Total LMWOA.

significant. Seedlings colonised by *H. longicaudum* had significantly lower concentrations of LMWOA than nonmycorrhizal seedlings. With seedlings with low P supply significantly higher concentrations of LMWOA were found than with seedlings with complete nutrient supply. All seedlings released appreciable amounts of oxalate and malonate (Table 5). Fumarate, tartrate, malate, succinate, and maleate were detected in some samples but there was no clear trend over the treatments.

Oxalate concentrations (Fig. 3, Table 5) were significantly affected by species ($P$<0.001) but not by nutrient supply ($P$=0.88), although there was a significant interaction effect ($P$<0.01). Seedlings colonised by *P. involutus* had significant higher amounts of oxalate than seedlings colonised by *H. longicaudum* or without mycorrhiza.

Malonate concentrations (Fig. 3, Table 5) were significantly affected by species ($P$<0.001) and nutrient supply ($P$<0.001), without a significant interaction effect ($P$=0.36). Highest concentrations of malonate were found with the nonmycorrhizal tree seedlings, with significantly lower concentration with seedlings colonised by *H. longicaudum* and *P. involutus*. Differences between nutrient supply treatments were not significantly different.

## Discussion

We found that both nonmycorrhizal and ectomycorrhizal seedlings exude oxalate,

**Table 5** Total LMWOA, oxalate and malonate concentrations in soil solution of *P. sylvestris* seedlings, either nonmycorrhizal (NM) or colonised by *P. involutus* or H. longicaudum, cultured for 6 months on quartz sand and nutrient solution (complete composition or without Mg or low P). Number of replicates given by n. Mean followed by standard error in italics. Values followed by the same letter in a column are not significantly different ($P$<0.05).

| EcM species | Treat-ment | n | LMWOA µM | | | Oxalate µM | | | Malonate µM | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NM | Complete | 8 | 360 | *120* | abc | 97 | *22* | a | 255 | *98* | abcd |
| NM | -Mg | 7 | 552 | *99* | c | 97 | *16* | a | 449 | *106* | a |
| NM | -P | 8 | 572 | *77* | c | 97 | *9* | ab | 454 | *75* | a |
| *H.longicaudum* | Complete | 8 | 192 | *30* | a | 87 | *11* | a | 106 | *32* | bcd |
| *H.longicaudum* | -Mg | 7 | 274 | *74* | ab | 74 | *12* | a | 166 | *52* | abc |
| *H.longicaudum* | -P | 7 | 288 | *26* | ab | 44 | *9* | a | 226 | *18* | ab |
| *P. involutus* | Complete | 8 | 247 | *39* | ab | 207 | *34* | bc | 30 | *9* | d |
| *P. involutus* | -Mg | 8 | 259 | *27* | ab | 204 | *27* | c | 47 | *8* | cd |
| *P. involutus* | -P | 8 | 352 | *33* | abc | 310 | *33* | c | 38 | *8* | d |

malonate and -in lower concentrations- fumarate. Oxalate is a by-product of the tricarboxylic acid cycle and its biosynthesis is well known (Gadd, 1999). It is found in root exudates of plants in response to aluminium toxicity (Ryan *et al.*, 2001), in solution cultures of fungi (Dutton & Evans, 1996), and has been found to be produced by ectomycorrhizal fungi before (Arvieu *et al.*, 2003, Casarin *et al.*, 2003, Cumming *et al.*, 2001, Gharieb & Gadd, 1999, Lapeyrie, 1988, Lapeyrie *et al.*, 1987, Paris *et al.*, 1996). To our knowledge this is, however, the first report on exudation of malonate by ectomycorrhizal fungi and *P. sylvestris* seedlings. The exact pathway of malonate biosynthesis is still unknown (Yu, 2003). *In vitro*, malonate is a competitive inhibitor of succinate dehydrogenase, which is a key enzyme of the tricarboxylic acid cycle (Li & Copeland, 2000, Yu, 2003). It is found in root exudates of several leguminous plants (Wouterlood *et al.*, 2005). Both oxalate and malonate have also been found in forest soil solutions (Strobel, 2001, Van Hees *et al.*, 2003, Van Hees *et al.*, 2005). They are strong weathering agents, with $\log K_{Al}$=6.5 for oxalate and $\log K_{Al}$=5.7 for malonate (Hue *et al.*, 1986). The concentrations of oxalate and malonate found in our experiments were a factor 10-100 higher than those commonly found in the soil solution, which typically range between 0.1 to 50 µM , but comparable to what might be expected in a rhizosphere (Jones *et al.*, 2003, Strobel, 2001).

The exudation of LMWOA by nonmycorrhizal and ectomycorrhizal seedlings was significantly influenced by limitation of P or Mg but not K. Limitation of P significantly increased the total concentrations of LMWOA (Fig. 3). This is in contrast to

Van Hees *et al.* (2003) who did not find any effect of omitting P from the nutrient solution. In their study, there was a very high turnover of organic anions, as evidenced from the low concentrations found (< 1 μM), and this may have obscured treatments effects.

Omission of Mg significantly increased the concentration of oxalate but not the total concentration of LMWOA when seedlings were cultivated on glass beads (Fig. 2). However, no significant effect of Mg omission from the nutrient solution was found when the seedlings were grown on sand as a rooting medium (Fig. 3). With the glass beads, the nutrient solution had been refreshed a few days before the end of the experiment, and the exudation of organic anions might be regarded as a direct response to the nutrient supply in solution. With the sand-grown seedlings, the exudation of organic anion may be expected to be a response to internal nutrient status of the seedlings. The seedlings were grown over a longer period. Apart from Mg deficiency in the seedlings of the treatment without Mg supply, all seedlings were also N deficient. As there was no effect of Mg deficiencies on the dry weight, we consider it likely that N was the growth-limiting element in our experiments and this may have suppressed plant response to the low internal Mg concentrations. For a wide range of fungi, oxalate production is stimulated by low N availability (Dutton & Evans, 1996). *P. involutus* also increased the concentration of oxalate in response to N limitation when grown in pure culture (Table 1).

In contrast to our expectations, ectomycorrhizal fungi did not increase the total amount of LMWOA in solution, although they did affect the type of LMWOA exuded. Differences among seedlings colonised by different ectomycorrhizal fungi were as big or bigger than between nonmycorrhizal and ectomycorrhizal seedlings. *H. longicaudum* decreased the concentrations of LMWOA and malonate, whereas *P. involutus* and *P. croceum* increased the concentrations of oxalate but not total LMWOA. Nonmycorrhizal seedlings exuded mainly malonate, whereas seedlings colonised by *P. involutus* exuded mainly oxalate (Fig. 2 and 3). In pure culture, *P. involutus* has been found to produce high concentrations of oxalate before (Lapeyrie *et al.*, 1987, Paris *et al.*, 1996). In contrast to our results, Ahonen Jonnarth *et al.* (2000) and Wallander and Wickman (1999) did not find any significant effect of colonisation by *P. involutus* on the concentrations of oxalate in the soil solution compared to nonmycorrhizal seedlings.

Differences among the ectomycorrhizal fungi were not as expected from their hypothesised role in mineral weathering and occurrence in soil layers. Colonisation by *H. longicaudum*, which was seen as a representative of species from the mineral soil horizon, significantly lowered the concentrations of LMWOA compared to nonmy-

corrhizal seedlings or seedlings colonised by *P. involutus*. The latter was considered as a representative of species from the organic horizon (Fig 2 and 3). This result should, however, be interpreted with caution. Knowledge on species occurrence and the distribution of their mycelium among the different soil horizons is still very limited. Moreover, the species used were not freshly isolated from sites with low supply of Mg, K or P but taken from lab collections.

The response of *P. sylvestris* seedlings and *P. involutus* grown in pure culture differed markedly from their response when grown in symbiosis. Omission of Mg significantly increased the concentrations of oxalate from the nonmycorrhizal or ectomycorrhizal seedlings (Table 3), but *P. involutus* in pure culture did not exude any oxalate when Mg was omitted from the nutrient solution (Fig. 1). Reduction of K did not have a significant effect on the organic anion concentration of either *P. involutus* in pure culture (Fig. 1) or nonmycorrhizal seedlings (Table 3), but significantly increased the concentration of oxalate when they were grown in the symbiosis (Table 3). Colonisation by *P. involutus* may have upregulated the oxalate concentration by the roots. Alternatively, the fungus may have been more deficient in symbiosis where the tree acts as a sink of nutrients than in pure culture. No growth reduction of *P. involutus* in pure culture was found in the K or Mg treatments. Paris *et al.* (1996) observed an increase in oxalate production by *P. involutus* when both Mg and K were omitted from the culture medium, but not when either K or Mg were omitted.

Results obtained with ectomycorrhizal fungi without their host seem meaningless at least in an ecological context. Two-compartments systems might be a useful alternative to study the response of the external mycelium compared to the (ectomycorrhizal) root system.

Under P deficiency, the concentration of malonate but not oxalate exuded by *P. involutus* in pure culture was significantly increased compared to complete nutrition or limited N, Mg or K (Fig. 1). Also, acidification was stronger with low P supply than with low N, Mg K or complete nutrient supply (Table 2). These findings can explain the high phosphate solubilising activity of *P. involutus* found by Lapeyrie *et al.* (1991). Arvieu *et al.* (2003) found no effect of P deficiency on oxalate exudation by six ectomycorrhizal isolates in pure culture. However, as in most studies with fungi in pure culture, no other LMWOA were determined. With sand-grown *P. sylvestris* seedlings, colonisation by *P. involutus* did not increase the concentrations of malonate in response to P deficiency (Table 5). As discussed above, however, the N deficiency of the seedlings may have been the overriding factor in the exudation of the sand-grown seedlings.

Most forest ecosystems are considered to be N limited. In areas with high levels of

nitrogen deposition, Mg, K and P can become limiting (Tamm *et al.*, 1999), and enhanced weathering of mineral grains by the increased exudation of LWM organic anions can become an important mechanism for nutrients supply and forest growth. The results of this study can not be directly extrapolated to the forest situation, because they were obtained under experimental conditions which are distant from the forest conditions. They do, however, show the potential of trees and ectomycorrhizal fungi to manipulate their environment and respond to nutrient deficiencies by increasing the exudation of LMWOA with high weathering ability.

## Acknowledgements:

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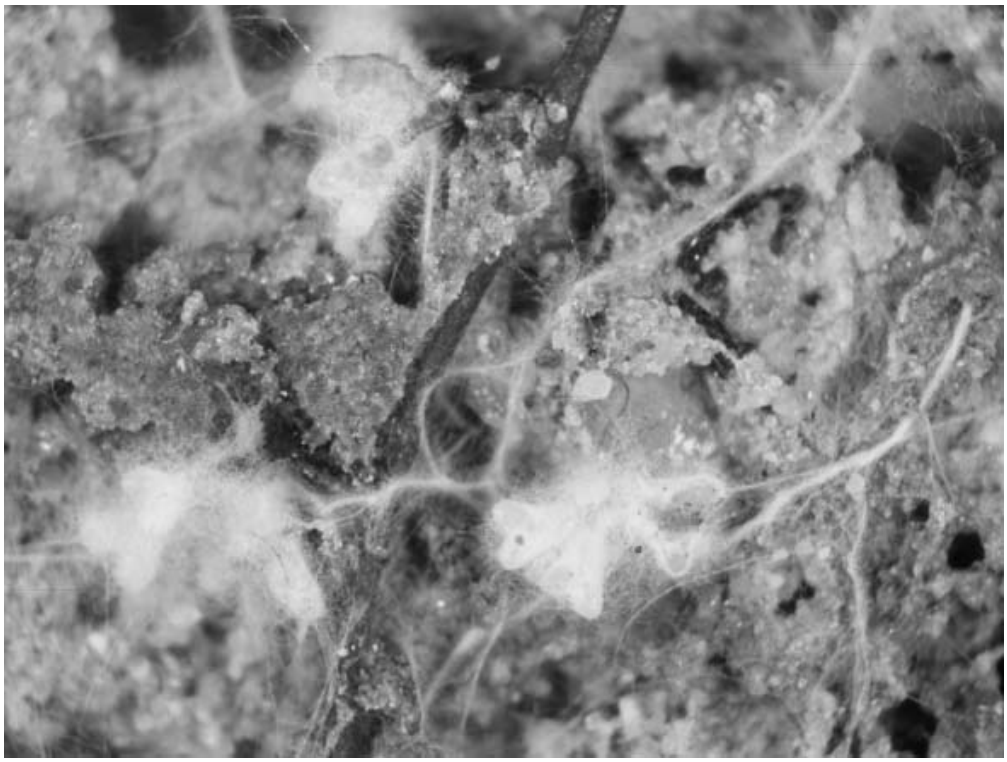
# 6

# Ectomycorrhizal weathering of muscovite and hornblende



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## Summary

Ectomycorrhizal fungi are hypothesized to enhance mineral weathering in forest soils. Several studies have shown an increased uptake of mineral-derived nutrients by trees when in symbiosis with ectomycorrhizal fungi. The combined effect of ectomycorrhizal fungi on nutrient uptake by the tree and mineral weathering, however, complicates interpretation of such studies. In a pot experiment *Pinus sylvestris* (Scots pine) seedlings were grown with or without ectomycorrhizal fungi, and with or without the mineral muscovite as the only K source or the mineral hornblende as the only Mg source. After 27 weeks, all pools of nonmineral-bound K or Mg were determined. The ectomycorrhizal fungus *Paxillus involutus* increased weathering of muscovite but not hornblende. The other ectomycorrhizal fungi tested, *Piloderma croceum* and *Suillus bovinus*, did not increase weathering of either muscovite or hornblende compared to the nonmycorrhizal trees. The *P. involutus*-mediated mobilisation of K from muscovite resulted in increased K content of root plus adhering hyphae, but not of shoots. In conclusion, ectomycorrhizal fungi may increase weathering of minerals in response to nutrient deficiencies, but this response is species specific.

## Introduction

There has been a growing interest in the role of ectomycorrhizal fungi in mobilising nutrients from soil minerals (Landeweert et al., 2001, Wallander & Hagerberg, 2004). Ectomycorrhizal fungi live in symbiotic relationship with trees, that provide the fungi with photosynthates in exchange for nutrients (Smith & Read, 1997). Most research dealing with ectomycorrhizal fungi has focused on the uptake of N, which is generally assumed to be the limiting element for tree growth in boreal forests (Tamm, 1985). In forests with high biomass removal and decreasing concentrations of K and Mg in the soil solution as a result of continuous acidification and leaching, K and Mg may become growth-limiting (Jönsson *et al.*, 2003, Übel & Heinsdorf, 1997). Mineral weathering is a primary source of K and Mg for plant nutrition. Ectomycorrhizal fungi can transport Mg and K (Jentschke et al., 2001), but knowledge on the effect of ectomycorrhizal fungi on the weathering of Mg-containing or K-containing soil minerals and subsequent uptake by their host plant is limited (Bücking *et al.*, 2002, Wallander, 2006).

Trees and their associated ectomycorrhizal fungi have a significant influence on the soil ecosystem. The influx of current photosynthates of trees is responsible for half of the total soil respiration, mainly through ectomycorrhizal fungal activity (Högberg *et al.*, 2001). The ectomycorrhizal hyphae and roots produce half of the total dissolved organic carbon in a forest soil, presumably for a large part in the form of organic anions (Högberg & Högberg, 2002) with a high weathering potential. Landeweert *et al.* (2001) proposed that the ectomycorrhizal fungi exude low molecular weight organic anions, like citrate and oxalate, near mineral surfaces, in that way accelerating mineral weathering. Both oxalate and citrate are strong ligands (Welch & Ullman, 1993) and are assumed to be the most potent biological weathering agents in the soil (Barker *et al.*, 1998, Ochs, 1996).

Several pot experiments have been done with tree seedlings and ectomycorrhizal fungi to investigate the effect of ectomycorrhizal fungi on mineral weathering (see (Wallander, 2006) for review). In most studies the effect on mineral weathering has been determined via the nutrient uptake into the tree seedlings (Casarin *et al.*, 2004, Leyval & Berthelin, 1989, Wallander, 2000, Wallander & Wickman, 1999, Wallander *et al.*, 1997). Interpretation of these experiments is complicated by the fact that

ectomycorrhizal fungi can enhance growth and nutrient uptake through factors other than increased weathering: (1) ectomycorrhizal fungi can enhance uptake of nutrients through better soil exploration by hyphae compared to roots; (2) ectomycorrhizal fungi can take up N and P from organic sources largely unavailable to nonmycorrhizal roots; (3) ectomycorrhizal and nonmycorrhizal seedlings may be affected differently by changes in the rooting medium caused by the addition of mineral grains (e.g. water availability, pH). These problems can be overcome by making a complete mineral budget in which all pools of mobile minerals are quantified. In that way, Van Hees *et al.* (2004) showed that tree seedlings can enhance mineral weathering, as indicated by increased leaching of Al, Si and Fe, and that ectomycorrhizal colonisation doubles mineral weathering. In the same way Bakker *et al.* (2004) showed that ectomycorrhizal seedlings of Scots pine and Douglas fir increased plagioclase weathering, but they did not distinguish between effects of trees and ectomycorrhizal fungi.

In this paper we quantify the effect of tree seedlings and ectomycorrhizal fungi on mineral weathering under K and Mg limited conditions. We focussed on the effect of different ectomycorrhizal fungal species on release of K from muscovite and release of Mg from hornblende. Muscovite and hornblende are both common silicate minerals in soils. We used pot experiments in such a way that we were able to measure all the pools of mineral-derived K and Mg.

## Material and Methods

*Minerals*

Muscovite from Tuftane (Norway, type nr. X01541) and hornblende from Kragerö (Norway, type nr. X00131) were obtained from Krantz Company, Bonn (Germany). The stones were ground and wet-sieved to a grain size between 150 and 250 µm. In order to remove fine dust, the minerals were shaken overnight in 0.1 M HCl. Next, the minerals were shaken for one week in demineralised water, which was daily adjusted to pH 4 with 0.1 M HCl to minimise the effects of high energy surface sites produced by grinding. Muscovite contained 8.9% K (w/w) (Kalinowski & Schweda, 1996), and hornblende contained 7.7% Mg (w/w) (determined on ICP-MS after aqua regia digestion).

Quartz sand (deposit Limburg, the Netherlands) with a grain size between 150 and 420 µm was incubated with 0.1 M HCl overnight to remove fine dust, followed by flushing with demineralised water for several days till the effluent reached the pH of

the demineralised water. The quartz contained traces of K (50 mg $kg^{-1}$) and Mg (30 mg $kg^{-1}$).

*Inoculation*

The ectomycorrhizal fungi *Paxillus involutus* (Batch: Fr.) Fr. (nr. 17 Sheffield, UK), *Suillus bovinus* (L.: Fr.) Roussel (BL97-14, Stadskogen, S) and *Piloderma croceum* J. Erikss. & Hjortst. (BL97-01, Gottsunda, S) (all kindly provided by R. Finlay, SLU, Sweden) were cultured on MMN agar (Marx, 1969). Seeds of *Pinus sylvestris* L. (Scots pine) were soaked overnight in demineralised water, surface sterilised in 30 % $H_2O_2$ with one drop TWEEN for 30 min., rinsed with autoclaved demineralised water and transferred to Petri dishes with water agar (8% agar, 10 g $l^1$ glucose). During the one week germination the seeds were checked on contamination.

