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Chelating organic substances in roots and root exudates and their potential role in aluminium resistance of Norway spruce (*Picea abies* [L.] Karst.)

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Abstract

A major constraint for plants growing on acid soils is the presence of high concentrations of Al^{3+}, which is released from soil minerals into soil solution by weathering processes. Adaptation to these adverse conditions has been extensively studied for crop species, where it is often achieved by root-induced changes in the rhizosphere, such as local pH changes or root exudation of chelators for Al. Conifers like Norway spruce (*Picea abies* [L.] Karst.) are widespread on naturally acid soils in Northern and Central Europe and are resistant to higher Al concentrations than most crop species. The aim of the present thesis was to investigate if Norway spruce owes its Al resistance to similar mechanisms as have been detected in several crop species. In particular, the effect of Al on the root accumulation and exudation of chelating compounds was studied, as the formation of organic Al complexes can greatly reduce the phytotoxicity of Al. Experiments were conducted both in hydroculture and in solid substrate and addressed the following aspects.

In a first experiment, root exudation of 3-year-old Norway spruce trees under non-sterile conditions in hydroculture was investigated quantitatively and qualitatively and the effect of Al on root exudation was studied. In addition, the distribution and binding forms of Al in roots were studied in detail by EDX analysis and extraction of fresh roots. In this system, no aliphatic organic acids were detected in the exudates, but chelating phenolic substances were present in the exudates and there were indications for low-charged Al-phenolic complexes adsorbed to the root exchange sites.

In order to check if the absence of organic acids in the exudates is a peculiarity of Norway spruce or if it can be attributed to rapid microbial degradation, a similar experiment was conducted with axenically grown seedlings, where maximum possible exudation was studied. This study focused especially on exudation and root content of organic acids. The proportion of organic acids in exudates was generally low. Acetate, oxalate, formate, and lactate were found in the exudates and in roots, while malate and citrate were only found in roots. No significant effects of Al on exudation of organic acids were detected. In roots, only acetate was enhanced by Al. These seedlings, which had been well supplied with P before, constitutively released phosphate from their roots, which precipitated as Al phosphate at the root surface in Al treatments.

In order to allow interpretation of these results with respect to natural conditions, where roots experience mechanical resistance during growth in solid substrate, and where mycorrhizal colonisation is frequent, an additional experiment was conducted, in which Norway spruce seedlings were grown for 5 months in perlite. In this experiment, the factors Al addition and ectomycorrhizal inoculation were varied. The amount of oxalate released by the roots was similar to the amounts of Al adsorbed to roots, indicating that the size of the exudate pool can have some effect on Al speciation in the rhizosphere. However, confirming the results from the hydroponic experiments, the release of oxalate was not affected by Al. Inoculation with *Hebeloma crustuliniforme* reduced oxalate accumulation in roots and also tended to reduce the amount of oxalate found in exudates. Nevertheless, inoculation had a beneficial effect on biomass production at high Al addition. Net P release occurred only from non-inoculated plants and was clearly enhanced by Al under the conditions in this experiment, suggesting that it might serve to reduce Al^{3+} activity in the rhizosphere. In inoculated roots, the fungus either prevented or compensated the P exudation from roots.

In order to investigate potential Al chelators other than aliphatic acids, a detailed study was included on effects of Al and inoculation on phenolic metabolites. Several minor substances were found to be positively affected by Al but remained mostly unaffected by inoculation, indicating that fungi and Al evoke different metabolic reactions. Another group of compounds reacted differently in inoculated and non-inoculated roots, indicating that Al interferes with some processes involved in mycorrhiza formation. Exudation of phenolics into the substrate was too low to be of significance for Al detoxification outside roots.

In summary, constitutive exudation of chelating compounds (oxalate, phenolic substances, phosphate) probably contributes in part to the detoxification of Al in Norway spruce. As Al does not increase exudation of organic chelators above the baseline level, although large concentrations of Al can occur in acid forest soils, it seems unlikely that such a process is of major importance for the Al resistance of Norway spruce. Exudation of phosphate can only be imagined to be of some relevance in the long term, if mycorrhizal symbiosis provides sufficient phosphate for the excretion process. By contrast, processes that detoxify Al in the apoplast, involving chelation by phenolic metabolites, may be of considerable importance in Al resistance of Norway spruce.

Zusammenfassung

Auf sauren Böden stellen hohe Konzentrationen an Al^{3+}, welches durch Verwitterungsprozesse aus den Bodenmineralen in die Bodenlösung freigesetzt wird, eine wesentliche Einschränkung für das Pflanzenwachstum dar. Die Anpassung an diese ungünstigen Bedingungen ist für landwirtschaftliche Nutzpflanzen gut untersucht. Bei diesen Arten wird sie in vielen Fällen durch wurzelinduzierte Veränderungen in der Rhizosphäre erreicht, wie zum Beispiel lokale pH-Wert-Veränderungen oder Wurzelausscheidungen von Substanzen, welche mit Al starke Chelatkomplexe bilden. Koniferen wie die Fichte (*Picea abies* [L.] Karst.) sind auf natürlicherweise sauren Böden in Nord- und Mitteleuropa weit verbreitet und vertragen höhere Al-Konzentrationen als die meisten landwirtschaftlichen Nutzpflanzen. Das Ziel der vorliegenden Arbeit war es, zu untersuchen, ob die Al-Resistenz der Fichte auf ähnliche Mechanismen zurückzuführen ist, wie sie in verschiedenen Nutzpflanzen beobachtet wurden. Ein Schwergewicht lag dabei auf der Auswirkung von Al auf die Wurzelgehalte und Ausscheidung von komplexierenden Verbindungen, da die Bildung von organischen Al-Komplexen die toxischen Wirkungen des Al auf Pflanzen stark herabsetzen kann. Es wurden Versuche in Hydrokultur und in einem festen Substrat durchgeführt, welche die folgenden Aspekte behandelten.

In einem ersten Versuch wurde die Wurzelausscheidung dreijähriger Fichten in Hydrokultur unter nicht-sterilen Bedingungen qualitativ und quantitativ betrachtet, und der Einfluss von Al auf das Exsudationsverhalten untersucht. Des weiteren wurden die Verteilung und die Bindungsformen von Al in den Wurzeln mit Hilfe von elektronendispersiver Röntgenmikroanalyse und der Extraktion frischer Wurzeln detailliert beschrieben. In diesem System wurden keine aliphatischen organischen Säuren in den Wurzelexsudaten gefunden. Phenolische Komplexbildner wurden jedoch in den Wurzelexsudaten gefunden und die Extraktion lieferte Hinweise darauf, dass niedrig geladene Komplexe zwischen Al und phenolischen Liganden an die Austauscherplätze der Wurzel adsorbiert sind.

Um zu überprüfen, ob das Fehlen organischer Säuren in den Wurzelexsudaten der Fichte eine artspezifische Besonderheit darstellt oder ob rascher mikrobieller Abbau dafür verantwortlich ist, wurde ein ähnlicher Versuch mit steril angezogenen Fichtensämlingen durchgeführt, womit die maximal mögliche Ausscheidung erfasst

werden sollte. Der Schwerpunkt dieses Versuchs lag auf der Wurzelausscheidung und den Wurzelgehalten organischer Säuren. Der Anteil dieser Säuren an den Wurzelexsudaten war einheitlich sehr niedrig. Acetat, Oxalat, Formiat und Lactat wurden in den Exsudaten und in den Wurzeln gefunden, während Malat und Citrat nur in den Wurzeln nachweisbar waren. Es wurde kein statistisch signifikanter Einfluss von Al auf die Exudation organischer Säuren gefunden. In den Wurzeln stieg lediglich der Acetatgehalt durch Al-Behandlung an. Es wurde jedoch beobachtet, dass diese Fichtensämlinge, welche zuvor gut phosphorversorgt gewesen waren, Phosphat aus ihren Wurzeln ausschieden, welches in Al-Behandlungen als Aluminiumphosphat auf der Wurzeloberfläche ausfiel.