After 6 days on water agar, individual seedlings (± 0.006 g dry weight) were transferred to incubation tubes (1.5 cm diam., length 15 cm) together with 3 plugs of agar, which were either sterile (nonmycorrhizal treatment) or taken from the front of an actively growing mycelium (ectomycorrhizal treatments). Growth tubes contained 20 gram of a mixture of quartz sand:perlite:nutrient solution (ratio 1 kg: 0.13 kg: 0.41 l) and had been autoclaved for 30 minutes at 120°C. Composition of the nutrient solution can be found Table 1. The incubation tubes were closed with autoclaved cotton wool and a plastic cap and the bottom was covered with aluminium foil. They were placed in a climate chamber (16 hours photoperiod at 70 Watts $m^{-2}$, day/night temperatures of 20/16°C and 80% humidity) for 3 months.

*Experimental setup*

Seedlings of *P. sylvestris* (dry weight ± 0.05 g), either nonmycorrhizal or inoculated with either of the ectomycorrhizal fungi *P. involutus*, *P. croceum* or *S. bovinus*, were transferred to and grown on pots (one seedling $pot^{-1}$) in the greenhouse from February 2005 until August 2005. Pots contained 500 g of either pure quartz sand or quartz sand amended with hornblende or muscovite (10% w/w), in all cases mixed with 85 ml nutrient solution (composition according to experimental treatment, see below). The nutrient treatments were: (1) Quartz sand with nutrient solution -K; (2) Quartz sand with muscovite and nutrient solution -K; (3) Quartz sand with nutrient solution -Mg; (4) Quartz sand with hornblende and nutrient solution -Mg, (5) Control: Quartz sand with complete nutrient solution. Weathering in the absence of trees was studied in pots without tree seedlings. All treatments were performed with 6 replicates.

To reduce evaporation and growth of algae, each pot was covered by PVC pellets

**Table 1** Nutrient solution composition (mM)

| Mineral | Pregrowth | Complete | –Mg | –K |
|---|---|---|---|---|
| $NH_4NO_3$ | 1.72 | 6.17 | 6.17 | 5.90 |
| $NH_4H_2PO_4$ | 0.10 | 0.40 | 0.40 | 0.94 |
| $(NH_4)_2SO_4$ | 0.07 | 0.29 | 0.29 | 0.29 |
| $KNO_3$ | | 1.45 | 1.45 | |
| $K_2SO_4$ | 0.10 | | 0.20 | |
| $KH_2PO_4$ | | 0.53 | 0.53 | |
| KCl | | 0.40 | | |
| $Ca(NO_3)_2$ | | | | 0.31 |
| $MgSO_4$ | | 0.48 | | 0.48 |
| $NaNO_3$ | | | | 0.83 |
| $Na_2SO_4$ | | | 0.27 | |
| $CaCl_2$ | 0.08 | 0.31 | 0.31 | |

and a sheet of plastic. Soil water content was kept constant by maintaining the original weight through addition of demineralised water and nutrient solution. The amount of nutrient solution added weekly increased from 0 ml $pot^{-1}$ at the start of the experiment, to 2 ml $pot^{-1}$ in weeks 7 to 15, to 8 ml $pot^{-1}$ in weeks 16 to 27. The composition of the nutrient solutions were based on optimal nutrient solution for *P. sylvestris* (Ingestad, 1979) and are described in Table 1. The amounts of nutrients other than K and Mg were equal among all treatments. Trace elements added to all treatments (mg $l^{-1}$): 10.6 Fe (as FeEDTA), 0.99 B, 0.04 Cu, 0.02 Mo, 1.01 Mn, 0.1 Zn. The nutrient solution mixed with the sand at the start of the experiment was ¼ strength of those in Table 1. For pregrowth in the tubes, trace elements in nutrient solution were reduced to ¼ strength.

*Harvest*

After 27 weeks a soil sample of ± 60 g was taken with an apple corer and centrifuged at 5500 x *g* to collect soil solution. The pH of the soil solution was measured immediately. Shoots were cut off. Roots with adhering fungal hyphae were collected from the remaining soil and rinsed gently with demineralised water to remove most of the adhering soil particles. Extramatrical mycelium was extracted by mixing the soil with demineralised water and collecting the floating hyphae over a 36 µm mesh (Wallander *et al.*, 2004). The extramatrical mycelium of three pots was collected together, dried at 60ºC and weighted.

*Analyses*

Concentrations of Mg and K in the soil solution were measured by ICP-AES. The shoots and roots were at dried at 60ºC for 48 hours, weighed and ground. Shoot and root material was digested in a $H_2SO_4$/salicylic acid/$H_2O_2$/Se mixture. Concentrations of K were measured by the flame-AES, Mg by flame-AAS and (for shoot samples only) N and P by spectrophotometry. Root biomass and concentration of K and Mg were corrected for sand adhering to the roots. For that purpose, a subsample of the ground root samples was ignited at 600ºC to combust the organic material. This 'loss on ignition' dry weight was used to correct dry weight of the roots for dry weight of adhering minerals. The remaining material was stirred in water to remove Mg and K originating from the combusted organic material. Concentrations of K and Mg in the remaining mineral material were analysed in the same way as the root samples. The amount of K and Mg found in this mineral fraction was subtracted from the amount in the original root sample. Concentrations of K and Mg in the remaining mineral material were in line with the K and Mg concentration of the quartz sand/muscovite mixture, validating our method. We were not able to measure the Mg and K content in the extramatrical mycelium samples due to low hyphal biomass. Total amount of nonmineral-bound K and Mg in the pots were defined as the sum of K or Mg contents in shoot, root and soil solution.

*Statistical analysis*

Statistical analyses was performed with SPSS 12.0.1 for WINDOWS (SPSS Inc. Chicago IL. USA). The muscovite and hornblende experiments were analysed separately. All data were analysed with a two-way ANOVA. Data were transformed ($^{10}$log or square root) before analysis where necessary to homogenise the variances among treatments (Levene's test). The dependent variables content of K or Mg in the shoot or root were analysed with as independent variables mineral treatment (no mineral added, mineral added), and ectomycorrhizal treatment (nonmycorrhizal, *P. involutus*, *P. croceum*, *S. bovinus*). For the dependent variables nonmineral-bound K and nonmineral-bound Mg in the pots and soil solution content of K and Mg, the treatment 'no tree' was added as an ectomycorrhizal treatment level. For the dependant variables dry weight and concentrations of K or Mg of shoot and root the independent variable 'complete nutrient supply' was added as a mineral treatment. The ANOVA was followed by a Tukey's honestly significant difference (HSD) *post hoc* test (homogeneous variances) or Games-Howell *post hoc* test (where variances remained heterogeneous) on treatment means. For the treatments 'no mineral added' and 'mineral added' *post hoc* test were performed on all pair wise combinations of treatment levels.

## Results

*Mycorrhizal colonisation and hyphal density*

We did not observe any ectomycorrhizal root tips on the tree seedlings of the non-mycorrhizal treatment. Within the treatments with ectomycorrhizal fungi, mycorrhizal colonisation ranged from sparse to almost complete. Mycorrhizal root tips were mostly concentrated in parts of the root system. Extramatrical hyphal biomass ranged from 10 to 137 mg per pot (Table 4).

*Muscovite weathering*

Nonmineral-bound K in the pots and K content of shoot, root and soil solution was significantly affected by muscovite addition, ectomycorrhizal treatment and (except for shoot) interaction (Table 2). Muscovite addition increased nonmineral-bound K compared to the treatment without muscovite, with significantly higher contents of K in pots with seedlings than in pots without seedlings, and significant differences among ectomycorrhizal treatments (Fig. 1). Pots with seedlings colonised by *P. involutus* had significantly higher amounts of nonmineral-bound K than pots with seedlings colonised by *S. bovinus* or *P. croceum* or without mycorrhiza (Fig.1).

Weathering of muscovite (calculated from nonmineral-bound K in pots with musco-

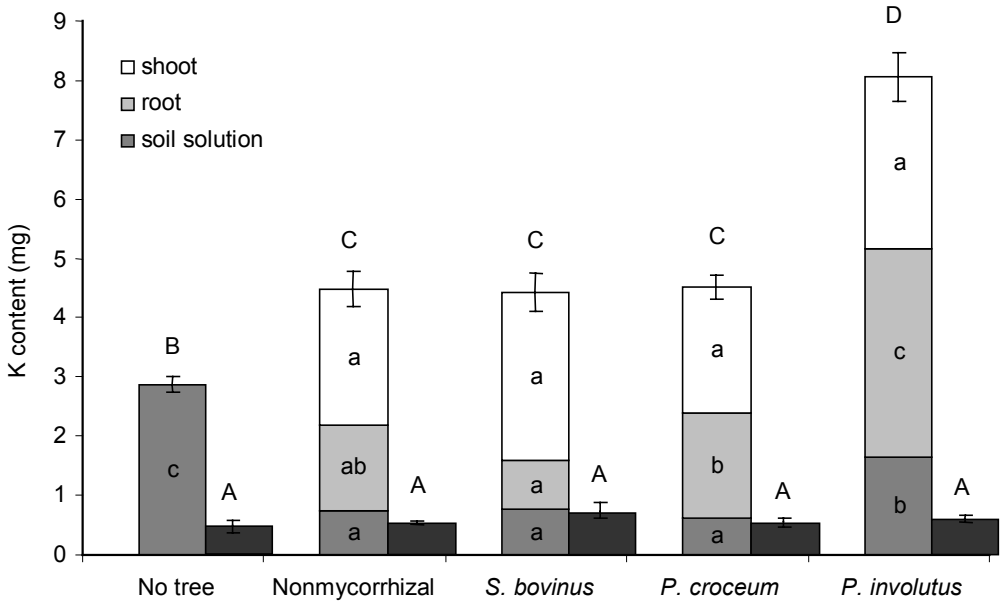



**Figure 1** Nonmineral-bound K in the pots with muscovite, distributed over the shoot, roots and soil solution. Black bars denote the nonmineral-bound K in pots without muscovite addition. Mean of six replicates. Error bars represent the standard error of the nonmineral-bound K. Bars with the same letter are not significantly different (P< 0.05); capital letters above bars denote total amount of nonmineral-bound K in pot.

vite minus nonmineral-bound K in pots with pure quartz) was increased by a factor of 1.7 by nonmycorrhizal tree seedlings or seedlings colonised by *S. bovinus* or *P. croceum*, and by a factor of 3.2 by seedlings colonised by *P. involutus*. Most of the nonmineral-bound K was found in the seedlings. In pots with seedlings colonised by *P. involutus*, the K content of the roots and soil solution were significantly higher, but K content of the shoots did not differ among the ectomycorrhizal treatments (Fig. 1). In the treatment without muscovite nonmineral-bound K did not significantly differ between pots with or without tree seedlings or among the different ectomycorrhizal treatments (Fig.1), indicating that influx of K by the pregrown seedlings or from the quartz sand was negligible.

The pH, at the end of the experiment, ranged from 2.7 to 3.1 in the pots without muscovite and from 2.9 to 3.5 in the pots with muscovite added. In the pots with muscovite, the pH showed a positive relationship with nonmineral-bound K in the pots which was not observed in the pots without muscovite (Fig. 2).

*Seedling growth on the muscovite*

Shoot and root dry weights were significantly affected by muscovite addition, ectomycorrhizal treatment and -for root dry weights- their interaction (Table 3). Shoot

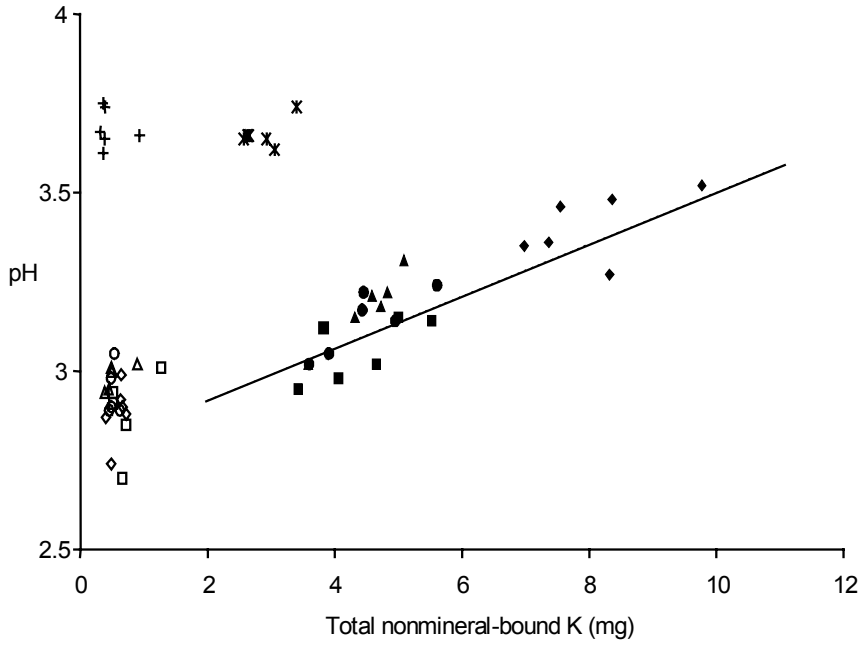



**Figure 2** The pH against nonmineral-bound K in the pots with muscovite (solid symbols) or without muscovite addition (open symbols). Markers denote no tree (+), nonmycorrhizal (o), *S. bovinus* (□), *P. croceum* (△) and *P. involutus* (◊). The trend line is based on linear regression of the muscovite treatments bulked together ($R^2$= 0.78, *P*< 0.001).

**Table 2** *P*-values from a two-way ANOVA, with ectomycorrhizal treatment (no tree, nonmycorrhizal, *S. bovinus*, *P. croceum* or *P. involutus*), mineral addition (no mineral added, muscovite or hornblende added) and their interaction as independent variables and nonmineral-bound K or Mg in the pots, and K or Mg content in the shoot, root or soil solution as dependant variables.

| | Factor | Nonmineral bound K (muscovite) or Mg (hornblende) | | | |
|---|---|---|---|---|---|
| | | Shoot | Root | Soil sol. | Total |
| Muscovite | mineral | <0.001 | <0.001 | <0.001 | <0.001 |
| | mycorrhiza | 0.008 | <0.001 | <0.001 | <0.001 |
| | interaction | 0.937 | 0.001 | <0.001 | <0.001 |
| Hornblende | mineral | <0.001 | <0.001 | <0.001 | <0.001 |
| | mycorrhiza | <0.001 | <0.001 | <0.001 | <0.001 |
| | interaction | 0.001 | 0.096 | <0.001 | 0.110 |

biomass differed significantly among the ectomycorrhizal treatments in the treatment with muscovite (Table 4). Nonmycorrhizal seedlings had higher shoot biomass than seedlings colonised by *P. croceum*. Compared to the seedlings in the treatment without muscovite, shoot biomass of the nonmycorrhizal seedlings but not of the ectomycorrhizal seedlings was significantly increased by muscovite addition. Root biomass of seedlings colonised by *P. involutus* or *P. croceum* or without mycorrhiza was significantly increased by muscovite addition, with significantly higher root biomass in seedlings colonised by *P. involutus* compared to seedlings colonised by *S. bovinus* or without mycorrhiza (Table 4). The K concentrations of the shoots of tree seedlings in the treatment with muscovite were higher than those in the treatment without muscovite (Table 4), and above the level required for adequate growth (3 mg K $g^{-1}$d.w., (Reuter *et al.*, 1997) for *Pinus* spp.), but shoot dry weights were significantly lower than in the control treatment with complete nutrient supply.

Dry weight of shoot or roots did not differ significantly among the ectomycorrhizal treatments in the treatment without muscovite (Table 4). Tree seedling growth was clearly K-limited showing characteristic yellow spots on the needles and K concentrations below the level required for adequate growth. Also, shoot and root dry weights were significantly lower compared to seedlings in the control treatments with complete nutrient solution.

*Hornblende weathering*

Nonmineral-bound Mg and Mg content of shoot, root and soil solution was signify-

**Table 3** *P*-values from a two-way ANOVA, with ectomycorrhizal treatment (nonmycorrhizal, *S. bovinus*, *P. croceum* or *P. involutus*), nutrient supply (complete nutrient solution, no mineral added, muscovite or hornblende added) and their interaction as independent variables and dry weight and K or Mg concentrations of the shoot and root as dependant variables.

| | Factor | Dry weight | | Concentration of K or Mg | |
|---|---|---|---|---|---|
| | | Shoot | Root | Shoot | Root |
| Muscovite | nutrient supply | <0.001 | <0.001 | <0.001 | <0.001 |
| | mycorrhiza | 0.002 | <0.001 | <0.001 | 0.006 |
| | interaction | 0.008 | 0.001 | 0.130 | <0.001 |
| Hornblende | nutrient supply | <0.001 | <0.001 | <0.001 | <0.001 |
| | mycorrhiza | <0.001 | <0.001 | 0.017 | 0.581 |
| | interaction | 0.437 | 0.057 | <0.001 | 0.349 |

cantly affected by hornblende addition, ectomycorrhizal treatment and (for Mg content of shoot and soil solution) their interaction (Table 2). Hornblende addition significantly increased the amount of nonmineral-bound Mg, with significantly higher contents in pots with than in pots without tree seedlings, but no differences among the ectomycorrhizal treatments (Fig. 3). Weathering of hornblende (calculated from nonmineral-bound Mg in pots with hornblende minus nonmineral-bound Mg from pots without hornblende) was increased with a factor 1.5-2 by the tree seedlings. Most of the nonmineral-bound Mg was found in the tree seedlings. Seedlings colonised by *P. croceum* had a significantly lower shoot Mg content and higher Mg root content compared to the other seedlings. Mg content of the soil solution did not significantly differ among the ectomycorrhizal treatments (Fig. 3).

Tree seedlings also increased the amount of nonmineral-bound Mg compared to the pots without tree seedlings in the treatment without hornblende, with a significantly higher content of nonmineral-bound Mg in pots with seedlings colonised by *P. involutus* than in pots with nonmycorrhizal seedlings (Fig. 3). This increase of nonmineral-bound Mg (0.11-0.24 mg Mg) can partly be explained by the influx of the tree seedlings (0.05 g dry weight at time of transplanting, estimated concentration 0.1 % Mg), and partly from release from the quartz sand.

Hornblende addition strongly increased pH. At the end of the experiment, the pH of the pots without hornblende ranged from 2.9 to 3.6 and in the pots with hornblende from 7.4 to 7.9. The pH did not show any relationship with nonmineral bound Mg in the pot (Fig. 4).

*Seedling growth on hornblende*

Shoot and root dry weights were significantly affected by hornblende addition and by ectomycorrhizal treatment (Table 3). Shoot and root weights did not differ among the ectomycorrhizal treatments in the treatment with hornblende (Table 4). Compared to the seedlings in the treatment without hornblende, shoot dry weights of seedlings colonised by *P. involutus*, and the root dry weights of the nonmycorrhizal seedlings were significantly increased by hornblende addition. Concentrations of Mg in the shoots and root in the seedlings of the treatment with hornblende were higher than those of the seedlings in the treatment without hornblende (Table 4), except for seedlings colonised by *P. croceum* in which the shoot Mg concentration was not increased and remained below the sufficiency level (0.6 mg Mg $g^{-1}$d.w. (Reuter *et al.*, 1997)). Compared to the seedlings in the control treatment with complete nutrient supply, shoot dry weights were not different and root dry weights were increased.

Nonmycorrhizal seedlings in the treatment without hornblende had higher shoot dry weights and lower root dry weights than the ectomycorrhizal seedlings (Table 4). At the end of the experiment, some seedlings showed the first signs of Mg deficiency, and shoot concentrations were below the sufficiency level. Also, shoot and root dry weights were lower than those of the control seedlings with complete nutrient supply.