Um diese Resultate auch im Hinblick auf natürliche Bedingungen interpretieren zu können, wo Wurzeln während des Wachstums in einem festen Substrat mechanischen Widerstand erfahren und häufig von Mykorrhizapilzen besiedelt sind, wurde ein weiterer Versuch durchgeführt, in welchem Fichtensämlinge für 5 Monate in Perlit angezogen wurden. Es wurde sowohl die Menge zugegebenen Aluminiums als auch die Beimpfung mit Mykorrhizapilzen variiert. In diesem Experiment wurde ähnlich viel Oxalat von den Wurzeln ausgeschieden, wie Al an die Wurzeln adsorbiert wurde. Daraus lässt sich schließen, dass die Menge ausgeschiedenen Oxalats einen gewissen Einfluss auf die Aluminiumspeziierung in der Rhizosphäre haben kann. Jedoch wurden die Ergebnisse aus der Hydrokultur bestätigt, dass Al keinen Einfluss auf die Oxalatausscheidung hat. Die Beimpfung mit *Hebeloma crustuliniforme* führte zu verringerten Oxalatgehalten in den Wurzeln und zu tendenziell verringerter Oxalatausscheidung. Dennoch hatte die Beimpfung eine positive Auswirkung auf die Biomasseproduktion bei hoher Al-Zugabe. Nur nicht-beimpfte Pflanzen gaben P ab, was bei hoher Al-Zugabe unter den Bedingungen in diesem Experiment verstärkt war. Diese Ausscheidung könnte also dazu dienen, die Al^{3+}-Aktivität in der Rhizosphäre zu verringern. In den beimpften Pflanzen verhinderte der Pilz entweder die P-Abgabe durch die Wurzeln oder er kompensierte sie durch vermehrte Aufnahme.

Eine weitere Untersuchung befasste sich innerhalb des gleichen Experiments mit einer weiteren Gruppe potentieller Al-Komplexbildner. Dabei standen der Einfluss von Al und Pilzbeimpfung auf phenolische Stoffwechselprodukte im Mittelpunkt. Die Gehalte einiger mengenmäßig untergeordneter Substanzen wurden durch Al positiv beeinflusst, während der Pilz keinen Einfluss auf sie hatte, was darauf hindeutet, dass Al und

Pilzbesiedlung unterschiedliche Stoffwechselreaktionen bei der Fichte bewirken. Eine weitere Gruppe von Verbindungen wurde unterschiedlich durch das Al beeinflusst, je nachdem ob die Pflanzen beimpft waren oder nicht. Diese Ergebnisse deuten darauf hin, dass Al in Prozesse der Mykorrhizabildung eingreift.

Die Ausscheidung phenolischer Substanzen in das Substrat war in diesem Versuch zu niedrig, um eine größere Bedeutung für die Entgiftung von Al außerhalb der Wurzeln zu haben.

Zusammenfassend lässt sich sagen, dass eine bestimmte Ausscheidung komplexierender Substanzen (Oxalat, phenolische Substanzen, Phosphat) ständig stattfindet und so teilweise zur Entgiftung von Al bei Fichten beiträgt. Da die Ausscheidung organischer Komplexbildner durch Al nicht erhöht wird, obwohl hohe Al-Konzentrationen in sauren Waldböden auftreten können, scheint es aber unwahrscheinlich, dass ein solcher Prozess eine wesentliche Rolle bei der Al-Resistenz der Fichte spielt. Die Ausscheidung von Phosphat kann langfristig nur dann eine gewisse Bedeutung haben, wenn symbiotische Mykorrhizapilze gleichzeitig ausreichend Phosphat für die Ausscheidung zur Verfügung stellen.

Dagegen könnten Prozesse, die zu einer Entgiftung des Al im Apoplasten der Wurzel führen, zum Beispiel durch Komplexierung mit phenolischen Liganden, von einiger Bedeutung für die Al-Resistenz der Fichte sein.

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Chapter 1
Introduction

1.1. Aluminium in soils

Following oxygen and silicium, aluminium is the third most abundant element in the earth's crust and in soils (Sposito 1989). A major environmental problem with aluminium is the toxic effect of some soluble Al species on plants growing in acid soils, when they are present at elevated concentrations in soil solution. The solution chemistry of Al is rather complex. At pH values above 5.5 the solubility of aluminium hydroxides is low enough that toxic effects of dissolved aluminium are eliminated (Sposito 1996). However, natural (mineralisation processes) or anthropogenic factors (atmospheric sulfate and nitrogen inputs) can cause acidification of a soil below that value. When the pH value is below 5.5, Al solubility increases 1000-fold when the pH drops by 1 unit. Depending on pH and the concentrations of possible ligands in solution, a wide variety of Al species with different toxicity can occur in solution. Among these species, the proportion of the phytotoxic $Al(H_2O)_6^{3+}$ ion, commonly referred to as Al^{3+}, increases with decreasing pH and dominates speciation below pH 4.5 (Kinraide 1991). There is evidence that complexation of Al by organic ligands can reduce the phytotoxicity of Al (Hue et al. 1986), although this is dependent on the ligand:Al ratio in solution (Suthipradit et al. 1990).

1.2. Aluminium toxicity

The toxic effects of Al on plant growth were already investigated several decades ago (Hartwell and Pember 1918, Trénel and Alten 1934) and have been the subject of scientific research since then.

The primary effect of Al is an inhibition of root elongation (Wagatsuma et al. 1987, Taylor 1988, Kochian 1995). Secondary effects that can arise as the result of the abnormal root system include inhibition of nutrient and water uptake (Kochian 1995). Although plasma membrane functions may be impaired by Al (Zhao et al. 1987, Taylor 1988), there are also indications that the membrane itself stays intact (Kinraide 1988,

Ryan et al. 1992). Inside cells, Al may bind to the phosphate groups of DNA thus interfering with its transcription (Matsumoto 1991).

Toxicity of Al to and Al resistance in crop plants have received much attention. Knowledge of the processes underlying these phenomena allows selection and breeding of resistant varieties thus ensuring food production in the tropics where acid soils are common (Foy 1974, Van Raij 1991). However, acid soils frequently occur in temperate climates, in particular under forests. Depending on the mineralogy of a particular soil, susceptibility or resistance to Al can be decisive factors that determine the stability of forest ecosystems under elevated inputs of acidity due to atmospheric pollution.

1.3. Mechanisms of Al resistance

1.3.1. Internal and external mechanisms

Plant species growing naturally on acid soils have developed strategies to cope with high concentrations of available Al in soil solution. Aluminium resistance mechanisms can be divided into internal and external mechanisms (Taylor 1991, Kochian 1995). Internal tolerance mechanisms detoxify Al in the symplasm, whereas external mechanisms prevent Al from crossing the plasma membrane and exclude it from the symplasm, where sensitive sites are located (Taylor 1991). Internal detoxification of Al by catechins occurs e.g. in tea (*Camellia sinensis* (L.) O. Kuntze) leaves (Nagata et al. 1992). In general, internal mechanisms include Al complexation by organic ligands in the cytosol, compartmentation in the vacuole, the formation of Al-binding proteins or the evolution of Al-tolerant enzymes. Possible exclusion mechanisms are immobilization at the cell wall, selective permeability of the plasma membrane, an active efflux of Al, or plant-induced changes in the rhizosphere or apoplast. The latter include an increase in pH, which decreases solubility of ionic Al^{3+} and thus leads to precipitation of Al hydroxides (Clarkson 1967). Roots also can alter Al^{3+} activity by exudation of organic chelators into the rhizosphere, thus promoting the formation of less toxic Al species (Hue et al. 1986).

1.3.2. Organic acid exudation as an Al exclusion mechanism in crop species

By the definition of Uren and Reisenauer (1988), the term root exudates includes all compounds that are released from different parts of root systems and for different

reasons. These authors further distinguish between diffusates which passively leak from cells, and excretions and secretions which are both released actively. Excretions and secretions are differentiated according to their purpose: while excretion describes the release of metabolic endproducts in order to facilitate internal metabolic processes, the term secretion is used for the active release of substances that promote external processes (nutrient uptake, mineral dissolution, detoxification of toxic ions). Uren and Reisenauer do, however, not include root debris and lysates in their definition.

In this thesis, the root exudates are considered to be all substances released by roots into the environment as it is usually impossible to distinguish between different mechanisms (Grayston et al. 1996). The chemical reactions of substances released by roots into the rhizosphere are the same irrespective of their release mechanism. Al-chelating properties of organic acid anions in soil and their efficiency in detoxification of Al have been illustrated by Hue et al. (1986). These findings suggest that exudation of organic acids can be involved in Al resistance mechanisms. Such resistance mechanisms can be either active or passive.