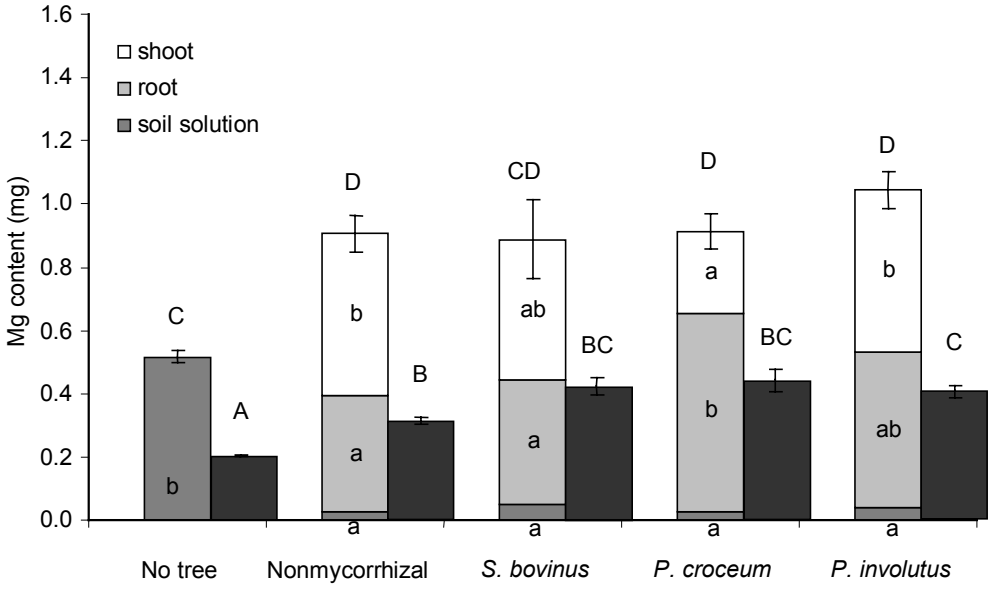



**Figure 3** Nonmineral-bound Mg in the pots with hornblende, distributed over shoot, roots and soil solution. Black bars denote the nonmineral-bound Mg in pots without hornblende addition. Mean of six relicates. Error bars denote the standard error of the nonmineral-bound Mg. Bars with the same letter are not significantly different ($P$< 0.05); capital letters above bars denote total amount of nonmineral-bound Mg in pot.

## Discussion

Weathering of muscovite and hornblende was increased by nonmycorrhizal and ectomycorrhizal tree seedlings. Tree seedlings colonised by the ectomycorrhizal fungus *P. involutus* almost doubled the weathering of muscovite compared to nonmycorrhizal seedlings or seedlings colonised by *S. bovinus* or *P. croceum*, but for hornblende, there was no additional effect of ectomycorrhizal fungi. Previously, contrasting results were reported for effects of *P. involutus* on mineral weathering. Van Hees *et al.* (2004) found a twofold increase in Al and Si mobilisation in forest soil E material due to *P. involutus* compared to nonmycorrhizal tree seedlings. *P. involutus* also increased apatite-derived P uptake in the shoot of tree seedlings (Wallander *et al.*, 1997), but did not increase uptake of K from microcline and biotite (Wallander & Wickman, 1999). In the latter study a partial weathering budget was made which did not show increased weathering by *P. involutus*, but unfortunately K content in the roots, which is shown in our study to be significantly higher in the tree seedlings colonised by *P. involutus*, was not determined.

Oxalate exudation by ectomycorrhizal fungi and tree seedlings in response to K and Mg deficiencies could explain the observed differences in weathering of muscovite and hornblende. In an axenic experiment with the same fungal isolates, K deficiency

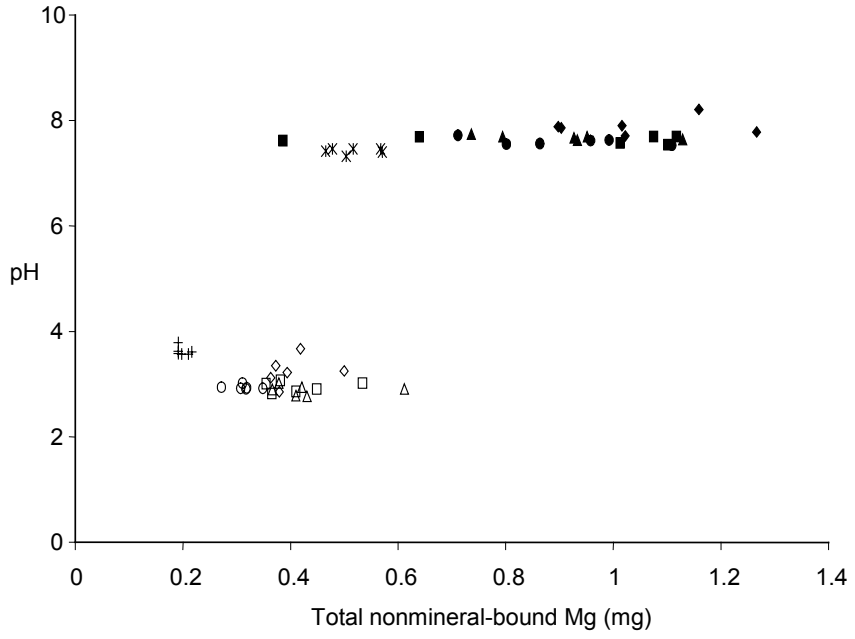



**Figure 4** The pH against nonmineral-bound Mg in the pots with hornblende (solid symbols) and without hornblende addition (open symbols). Markers denote no tree (+), nonmycorrhizal (o), *S. bovinus* (□), *P. croceum* (△) and *P. involutus* (◊).

**Table 4** Dry weight and concentration of K and Mg in shoot, root and external hyphae. Data are means of six replicate pots. Data on hyphal biomass are the mean of two bulk samples (3 pots). Means followed by the same letter within a column are not significantly different ($P$<0.05). The muscovite and hornblende experiments were analysed separately. nd= not determined

| **Treat-ments** | | **Measurements** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Mineral (Nut. sol.) | Mycorrhiza | Hyphal dry weight (mg) | Shoot d.w. (g) | Shoot K (mg $g^{-1}$) | Shoot Mg (mg $g^{-1}$) | Root d.w. (g) | Root K (mg $g^{-1}$) | Root Mg (mg $g^{-1}$) |
| ***Control treatment*** | | | | | | | | |
| No mineral | NM | | 0.77 | 6.3 | 1.19 | 0.36 | 2.7 | 1.70 |
| (Complete) | *S. bovinus* | 136 | 0.63 | 7.3 | 1.14 | 0.48 | 2.1 | 1.67 |
| | *P. croceum* | 45 | 0.61 | 7.2 | 1.23 | 0.53 | 2.4 | 1.22 |
| | *P. involutus* | 48 | 0.49 | 7.3 | 1.17 | 0.52 | 2.5 | 1.88 |
| ***Muscovite weathering*** | | | | | | | | |
| No mineral | NM | | 0.33 ab | 1.1 a | nd | 0.16 a | 0.45 a | nd |
| (-K) | *S. bovinus* | 10 | 0.33 ab | 1.3 a | nd | 0.14 a | 0.53 a | nd |
| | *P. croceum* | 19 | 0.32 ab | 1.1 a | nd | 0.23 a | 0.48 a | nd |
| | *P. involutus* | 23 | 0.35 ab | 1.2 a | nd | 0.22 a | 0.38 a | nd |
| Muscovite | NM | | 0.52 c | 4.5 b | nd | 0.46 b | 3.0 bc | nd |
| (-K) | *S. bovinus* | 70 | 0.48 bc | 6.0 bc | nd | 0.31 ab | 2.7 bc | nd |
| | *P. croceum* | 48 | 0.34 ab | 6.3 c | nd | 0.48 bc | 3.9 c | nd |
| | *P. involutus* | 60 | 0.46 abc | 6.5 c | nd | 0.66 c | 5.4 d | nd |
| ***Hornblende weathering*** | | | | | | | | |
| No mineral | NM | | 0.63 c | nd | 0.30 a | 0.31 a | nd | 0.30 a |
| (-Mg) | *S. bovinus* | 105 | 0.54 abc | nd | 0.32 a | 0.66 b | nd | 0.32 a |
| | *P. croceum* | 101 | 0.49 ab | nd | 0.34 a | 0.71 b | nd | 0.34 ab |
| | *P. involutus* | 137 | 0.44 a | nd | 0.34 a | 0.65 b | nd | 0.33 ab |
| Hornblende | NM | | 0.74 c | nd | 0.70 b | 0.71 b | nd | 0.5 bc |
| (-Mg) | *S. bovinus* | 109 | 0.67 bc | nd | 0.70 ab | 0.69 b | nd | 0.6 cd |
| | *P. croceum* | 81 | 0.69 abc | nd | 0.38 a | 0.92 b | nd | 0.7 cd |
| | *P. involutus* | 134 | 0.65 bc | nd | 0.80 b | 0.71 b | nd | 0.7 d |

increased oxalate production of tree seedlings colonised by *P. involutus* but not of nonmycorrhizal seedlings or seedlings colonised by *P. croceum*, while Mg deficiency increased the oxalate production of nonmycorrhizal and ectomycorrhizal seedlings alike (Van Schöll *et al.*, 2006: Chapter 5). Increased oxalate production by *P. involutus in vitro* under K and Mg-limiting conditions was also found by Paris *et al.* (1996). An indication for a role of oxalate, or another chelating compound, in the *P. involutus*-mediated increased weathering of muscovite is the positive relationship between pH and nonmineral-bound K (Fig. 3). If proton exudation would be the main weathering agent produced by the tree or fungus, this relationship would be either negative or nonexistent. No such pH increase was observed in the treatment without muscovite. The difference between the effect of *P. involutus* on muscovite and hornblende weathering may, however, also be linked to more severe K deficiency compared to the Mg deficiency, as evidenced by the differences in dry weights between the treatments (Table 4). The latter explanation would also be supported by the observation that in the treatment without hornblende, in which the seedlings were Mg deficient, pots with seedlings colonised by *P. involutus* contained more nonmineral-bound Mg, presumably mobilised from impurities in the quartz sand.

Weathering of muscovite and hornblende was sufficient to ameliorate K and Mg deficiency and to increase the concentrations of K and Mg in the shoots to levels above those required for adequate growth at the end of the experiment. In the treatment with muscovite, however, shoot dry weights remained lower than those of the treatment with complete nutrient supply, and only for the nonmycorrhizal seedlings was the shoot dry weights increased compared to those in the treatment without muscovite (Table 4). Probably the positive effect of the tree seedlings and ectomycorrhizal fungi on muscovite weathering only became effective some time after transplanting of the seedlings when the root system and ectomycorrhizal fungi were well developed, causing K limitation and growth reduction in the early stage of the experiment. The higher shoot dry weights of the nonmycorrhizal seedlings compared to the ectomycorrhizal seedlings is probably caused by the carbon demand of the fungus. Competition for other resources seems a less likely explanation, as the N and P concentrations of the shoots were above the deficiency level (10 mg N $g^{-1}$d.w. and 1 mg P $g^{-1}$d.w. (Reuter et al., 1997) for *Pinus* spp.) in all treatments (data not shown). In the hornblende treatment, weathering was sufficient throughout the experiment, as no growth reduction was seen compared to seedlings in the complete nutrient treatment, nor were there significant differences in dry weights among the ectomycorrhizal treatments (Table 4). Mg is needed in lower amounts than K, and growth reductions only occur after the Mg content has been diluted strongly.

The impact of ectomycorrhizal fungi on mineral weathering is not *per se* reflected by increased nutrient uptake into the shoot. For tree seedlings colonised by *P. croceum* in the hornblende treatment, most nonmineral-bound Mg was retained in the roots with adhering hyphae, resulting in Mg concentrations in the shoot that were below the level considered adequate for growth. The increased weathering of muscovite by *P. involutus* did not result in higher K content of the shoot. The K that was mobilised in excess of the demand by the shoot was retained in the roots with adhering hyphae, which had significant higher biomass and K concentration than the nonmycorrhizal seedlings, and in the soil solution. The increase in biomass and K content of roots plus adhering hyphae may have been caused largely by an increase in hyphal biomass; visual estimation indicated that the biomass of hyphae adhering to the roots of seedlings colonised by *P. involutus* was several times higher than biomass of hyphae from the soil. The high contents in the soil solution may have partly originated from roots or hyphae in the soil sample, as soil centrifugation can cause leaching of living cell contents (Nambu *et al.*, 2005).

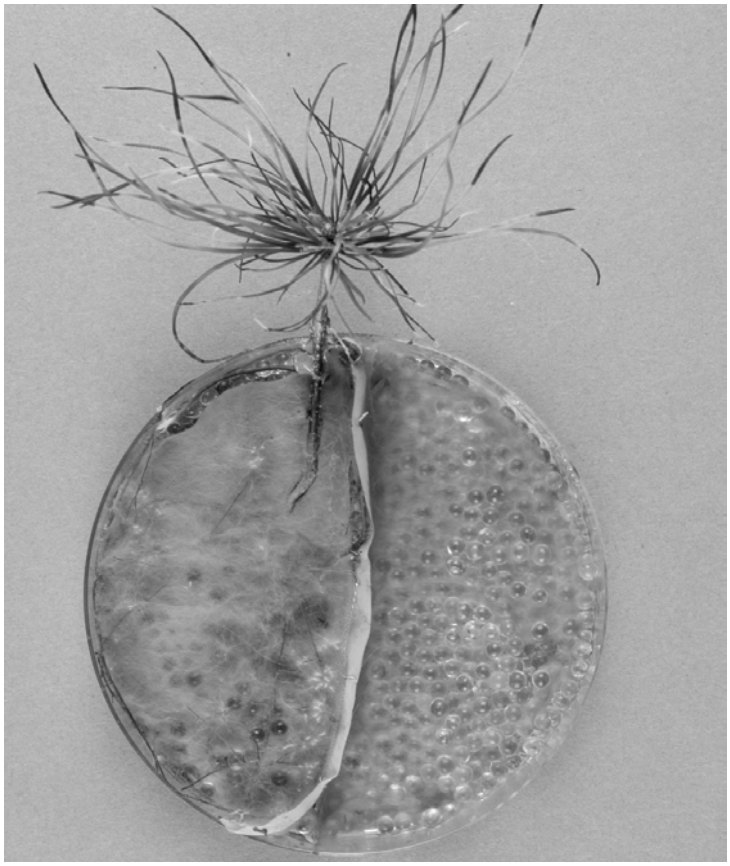
The increased weathering by *P. involutes* provides a net influx of nonmineral-bound K into the system that will probably be more important under natural conditions than in our pot experiment. This influx will be especially important in forests where K is growth-limiting, due to a combination of high biomass removal and decreasing concentrations of K in the soil solution as a result of continuous acidification and leaching (Jönsson *et al.*, 2003, Übel & Heinsdorf, 1997). Under natural conditions, ectomycorrhizal fungi generally enhance shoot growth of their host tree, and it can be expected that mobilised K will be transported to the shoot. In our experiment, shoot growth was not limited by K at time of harvest, and the effect of ectomycorrhizal colonisation on shoot growth was none of negative. By retaining K in the hyphae, the K is protected against leaching. Decreased leaching of K and other base cations under ectomycorrhizal seedlings compared to nonmycorrhizal seedlings has been shown by Ahonen Jonnarth *et al.* (2003). After decomposition, the K retained in the roots and hyphae will become available for uptake.

## Acknowledgements

The authors like to thank Monique Driessen, Gerlinde Vink, Peter Nobels and Carry van Mameren for chemical analysis of the samples, Willem Menkveld and Arie Brader for technical assistance, Peter Buurman for helpful suggestions on the minerals, Nico van Breemen for useful comments on drafts of the manuscript. Roger Finlay is acknowledged for kindly providing the EcM fungi. M.M.S. and L.v.S. were funded by the Netherlands Organisation of Scientific Research (NWO).

# 7

# Ectomycorrhizal foraging and transport of Mg under Mg deficiency



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## Introduction

In boreal and temperate forest, the root systems of most trees are associated with ectomycorrhizal fungi. The role of ectomycorrhizal fungi in the uptake of N and P by their host plant is well established, but knowledge on the effect of ectomycorrhizal fungi on uptake of base cations is scarce and conflicting (Bücking *et al.*, 2002). Generally, biomass production in forest ecosystems is supposed to be N-limited or P-limited, but due to atmospheric deposition of N and intense harvesting, Mg may also become litimed (Jönsson *et al.*, 2003, Landman *et al.*, 1997, Übel & Heinsdorf, 1997).

The extraradical mycelium of ectomycorrhizal fungi plays an important role in the uptake and translocation of water and nutrients. Increased hyphal proliferation in patches of relatively high supply of mineral or organic N and P has been observed (Bending & Read, 1995, Brandes *et al.*, 1998, Jentschke *et al.*, 2001a, Jentschke *et al.*, 2001b, Perez Moreno & Read, 2000). Increased ectomycorrhizal hyphal proliferation and carbon allocation to patches of feldspar compared to patches with pure quartz in a microcosm might indicate that ectomycorrhizal fungi actively respond to base cations supply by increased growth (Rosling *et al.*, 2004). In response to Mg deficiency, the ectomycorrhizal fungus *Paxillus involutus*, in symbiosis with *Pinus sylvestris* seedlings increases the exudation of oxalate, which is a strong biological weathering agent (Van Schöll *et al.*, 2006: Chapter 5).

Jentschke *et al.* (2000, 2001a) demonstrated that Mg can be transported through the hyphae of *P. involutus* to P-deficient seedlings using a two-compartment system. Uptake and transport of Mg was linked to higher uptake and transport of P supplied in high concentrations in a hyphal compartment that was accessible to hyphae but not to roots. At low concentrations of P in the hyphal compartment, hyphal density and transport of Mg and P were lower. Jentschke et al. (2001a) concluded that co-transport of P with Mg serving for charge balance, was likely. Hyphal uptake contributed to 3-4% of total plant uptake of Mg. Magnesium was, however, not a limiting element for plant growth.

We expect that ectomycorrhizal foraging, transport and uptake of Mg can be important if Mg is the growth-limiting element. In this study we therefore wanted to answer the following questions:

1. do hyphae actively forage into Mg rich patches?
2. does hyphal foraging and transport of Mg improve host Mg status and growth?
3. is transport of Mg through hyphae driven by Mg deficiency, or is it the result of a passive co-transport with P?

## Material and methods

*Plant and fungal material*

The ectomycorrhizal (EcM) fungus *Paxillus involutus* (Batch Fr.) Fr. ('nr 17', kindly provided by Roger Finlay, SLU, Uppsala, Sweden) was maintained on MMN medium (Marx, 1969). Seeds of *Pinus sylvestris* L. (Scots pine) were soaked overnight in demineralised water, surface-sterilised in 30% $H_2O_2$ for 30 min. and rinsed with autoclaved demineralised water. To check for eventual microbial contamination they were transferred to water agar plates (8 g $l^{-1}$) with glucose (10 g $l^{-1}$) for germination.

Six days after transfer, individual seedlings of *P. sylvestris* (± 0.006 g dry weight) were transferred to growth tubes together with 3 plugs of agar, which were sterile (nonmycorrhizal treatment) or taken from the front of an active growing mycelium (*P. involutus* treatment). Growth tubes contained 20 gram of a mixture of 2 kg of fine quartz sand, 0.13 kg of perlite and 0.685 l of a nutrient solution that had been autoclaved for 30 minutes at 120°C. For composition of the nutrient solution see Table 1 (modified from Ingestad (1979)). The growth tubes were closed with cotton wool and a loose-fitting plastic cap, and the outside bottom was covered with aluminium foil. They were placed in a climate chamber (16 hours photoperiod at 70 Watts/$m^2$, day/night temperatures of 20/16°C and 80% humidity).

*Experimental set up*

Nine weeks after inoculation, the seedlings (± 0.05 g dry weight) were transferred to two-compartment Petri dishes (9 cm diam.). A fine mesh (SEFAR NITEX 03-31/24, mesh opening 30 μm), permeable to hyphae but not to roots, had been melted against the plastic divider in the middle to prevent roots from entering the hyphal compartment (see picture at page 91). Each compartment contained 24 g (two layers) of glass beads (3mm diam.) and 8 ml (root compartment) or 7 ml (hyphal compartment) of nutrient solution (composition see Table 1). The composition of the basal nutrient solution was based on Ingestad (1979), with Mg omitted in the root compartment in all treatments, and Mg and P added in the hyphal compartment according to treatment. Treatments in the hyphal compartment were: (1) Control: nu-

**Table 1** Composition of the basal nutrient solutions used (mM) (modified from Ingestad (1979)).

| Salt | Incubation | Root comp. | Hyphal comp. | Hyphal comp. after refreshing |
|---|---|---|---|---|
| $NH_4H_2PO_4$ | 0.10 | 0.10 | | |
| $KNO_3$ | | 0.36 | 0.26 | |
| $(NH_4)_2SO4$ | 0.07 | 0.07 | 0.07 | 0.21 |
| $NH_4NO_3$ | 1.72 | 1.54 | 1.64 | |
| $CaCl_2$ | 0.08 | 0.08 | 0.08 | |
| $Ca(NO_3)_2$ | | | | 0.21 |
| $KH_2PO_4$ | | 0.13 | | |
| $K_2SO_4$ | 0.10 | 0.05 | 0.12 | |
| KCl | | | 0.10 | 0.84 |
| $Na_2SO_4$ | | 0.07 | | |

**Note** In addition, all nutrient solutions contained micro-elements in concentration (mg $l^{-1}$): 2.65 Fe; 0.25 B; 0.01 Cu; 0.005 Mo; 0.25 Mn; 0.03 Zn.

trient solution without Mg or P (2) +Mg: nutrient solution with 0.514 mM $MgCl_2$ (3) +Mg+P: nutrient solution with 0.51 mM $MgCl_2$ and 1 mM $NaH_2PO_4$. There were 5 replicates for every treatment, but due to insufficient colonisation or growth, for most treatments only 4 replicates could be used for final measurements. Even though all solutions and material used were sterilised before usage, and work was conducted in a sterile flow hood, we did not expect the root systems to remain aseptic throughout the experiment.

The roots were washed in autoclaved, demineralised water and spread out over the beads in the root compartment. The shoot protruded from a notch cut out along the side of the dishes. The dishes were sealed with anhydrous lanolin around the base of the stem and TESA painters tape, covered with aluminium foil and placed horizontally in a growth box. They were left in the laboratory so that the seedlings could acclimatise for two weeks, and then placed in a climate chamber (specifications as above). To correct for transpiration losses, nutrient solution and sterile demineralised water was added 2 times a week to the root compartment of nutrient solution up to original weight of the Petri dishes. The amount of nutrient solution added weekly increased from 0 ml at the start of the experiment, to 2.75 ml in weeks 4-9, and 4 ml in weeks 10 to 14. From week 15 on each Petri dish received 3 ml of nutrient solution that was concentrated twice.

After 19 weeks, the Petri dishes were opened, and the nutrient solution was extracted from the hyphal compartment. The glass beads were washed by the addition of 5 ml of demineralised water, gentle agitation of the dishes and removal of the water again for 3 times. Next, 5 ml of fresh nutrient solution was added (composition of basal nutrient solution Table 1) with, according to treatment (1) Control: no Mg or P,

(2) +Mg: 0.72 $MgSO_4$ mM and (3) +Mg+P: 0.72 mM $MgSO_4$ and 1.40 mM $NaH_2PO_4$. Not all solution could be removed from the hyphal compartment, therefore the final concentrations in the hyphal compartment were lower.

Four weeks after refreshment of the nutrient solution in the hyphal compartment, the experiment ended. The mycelium from the hyphal compartment was collected, and shoot and root (with adhering hyphae from root compartment) were separated. All tissues were dried at 70°C for 48 hours and weighted.

Shoot and root concentrations of Mg were determined by ICP-MS after wet-ashing the plant material with 65% (w/w) $HNO_3$ in closed Teflon vessels at 180°C.

*Statistical analysis*

Statistical analyses were performed with SPPS 12.0.1 for WINDOWS (SPSS Inc., Chicago, IL, USA). Data on total dry weight, Mg content and Mg concentration of shoot and root (with adhering hyphae from root compartment) were analysed with a two-way ANOVA, with mycorrhizal status (nonmycorrhizal, ectomycorrhizal) and composition of the nutrient solution in the hyphal compartment (Control, +Mg, +Mg+P) as independent variables, followed by Tukey's honestly significant differences (HSD) *post hoc* test. Data on hyphal dry weight in the hyphal compartment were analysed with a one-way ANOVA, with composition of the nutrient solution (Control, +Mg, +Mg+P) as independent variable, followed by Tukey's HSD *post hoc* test. Data were transformed ($^{10}$log) where necessary to homogenise variances (Levene's test) prior to analysis.

## Results

Seedlings and fungus grew well in the experimental system. Total dry weight of the seedlings and adhering hyphae from the root compartment (Fig.1) increased significantly by colonisation with *P. involutus* ($P$=0.015) with a weak effect of nutrient solution ($P$=0.062) or interaction effect ($P$=0.113). Dry weight of the shoots (Fig.1) were not significantly ($P$>0.1) influenced by either colonisation or nutrient solution. The root dry weight was significantly affected by mycorrhizal colonisation ($P$=0.008), nutrient composition ($P$=0.047) and interaction between those two factors ($P$=0.047) (Fig.1).

The hyphae extended into the hyphal compartment, forming a thin mycelial layer on the surface of the glass beads and nutrient solution. Hyphal dry weight in the hyphal compartment was significantly increased ($P$=0.007) by adding Mg or Mg+P (Fig. 2).

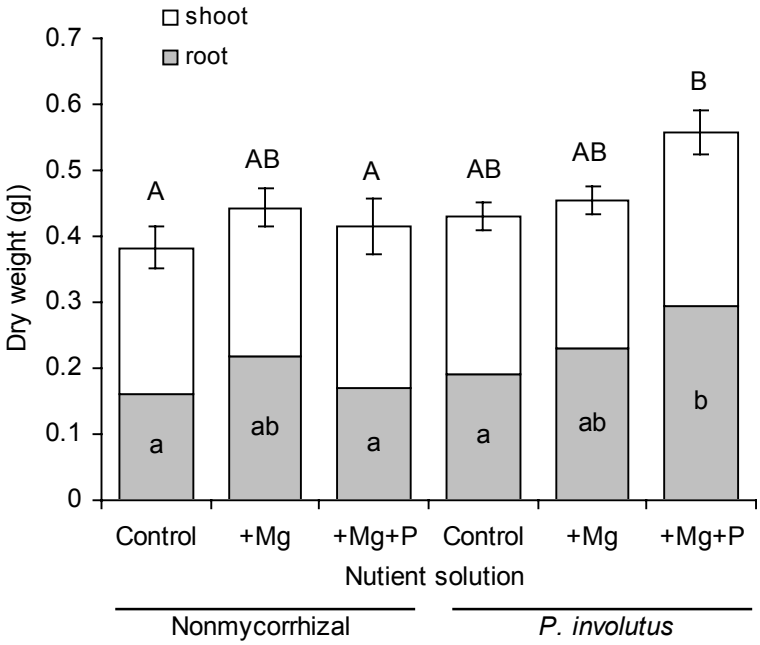



**Figure 1** Dry weight of shoot and root (with adhering hyphae) of *P. sylvestris*, either nonmycorrhizal or colonised by the ectomycorrhizal fungus *P. involutus*. Bars with the same letter are not significantly different ($P$<0.05). Mean of 4-5, standard error for the total dry weight.

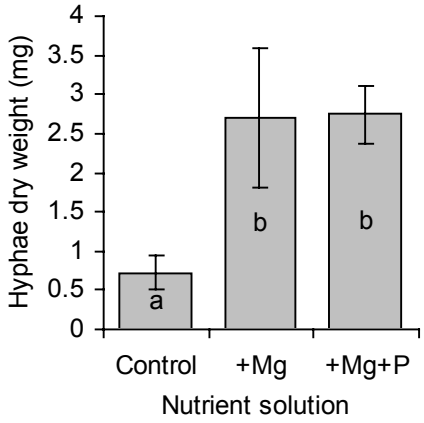



**Figure 2** Dry weight of mycelium of *P. involutus* in the hyphal compartment. Bars with the same letter are not significantly different ($P$<0.05). Mean of 4 with standard error.

The Mg concentration of the shoot was significantly increased by ectomycorrhizal colonisation and nutrient supply, but for the Mg concentration in the roots there was no significant effect (Table 2).

Total Mg content of the seedlings (Fig. 3) was significantly increased by colonisation with *P. involutus* ($P$=0.001 for main effect), with a weak main effect of nutrient supply to the hyphal compartment ($P$=0.065) or interaction between those two factors ($P$=0.098). The Mg content of the shoots (Fig. 3) were significantly increased by colonisation with *P. involutus* ($P$=0.006) but unaffected ($P$>0.1) by nutrient sup-

**Table 2** Mg concentration (µg $g^{-1}$) in seedlings (with adhering hyphae) of *P. sylvestris*, either nonmycorrhizal or colonised by the ectomycorrhizal fungus *P. involutus*. Values with the same letter within a column are not significantly different ($P$<0.05);

| Ectomycorrhizal colonisation | Nutrient supply | n | Shoot | | Root |
|---|---|---|---|---|---|
| Nonmycorrhizal | Control | 4 | 121 | a | 177 |
| | +Mg | 5 | 149 | ab | 160 |
| | +Mg+P | 4 | 121 | a | 127 |
| *P. involutus* | Control | 4 | 155 | ab | 145 |
| | +Mg | 4 | 235 | b | 169 |
| | +Mg+P | 4 | 152 | ab | 194 |
| ANOVA | | DF | | | |
| EcM status | | 1 | 0.012 | | 0.366 |
| Nutrient supply | | 2 | 0.033 | | 0.975 |
| Interaction | | 2 | 0.381 | | 0.067 |

**Note**: n= number of replicates, DF =degree of freedom

ply or their interaction. The Mg content of the roots (with adhering hyphae from the root compartment) was significantly increased by mycorrhizal colonisation ($P$=0.032) and the interaction effect with nutrient supply ($P$ =0.015), which did not have a significant effect itself ($P$=0.236)(Fig. 3).

## Discussion

Hyphae actively forage for Mg and respond to Mg supply by increasing their dry weight (Fig. 2). Addition of P to the hyphal compartment did not further affect hyphal dry weight (Fig. 2). Hyphal proliferation in patches with high supply of P (Jentschke *et al.*, 2001a) or inorganic or organic N (Bending & Read, 1995, Brandes *et al.*, 1998, Jentschke *et al.*, 2001b) has been found before, but this is the first report on active ectomycorrhizal foraging for Mg under Mg deficient conditions. These findings support the hypothesis that preferential growth of ectomycorrhizal fungi on mineral substrates and feldspars is driven by nutrient availability (Rosling *et al.*, 2004).

Ectomycorrhizal colonisation increased the Mg content of the seedlings (Fig. 3) which is probably an effect of foraging and transport of Mg. Further proof should be provided by tracer experiments with addition of $^{25}$Mg to the hyphal compartment.

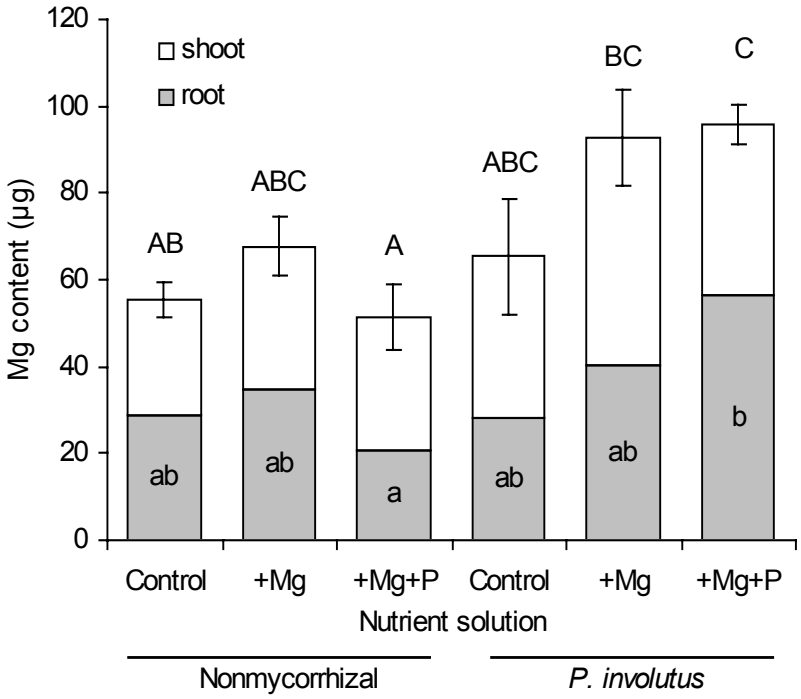



**Figure 3** Mg content (µg) in shoot and root of *P. sylvestris*, either nonmycorrhizal or colonised by the ectomycorrhizal fungus *P. involutus*. Bars with the same letter are not significantly different ($P$<0.05); Capital letters refer to total Mg content of seedling. Mean of 4-5, standard error for total Mg content.

Transport of Mg through ectomycorrhizal hyphae and subsequent uptake by the plant was shown before by Jentschke *et al.* (2000, 2001a). The dry weight of the seedlings did not reflect the increased contents or concentrations of Mg. All seedlings were extremely Mg deficient, with tissue concentrations well below the level considered necessary for growth (0.6 mg Mg $g^{-1}$ dry weight for *Pinus* spp. (Reuter *et al.*, 1997)). Shoot concentration of Mg was increased by ectomycorrhizal colonisation and by addition of Mg, but surprisingly, not addition of Mg plus P to the hyphal compartment. Dry weight of the seedlings and roots was increased by ectomycorrhizal colonisation, most notably in the treatment with both Mg and P added to the hyphal compartment, indicating that apart from Mg the seedlings might also have been growth-limited by P.

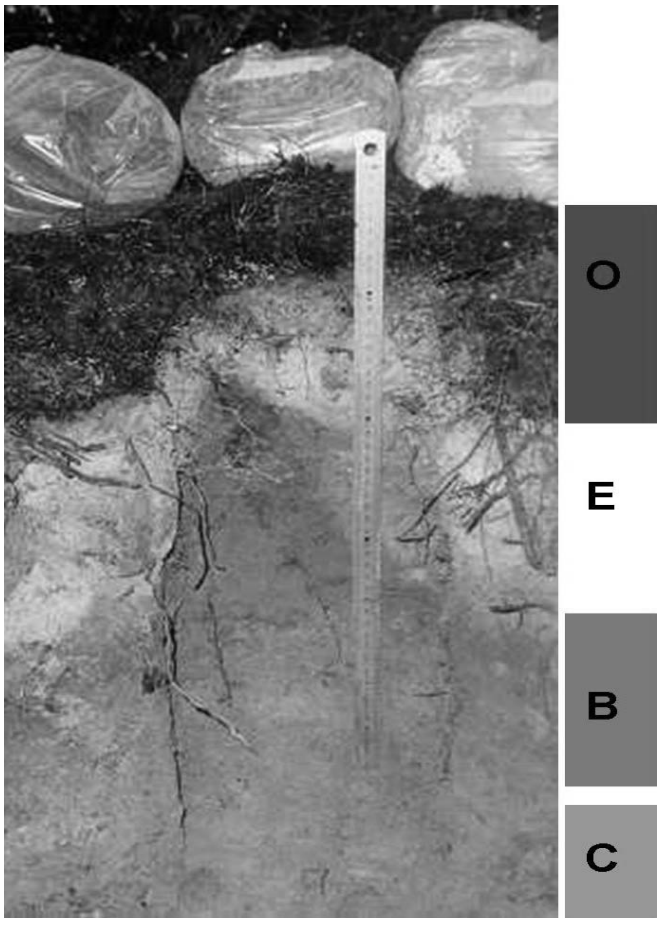
In contrast to Jentschke *et al.*(2001a), we observed active ectomycorrhizal hyphal transport of Mg, rather than a passive co-transport of Mg with P: the Mg content of the ectomycorrhizal seedlings was not affected by omission or addition of P in the hyphal compartment (Fig.3). The apparent inconsistency is probably caused by differences in the growth-limiting element: in Jentschke *et al.* (2001a) P limited growth, and hyphal foraging and biomass was strongly reduced by omitting P from the hyphal compartment. In our experiment, Mg limited growth, and hyphal foraging

and biomass was not affected by P omission in the hyphal compartment. Possibly P transport results in co-transport of Mg, but here we show that Mg transport independent of P transport also occurs.

These results, in combination with the earlier finding that *P. involutus* (in symbiosis with *P. sylvestris* seedling) increases the exudation of oxalate in response to Mg deficiencies (Van Schöll *et al.*, 2006: Chapter 5), are supportive for a role of ectomycorrhizal fungi in mobilising Mg from mineral under Mg deficiency.

# 8

# A short history of Rock-eating Mycorrhizae: their role in ecosystem processes




Laura van Schöll, Thomas W. Kuyper, Mark M. Smits, Renske Landeweert,
Ellis Hoffland, Nico van Breemen

The work presented in the preceding chapters was conducted within the research programme ‘Rock-eating Mycorrhiza; Where, why, how?’ that started at Wageningen University at 1998 and ends with this review paper. In this review, the results of the whole research programme are presented and discussed, and is therefore broader than the general conclusions of the preceding chapters.

# 1. Rock-eating mycorrhizae

## *1.1 Rock-eating mycorrhizae and tunnelled minerals*

The term *Rock-eating fungi* was introduced in 1997, when it was discovered that feldspar and hornblende grains in the E horizon of Swedish podzol soils contained numerous microscopic small tunnels, presumably formed through organic anions exuded at hyphal tips (Jongmans *et al.*, 1997). These tunnels had smooth and parallel-oriented walls with a constant diameter (3-10 µm) and rounded ends, which distinguished them from (coalesced) etch pits and cracks caused by chemical weathering processes known so far (see picture on page 123). The size and shape of the tunnels seemed to perfectly fit fungal hyphae, and some tunnels were colonised by fungal hyphae.

Ectomycorrhizal fungi (*Rock-eating Mycorrhiza*) were seen as the most likely candidates responsible for this tunnel formation. Ectomycorrhizal fungi form a symbiotic association with the roots of coniferous trees, which are the dominant vegetation on podzol soils where the tunnels were found. Ectomycorrhizal fungi are known to exude low molecular weight organic anions (LMWOA), that are strong weathering agents (Drever & Stillings, 1997, Gadd, 1999). Because of their association with tree roots, ectomycorrhizal fungi have a source of photo-synthetically produced carbohydrates available (Högberg *et al.*, 2001). Saprotrophic fungi and bacteria, in contrasts, are mostly carbon-limited, especially in the mineral E horizon where the tunnels were found, which has a low content of organic matter.