Root exudation can be both an active and a passive resistance mechanism. It is active, when exudation is specifically induced by a stress factor. In fact, it has been found that in various crop plants exudation of organic acid is induced by Al and correlates with Al resistance (Pellet et al. 1995, Ryan et al. 1995, Pellet et al. 1997, Ma and Miyasaka 1998, Zheng et al. 1998). On the other hand, exudation can be a passive resistance mechanism when high exudation in a particular plant is a constitutive property, i. e. present under all conditions, and a high exudation rate presents an advantage under certain conditions, e.g. by detoxifying metal ions in the rhizosphere. For example, aluminium resistant mutants of *Arabidopsis thaliana* were characterised by higher constitutive organic acid release than wild type plants, but the organic acid release was not induced by Al (Larsen et al. 1998). Similarly, overproduction of citrate in transgenic tobacco and papaya plants resulted in increased Al resistance (De la Fuente et al. 1997). Besides organic acids, exudation of polypeptides (Basu et al. 1994, 1997) and mucilage (Puthota, et al. 1991) has been found to be related to differential Al resistance of wheat cultivars. However, Li et al. (2000) found that strong binding of Al to maize (*Zea mays* L.) mucilage did not prevent Al injury in this species.

1.3.3. Al resistance in trees with special emphasis on Norway spruce

Until now, the work on Al resistance and on root exudation has focused on crop plants (Pellet et al. 1995, Ryan et al. 1995, Pellet et al. 1997, Ma and Miyasaka 1998, Zheng et al. 1998). However, many tree species withstand much higher Al concentrations in soil solution than do crop plants (Horst and Göppel 1986, Schaedle et al. 1989). Norway spruce (*Picea abies* [L.] Karst.) is a conifer that naturally occurs on acid soils in Northern and Central Europe and is known to tolerate Al concentrations up to 0.3 mM before root growth is affected (Göransson and Eldhuset 1991), which is about one order of magnitude higher than the value reported for an Al-tolerant wheat cultivar (Pellet et al. 1996).

Little is known on the Al resistance mechanisms of trees. The major finding of previous work has been that Al is retained in the root system, and thus uptake into the shoot is strongly reduced e.g. (Jentschke et al. 1991, Hentschel et al. 1993). Aluminium binding in root cortical cell walls occurs in healthy as well as in nutrient-deficient spruce trees (Bauch and Schröder 1982). Forest trees frequently live in symbiosis with mycorrhizal fungi. The main role of these fungi is believed to be the improvement of mineral nutrition as the fungal hyphae strongly increase the area of nutrient absorption compared with a non-mycorrhizal root system (Marschner 1996). In addition, a role of mycorrhizal fungi in metal tolerance has been postulated (Wilkins 1991, Colpaert and van Assche 1992, 1993), but ameliorating effects were often dependent on fungal species (Jones and Hutchinson 1986, Colpaert and van Assche 1993). In a review on the role of ectomycorrhizas in ameliorating metal stress in trees, Godbold et al. (1998) conclude, that a general answer cannot be given, but that the ameliorating effect depends on metal, tree species and fungal strain. Some fungal species excrete high amounts of organic acids (Lapeyrie et al. 1987) and, thus, could contribute to Al tolerance of the host tree by metal complexation, but the mechanism most commonly quoted is sequestration of metals in the fungal tissue, especially the extramatrical mycelium (Colpaert and van Assche 1992, 1993, Godbold et al. 1998). *Pisolithus tinctorius* Coker and Couch was effective in reducing Al uptake of *Pinus rigida* Mill. (pitch pine) and compensated growth reductions caused by 200 µM Al (Cumming and Weinstein 1990). By contrast, in ectomycorrhizal Norway spruce treated in sand culture with 0.8 mM $Al(NO_3)$, *Lactarius rufus* (Scop.) Fr. was unable to compensate negative effects of Al on root growth, Mg uptake and chlorophyll content of needles (Jentschke

et al. 1991). Inoculation with *Paxillus involutus* Fr. increased Al concentration in cortical cell walls compared with non-inoculated roots of Norway spruce grown on perlite (Hodson and Wilkins 1991). The effect of *Paxillus involutus* on Al uptake into shoots was dependent on the spruce population. In spruce seedlings from an acidic site, the fungus increased Al uptake into shoots, while the opposite was observed for plants originating from a calcareous site (Wilkins and Hodson 1989). Brunner and Frey (2000) localized Al in ectomycorrhizas of Norway spruce with *Hebeloma crustuliniforme* (Bull.: St. Amans) Quél. that had been grown at Al levels above the naturally occurring range. Aluminium was found in the fungal hyphae and in cortex cell walls at similar concentrations.

For trees, it has not been shown so far that exudation of organic chelators, which is a major tolerance mechanism for crops (see above), prevents Al uptake into the shoot. Moreover, data on root exudation by trees are generally rare. In laboratory experiments, organic acids have been detected in exudates of various pine species (Smith 1969) as well as in the exudates of deciduous trees (reviewed by Grayston et al. 1996). Until now, there has been only one report on root exudates of Norway spruce (Eltrop 1993). The author reported the presence of phenolic substances in the exudate of Norway spruce grown in sand culture. Organic acids were below the detection limit in the system used. This study, however, had been designed to study the role of ectomycorrhizas in mineral nutrition of Norway spruce and did not include Al treatments. The importance of organic acids in forest ecosystems has been shown by the following studies. A wide range of organic acids were detected in the topsoil of a beech forest in Sweden (Shen et al. 1996). The authors found total organic acid concentrations in soil solution in the range of 20-30 µM and observed a rapid degradation or sorption of most acids in the soil. Concentrations were higher at sites with ground floor vegetation than in pure beech stands, indicating that a proportion of the organic acids in this forest originated from the ground floor vegetation. Fox and Comerford (1990) identified oxalic and formic acids as the most abundant aliphatic organic acids in various forest soils of the USA under pine and oak stands. Oxalic acid reached concentrations of up to 1 mM in these soil solutions.

1.4. Phenolic substances as stress indicators and their potential role in metal tolerance of trees

A wide variety of secondary metabolites occurs in plants. Among these are phenolics, many of which, by their chemical structure, are potential chelators for Al. The pathways of phenolic biosynthesis have been elucidated to some extent (Higuchi 1985, Hrazdina 1992). A variety of functions is attributed to these substances. From an ecological point of view, phenolics act as pigments, allelopathic agents, feeding deterrents, anti-fungal agents and phytoalexins (Harborne 1980). Various abiotic and biotic stresses induce the formation of plant phenolics, such as UV radiation, low temperature, nutrient deficiency, pathogen attack and herbivore feeding (Dixon and Paiva 1995). Air pollutants like sulfuric acid lead to an accumulation of phenolic substances in needles of pine species, before macroscopic damage can be observed (Zobel and Nighshwander 1991). During the last years, the role of plant phenolics as anti-fungal agents has become a topic in mycorrhizal research, as coniferous trees appear to regulate the degree of mycorrhizal infection by the levels of fungistatic phenolic compounds in their root tissue (Münzenberger et al. 1990, 1995, Weiss et al. 1997, 1999). Little work has been done on reactions in plant phenolic metabolism to metal stress. In wheat, Cd treatment increased total soluble phenolic content, while Pb did not (Öncel et al. 2000). Jung (2000) found that Cu excess and Fe deficiency affected isoflavonoid metabolism in roots of white lupin (*Lupinus albus* L.) and enhanced root exudation of soluble phenolics. McQuattie and Schier (1990) found accumulation of phenolics by microscopical observation of Al treated red spruce (*Picea rubens* Sarg.) root cells. Karolewski and Giertych (1994) studied total phenolic levels in Scots pine (*Pinus sylvestris* L.) after treatment with various metals. Levels of total phenolics increased in needles and decreased in roots of Scots pine after Pb or Cd treatment, but were not significantly affected by Al. The catechol (*o*-dihydroxyphenyl) unit of many phenolic substances is a potent chelator of Al (Harborne 1980, Tang et al. 1992). The effectiveness of phenolic substances in chelating Al was demonstrated by Luster et al. (1996), who studied complex formation between Al and phenolic substances in aqueous leaf litter extracts. The binding of copper and zinc to plant polyphenols and precipitation of the complexes have been studied as well (McDonald et al. 1996). Phenolic constituents in tree bark have been shown to effectively bind heavy metals (Gaballah and Kilbertus 1998). Recently, a report by Malinowski and Beleski (1999) suggested

that increased exudation of phenolic-like compounds from roots of endophyte-infected tall fescue may be involved in Al tolerance of this herbaceous species. Similar reports for trees, however, are missing, although phenolics are important constituents of tree tissues, reaching, e.g. 7% of the dry matter in Norway spruce needles (Hättenschwiler and Schafellner 1999).

1.5. Questions and experiments

The present thesis mainly wants to contribute knowledge concerning the potential role of organic chelators in roots and root exudates in mediating Al tolerance in conifers. Norway spruce, which is an important conifer species in Central and Northern Europe was chosen as a model.