Podzols typically develop under boreal forests and are characterised by four distinct soil horizons: (1) a dark-coloured organic (O) horizon underlain by (2) a white/ash-coloured eluvial (E) horizon, overlying (3) a usually dark-coloured illuvial (B) horizon on top of (4) the unaltered parent (C) material (see picture on page 101).

## *1.2 Rock-eating Mycorrhiza: Where, Why, How? research programme*

The tunnelled minerals were seen as an indication of the weathering capacity of ectomycorrhizal fungi, and this raised some intriguing questions regarding the potential role of ectomycorrhizal fungi in biogeochemical cycles and plant uptake of base cations (Van Breemen *et al.*, 2000a, Van Breemen *et al.*, 2000b). These questions

have been addressed in the "*Rock-eating Mycorrhiza: Where, Why, How?*" research programme. It also induced a shift in the focus of ectomycorrhizal research from the organic soil horizon to the mineral soil horizon, and from the ectomycorrhizal root tip approach to the ignored extraradical hyphae in the bulk soil. The results of the programme are presented and discussed below. The research questions are introduced in the following paragraph, grouped into three themes.

*I. Linking tunnels, podzols and ectomycorrhizal species distribution*

- How are tunnelled minerals distributed and is this related to occurrence of ectomycorrhizal fungi?

The tunnelled minerals were a newly discovered phenomenon, and had so far been found only in mineral grains from podzol soils. A more thorough knowledge on their occurrence and on their development was considered necessary to link them to ectomycorrhizal fungi.

- Which ectomycorrhizal species are responsible for mineral weathering and tunnelling?

Data on the species composition and abundance of ectomycorrhizal fungi in the mineral E horizon, where most tunnels were found, were lacking at the start of the programme. To relate ectomycorrhizal fungi to mineral tunnelling we considered it necessary to study mycelial distribution rather than root tip distribution. This required the development of molecular methods to quantitatively and qualitatively determine the occurrence of ectomycorrhizal fungal hyphae in soil.

*II. Role of ectomycorrhizal fungi in biogeochemical cycles*

- Role in podzolisation process: do ectomycorrhizal fungi transport Al from mineral horizon to overlying organic horizon?

Mineral tunnelling seems restricted to the E horizon of podzol soils, and is almost absent in the underlying B horizon, suggesting a link to the podzolization process. Possibly, ectomycorrhizal fungi transport Al from the E horizon to the O horizon through hyphae that exploit the E horizon while being attached to the ectomycorrhizal root tips in the O horizon.

- What is the contribution of tunnels and ectomycorrhizal fungi total weathering?

The role of plants in mineral weathering through root respiration and exudation has been long established, but the role of fungi is more controversial. This question was addressed by the quantification of the contribution of tunnels and ectomycorrhizal fungi to total weathering budget.

*III. Role of ectomycorrhizal fungi in plant nutrition*

- Do ectomycorrhizal fungi respond to base cations deficiencies?

Ectomycorrhizal fungi may actively respond to base cation deficiencies through the enhanced exudation of LMWOA and mineral weathering, with subsequent transport and plant uptake of the weathering products?

- Can ectomycorrhizal fungi protect their host from aluminium toxicity?

Ectomycorrhizal fungi may increase plant tolerance to Al toxicity by an increased supply of base cations, by exclusion of Al from the root cortex by the hyphal network enveloping the root tip or by the exudation of LMWOA.

## 2. Linking ectomycorrhizal fungi to rocks: are ectomycorrhizal fungi responsible for tunnel formation?

*2.1 Relation between ectomycorrhizal fungi, podzols and tunnelled minerals*

Evidence to link ectomycorrhizal fungi to mineral weathering was lacking at the start of the programme. Mineral tunnelling and weathering are slow processes and, therefore, a causal relationship between ectomycorrhizal fungi and tunnel formation cannot be determined under controlled laboratory conditions. The relation between ectomycorrhizal fungi and tunnelled minerals was therefore investigated in a range soil samples and field studies.

Mineral tunnelling seems restricted to soils that have developed under a vegetation with ectomycorrhizal fungi (Hoffland *et al.*, 2005). Vegetation pattern can change over time, and present-day vegetation is no evidence for past vegetation. Podzols typically develop under a vegetation of coniferous trees, with an undergrowth of ericaceous shrubs, and develop in a time-scale of several hundred to thousand years (Lundström et al., 2000). Podzols are therefore indicative for the long-term presence of ectomycorrhizal fungi. An extensive survey of 75 soils from Europe, Asia, North America and Australia revealed that tunnelled mineral grains occur almost exclusively in podzols in temperate and boreal zones, and also sometimes in acid brown forest soils. No tunnelled minerals were observed in podzol soils that had developed under *Agathis australis* (Kauri tree) in New Zealand, which do not associate with ectomycorrhizal fungi.

Mineral tunnelling is related to ectomycorrhizal fungi, but not to ericoid or sapotrophic fungi. Ectomycorrhizal root tip density was positively related to tunnel frequency in a field study along two natural productivity gradients (Hoffland *et al.*, 2003). The productivity gradients consisted of a toposequence where the pH, N sup-

ply and vegetation vary strongly over a short distance (<100 m), but with similar mineralogy. No relation was found with arbuscular mycorrhizal plants. Ericoid fungi associate with the understory of ericoid mycorrhizal *Vaccinium* species in boreal forest, and therefore often co-occur with ectomycorrhizal fungi. Ericoid fungi can also produce high amount of LMWOA (Martino *et al.*, 2003), and may thereby contribute to tunnel formation. It is unlikely, however, that ericoid mycorrhizal fungi are solely responsible for tunnel formation, as tunnels were found in sites with no ericoid plants, and where they are unlikely to have grown considering the rapid establishment of the productivity gradient following deglaciation (Giesler *et al.*, 1998).

Mineral tunnelling increases with soil age and podzol formation. Relation of tunnels with soil age and podzol formation was studied in soil chronosequence (Hoffland *et al.*, 2002). A chronosequence is a series of soils that differ in starting time of development and thus age, but have similar parent material and other biotic and abiotic factors that determine soil development. The North Sweden chronosequence studied here was formed through glacial rebound followed by gradual uplifting of land from sea (9-50 mm $year^{-1}$). Tunnel length and frequency increased with soil age. Up to 25% of the feldspars from the uppermost 2 cm of the E horizon contained tunnels, and this percentage decreased rapidly with soil depth.

Together with the finding of fungal hyphae inside the tunnels, this coincidence of vertical distribution pattern of tunnels with highest concentration of ectomycorrhizal root tips strongly suggest that ectomycorrhizal hyphal activity is involved in mineral tunnelling.

### *2.2 Ectomycorrhizal fungal species in podzol soil horizons*

In order to study ectomycorrhizal weathering it was necessary to establish which ectomycorrhizal species occurred in the mineral E horizon in which the tunnels were found. Ectomycorrhizal species richness in podzols is high, but little was known on the vertical distribution of ectomycorrhizal fungi over the podzol soil profile at the start of the programme. Most recent[1] studies on ectomycorrhizal communities have been confined to the organic horizon, where ectomycorrhizal root tip density is highest. The chemical and mineralogical properties of the soil horizons differ strongly, and it is likely that this vertical zonation provides different niches for ec-

[1] After the first description of ectomycorrhizae by Frank, attention was initially focused on the possible role of ectomycorrhizae in the uptake of organic sources of nutrients, because ectomycorrhizas were most commonly distributed in the organic parts of the soil profile. Gradually, however, attention was drawn towards the role in the absorption of mineral nutrients (P, $NH^{4+}$, base cations). Read (1987) drew again to the role in the uptake of more complex organic nutrient sources. Subsequent work with the stable isotope $^{15}N$ further supported Frank's organic nitrogen theory.

tomycorrhizal fungi with different functional roles. Data on the species composition and abundance of ectomycorrhizal fungi in the mineral E horizon were lacking.

Vertical niche differentiation of ectomycorrhizal fungal root tips across soil horizons was shown for a Swedish podzol (Rosling *et al.*, 2003). Root tips were sampled from all horizons of three soil columns, sorted into groups on the base of morphological characteristics and counted. Ectomycorrhizal fungal species on the root tips were identified molecularly by sequencing the ITS-rDNA region. Two thirds of the root tips were found in the mineral soil horizons. The percentage of root tips colonised ranged between 60 and 98%, but this was not related to soil horizon. A significant relationship between ectomycorrhizal fungal species composition and soil horizon was found, but species compostion could not be related to specific chemical properties of the mineral E, B or C horizons. Most taxa typically occurred in one part of the soil profile only. *Tomentellopsis submollis*, three *Piloderma* species, *Cortinarius* subgen. and *Dermocybe* spp. were found predominantly in the upper horizons while other species within *Piloderma* spp., *Cortinarius* spp., and *Suillus luteus* were associated with the lower mineral horizons. As much as half of the distinguished ectomycorrhizal fungal species were restricted to the mineral horizons. Thus, ectomycorrhizal community studies with limited sampling depth could strongly underestimate ectomycorrhizal diversity.

### *2.3 From root tip to mycelial view*

Data on abundance and occurrence of ectomycorrhizal fungi on root tips do not necessarily reflect the simultaneous occurrence of extraradical mycelium in the bulk soil. Ectomycorrhizal weathering is expected to take place at the hyphal tips by exudation of LMWOA. To establish which ectomycorrhizal fungal hyphae occur in the mineral horizons, where mineral tunnelling and weathering take place, it was necessary to develop a method to identify ectomycorrhizal hyphae from soil samples.

Ectomycorrhizal mycelium from soil samples was identified with the novel application of molecular techniques (Landeweert *et al.*, 2003a). This method involved extracting DNA from soil layers, selectively amplifying fungal DNA or more specifically amplifying basidiomycete DNA, cloning the ITS fragments, and sequencing these clones, after which their identity (the ribosomal spacers ITS) could be established. The method was applied to identify the diversity of ectomycorrhizal fungal hyphae in a Swedish podzol, using soil samples from the same soil columns and soil horizons as in the accompanying root tip study described above. DNA was successfully extracted, with amounts of DNA decreasing with soil depth. The distribution of the fungal types showed a vertical niche differentiation, with 16 out of 25 fungal

taxa occurring exclusively in the mineral layers of which 7 fungal taxa occured exclusively in the E horizon. At least 16 out of 25 fungal types[2] were found to be ectomycorrhizal basidiomycetes, suggesting a dominance of ectomycorrhizal over saprotrophic basidiomycetes in most soil layers.

*2.4 Comparison between root tip and soil mycelium DNA identification*

Comparison between the root tips study and the soil mycelium study showed that abundance of root tips and the extramatrical mycelium were only partly correlated, (Landeweert *et al* 2003a) an observation also made by Dickie *et al.* (2002). In general, the same species were detected when comparing the ectomycorrhizal fungal species composition on root tips with the species composition of the extraradical mycelia. Of the 16 ectomycorrhizal fungal taxa identified in DNA extracts from root free soil, all but one were also found on root tips. In several cases, however, species that were abundant on root tips were not detected in soil extracts, and others that were abundant in soil extracts were rarely detected on the roots.

The use of soil DNA extracts can avoid the time-consuming root tip morhotyping and reduce several sample biases in diversity studies related to root tips sampling and morphological screening (see Horton & Bruns, 2001, Taylor, 2002). The fungal diversity on root tips samples and soil mycelial identification, taken from the same bulk sample was compared without initial morphotyping of the root tips (Landeweert 2005). From six soil samples from a *Pinus sylvestris* (Scots pine) forest in the Netherlands, the roots and soil were separated on a sieve, DNA was amplified and basiodiomycete diversity was analysed by DGGE. The number of fungal taxa distinguished in soil was a little higher (14) than the number from root tips (11). No fungal species were found exclusively on the root tips, indicating that the mycelia of most species (including *Russula* and *Laccarius*, which have smooth hyphal mantles without large amounts of emanating hyphae) occur in soil in sufficient quantities for detection by the molecular methods. Molecular identification methods of ectomycorrhizal hyphae in soil permit *in situ* testing of hypotheses on resource partitioning and niche differentiation of individual fungal species.

*2.5 Vertical niche differentiation of ectomycorrhizal hyphae*

The data from the Swedish podzol provided evidence for vertical niche differentiation by ectomycorrhizal fungi (Rosling *et al.* 2003, Landeweert *et al.* 2003a). The

[2] At that time, the BLAST database was still incomplete, and various fungal taxa could not be identified. Since that publication another sequences could be assigned to the fungal generus: *Albatrellus*

observed niche differentiation is consistent[3] with similar data provided by Dickie *et al.* (2002). Interestingly, the mycelial view did not suggest niche partitioning of ectomycorrhizal hyphae across the organic versus mineral layers: in fact, the organic horizon and the E horizon were more similar than the E horizon and the B or C horizons. Two ectomycorrhizal fungal species, *Cortininarius collinitus* and *Russula decolorans*, showed high hyphal occurrence in the E horizon, and the latter was also abundant in the O horizon. As most of the tunnelling occurred in the uppermost E layer, candidate fungi for tunnelling are more likely to occur in the E and O horizon than in the lower mineral horizons. The lower tunnel density in the mineral layers does not exclude the possibility that ectomycorrhizal species from the mineral B horizon are involved in mineral weathering. The lower tunnel density may result from the lower hyphal density in the B horizon. The co-occurrence of ectomycorrhizal fungi in the O and E horizon may seem somewhat surprising in view of the large differences in soil chemistry between both horizons, but may be a consequence that mycelial growth is three- rather than two-dimensional.

The data from the Swedish conifer forest with an understory of ericoid mycorrhizal *Vaccinium* species do not allow us to rule out the possibility that ericoid mycorrhizal ascomycetes that are involved in tunnelling. The molecular methods used by Landeweert *et al.*(2003a) only amplified basidiomycete DNA, no ascomycete DNA was amplified. While quantitatively this is unlikely to be a problem (Rosling's *et al.* (2003) accompanying study suggests that over 95% of all root tips belonged to basidiomycetes), oversight of ascomycetes, especially a group of dark septate endophytes that can both form ectomycorrhizal and ericoid mycorrhizal relationships, is somewhat unfortunate, as several of these fungi have been reported to be effective producers of LMWOA (Martino *et al.*, 2003).

### *2.6 Quantification of ectomycorrhizal fungal hyphae in soil*

Existing methods to determine mycelial biomass in soil do not allow species-specific identification, or distinction between ectomycorrhizal and other fungi. The molecular identification methods described Landeweert *et al.* (2003a) or by Dickie *et al.* (2002) (based on T-RFLP instead of cloning and sequencing) enable identification of mycelia directly form the soil. They can be called semi-quantitative at best, because the relationship between abundance of extraradical hyphae and clone num-

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[3] Dickie *et al.* suggests much more statistical support for vertical niche differentiation than Landeweert *et al.*; however, the difference is due to the statistics applied (Kuyper & Landeweert 2002). Whereas Dickie *et al.* assumed that the samples from the different layers could effectively be treated as independent samples, Landeweert *et al.* cautiously avoided that assumption, reducing the power of their test, but not the validity of the outcomes.

bers or size of T-RFLP peaks is not straightforward. Quantitative PCR is a way forward.

Fungal biomass of individual ectomycorrhizal species was quantified by three molecular methods and compared to conventional methods (Landeweert *et al.*, 2003b). Fungal presence was determined over time in the soil of a pot experiment where *P. sylvestris* seedlings were grown, inoculated with none, one or two ectomycorrhizal fungal species (*Suillus bovinus* and *Paxillus involutus*). The conventional methods (hyphal length estimation and determination of phospholipid fatty acids (PLFAs)) showed similar trends over time. These methods failed, however, to distinguish between the two ectomycorrhizal species. Fungal biomass in the pots with both ectomycorrhizal species did not equal the sum of the amount of biomass in the pots with only one species. The three molecular methods (denaturing gradient gel electrophoresis (DGGE), a cloning technique and Real-time quantitative PCR) enabled identification and (relative) quantification of both species and showed consistent results. The methods therefore have high potential for studying dominance, succession and interaction among fungal species in soil, which is a prerequisite to study the contribution of individual species and functional roles in ecosystem properties.

Molecular quantification methods require specific primers through which DNA of one fungal species can be amplified. While such methods can be successfully applied in microcosms where the researcher has almost complete control over ectomycorrhizal species composition, as in our study (Landeweert *et al.*, 2003b), they haven't yet been applied under field conditions. Primer specificity is still a major issue to be addressed: some primers do not amplify all individuals of a certain species, or show (slight) co-amplification of several other species. Further research should therefore be directed towards developing a larger set of species-specific primers to quantitatively assess fungal occurrence in the upper layers of the E-horizon where tunnelling takes place. Another prospect of future research could be the development of a set of primers that allow amplification of genes that could be associated with the tunnelling processes, e.g. genes involved in the synthesis and/or exudation of LMWOA. Quantification of the ectomycorrhizal or ericoid mycorrhizal fungi that possess the required genes, or even better quantification of RNA products thereby directly quantifying activities, would be another interesting possibility.

## 3. Ectomycorrhizal fungi in biogeochemical processes

### *3.1 Ectomycorrhizal fungi in the podzolisation process*

Ectomycorrhizal fungi were also hypothesised to play a role in the podzolisation process. Existing theories on the podzolisation process focus on the formation of the mineral E and B horizon, but do not explain the high concentrations of Al observed in the organic O horizon. The formation of the distinct soil horizons in podzols is linked to mobilisation of aluminium (Al) and iron (Fe) in the E horizon and subsequent precipitation in the B horizon. Budget studies on Al and Fe in podzols demonstrate that much of the Fe and Al leached out of the E horizon into the B horizon originates in the O horizon (Giesler *et al.*, 2000). Known input routes of Al and Fe into the O horizon, i.e. litter fall and atmospheric deposition, are insufficient to explain the high concentrations of Al in the O horizon and output rates of Al from the O horizon to the E horizon. Possibly ectomycorrhizal activity in the E horizon is associated with uptake and transport of mineral elements, including Al and Fe, from the E horizon towards the O horizon through hyphae that exploit the E horizon while being attached to root tips in the O horizon.

A prerequisite for a role of ectomycorrhizal fungi in the upward transport of Al is that ectomycorrhizal fungi are able to transport Al through their hyphae. An *in vitro* test of five different ectomycorrhizal fungal isolates, growing in two-compartment Petri dishes demonstrated that 2 of the 5 isolates used were able to transport Al (Smits, 2005). In plants, transport of Al has been linked to complexes of Al with LMWOA, mostly citrate. For ectomycorrhizal fungi, complexation of Al to small soluble polyphosphate chains has also been suggested. Polyphosphates are, besides organic anions like citrate, the strongest Al binders in biological systems (Martin *et al.*, 1994). An *in vitro* test did, however, not indicate any effect of P supply on the transport of Al through ectomycorrhizal hyphae (Smits, 2005).