The main hypothesis is that Norway spruce roots actively increase the production and release of chelating substances, when elevated concentrations of Al^{3+} ions are present in soil solution. Complex formation in the rhizosphere or in the apoplast then reduces the activity of the toxic Al^{3+} species, inhibits its uptake into the symplast and thus significantly contributes to Al resistance in Norway spruce.

The thesis focuses on two main groups of chelating compounds, aliphatic organic acids and phenolic substances. Other substances that have been proposed to chelate Al (chapter 1.3.2.) will not be studied in detail.

Starting from the knowledge on Al accumulation in roots, a first experiment was performed with three year old non-sterile Norway spruce trees in hydroculture in order to study in detail the compartmentation and binding forms of Al in roots. This study was further intended to give a first overview over the amount and composition of root exudates and the effect of Al on these parameters. This experiment is described in chapter 2.

Based on the results of this experiment, the following questions were considered essential for testing the main hypothesis:

Which amounts and types of organic acids are present in root exudates of Norway spruce? What is the effect of Al on root content (i.e. production) and exudation of these acids? How are these parameters affected when Norway spruce roots experience mechanical resistance in a solid medium and are colonised by ectomycorrhizal fungi as under natural conditions? How are root content and exudation of plant phenolics, the

other main group of chelating compounds in plant roots apart from aliphatic organic acids, affected under these conditions?

These questions were addressed in two steps.

- In a first step, the effect of Al on maximum exudation and root content of organic acids was studied in a hydroponic system under sterile conditions (chapter 3).
- In a second step, a solid substrate (perlite) was chosen as the growth medium in order to include mechanical resistance in the experimental system, as mechanical forces have been observed to increase root exudation (Barber and Gunn 1974, D'Arcy-Lameta 1982, Mozafar 1991). Data from hydroponic systems might therefore underestimate root exudation. This design also allowed inoculation with an ectomycorrhizal fungus, which is closer to natural conditions, where conifers are frequently ectomycorrhizal (Newman and Reddell 1987). This cannot be accounted for in hydroponic systems, where the extramatrical fungal mycelium is absent. The studies in perlite culture focused on the individual and combined effects of Al and inoculation on root content and root exudation of organic acids (chapter 4) and phenolic substances (chapter 5).

Chapter 2

Effects of aluminium treatment on Norway spruce roots: Aluminium binding forms, element distribution, and release of organic substances

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2.1. Abstract

In order to investigate if Al resistance in Norway spruce (*Picea abies* [L.] Karst.) can be attributed to similar exclusion mechanisms as they occur in several crop plants, three-year-old Norway spruce plants were treated for one week in hydroculture with either 500 µM $AlCl_3$ or $CaCl_2$ solutions at pH 4.

Sequential root extraction with 1 M NH_4Cl and 0.01 M HCl and EDX microanalysis revealed that Al and Ca in cell walls and on the surface participated in exchange processes. About half of the Al extracted by the sequential extraction was not exchangeable by 1 M NH_4Cl. Phenolics and P present in the root extracts are possible ligands for Al adsorbed to or precipitated at the root in a non-exchangeable form.

In both treatments, C release during the first period of 2 d was much higher than during the remaining time of the experiment. Al treated plants released less total C, carbohydrates and phenolics than did Ca treated plants. Acetate was the only organic acid anion that could be detected in some samples of both treatments. Free amino acids were present at micromolar concentrations but as hydrolysis did not increase their yield, there was no evidence of peptide release. One to two thirds of the released C were large enough not to pass a 1 kDa ultrafilter.

The results suggest that exudation of soluble organic complexors is not a major Al tolerance mechanism in Norway spruce, although complexation of Al by phenolic substances released by the root could be detected by fluorescence spectroscopy. Aluminium tolerance could rather be attributed to immobilisation in the root apoplast, where strong binding sites are available or precipitation may occur.

Key words

Aluminium, binding forms, element compartmentation, *Picea abies*, resistance, root exudates

2.2. Introduction

Norway spruce (*Picea abies* [L.] Karst.) is a common forest tree in Northern and Central Europe, which is found mostly on acid soils of pH 4-5 (Leibundgut 1984). At such acidic sites proton buffering by the mineral phase leads to a release of Al^{3+}, which can result in high concentrations of toxic Al species in the soil solution. Free Al^{3+} ions potentially are toxic to plants as they can inhibit root growth and impair nutrient uptake (Kinraide 1991, Delhaize and Ryan 1995, Kochian 1995). However, in contrast to agricultural crops, where Al^{3+} concentrations at the low micromolar level cause growth reductions in sensitive wheat and maize varieties (Pellet et al. 1995, 1996), Norway spruce is able to tolerate concentrations of up to 0.3 mM Al^{3+} before growth is reduced. Lethal concentrations are even 30 times higher (Göransson and Eldhuset 1991). Only very little is known on the mechanism of the Al resistance in Norway spruce as studies focused rather on nutritional and growth effects of high Al concentrations (e.g. Göransson and Eldhuset 1991, Godbold and Jentschke 1998). Several plant species have developed strategies to avoid or tolerate Al toxicity. The proposed mechanisms of Al resistance can be classified into internal tolerance mechanisms and exclusion mechanisms (Taylor 1991, Kochian 1995). The main difference between these two mechanisms is the site of Al detoxification: symplasm (internal) or apoplasm (exclusion). The internal tolerance mechanism immobilises, compartmentalises or detoxifies Al entering the symplasm. Recently, complexation by citrate in hydrangea (*Hydrangea macrophylla* (Thunb.) Ser.) and by oxalate in buckwheat (*Fagopyrum esculentum* Moench.) has been shown to detoxify Al internally (Ma et al. 1997, 1998). By contrast, exclusion mechanisms prevent Al from entering the symplasm, where sensitive intracellular sites are located (Taylor 1991). A proposed exclusion mechanism is the excretion of chelating organic substances as these can form stable complexes with Al^{3+} ions in the soil solution, which are less phytotoxic than free Al^{3+} ions (Hue et al. 1986). For Al resistant varieties of wheat (Delhaize et al. 1993), maize (Pellet et al. 1995), and buckwheat (Zheng et al. 1998), exudation of organic acids was found as a reaction to Al exposure. Beside organic acids, polysaccharides and polyuronic acids secreted by the root cells and forming the mucilage around root tips have been proposed to be involved in Al tolerance of cowpea (Horst et al. 1982). It is not known if these processes have any importance for the Al resistance of Norway spruce. There is generally very little and partly contradictory information on exudation of organic

substances by forest trees. While Smith (1969) reported detailed patterns of organic acids exuded by seedlings of various pine species, Eltrop (1993) found exudation of organic acids by Norway spruce seedlings in semi-hydroponic culture to be below the detection limit of modern analytical tools. However, organic acid exudation of a given tree species may increase with age (Smith 1970). In forest soils, organic acids have been detected in varying concentrations depending on soil type and vegetation, but their origin (i.e. plant or microbial) is unknown (Fox and Comerford 1990, Shen et al. 1996, Jones 1998).

An exclusion mechanism does not necessarily have to work completely outside the root. In the apoplast, binding of Al to non-sensitive sites can be equally efficient in excluding Al^{3+} ions from sensitive symplastic sites (Horst 1995). It is known that Al mainly is retained in the roots of Norway spruce and only very little is translocated to the shoot (Göransson and Eldhuset 1991, Hentschel et al. 1993). However, only little information is available on the compartmentation and chemical form of Al accumulated in the root (Jentschke 1990). Dahlgren et al. (1991) discussed co-precipitation of Al with oxalate and phosphate as possible retention mechanisms of Al in fine roots of a Northwest American conifer stand of *Abies amabilis* (Dougl.) Forbes with *Tsuga mertensiana* (Bong.) Carr. as an associated species. Mycorrhizal infection could also be involved in preventing Al transport to the shoot as inoculation with the mycorrhizal fungus *Pisolithus tinctorius* increased Al resistance in pitch pine seedlings (Cumming and Weinstein 1990) and Al retention in Norway spruce roots was increased by inoculation with *Paxillus involutus* (Hentschel et al. 1993).

The aim of this study was on one hand to examine if root exudation of organic substances is likely to play a role in the Al resistance of Norway spruce, and on the other hand to get more insight into the distribution and chemical form of Al accumulated in the roots of this plant species. Treatments of three year old soil-grown tree individuals in hydroponic culture were chosen because organic acid release from older trees can be expected to be substantially higher than from few month old seedlings (Smith 1970). The use of single-salt solutions eliminated unwanted amelioration of Al toxicity by nutrient cations (Grauer and Horst 1992). Since changes in the exudation pattern and reactions of Al with the root surface are likely to occur within a few hours or days after the stress is imposed (Delhaize et al. 1993, Pellet et al. 1995), one week

treatments were considered sufficient. Within such short periods it can be expected that no nutrient deficiencies and only limited microbial growth occur.

2.3. Material and Methods

2.3.1. Plant material

Norway spruce (*Picea abies* [L.] Karst.) seedlings from a parent tree near Bremgarten (canton of Aargau, Switzerland) were grown for three years in a tree nursery and for another half year in a greenhouse in pots filled with a fertilised mixture of peat and wood chips. Upon transplantation to the pots, the root system was cut back to about half of its size to induce formation of new roots.

2.3.2. Treatments

For the treatments the plants were removed from the pots and a shower fed with tap water was used to remove substrate adhering to the roots. The root system was bathed in 10^{-4} M HCl for 10 min in order to neutralise carbonates originating from the tap water. The plants were transferred to 250 ml Erlenmeyer flasks where the root system was treated with 250 ml of treatment solution while the shoot remained outside the flask. The treatment solutions were either 0.5 mM $AlCl_3$ or $CaCl_2$ (control) solutions acidified to pH 4.0 with HCl and sterile filtered before use. Treatments were done in triplicates. In both treatments, the pH of the system was kept between 3.8 and 4.0 by a titroprocessor (Metrohm 670). While in the Al treatments only negligible amounts of HCl needed to be added to keep pH at this level, about 100 µM HCl had to be added in the Ca treatments. At this pH, Al speciation is dominated by the trivalent Al^{3+} ion. During the experiment, the solutions were aerated with 0.2 µm filtered air. The treatments lasted for one week, which was divided into three periods of 2, 2, and 3 d. After each of these periods the treatment solutions were replaced by fresh ones. The purpose of this pattern was to separate rapid effects caused by the treatment shock at the beginning of the experiment from longer-term plant reactions. The experiments were carried out in a growth chamber (20°C, 50% relative humidity, 16 h photoperiod).

2.3.3. Sampling

Solution samples were taken three times a day during the first and second period and once a day during the third period. Ten ml were sampled at each sampling date, and the liquid in the Erlenmeyer flask lost by sampling and evapotranspiration replaced with sterile distilled water. The samples were filtered immediately through 0.45 µm syringe filters (Spartan 30 B; Schleicher & Schuell) and stored at –20°C until analysis. At the end of each treatment period the remaining solutions also were 0.45 µm filtered and stored frozen.

2.3.4. Solution analysis

On all solutions the following analyses were performed: Cations were measured by capillary electrophoresis (CE) (BioFocus 3000, BioRad, 40 cm x 50 µm fused silica capillary) using the metol buffer of Göttlein and Blasek (1996). Dissolved organic carbon (DOC) was determined with a TOC-Analyzer (Shimadzu TOC-500). Blank values of DOC due to release from the syringe filters were determined separately (5 replicates) and subtracted from the measured values. UV absorption was measured at 215 nm, 254 nm and 280 nm with a UV-VIS spectrophotometer (Shimadzu UV-240), and total phenolics were analysed with a colorimetric method according to Swain and Hillis (1959) using phenol as standard.

On the solutions remaining at the end of each treatment period, additional analyses were performed. Forty ml were freeze-dried and redissolved in 1 ml of 0.1 M HCl. Chloride and cations in the concentrated solutions were removed by passing over a cation exchange resin saturated with Ag^+ ions, and organic acids were analysed using CE (buffer: 10 mM potassium hydrogen phthalate; 2.5% Waters OFM Anion-BT; pH 5.6). Oxalate was determined separately by CE using the anion method of Göttlein and Blasek (1996). During lyophilisation calcium oxalate can precipitate. In order to dissolve such precipitates, after removal of the concentrated sample solutions the bottles used for freeze-drying were shaken with 1 ml of 1 mM EDTA. Organic acids and oxalate in these solutions were analysed by CE as mentioned above. Experiments with standards showed that this also improves malate and citrate recovery in the presence of Al. Total carbohydrates were determined in 10-fold concentrated solutions by the phenol-sulfuric acid assay according to Chaplin (1994) using glucose as standard. Total amino acids were determined in 5-fold concentrated solutions before and after alkaline

hydrolysis using the ninhydrin method (Allen 1981). Twelve ml of the non-concentrated final solutions were filtered through a 1 kDa filter (Macrosep™ centrifugal concentrator, Pall Filtron, USA) at 5000 min^{-1} for about 2.5 h. Retentate and filtrate were analysed for DOC and for total phenolics as above. The amounts of high molecular C and high molecular phenolic substances were calculated as difference between retentate and filtrate amounts. The fluorescence spectra of selected samples were recorded using quartz cells (path length 1 cm) on a Shimadzu RF 5000 spectrometer. Excitation and emission monochromator entrance and exit slit widths were set to 3 nm. Excitation wavelength was varied from 250 nm to 550 nm.

2.3.5. Root extraction

At the end of each treatment, a four-step sequential extraction of the roots was performed. One hundred mg of fresh fine roots were extracted for 30 min with 10 ml of 1 M NH_4Cl on an end-over-end shaker to yield exchangeable cations. The supernatant was sampled by pipetting. Since a small volume of extract still adhered to the roots, it was necessary to perform a second extraction with fresh NH_4Cl in order to obtain a complete recovery of exchangeable cations and to avoid carry-over into the following extract. In a third step, 10 ml of 0.01 M HCl were added to the roots and the bottles were shaken again for 30 min and the supernatant sampled with a pipette. This extract was chosen to dissolve potential precipitates of Al (Dahlgren et al. 1991). Finally, the HCl extraction procedure was repeated once to obtain complete recovery of the acid soluble fraction.

In all extracts total element concentrations were determined using inductively coupled plasma atomic emission spectrometry (Optima 3000, PE), and total phenolics were determined with the method described above. After removal of chloride, oxalate was determined with the method described above.

2.3.6. Electron microscopy and X-ray microanalysis

Fine root samples of newly formed lateral roots were taken from treated plants and from a control plant in a pot. Lateral roots of approx. 5 mm length were fixed vertically in slits of supporting brass stubs using a cryo glue, frozen in liquid nitrogen and freeze-fractured with a rotating microtome at –90°C in the preparation chamber (Balzers SCU 020) of the cryo scanning electron microscope (Philips 515). For surface analyses, the

samples were fixed horizontally with double-sided adhesive tape and frozen in liquid nitrogen. Elemental concentrations on the root surface, in cell walls and lumina of epidermis and cortex, and in the stele were assessed by energy-dispersive X-ray microanalysis in the cryo scanning electron microscope (Brunner et al. 1996). The microscope was operated at an acceleration voltage of 20 kV with a beam current of 80 µA and a take-off angle of 15°. Working distance was 12 mm. All spectra were acquired for 120 s (live time) and a dead time of 20 %. Spot analysis was carried out with a maximum magnification of 10 000. Spectra were analysed using the Voyager software package and results for individual elements are presented as background corrected net counts. These net counts are semi-quantitative measures of concentration, but as analysis conditions and the etching process were not controlled rigorously and because of the problems of obtaining fully quantitative results from bulk-frozen hydrated samples (Van Steveninck and van Steveninck 1991), they were not converted to absolute concentrations. Three samples per treatment were analysed; on each sample 4-5 analyses per compartment were performed.

2.3.7. Statistical analysis

In order to compare treatment effects in a given period, one-way ANOVA tests were performed. The unequal variances between first and later treatment periods did not allow a two-way analysis over all data on the factors treatment and period. Statistical analysis was done using Data Desk 6.0.2. for Macintosh (Data Description Inc.).

2.4. Results

2.4.1. Ion exchange

During the first 2-day period, the concentration of Al^{3+} in the Al treatment solutions, as measured by CE, decreased very rapidly from the initial level of 500 µM to approximately 100 µM (fig. 2.1). A less pronounced decrease in Al^{3+} concentrations was observed during the second and third period. Minimum Al^{3+} concentrations were about 250 µM in the second period and only in one case dropped below 400 µM during the third period.