Ectomycorrhizal hyphae may play a role in the upward transport of Al from the mineral E horizon to the overlying organic O-horizon (Smits, 2005). Since Al is abundant in podzols, transport of Al was studied using gallium (Ga) as a tracer. The similarity of Ga and Al transport through ectomycorrhizal hyphae was tested *in vitro*, using two-compartment Petri dishes with either Al or Ga (Smits, 2005). Ectomycorrhizal tree seedlings were grown in pots, filled with material from a Swedish podzol. Pots without tree seedlings were used as a control for abiotic upward transport. In half of the pots, downward growth of roots from the organic top layer into the mineral soil layer was inhibited with a mesh, which was permeable to fungal hyphae. During the experiment, Ga was injected into the reconstructed mineral soil

layer. Gallium content in the tree seedlings increased in the pots with Ga addition compared to the control pots without Ga, but Ga content was not different between pots where tree roots were allowed or inhibited to grow into the mineral soil layer.

*3.2 Rock-eating mycorrhizae: contribution to mineral weathering*

Ectomycorrhizal fungi may contribute to ecosystem influx of base cations through the dissolution of mineral grains by the exudation of low molecular weight organic anions (LMWOA). Mineral weathering is a primary source of base cations in forest ecosystems (April & Newton, 1992, Likens & Bormann, 1995) and can be enhanced by LMWOA (Drever & Stillings, 1997). Past research has shown that ectomycorrhizal fungi can actively dissolve mineral grains and produce LMWOA (see reviews by Gadd (1999), Landeweert *et al.* (2001) and Hoffland *et al.* (2004)). The LMWOA in the soil solution are the product of decaying plant material, root exudation and microbial activity. Concentrations of LMWOA in the bulk soil solution typically range between 0.1 and 50 µM, but concentrations in the rhizosphere are expected to be higher (Jones, 1998, Strobel, 2001).

The quantitative importance of LMWOA and the contribution of the biota in the weathering process is, however, still under debate. Van Breemen *et al.* (2000a) estimated the contribution of ectomycorrhizal fungi to mineral weathering in a podzol soil via tunnelling alone to be 50%. Sverdrup *et al.* (2002) estimated the contribution of organic ligands to mineral weathering to be 15 % and the direct contribution of biota to be maximally 2%. This estimate seems very conservative considering that 50% of total dissolved carbon in the soil is the product of current photosynthesis, exuded by roots and associated ectomycorrhizal hyphae, presumably for a large part in the form of LMWOA (Högberg & Högberg, 2002). Quantification of ectomycorrhizal weathering and production of LMWOA is complicated by our inability to separate contribution of ectomycorrhizal fungi from other contributors and processes and by the slow rate of the tunnelling and weathering process.

Fungal tunnels in mineral grains can be distinguished visually from other weathering phenomena (Hoffland *et al.*, 2002) and can therefore be used as a tool to recognise and quantify one aspect of ectomycorrhizal weathering. Quantification of tunnel weathering revealed that fungal tunnelling accounted for less than 0.5% of total feldspar weathering in the upper 2 cm of the mineral soil (Smits *et al.*, 2005). Weathering by tunnelling was quantified via image analysis in soil thin sections, which were taken from a North Michigan chronosequence. Total weathering was quantified by comparing the mineralogy of the surface soil with the underlying parent material. Mineral tunnelling is, however, only one aspect of ectomycorrhizal weathering.

Mineral surface weathering due to ectomycorrhizal fungi may be quantitatively more important.

Modelling results show that ectomycorrhizal exudation of oxalate, an important weathering agent, could account for up to 15% of total feldspar weathering in the upper 2 cm of the mineral horizon of the Lake Michigan soil (Smits, 2005). Ectomycorrhizal feldspar weathering through oxalate exudation was modelled by considering the kinetics of oxalate production, biodegradation and complexation. Ectomycorrhizal production of oxalate in the vicinity of feldspars was estimated from literature data on ectomycorrhizal biomass in E horizons of podzols and *in vitro* oxalate production by ectomycorrhizal fungi, combined with data on fungal distribution over the different minerals. Fungal distribution was quantified microscopically in stained soil thin sections from the Lake Michigan dune chronosequence. The relative abundance of hyphae in direct contact with the mineral surface was in the order Na/Ca-feldspar > K-feldspar > quartz. Comparing the modelling outcomes to the total feldspar weathering showed that mineral surface weathering is probably substantially higher than mineral tunnelling. The model did not include other fungal weathering agents like citrate and protons, therefore the total effect of ectomycorrhizal fungi on mineral weathering could be even higher.

## 4. Role of ectomycorrhizal fungi in plant nutrition.

### *4.1 Ectomycorrhizal weathering under base cation deficiencies*

Ectomycorrhizal weathering of minerals containing base cations through the exudation of LMWOA might have a profound effect on plant uptake. Trees in forest ecosystems are generally assumed to be growth-limited by N availability, but under impact of atmospheric deposition, causing increased leaching of base cations, combined with (intensive) harvesting, base cations deficiencies may develop. Ectomycorrhizal fungi are known to be involved in the uptake of N and P, but in the mineral horizons these are only present in low concentrations. Tunnels were found exclusively in feldspar and hornblende grains, which contain a.o. K, Ca and Mg (Hoffland *et al.*, 2002, Hoffland *et al.*, 2005). Mineral tunnelling is faster in soils with low supply of base cations than in soils with higher supply (Smits *et al.*, 2005), and in chronosequences, tunnels only emerged when easily weatherable minerals had disappeared or were strongly weathered (i.e. biotite and hornblende resp.) (Hoffland *et al.*, 2002), indicating that tunnelling may be driven by the (low) bioavailability of base cations K, Ca and Mg.

*4.2 Organic anions exudation and hyphal foraging under nutrient deficiencies*

Ectomycorrhizal fungi can actively and selectively accelerate mineral weathering by changing the amount and type of LMWOA exuded, but differences among ectomycorrhizal species are as big or bigger than between nonmycorrhizal and ectomycorrhizal (Van Schöll *et al.*, 2006: Chapter 5). The exudation of di-carboxylic[4] LMWOA by tree seedlings and ectomycorrhizal fungi, grown in symbiosis or in isolation, in response to base cation and P deficiencies was measured in axenic culture systems, where roots and fungi grew on a layer of glass beads with nutrient solution or in sand mixed with nutrient solution. Surprisingly, ectomycorrhizal colonisation did not affect or even decrease the total amount of LMWOA exuded compared to nonmycorrhizal tree seedlings. Deficiency of P increased the total amount of LMWOA exuded by nonmycorrhizal and ectomycorrhizal seedlings alike, but there was no effect of Mg or K omission from the nutrient supply on the total amount of LMWOA.

Ectomycorrhizal fungi and nutrient omissions did affect the type of LMWOA exuded. Nonmycorrhizal seedlings exuded mainly malonate. Ectomycorrhizal colonisation with *Paxillus involutus* and *Hebeloma longicaudum* significantly decreased the exudation of malonate, whereas the exudation of oxalate was increased by colonisation with *P. involutus* and *Piloderma croceum*. Oxalate is probably the most potent weathering agent known to be exuded by plant roots and ectomycorrhizal hyphae. The weathering potential of oxalate via complexation with Al, as indicated by the stability constant (log $K_{Al}$ = 6.5), is higher than that of malonate (log $K_{Al}$ =5.7) (Fox *et al.*, 1990). Omission of Mg from the nutrient solution increased the exudation of oxalate by nonmycorrhizal and ectomycorrhizal seedlings alike. Seedlings colonised by *P. involutus* significantly increased the exudation of oxalic acid when K was omitted from the nutrient solution, whereas there was no effect of K omission on oxalate exudation by nonmycorrhizal seedlings or seedlings colonised with *P. croceum* or *H. longicaudum*. Base cation deficiencies and ectomycorrhizal fungi may enhance weathering by changing the type of LMWOA, without changing the total amount of LMWOA exuded.

Ectomycorrhizal hyphae respond to Mg deficiency by increased foraging and active transport of Mg to their host tree (Chapter 7). The foraging behaviour and transport of Mg through the ectomycorrhizal hyphae in response to Mg deficiencies of their host plant was investigated in a two-compartment system, with addition of Mg or Mg+P to the hyphal compartment (not accessible to roots). Transport of Mg through

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[4] citrate, which is another LMWOA with strong weathering capacity, could unfortunately not be determined by the CE method used.

ectomycorrhizal fungi has been shown before, but was assumed to be a passive co-transport with P, driven by P deficiency (Jentschke *et al.*, 2001). We observed active ectomycorrhizal hyphal transport of Mg, rather than a passive co-transport of Mg with P. Hyphal proliferation of the hyphal compartment significantly increases in response to Mg addition. These findings are supportive for the hypothesis that preferential growth of ectomycorrhizal fungi on mineral substrates and feldspars is driven by base cation availability (Rosling *et al.*, 2004), parallel to the intense proliferation of litter patches (Bending & Read, 1995) or patchily supplied P or N (Brandes *et al.*, 1998, Jentschke *et al.*, 2000) under N or P deficiency.

The high amount of ectomycorrhizal root tips in the mineral soil horizons and the vertical niche differentiation of ectomycorrhizal fungi indicate that species in the mineral soil layers have a functional role in the mineral soil horizons that is different from the role of species in the organic layer. Our first tentative efforts to link LMWOA exudation and weathering capacity to species selectivity for the mineral versus the organic soil horizon did not show a positive correlation: *P. involutus*, seen as a representative for the organic horizon (Laiho, 1970), increased the exudation of oxalate and the weathering of muscovite in response to K deficiency. *H. longicaudum*, taken as a representative for the mineral soil layer (Rosling *et al.*, 2003), decreased the total amount of LMWOA. The fungi used were, however, lab-cultured for a long period. In future studies, ectomycorrhizal species and isolates obtained from different soil horizons, and from soils with low base cation versus high base cation supply should be used.

### *4.3 Mineral weathering by nonmycorrhizal and ectomycorrhizal tree seedlings*

The effect of nonmycorrhizal and ectomycorrhizal tree seedlings on the exudation of oxalate in response to omission of Mg and K corresponds well to their weathering of Mg and K containing minerals as determined in a pot experiment (Van Schöll *et al. in press*: Chapter 6). Nonmycorrhizal and ectomycorrhizal tree seedlings were grown on pots filled with quartz sand mixed with nutrient solution, with the K-containing mineral muscovite as K source or the Mg-containing mineral hornblende as the only Mg source. Weathering of muscovite was increased by tree seedlings, and was increased even further when tree seedling were colonised by *P. involutus*. Compared to pots without tree seedlings, the release of K from muscovite was increased by a factor of 3.3 in the pots with seedlings colonised by *P. involutus* and by a factor of 1.7 in pots with seedlings colonised by *Suillus bovinus* or *P. croceum* or without mycorrhiza. It seems likely that *P. involutus* enhanced the weathering of muscovite by higher exudation of oxalate, which was stimulated by the K deficiency. Weathering of hornblende was increased by tree seedlings, but this was not

affected by ectomycorrhizal fungi. Compared to pots without tree seedlings, the release of Mg from hornblende was increased by a factor 1.5-2 by ectomycorrhizal and nonmycorrhizal tree seedlings.

*4.4 Who is in the driver's seat of the mycorrhizal symbiosis?*

Ectomycorrhizal fungi and tree seedlings can adapt to base cations deficiencies by increasing the exudation of LMWOA and mineral weathering, but the question remains who of the two symbionts is driving this response.

In the pot experiment, (Van Schöll *et al. in press*: Chapter 6) increased weathering was not *per se* reflected by increased nutrient uptake into the shoot, and with some ectomycorrhizal fungi the weathering products may have been retained partly in the fungal mycelium. *P. involutus* doubled the dissolution K from muscovite but this did not result in increased K content of shoots compared to other tree seedlings. *P. croceum* decreased allocation of hornblende dissolved Mg to the shoot. The impact of ectomycorrhizal fungi on base cation availability will be more important in the field than in our pot experiment. By mobilising and retaining the K and Mg in the roots and hyphae, they are protected from leaching. After decomposition, the K and Mg retained in the roots and hyphae will become available for uptake, thereby increasing the total amount that is bioavailable in the system.

The exudation of oxalate by *P. sylvestris* seedlings and *P. involutus* differs when grown in pure culture or symbiosis (Van Schöll *et al.* 2006: Chapter 5). Omission of Mg significantly increased the exudation of oxalate from the nonmycorrhizal and ectomycorrhizal seedlings, but *P. involutus* in pure culture did not exude any oxalate when Mg was omitted from the nutrient solution. Reduction of K supply did not significantly affect the LMWOA exudation of either *P. involutus* in pure culture or nonmycorrhizal seedlings, but significantly increased the exudation of oxalate when they were grown in symbiosis. Reduction of P supply increased the exudation of malonate by *P. involutus* in pure culture, whereas in symbiosis *P. involutus* strongly reduced malonate exudation compared to nonmycorrhizal seedlings under P deficiency in favour of oxalate. This emphasizes the need to do experiments with both symbiotic partners. By using two-compartment systems, we could gain more insight into the spatial distribution of the LMWOA exudation: ectomycorrhizal root system versus the external hyphae.

*4.5 Al toxicity in forest ecosystems*

Improved uptake of base cations and enhanced exudation of LMWOA could be important in counteracting the toxic effect of aluminium on tree growth. Aluminium

toxicity has been proposed as one of the explanations for the so-called 'new type forest decline' in Europe and North America in the last decades of the last century. Due to atmospheric deposition of acidifying pollutants, the pH of soil solution declines, and the concentration of dissolved Al increases while dissolved base cations (K, Ca, Mg) are leached from soil (Mulder *et al.*, 1987, Ulrich *et al.*, 1980). At a pH below 4, dissolved aluminium is present mainly in the form of the phytotoxic $Al^{3+}$ (Kinraide, 1991). $Al^{3+}$ reduces root growth, which results in impaired uptake of water and nutrients, and more specifically inhibits the uptake of Ca and Mg by kinetic processes at the root cell wall. Aluminium toxicity can be –partly- mitigated by increasing the concentrations of dissolved Ca and Mg (Kinraide, 2003) or by complexation of aluminium with LMWOA (Ryan *et al.*, 2001).

The interactive effect of base cations and aluminium on tree growth is often described by the ratio of base cations to aluminium (BC:Al) in the soil solution, but the use of this criterium has been strongly criticised (Binkley & Högberg, 1997, Falkengren Grerup *et al.*, 1995, Högberg & Jensén, 1994, Løkke *et al.*, 1996). It assumes a chain of direct causal relationships between tree growth, base cation uptake, aluminium versus base cation adsorption at the root cell walls and ratio of base cations to aluminium in the soil solution that has not been validated by experimental evidence. Most experimental studies on the effect of BC in Al toxicity show a strong bias, because generally high ratios coincide with high BC values, and low ratios with high concentrations of Al. Moreover, in most experiments the ratio was manipulated by varying either BC or Al, which therefore cannot be used to show an interactive effect of BC and Al.

Our results show that the BC:Al ratio and the threshold value BC:Al =1 are insufficient to describe the direct effect of Al toxicity (Van Schöll *et al.*, 2004: Chapter 2). We grew seedlings of *Pinus sylvestris* and *Picea abies* in hydroponics, on a range of Al and BC concentrations, establishing BC:Al ratio's between 0.25 and 8 at several concentrations of Al. Shoot dry weight decreased significantly with BC:Al ratio's of 1 but with increasing concentrations of Al in solution. Aluminium concentrations were the overriding factor determining shoot and root dry weight and uptake of BC and Al. The assumption that Al toxicity is determined by reduced uptake of BC is not supported. Shoot growth did not decrease due to reduced uptake of BC alone, because growth reduction occurred when tissue concentrations of BC were adequate. Increases of BC in the solution resulted in increased in shoot concentrations of BC to above the deficiency level, but this was not enough to counteract the toxic effects of Al. The concentrations of BC in solution had a positive effect on the uptake of BC and –for *Picea abies* but not *P. sylvestris*- growth, which could partly alleviate

the negative effect of Al. Shoot growth decreased significantly with increasing Al concentration in solution with constant BC:Al ratio of 1. Growth and concentrations of Al and BC were better described by assuming an additive effect of Al and BC than by an interactive effect via the BC:Al ratio.

These results may help to explain why correlation between forest health and calculated critical loads of atmospheric deposition in large scale studies is weak (Klap *et al.*, 2000). Critical load maps of atmospheric deposition are important instruments for policy making and the implementation of control measurements (Hettelingh *et al.*, 1995). The BC:Al ratio is widely used as a chemical criterium in the calculation of critical loads of atmospheric deposition (De Vries *et al.*, 2000, Posch *et al.*, 1997, Sverdrup & De Vries, 1994).

### *4.6 Do ectomycorrhizal fungi protect their host tree from $Al^{3+}$ toxicity?*

Ectomycorrhizal fungi may contribute to higher tolerance of their host tree to Al. Most experiments on the effect of aluminium on tree growth have been done with nonmycorrhizal tree seedlings, whereas in the natural situation most roots are colonised by ectomycorrhizal fungi. In several experiments ectomycorrhizal tree seedlings maintained higher growth rates than nonmycorrhizal seedlings under Al toxicity (Cumming & Weinstein, 1990a, b, Göransson & Eldhuset, 2001, Hentschel *et al.*, 1993, Schier & McQuattie, 1995, 1996). This does not necessarily mean that ectomycorrhizal fungi *protect* the tree, as they generally improve growth of their host tree through better nutrient and water supply, and it is impossible to separate these effects in pot experiments (Jentschke & Godbold, 2000, Meharg, 2003). As shown by Schier and McQuattie (1996) increased nutrient supply alone can partly mitigate Al toxicity effects of tree seedlings

Mineral tunnelling by ectomycorrhizal fungal hyphae will give the tree a direct access to base cations (Van Breemen *et al.*, 2000a). By directly translocating the weathering products Ca, Mg and K from these protected microsites to the roots of their host plants, the uptake is uncoupled from the toxic bulk soil solution. By quantifying the contribution of tunnelling to total weathering it could be calculated that release of Ca and K from feldspar tunnelling in the upper most 2 centimeters of the mineral soil is at the most 1 resp 1.7 g $ha^{-1}year^{-1}$ (Smits *et al.*, 2005). The total amount of weathering products from tunnelling is not expected to be much higher if the whole soil profile is considered, as tunnel intensity declines rapidly with soil depth. The total release of base cations from mineral tunnelling is negligible compared to forest tree uptake (on average 13 resp 21 kg $ha^{-1}year^{-1}$ for Ca and K respectively (Johnson & Lindberg, 1992b).

Ectomycorrhizal fungi have been hypothesised to protect the tree roots from aluminium toxicity by excluding aluminium from the root apoplast and improving the uptake of base cations (Finlay, 1995). Al toxicity is confined to root apex. Uptake of Ca and Mg, which is impaired by Al, occurs mainly at the root apex (Häussling *et al.*, 1988). In the ectomycorrhiza, the root tip is enveloped by network of hyphae (sheath), which extends into the apoplast of the root cortex (Hartig net). It seems likely that the uptake of Ca, Mg and Al is modified by the presence of the sheath and Hartig net. Lower shoot concentrations of Al in ectomycorrhizal trees cannot be used to show Al exclusion. Ectomycorrhizal seedlings generally have higher growth rates at both control and toxic Al treatments, which may caused dilution of Al, so that concentrations do not reflect the uptake or exclusion capacity.

Ectomycorrhizal colonisation does not physically protect the host tree from aluminium toxicity (Van Schöll *et al.*, 2005: Chapter 3). We compared the uptake and exclusion capacity of ectomycorrhizal and nonmycorrhizal tree seedlings with a semi-hydroponic experimental system. Hereby, optimal nutrient supply to both nonmycorrhizal and ectomycorrhizal seedlings is ensured and the effect of growth differences through better soil exploration the ectomycorrhizal hyphae is avoided. Under these conditions, colonisation by ectomycorrhizal fungi did not protect the seedlings from aluminium toxicity. Uptake of Ca and Mg was not affected by ectomycorrhizal colonisation, but surprisingly, shoot concentration of Al was slightly increased in the ectomycorrhizal seedlings. This must have been the result of higher translocation, as shoot dry weights of ectomycorrhizal and nonmycorrhizal seedlings were equal.