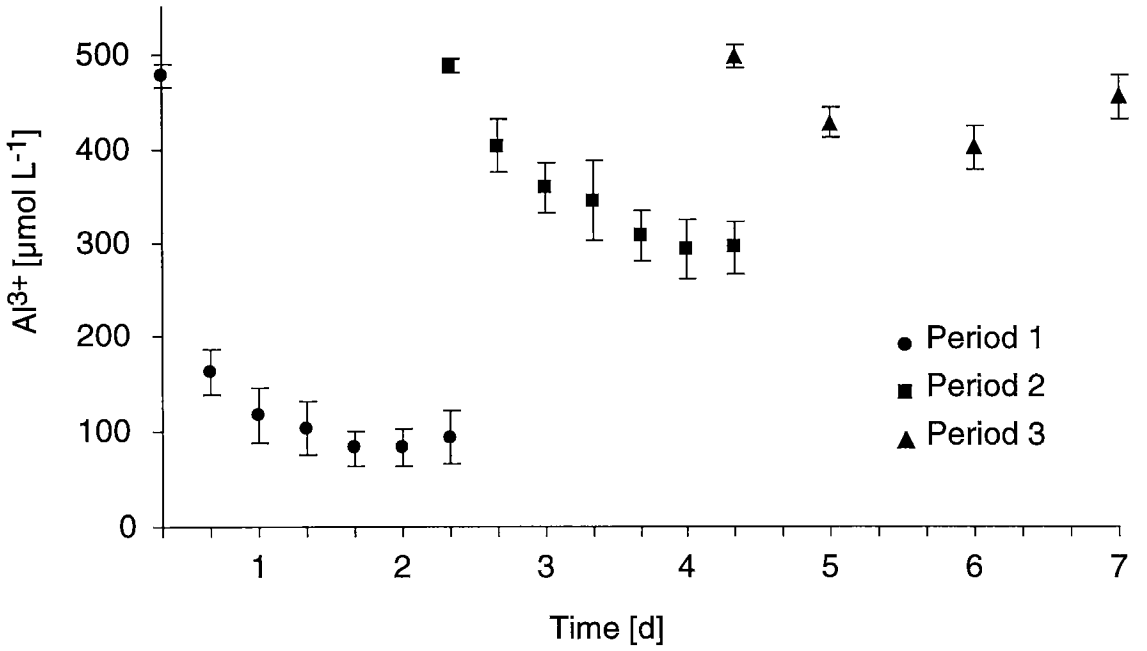

Figure 2.1. Concentrations of Al^{3+} in Al treatment solutions as measured by CE (mean of three replicates ± SE).

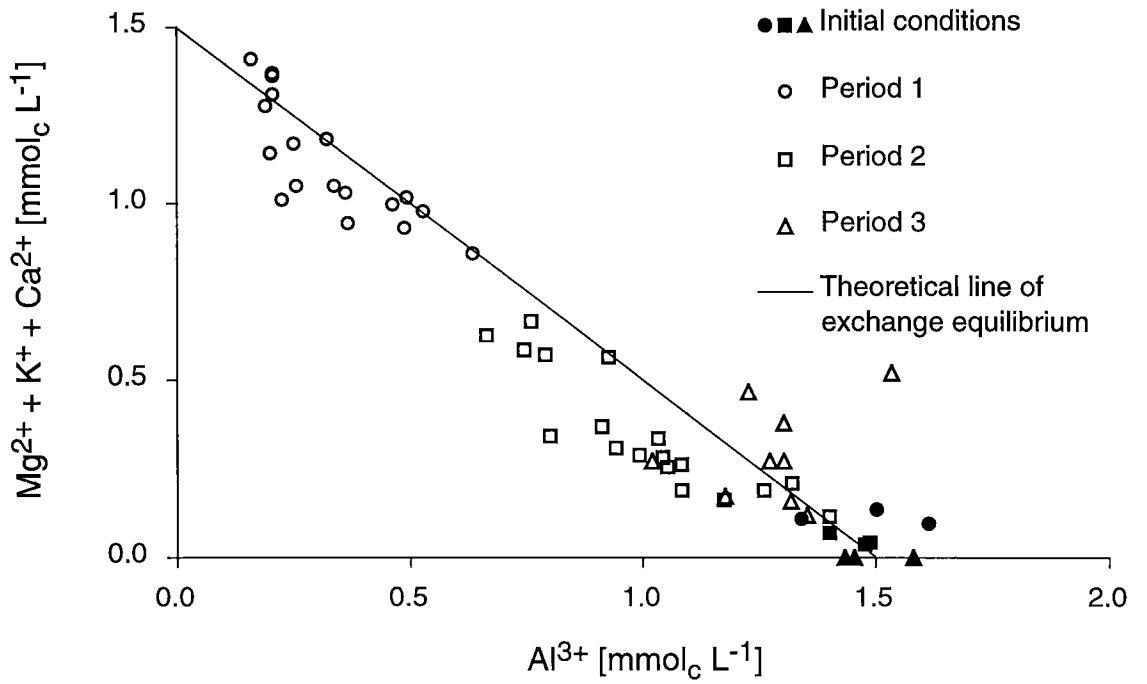

Figure 2.2. Exchange of nutrient cations against Al^{3+} ions at the root surface: Solution equivalent concentration of $Mg^{2+} + K^{+} + Ca^{2+}$ *vs.* solution equivalent concentration of Al^{3+}.

Figure 2.2 compares the concentrations of Al^{3+} ions with the sum of the concentrations of the major nutrient cations (Ca^{2+}, Mg^{2+}, K^{+}) in all samples of the Al treatments. Concentrations are given in micromoles of cationic charge in order to allow easy comparison. As only Al^{3+} was added to the treatment solutions, all nutrient cations must have been exchanged from the root surface. The line indicates a value of 1500 µM total cationic charge, which should be reached if only equivalent exchange happened between solution cations and adsorbed cations. This was the case during the entire experiment. Sodium and NH_4^+ ions were present at concentrations below the limit of quantification. Together, they did not exceed 3% of total cationic charge and could be neglected for the calculation of total cationic charge.

2.4.2. Sequential extraction

When comparing exchangeable cations at the root surface (fig. 2.3) at the end of the experiment, there were marked differences between the treatments. In Ca treatments, Al was mostly below the detection limit and never exceeded 5% of the exchangeable cations, whereas in Al treatments it represented about 30% of the exchangeable cations. Accordingly, there was about 2.5 times more exchangeable Ca in Ca treatments than in Al treatments. A higher amount of exchangeable K was found for Al-treated roots. Due to the large variability of exchangeable K values, this difference is not significant, however. No significant differences between treatments could be found for exchangeable Mg and Na.

The amount of acid soluble but non-exchangeable Al found in the 0.01 M HCl extract of Al treated roots (fig. 2.4) was similar to the amount of NH_4Cl-exchangeable Al in these roots. In Ca treatments, acid extractable Al was at the detection limit. There were no large differences in acid extractable Ca between the treatments. Acid extractable K was below the detection limit in most samples of the Al treatment whereas in the Ca treatments concentrations of exchangeable and acid extractable K were similar. There were no significant differences in acid extractable Mg and Na between the treatments. When comparing total extractable amounts of the cations, significant differences between the treatments could be found for Al and Ca only.

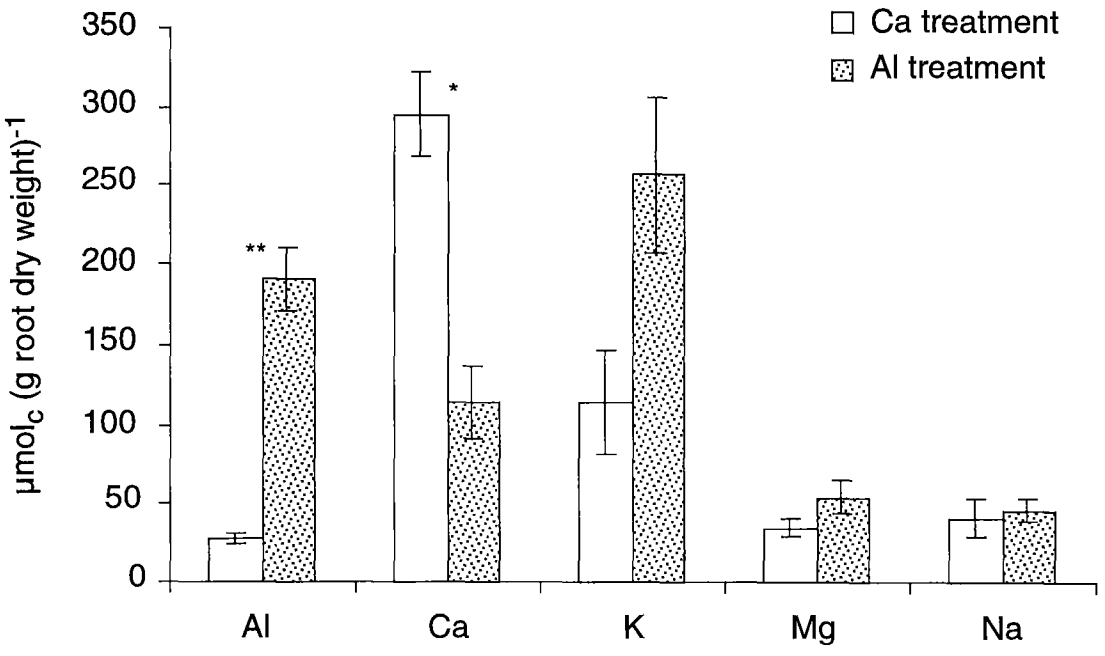

Figure 2.3. Cations exchangeable from Norway spruce roots by 1 M NH_4Cl. Values are means of three plants per treatment and three replicate extractions per plant. Bars indicate SE. Differences between the treatments: * = significant at $p < 0.05$; ** = significant at $p < 0.01$.

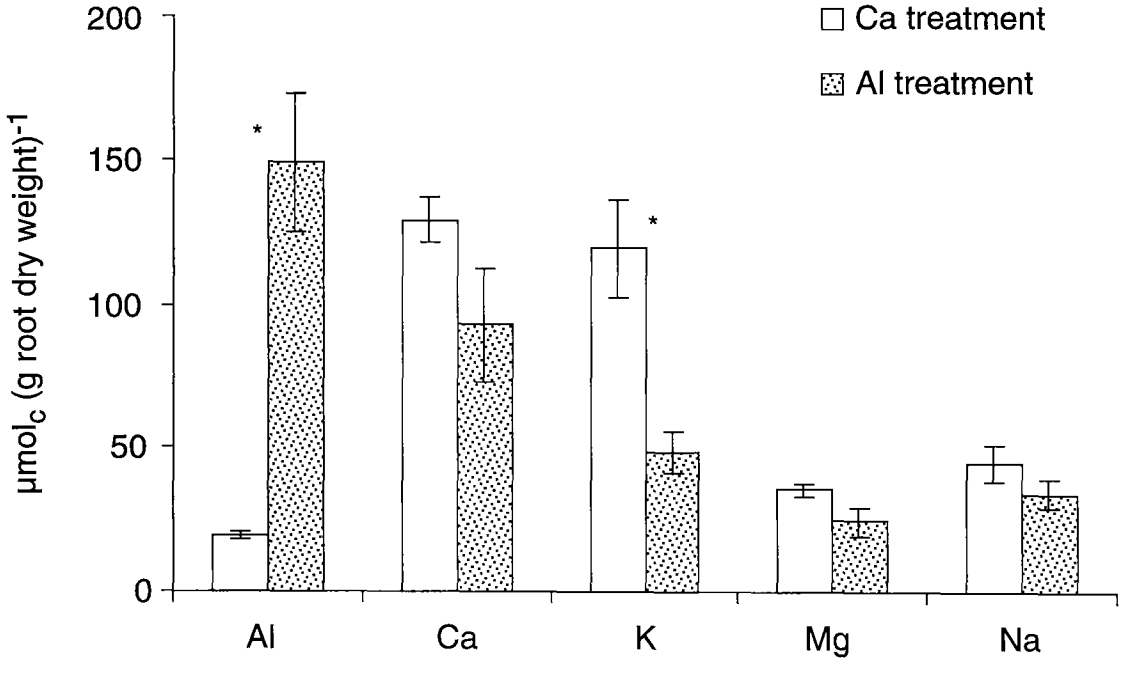

Figure 2.4. Cations extractable from Norway spruce roots by 0.01 M HCl during a sequential extraction. Values are means of three plants per treatment and three replicate extractions per plant. Bars indicate SE. For some samples, Al, K or Na were below the detection limit. In these cases, the detection limit was used for calculations. Differences between the treatments: * = significant at $p < 0.05$.

The total amount of phenolics extractable from the root surface by the sequential extraction did not differ between the treatments (fig. 2.5). However, while in Al treatments between 50 and 67% of all extracted phenolics were extractable with the first NH_4Cl extraction step, this percentage was only 10-33% in Ca treatments. More than 60% of the phenolics at the surface of Ca-treated roots could not be extracted with 1 M NH_4Cl, but only with 0.01 M HCl.

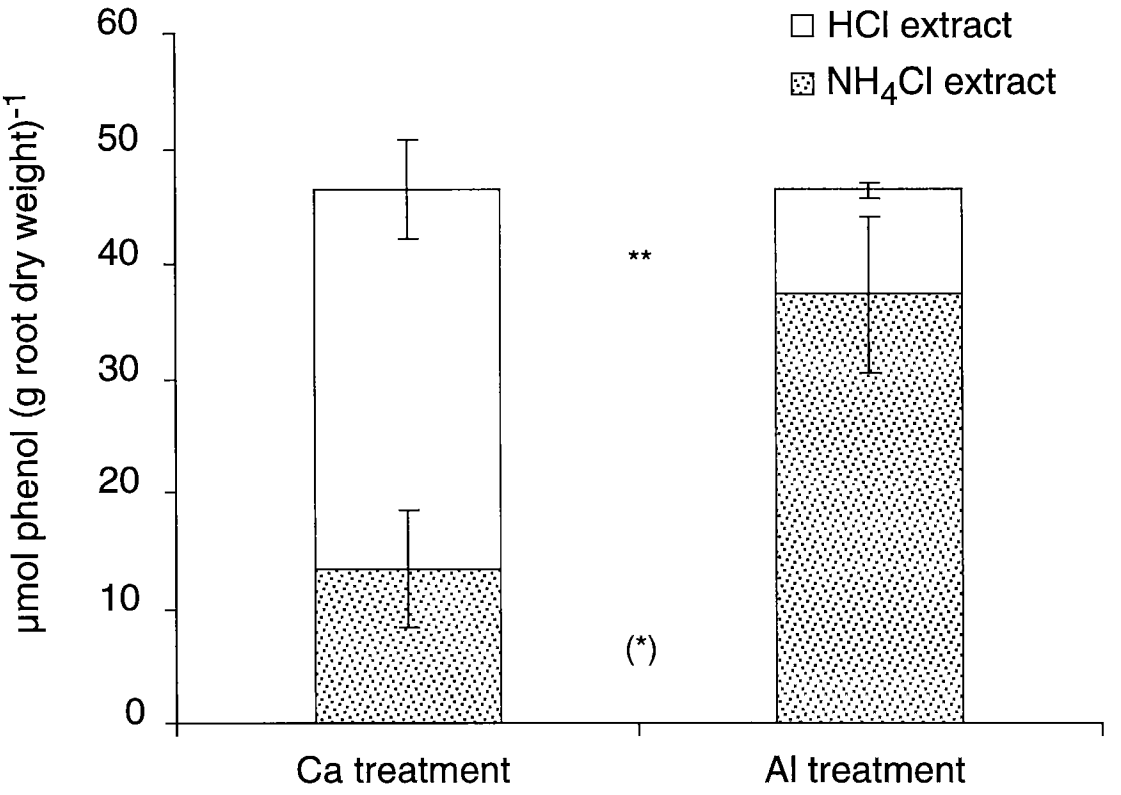

Figure 2.5. Fractions of phenolic substances extractable from the root surface of Norway spruce (means of three replicates per plant and three plants per treatment, error bars denote SE). Differences between the treatments for a given extract: (*) = significant at $p < 0.1$; ** = significant at $p < 0.01$.

2.4.3. Localisation of elements

Lateral roots investigated in the electron microscope showed no mycorrhizal structures, although single fungal hyphae were present. Elemental analysis revealed that a large proportion of Al was bound to the root surface (table 2.1). In Al treated roots, Al was also found in cell walls of the epidermis and cortex, but not in the stele (table 2.2). In the lumen of epidermal and cortical cells of Al treated plants, Al was present at concentrations near the detection limit. Calcium was found throughout the root, with the highest counts in the cell walls of Ca treated and untreated control plants. In cell walls of Al treated plants, however, Ca was drastically reduced. Phosphorus was slightly more abundant in the lumen than in cell walls, especially in control and Ca treated plants. In epidermal cells of Al-treated plants the count rate in the lumen was as low as in cell walls. However, surface analysis revealed an increase of P on Al treated roots compared with Ca treated or control plants. Potassium was found mainly in the lumina and less often in cell walls. Net counts in epidermal cells and in the stele of samples from Ca treated plants were very high. Elevated Cl concentrations were found in cortical lumina of both treatments when compared to the control. In Al treated plants both epidermal lumina and cell walls were low in Cl, while in Ca treated plants epidermal lumina reached five times more Cl net counts than the cell walls.