Ectomycorrhizal fungi can transport Al (Smits, 2005), but this is not likely to have caused the higher shoot concentrations, as root concentrations of Al in ectomycorrhizal and nonmycorrhizal seedlings did not differ significantly. Concentrations of Al in hyphae (up to 0.4 mg Al $g^{-1}$ d.w.) are below the concentrations encountered in tree roots (up to 18 mg Al $g^{-1}$ d.w root). Furthermore, transport of Al through the ectomycorrhizal hyphae is presumably in the form of Al-organic anions complexes, which do not bind to the cell wall, and are not taken up by the tree roots.

### *4.7 Exudation of LMW organic anions in response to Al toxicity*

Ectomycorrhizal fungi might protect their host tree from aluminium toxicity by the exudation of LMWOA. Al-induced exudation of LMWOA seems a general response mechanisms of Al tolerant varieties of crop plants (Ryan *et al.*, 2001). Tree seedlings have also been found to increase the exudation of oxalate under Al toxicity, and this might be increased by ectomycorrhizal fungi (Ahonen Jonnarth *et al.*, 2000). In forest ecosystems, aluminium toxicity normally coincides with low P and

Mg availability, which can also enhance the exudation of LMWOA (Van Schöll *et al.*, 2006: Chapter 5).

Aluminium induced the exudation of oxalate and this was increased by P and Mg deficiencies (Chapter 4). We used an axenic system to compare the separate the effects of aluminium toxicity and P and Mg deficiency on LMWOA exudation by ectomycorrhizal and nonmycorrhizal tree seedlings. Furthermore, we tested if simultaneous Al toxicity and Mg and P deficiency had a synergistic effect on the exudation of LMWOA. Oxalate exudations was induced by Al, and exudation was further enhanced by simultaneous Al toxicity and Mg and P deficiency. This latter effect was most profound in nonmycorrhizal seedlings, which only exuded low amounts of oxalate under Al toxicity alone, and in seedlings colonised by *Rhizopogon roseolus*. The enhanced exudation was not an effect of Mg of P deficiency alone, as Mg or P deficiency without Al did not induce the exudation of oxalate. This is in contrast to the results obtained before (Van Schöll *et al.*, 2006: Chapter 5), where we observed an increased exudation of oxalate in response to Mg and P deficiency. This discrepancy is attributed to differences in timing and experimental set up, which might also explain the substantial lower concentration of LMWOA found here. Differences among seedlings colonised by different species of ectomycorrhizal fungi were as big or bigger than between nonmycorrhizal and ectomycorrhizal seedlings. It seems that although ectomycorrhizal fungi can increase oxalate exudation, both symbionts have the ability to respond to external stresses. It appears unlikely that trees completely depend on ectomycorrhizal fungi for protection against aluminium toxicity. Some species, however, may increase Al tolerance through an enhanced exudation of oxalate. Further research with ectomycorrhizal species from sites with high versus low aluminium in the solution can elucidate if this is a general mechanism in Al tolerance.

## Concluding remarks

Our results strongly support a role of ectomycorrhizal fungi in weathering, podzolisation, plant tolerance to Al and nutrition. The generalisation of the effects of ectomycorrhizal fungi as observed in our experiments to the natural situation is, however, limited by the species-specificity of the responses of ectomycorrhizal fungi (summarised in Table 1) and our still limited knowledge on ectomycorrhizal species distribution. The use of molecular methods, as developed within this research programme, will enable studies linking the distribution and abundance of ectomycorrhizal fungi to their functional roles in weathering and plant nutrition under changing environmental conditions.

**Table 1** Ectomycorrhizal species-specific responses. Symbols denote enhanced (+), reduced (--) or no effect (0) of ectomycorrhizal *P. sylvestris* seedlings compared to nonmycorrhizal seedlings, except for Al transport, where figures denote transport (+) or no transport (0) of fungi in pure culture

| | Al transport | Muscovite weathering | Hornblende weathering | Oxalate | Malonate | Total LMWOA |
|---|---|---|---|---|---|---|
| *Paxillus involutus UK* | + | + | 0 | + | -- | 0 |
| *Paxillus involutus* | 0 | . | . | . | . | . |
| *Suillus bovinus* | 0 | 0 | 0 | . | | |
| *Piloderma croceum* | 0 | 0 | 0 | + | 0 | 0 |
| *Rhizopogon roseolus* | + | . | . | . | . | . |
| *Hebeloma longicaudum* | . | . | . | 0 | -- | -- |

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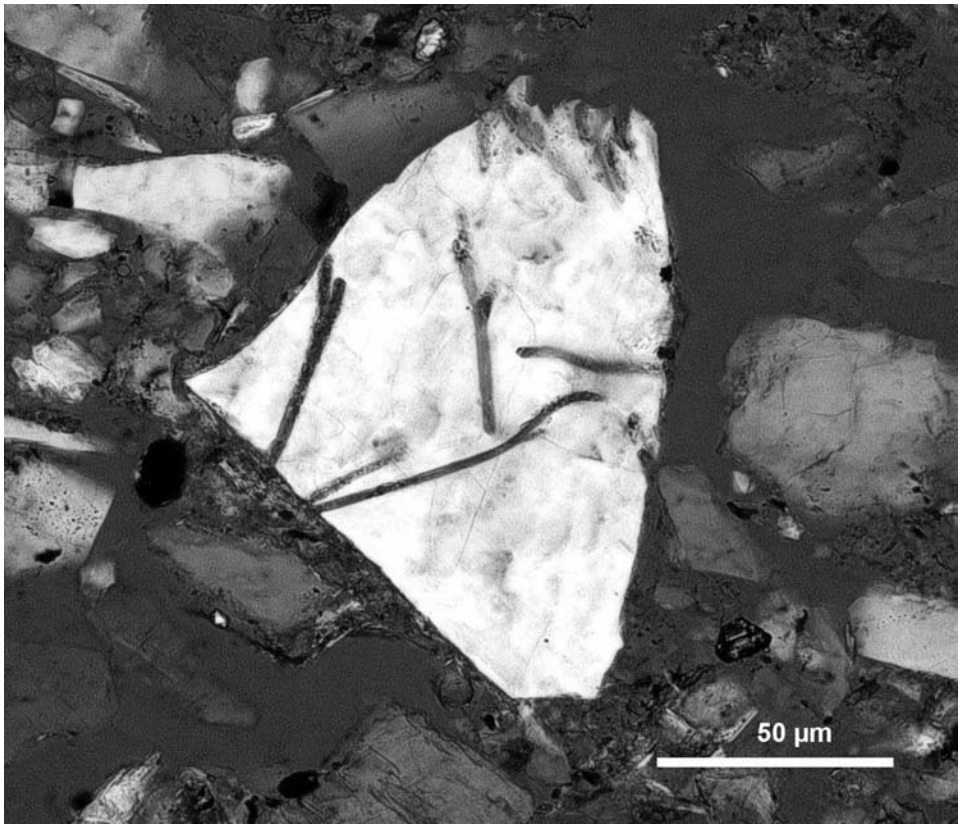

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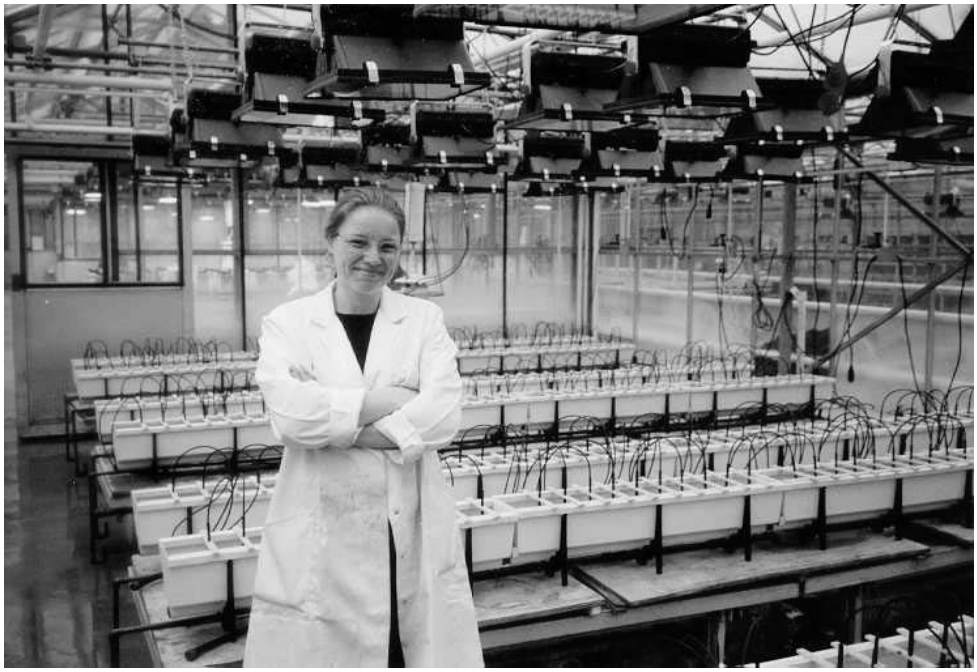
# Summary/Samenvatting



## Summary

Ectomycorrhizal fungi are soil fungi that form a mutualistic association with roots of host plants, mostly trees and shrubs. Ectomycorrhizal fungi are, with few exceptions, completely dependent on their host for carbohydrates, while the host plants receive water and nutrients taken up by the fungus. The finding of microscopic-small tunnel-like pores in mineral grains has triggered the interest in the role of ectomycorrhizal fungi in mineral weathering and plant uptake of base cations. Mineral weathering is a primary source of the base cations potassium (K), calcium (Ca) and magnesium (Mg) in forest ecosystems. Low molecular weight organic anions (LMWOA) like citrate and oxalate are strong biological weathering agents. Ectomycorrhizal fungi were hypothesised to be responsible for mineral tunnelling through the exudation of LMWOA at their hyphal tips, with mineral tunnelling reflecting the weathering capacity of ectomycorrhizal fungi. If ectomycorrhizal fungi can improve plant uptake of base cations and enhance the exudation of LMWOA, this might play an important role in counteracting the negative effect of Al toxicity and base cation deficiencies.

Aluminium toxicity is considered an important factor in forest deterioration attributed to atmospheric deposition of acidifying pollutants. Symptoms of Al toxicity are stunted root growth and deficiencies of phosphorus (P), calcium (Ca) and magnesium (Mg). Aluminium blocks ion channels over the root cell wall, and decreases the uptake of Ca and Mg specifically by kinetic processes in the root cell walls. Increased supply of Ca and Mg can -partly- mitigate Al toxicity. Aluminium toxicity in trees is often described by the ratio of base cations (BC) to Al in the soil solution, with a threshold level of 1 for BC:Al below which potentially adverse effects on tree growth are expected.

In chapter 2 we evaluated the validity of the assumptions that Al toxicity is determined reduced uptake of BC, and that the BC:Al ratio accurately describes uptake of BC and Al. Seedlings of *Pinus sylvestris* (Scots pine) and *Picea abies* (Norway spruce) were grown in nutrient solution with a range of Al and BC (here Ca+Mg) concentrations, giving a range of BC:Al ratios from 0.25 to 8. Increasing concentrations of dissolved Al reduced growth of *P. sylvestris* and *P. abies*. These growth reductions were not (*P. sylvestris)* or only partly (*P. abies*) counteracted by increasing

concentrations of dissolved BC. Concentrations of BC in shoot and root decreased with increasing Al in solution, and increased with increased concentrations of BC in solution. Growth reductions were, however, not solely a result of BC deficiencies, as growth reduction already occurred in tree seedlings that maintained adequate concentrations of BC. All growth and uptake variables were better described by the absolute concentrations of Al and BC than by the BC:Al ratio in solution. Shoot growth decreased significantly as Al increased at a constant BC:Al ratio of 1. Our results show that the BC:Al ratio and the threshold value BC:Al =1 are insufficient to describe the direct effect of Al toxicity.

A factor that is not accounted for in the determination of Al toxicity on tree growth is that most tree roots are ectomycorrhizal. Ectomycorrhizal tree seedlings have higher growth rates and lower Al concentrations than nonmycorrhizal seedlings under Al toxicity. Ectomycorrhizal fungi may enhance growth by improved water and nutrient uptake through better soil exploration by the hyphae, thereby counteracting the effect of Al-induced stunted root growth. Ectomycorrhizal fungi may also *protect* their host trees against the negative effects of Al toxicity by the exclusion of Al and improved uptake of base cations or by the exudation of LMWOA that can detoxify Al.

We tested if ectomycorrhizal fungi can physically exclude Al and improve uptake of Ca and Mg (Chapter 3). The root apex is the main site of Al toxicity and Ca and Mg uptake. Because ectomycorrhizal fungi form a dense network around the root tip and in the apoplast of the root cortex, they may affect uptake of Ca, Mg and Al. Nonmycorrhizal or ectomycorrhizal seedlings of *P. sylvestris* were continuously drip-irrigated with nutrient solution with or without Al. Adding aluminium significantly depressed growth and Ca and Mg concentrations of seedlings, and increased Al concentration and mycorrhizal colonization of the seedlings. Yet growth was not affected by mycorrhizal colonization. In contrast to our expectations, ectomycorrhizal fungi did not physically exclude Al. Ectomycorrhizal colonisation increased the Al concentration in the needles, but did not affect concentrations of Ca and Mg.

In chapter 4 we tested the effect of aluminium toxicity with or without simultaneous P and Mg deficiency on the exudation of LMWOA by ectomycorrhizal fungi. LMWOA can reduce Al toxicity by forming strong non-toxic complexes with Al. In forest ecosystems, aluminium toxicity normally coincides with low P and Mg availability, which may also enhance the exudation of LMWOA. Nonmycorrhizal or ectomycorrhizal seedlings of *P. sylvestris* were cultured on a layer of glass beads with nutrient solution. High concentrations of dissolved aluminium increased exudation of oxalate in both nonmycorrhizal and ectomycorrhizal seedlings. Colonisation by

the ectomycorrhizal fungi *Paxillus involutus* and *Rhizopogon roseolus* increased the exudation of oxalate under Al toxicity compared to the nonmycorrhizal seedlings, but colonisation by *Laccaria bicolor, Piloderma croceum* or *Suillus bovinus* had no significant effect. Simultaneous Al toxicity with Mg and P deficiency significantly enhanced this exudation, especially in nonmycorrhizal seedlings, which produced only low amounts of oxalate under Al toxicity alone. Without Al, Mg or P deficiency did not induce the exudation of oxalate.

We expected ectomycorrhizal fungi to enhance mineral weathering under BC deficiencies by the exudation of LMWOA. In chapter 5 we tested the effect of deficiencies of Mg, K or P on the exudation of LMWOA by ectomycorrhizal fungi and tree seedlings. Ectomycorrhizal fungi and *Pinus sylvestris* seedlings were cultured in symbiosis and in isolation on glass beads with nutrient solution or with sand as a rooting medium. The concentrations of LMWOA were substantially higher than in the experiment described in chapter 4, which is attributed to differences in timing and experimental set up. Ectomycorrhizal colonisation did not affect or even decreased the total amount of LMWOA exuded compared to nonmycorrhizal tree seedlings. Deficiency of P, but not of Mg or K, increased the exudation of total LMWOA. Nonmycorrhizal seedlings exuded mainly malonate. Ectomycorrhizal colonisation with *P. involutus* and *Hebeloma longicaudum* significantly decreased the exudation of malonate, whereas the exudation of oxalate was increased by colonisation with *P. involutus* and *P. croceum*. Oxalate has a higher weathering capacity than malonate. Omitting Mg from the nutrient solution increased the exudation of oxalate by nonmycorrhizal and ectomycorrhizal seedlings alike, but in pure culture, *P. involutus* did not exude oxalate under Mg deficiency. Reduction of K supply did not significantly affect the oxalate exudation of either *P. involutus* or *P. sylvestris* in pure culture, but significantly increased the exudation of oxalate when they were grown in symbiosis. Reduced P supply increased the exudation of malonate by *P. involutus* in pure culture, whereas in symbiosis *P. involutus* strongly reduced malonate exudation compared to nonmycorrhizal seedlings under P deficiency in favour of oxalate.

The effect of ectomycorrhizal fungi on the oxalate exudation in response to Mg and K deficiencies correlated well to their effect on mineral weathering of K and Mg containing minerals as found in a pot experiment described in chapter 6. Nonmycorrhizal and ectomycorrhizal *P. sylvestris* seedlings were grown with or without the mineral muscovite as the only K-source or the mineral hornblende as the only Mg-source. Muscovite weathering was increased by tree seedlings by a factor of 1.7, and this weathering was even further increased to a factor of 3.3 after colonisation by

*P. involutus*, whereas colonisation by *S. bovinus* or *P. croceum* had no additional effect. Hornblende weathering increased by a factor 1.5-2 by tree seedlings, but without any additional effect of ectomycorrhizal fungi. The increased weathering was not *per se* reflected by increased uptake in the plant shoot. *P. involutus* enhanced dissolution K from muscovite but this did not result in increased K content of shoots compared to other tree seedlings. *Piloderma croceum* decreased allocation of Mg, dissolved from hornblende, to the shoot.

In chapter 7 we tested whether the *P. involutus* actively transports and forages for Mg under Mg deficiencies. Nonmycorrhizal and ectomycorrhizal *P. sylvestris* seedlings were grown in two-compartment dishes on a layer of glass beads and nutrient solution without any Mg. The two compartments were separated by a fine mesh, permeable to hyphae but not roots. Addition of Mg to the hyphal compartment stimulated hyphal foraging. Ectomycorrhizal seedlings had higher Mg contents than nonmycorrhizal seedlings. Hyphal foraging and transport of Mg was not the result of P supply or a passive co-transport of Mg with P, as addition of P to the hyphal compartment did not increased hyphal foraging or Mg content of the seedlings.

The work presented in this dissertation was conducted as part of the *"Rock-eating mycorrhizae: where, why how?"* research programme, that focussed on the formation of mineral tunnels and the role of ectomycorrhizal fungi in weathering. The results of the entire research programme are presented and discussed in chapter 8.

## Samenvatting

Ectomycorrhizaschimmels zijn bodemschimmels die een mutualistische verbinding aangaan met de wortels van gastplanten, grotendeels bomen en struiken. Ectomycorrhizaschimmels zijn, op enkele uitzonderingen na, voor hun voorziening van koolhydraten volledig afhankelijk van hun gastplant, waarbij de gastplanten water en voedingstoffen van de schimmels ontvangen. De vondst van microscopisch kleine, tunnelachtige poriën in mineraalkorrels heeft de interesse aangewakkerd voor de rol van ectomycorrhizaschimmels in mineraalverwering en in de opname van basische kationen door de plant. Mineraalverwering is een belangrijke bron van de basische kationen kalium (K), calcium (Ca) en magnesium (Mg) in bosecosystemen. De hypothese was dat ectomycorrhizaschimmels verantwoordelijk waren voor de tunnelvorming in de mineralen door de uitscheiding van organische anionen met laag moleculair gewicht (LMG). LMG organische anionen, zoals citraat en oxalaat, zijn namelijk sterke mineraalverweerders.

Als ectomycorrhizaschimmels de opname van basische kationen en de uitscheiding van LMG organische kationen kunnen verbeteren, kan dit een belangrijke rol spelen in het tegengaan van de negatieve effecten van aluminium (Al) toxiciteit en van tekorten aan basische kationen. Aluminium toxiciteit en tekorten aan basische kationen worden gezien als belangrijke factoren in de achteruitgang van bossen, die wordt toegeschreven aan de atmosferische depositie van verzurende elementen. Verzuring van de bodemoplossing veroorzaakt verhoging van de concentraties opgelost Al en de uitspoeling van basische kationen. Symptomen van Al toxiciteit zijn een geremde wortelgroei en een tekort aan fosfor (P), calcium (Ca) en magnesium ((Mg). Aluminium blokkeert het transport van ionen door de celwand en verlaagt de opname van met name Ca en Mg door kinetische processen op de celwanden van de wortelcellen. Een verhoogd aanbod van Ca en Mg kan de Al toxiciteit verminderen. De Al toxiciteit bij bomen wordt vaak beschreven met de ratio tussen basische kationen (BK) en Al in de bodemoplossing, waarbij de waarde 1 voor BK:Al geldt als een kritische waarde waaronder negatieve effecten op de groei van bomen worden verwacht.

In hoofdstuk 2 evalueren we de onderliggende veronderstellingen dat Al toxiciteit bepaald wordt door een gereduceerde opname van BK, en dat opname van BK en Al

accuraat beschreven wordt door de BK:Al ratio in de bodemoplossing. Zaailingen van *P. sylvestris* (grove den) en *Picea abies* (fijnspar) werden gekweekt in voedingsoplossingen met een reeks van Al en BK (hier Ca+Mg) concentraties, met BK:Al ratios van 0,25 tot 4. Oplopende Al concentraties in de voedingsoplossing veroorzaakten een afname van groei, die bij *P. sylvestris* niet werden opgeheven door oplopende concentraties van BK in oplossing, en bij *P. abies* slechts gedeeltelijk. De concentraties van BK in de spruit (naalden en stam) en wortel werden lager bij oplopende concentraties van Al in voedingsoplossing, en hoger bij oplopende concentraties van BK in oplossing. De lagere groei werd echter niet geheel veroorzaakt door een tekort aan BK, want groei was ook lager in zaailingen met voldoende hoge BK concentraties. Alle groei- en opnamevariabelen werden beter beschreven door de werkelijke concentraties van Al en BK in oplossing dan door de ratio van BK:Al in oplossing. Groei van de spuit was lager bij oplopende concentraties van Al in oplossing waarbij de ratio van BK:Al constant 1 bleef. Onze resultaten laten zien dat de BK:Al ratio en de kritieke waarde van 1 voor BK:Al ratio het directe toxische effect van Al onvoldoende beschrijven.

Een factor waarmee in de beschrijving van aluminium toxiciteit bij bomen geen rekening wordt gehouden, is dat de meeste boomwortels in symbiose groeien met ectomycorrhizaschimmels. Gemycorrhizeerde boomzaailingen hebben een hogere groeisnelheid en een lagere Al concentratie dan niet-gemycorrhizeerde boomzaailingen. Ectomycorrhizaschimmels kunnen de groei verbeteren door een verhoogde toelevering van water en voedingsstoffen vanuit de schimmeldraden die de bodem beter "doorwortelen", waarmee het negatieve effect van Al op de wortelgroei wordt gecompenseerd. De hypothese was dat ectomycorrhizaschimmels hun gastplant ook *beschermen* tegen de negatieve effecten van Al, door het buitensluiten van Al en verhogen van de opname van BK, of door het uitscheiden van LMG organische anionen die Al detoxificeren.

In hoofdstuk 3 hebben we getest of ectomycorrhizaschimmels Al kunnen buitensluiten en de opname van Ca en Mg kunnen verhogen. Aluminium toxiciteit en opname van Ca en Mg vinden vooral plaats in de worteltop. Ectomycorrhizaschimmels vormen een netwerk van schimmeldraden rond de worteltop en in de ruimte tussen de cellen van de buitenste lagen van de wortel. Het daarom lijkt waarschijnlijk dat ectomycorrhizaschimmels de opname van Al, Ca en Mg beïnvloeden. Niet-gemycorrhizeerde en gemycorrhizeerde zaailingen van *P. sylvestris* werden, middels druppelirrigatie, gevoed met een voedingsoplossing met of zonder Al. Toevoeging van Al leidde tot een significante groeiafname van de zaailingen, verlaagde concentraties van Ca en Mg, verhoogde concentraties van Al en een toename van het aan-

deel worteltoppen dat gemycorrhizeerd was. Groei werd echter niet beïnvloed door ectomycorrhizaschimmels. In tegenstelling tot wat we verwacht hadden, werd Al niet buitengesloten door ectomycorrhizaschimmels. Ectomycorrhizaschimmels verhoogden de Al concentraties in de naalden, maar hadden geen effect op de concentraties van Ca en Mg.

In hoofdstuk 4 hebben we getest of ectomycorrhizaschimmels onder invloed van Al toxiciteit de uitscheiding van LMG organische anionen verhogen, en of deze uitscheiding beïnvloed wordt door gelijktijdige tekorten aan P en Mg. LMG organische anionen kunnen Al toxiciteit tegengaan door sterke, niet-toxische complexen te vormen met Al. In bosecosystemen valt Al toxiciteit gewoonlijk samen met een lage P en Mg beschikbaarheid. Tekorten aan P en Mg worden ook verondersteld de uitscheiding van LMG organische anionen te verhogen. Niet-gemycorrhizeerde en gemycorrhizeerde zaailingen van *P. sylvestris* werden gekweekt op een laag glasparels met voedingsoplossing. Aluminium leidde tot een uitscheiding van oxalaat in zowel gemycorrhizeerde als niet-gemycorrhizeerde zaailingen. Mycorrhisatie door de schimmels *Paxillus involutus* en *Rhizopogon roselus* verhoogde de oxalaatuitscheiding ten opzichte van niet-gemycorrhizeerde zaailingen onder Al toxiciteit, maar mycorrhisatie met *Laccaria bicolor, Piloderma croceum* en *Suillus bovinus* leidde niet tot een significante verhoging. Gelijktijdige Al toxiciteit met Mg en P tekorten verhoogde de oxalaatuitscheiding, met name in de niet-gemycorrhizeerde zaailingen, die slechts weinig oxalaat uitscheidden in het geval van Al toevoeging zonder Mg en P tekorten.

De hypothese was dat ectomycorrhizaschimmels mineraalverwering versnellen door de uitscheiding van LMG organische anionen bij met tekorten aan basische kationen. In hoofdstuk 5 hebben we getest of ectomycorrhizaschimmels en boomzaailingen de uitscheiding van LMG organische anionen verhogen in reactie op tekorten aan MG, K of P. Ectomycorrhizaschimmels en P. sylvestris zaailingen werden zowel alleen als in symbiose opgekweekt, op glasparels of in zand gemengd met een voedingsoplossing. De gevonden concentraties aan LMG organische anionen waren aanzienlijk hoger dan die in het experiment beschreven in hoofdstuk 4. Dit kan waarschijnlijk worden verklaard door verschillen in tijdsbestek en in proefopzet. Gemycorrhizeerde zaailingen hadden een gelijke of lagere concentratie aan totaal LMG organische anionen dan niet-gemycorrhizeerde zaailingen. Tekorten aan P, maar niet aan Mg of K, gaven een verhoogde uitscheiding van LMG organische anionen. Niet-gemycorrhizeerde zaailingen scheidden voornamelijk malonaat uit. Mycorrhisatie met *P. involutus* of *Hebeloma longicaudum* leidde tot een verlaagde uitscheiding van malonaat, terwijl de uitscheiding van oxalaat verhoogd werd door

mycorrhisatie met *P. involutus* of *P. croceum*. Oxalaat heeft een hogere verweringscapaciteit dan malonaat. Weglaten van Mg uit de voedingsoplossing verhoogde de uitscheiding van niet-gemycorrhizeerde en gemycorrhizeerde zaailingen in gelijke mate. Verlaging van de K toevoeging leidde niet tot een significant effect op de oxalaat uitscheiding van *P. involutus* of *P. sylvestris* als zij alleen werden opgekweekt, maar gaf een significante verhoging van oxalaat uitscheiding als zij in symbiose groeiden. Reductie van de P toevoeging verhoogde de uitscheiding van malonaat door *P. involutus* als die alleen groeide, maar in symbiose met *P. sylvestris* veroorzaakte *P. involutus* een afname van malonaat onder P tekort in het voordeel van oxalaat uitscheiding.

De oxalaat-uitscheiding door gemycorrhizeerde en niet-gemycorrhizeerde zaailingen in reactie op Mg en K tekorten kwam goed overeen met hun verweringscapaciteit in een pot experiment, beschreven in hoofdstuk 6. Niet-gemycorrhizeerde en gemycorrhizeerde zaailingen werden opgekweekt met of zonder het mineraal muscoviet als enige bron van K, en met of zonder het mineraal hoornblende als enige bron van Mg. Verwering van muscoviet werd door niet-gemycorrhizeerde zaailingen met een factor van 1,7 verhoog., Bij mycorrhisatie met *P. involutus* werd deze factor verhoogd tot 3,3. Mycorrhisatie met *S. bovinus* of *P. croceum* had geen extra effect had. Verwering van hoornblende werd door zowel niet-gemycorrhizeerde als gemycorrhizeerde zaailingen verhoogd met een factor 1,5-2. De verhoogde verwering leidde niet *per se* tot een verhoogde opname van K en Mg in de spruit. De extra muscoviet verwering door *P. involutus* leidde niet tot een hoger K gehalte in de spruit vergeleken met andere zaailingen. *Piloderma croceum* verlaagde zelfs de opname van Mg, afkomstig uit hoornblende, in de spruit.

In hoofdstuk 7 hebben we getest of *P. involutus* bij Mg tekorten ook actief op zoek gaat naar Mg en dit transporteert door de schimmeldraden. Niet-gemycorrhizeerde en gemycorrhizeerde zaailingen van *P. sylvestris* werden opgekweekt in tweecompartiment Petri schaaltjes, op een laag glasparels met voedingsoplossing zonder Mg. De twee compartimenten werden gescheiden door een fijnmazig net, doorlaatbaar voor schimmeldraden maar niet voor wortels. De toevoeging van Mg aan het schimmel-compartiment (niet toegankelijk voor wortels) verhoogde de groei van schimmeldraden vanuit het wortelcompartiment. Gemycorrhizeerde zaailingen hadden de hoger Mg gehalte dan niet-gemycorrhizeerde zaailingen. De toename van schimmeldraden in het schimmelcompartiment, alsook het transport van Mg, waren niet het gevolg van een verhoogd P aanbod of een passief co-transport van Mg met P: toevoeging van P aan het schimmelcompartiment leidde niet tot verhoogde schimmelgroei of een verhoogd Mg gehalte in de zaailingen.

Het werk dat in dit proefschrift is beschreven werd uitgevoerd als onderdeel van het onderzoeksprogramma "Gesteente-etende mycorrhizae: waar, waarom hoe?", dat zich richtte op de vorming van tunnels in mineralen en de rol van ectomycorrhiza-schimmels in mineraal verwering. De resultaten van het volledige onderzoeksprogramma worden besproken in hoofdstuk 8.

## Dankwoord

Mijn onderzoek begon in de kas, met grote bakken en veel potjes. De verzameling groene sprietjes noemde ik hoopvol Laura's Bos. Langzaamaan verschoof het werk naar steeds kleinere potjes in de klimaatkamer en uiteindelijk zelfs naar steriele buisjes en petrischaaltjes. Totdat ik uiteindelijk het lab bijna niet meer uitkwam. Plastic korrels, grind, zand en perliet werden vervangen door glasparels. Sommige proeven lukten wonderwel, anderen waren minder bruikbaar. Het werken met meerdere organismen is extra leuk maar geeft soms ook extra complicaties. Uiteindelijk is het proefschrift af, en kijk ik terug op een periode waarin ik veel heb geleerd, niet alleen op het wetenschappelijke maar ook op het persoonlijke vlak. Nu is het dan ook tijd om alle mensen te bedanken die er op directe of indirecte wijze een bijdrage geleverd hebben.

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Door de jaren heen heb ik ontzettend veel experimenten uitgevoerd, waarbij ik kon rekenen op de hulp en inzet van een grote groep mensen. Jaap Nelemans, ik kon

altijd rekenen op je hulp en op je brede ervaring. Je bleef altijd heel geïnteresseerd in mijn onderzoek, ook toen ik buiten jouw expertiseveld verder ging werken. Willeke van Tintelen, samen hebben we heel wat boompjes geplant. Je hebt veel werk met en voor mij gedaan, zonder welke dit proefschrift minder dik zou zijn. Het was altijd prettig om met jou te werken. Eef Velthorst, de metingen aan organische anionen waren voor onschatbare waarde voor dit proefschrift en voor mijn motivatie op een moment dat het allemaal niet meer leek te lukken. Bedankt ook voor de goede gesprekken. Willem Menkveld, zonder het zware werk dat jij voor mij hebt gedaan was het me een stuk zwaarder gevallen. Het aantal kilo's zand dat jij voor mij heen en weer gesleept en behandeld hebt loopt in de honderden. Daarnaast heb je met eindeloos geduld geholpen potjes te vullen en boompjes water te geven, ook in de weekenden. Peter Pellen, naast de verzorging van mijn proeven heb je ook prachtige foto's gemaakt, die algemeen veel bewondering oogsten en dit proefschrift verfraaien. Rijndert de Fluiter, jij hebt duizenden gaatjes in de twee-compartiment potten geboord. Jammer dat de resultaten niet in dit proefschrift terecht zijn gekomen. Arie Brader, bedankt voor de verzorging van mijn proeven. Simon Maasland, voor het maken van handige materialen en proefsystemen. Tamas Salanki, voor de onderhoudende gesprekken tijdens eentonig werk. De mensen van het chemisch lab, en in het bijzonder Arie van de Berg, Monique Driessen Gerdine Gaikhorst, Peter Nobel en Gerlinde Vink: bedankt voor alle metingen die jullie verricht hebben en voor alle geduldige uitleg en aanwijzingen. Ik voelde me altijd welkom op het lab.

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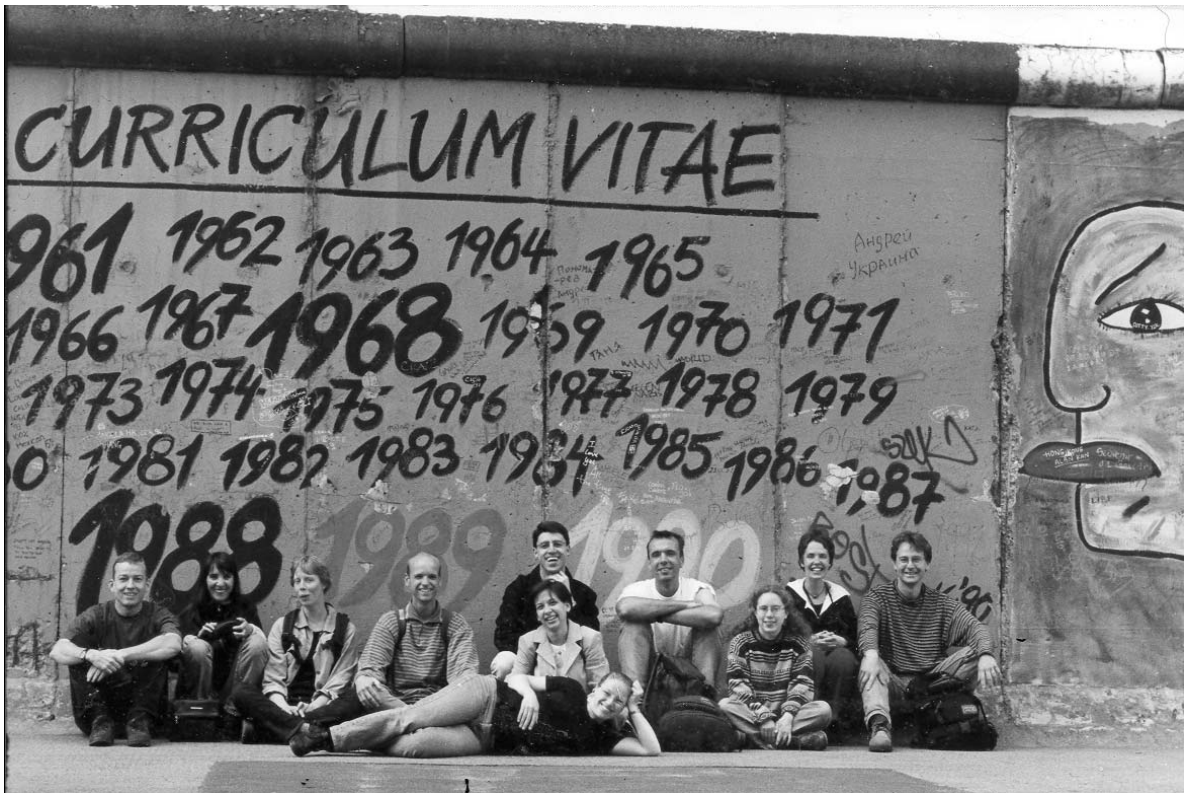
Laura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## Curriculum vitae

Laura van Schöll was born on November 27th 1967 in Rotterdam, The Netherlands. In 1990 she started the study "Tropical Landuse" at Wageningen Agricultural University (now Wageningen University). She conducted two thesis projects. The first was on the effect of Cd and Cu on plant uptake, plant growth and the induction of phytochelatine, at the Chair group of Soil Fertility and Plant Nutrition, under the supervision of Willem Keltjens. The second concerned the effect of low temperatures on the mineralisation of organic material, combining a modelling and experimental approach, at the Chair group of Theoretical Production Ecology, supervised by Peter Leffelaar and Anne-Marie van Dam. She graduated in 1995 with the degree of ir. (equivalent to M.Sc.).

Following graduation, she wrote a handbook on Soil Fertility Management in the series AGRODOK, published by the Agromisa foundation, Wageningen, aimed at people working directly with small subsistence farmers in the (sub)tropics.

In 1997, she conducted a literature study on the dynamics of soil micro-organisms after addition of organic matter and the resulting effect on nitrogen mineralization. This study produced a Ph.D. research proposal, which was supported by Rudy Rabbinge and Peter Leffelaar of the Chair group of Theoretical Production Ecology and Lijbert Brussaart of the Chair group Soil Biology, Wageningen University. In 1998, she was employed at the Chair of Theoretical Production Ecology, where she worked on a database to predict the effect of soil-born diseases on crop production in order to optimise crop rotation.

From September 1998 on she worked as a 'Ph.D. research trainee' (Onderzoeker in Opleiding) at the department of Soil Quality, Wageningen University. The focus of her research was the response of tree seedlings and ectomycorrhizal fungi to aluminium toxicity and base cation deficiencies. The main outcomes are described in this thesis.

## Publication list of Laura van Schöll

**Hoffland E, Smits MM, van Schöll L, Landeweert R. 2005.** Rock-eating mycorrhizas: mobilizing nutrients from minerals? *Plant Nutrition for Food Security, Human Health and Environmental Protection*, edited by Li CJ, Zhang FS, Doberman A, Hinsinger P, Lambers H, Li XL, Marschner P, Maene L, McGrath S, Oenema O, Peng SB, Rengel Z, Shen QR, Welch R, Wirén NV, Yan XL and Zhu YG, Beijing, China. Tsinghua University Press, p. 802-803.

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