Table 2.1. X-Ray net counts of selected elements on the surface of Norway spruce fine roots. Values are mean ± SD of 3-4 samples per treatment and of five spectra per sample.

	Net counts				
	Al	*Ca*	*K*	*P*	*Cl*
Ca treatment	215 ± 82	3220 ± 611	1306 ± 389	219 ± 71	358 ± 106
Al treatment	1431 ± 449	254 ± 96	667 ± 274	640 ± 287	916 ± 188
untreated control	323 ± 67	2189 ± 590	1087 ± 590	311 ± 56	214 ± 71

Table 2.2. X-Ray net counts of selected elements in various cell types and compartments of freeze-fractured Norway spruce fine roots. Values are mean ± SD of 3-4 samples per treatment and of four spectra per cell type, n.d. = not detected.

Cell type	Net counts				
	Al	*Ca*	*K*	*P*	*Cl*
Ca treatment					
Epidermal cell wall	151 ± 20	6125 ±1084	2239 ± 296	258 ± 87	449 ± 135
Epidermal lumen	n.d.	1165 ± 238	5976 ± 781	592 ± 361	2292 ± 472
Cortical cell wall	n.d.	1731 ± 380	1398 ± 255	241 ± 65	228 ± 62
Cortical lumen	n.d.	254 ± 147	3378 ± 638	629 ± 271	781 ± 170
Stele	n.d.	1383 ± 275	6207 ± 508	511 ± 170	859 ± 211
Al treatment					
Epidermal cell wall	627 ± 138	188 ± 68	522 ± 91	278 ± 94	577 ± 208
Epidermal lumen	88 ± 27	117 ± 25	1961 ± 582	316 ± 70	391± 78
Cortical cell wall	552 ± 145	350 ± 77	1659 ± 388	295 ± 87	455 ± 176
Cortical lumen	24 ± 8	186 ± 53	2831 ± 605	480 ± 217	1269 ± 331
Stele	n.d.	174 ± 49	2199 ± 157	293 ± 58	1440 ± 142
untreated control					
Epidermal cell wall	93 ± 42	2293 ± 436	702 ± 147	688 ± 297	75 ± 21
Epidermal lumen	n.d.	98 ± 30	1855 ± 528	1508 ± 603	34 ± 14
Cortical cell wall	n.d.	1027 ± 473	905 ± 216	246 ± 117	234 ± 66
Cortical lumen	n.d.	137 ± 66	2299 ± 725	891 ± 260	155 ± 79
Stele	n.d.	420 ± 128	3084 ± 609	376 ± 67	182 ± 76

2.4.4. Amount of released organic substances

The amount of C released by individual plants varied strongly. On a fresh weight basis, the roots of the Ca treated plants released more C than those of the Al treated plants (table 2.3). For periods 1 and 2, the differences were significant. In both treatments, the amount of released C during the first period was higher than during the two later periods. This resulted in mean solution concentrations of 1 mM C (Ca treatments) and 0.5 mM C (Al treatments) during the first period and about four times lower concentrations during the second and third period.

UV absorption data of all sampling dates are shown in fig. 2.6. UV absorption usually correlates closely with DOC for samples of the same origin, and thus, absorption data can be used as a measure of DOC (Buffle et al. 1982). In our experiment, we found similar molar absorptivity at 280 nm in both treatments (table 2.3). At the low levels of DOC in the second and third period, absorption data were more sensitive than DOC measurements. For both treatments, UV absorption at 280 nm increased during all periods. During the first periods, this occurred rapidly during the first hours, after which absorption remained almost constant for the next two days. In the following periods, this increase was less pronounced and more gradual.

Table 2.3. Release of organic substances by roots of 3-year-old Norway spruce during treatments in hydroculture (mean ± SE). Data were calculated from concentrations in the solutions remaining after each period. For a given period, release by Al treated plants was different from release by Ca treated plants at the following levels of significance: **: $p < 0.01$; *: $p < 0.05$; (*): $p < 0.1$; ns: not significant (n=3).

	Ca treatment			Al treatment					
	1st period Day 2	2nd period Day 4	3rd period Day 7	1st period Day 2		2nd period Day 4		3rd period Day 7	
	µmol C (g root fresh weight)$^{-1}$								
Total carbon	20.2 ± 2.3	5.3 ± 0.2	4.3 ± 0.1	9.8 ± 1.5	*	2.7 ± 0.3	**	2.8 ± 0.7	ns
Total carbohydrates	8.0 ± 1.1	1.8 ± 0.1	1.6 ± 0.1	3.5 ± 0.4	*	0.9 ± 0.1	**	0.9 ± 0.2	(*)
Total phenolics	1.3 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.8 ± 0.1	*	0.5 ± 0.1	ns	0.5 ± 0.1	ns
Amino acids unhydrolysed	1.2 ± 0.3	0.4 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	ns	0.4 ± 0.1	ns	0.2 ± 0.0	ns
hydrolysed	1.3 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	(*)	0.5 ± 0.1	ns	0.3 ± 0.1	ns
HMW [a] carbon	14.3 ± 2.2	2.3 ± 0.6	1.3 ± 0.4	4.3 ± 1.1	*	0.6 ± 0.3	ns	0.6 ± 0.1	ns
HMW phenolics	0.8 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	**	0.1 ± 0.0	*	n. a.[b]	
	L (mol C)$^{-1}$ cm^{-1}								
Molar absorptivity	158 ± 26	211 ± 25	216 ± 34	135 ± 5	ns	177 ± 21	ns	173 ± 14	ns

[a] high molecular weight (> 1kDa); [b] not analysed

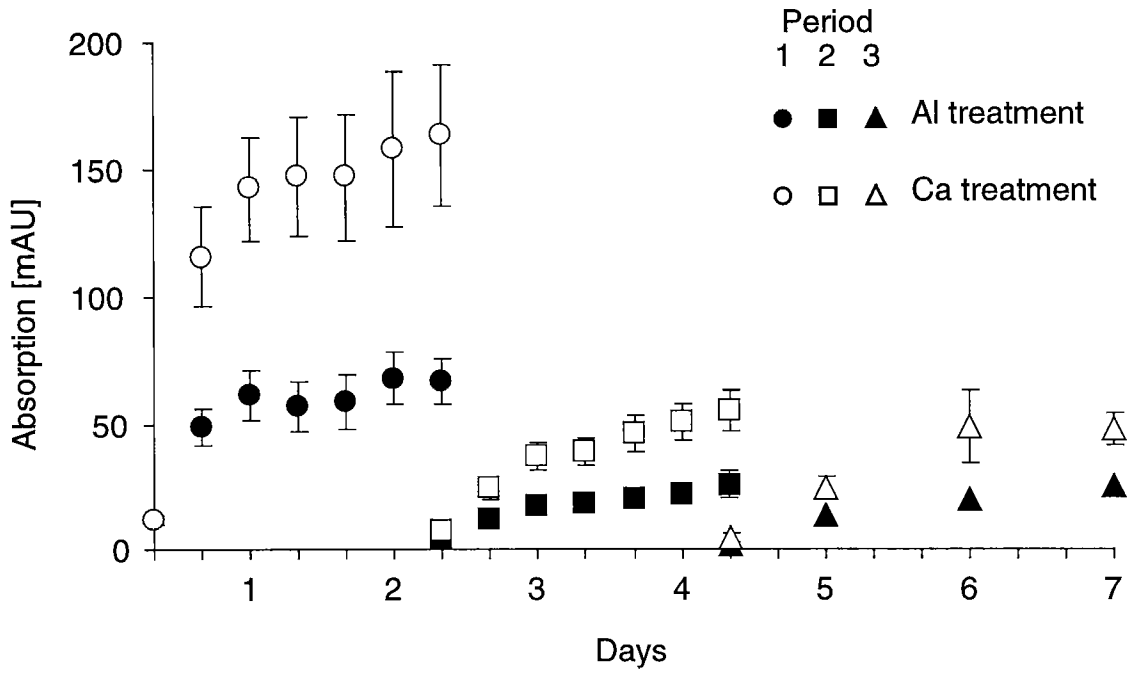

Figure 2.6. UV Absorption of Norway spruce treatment solutions at 280 nm (mean of three replicates ± SE).

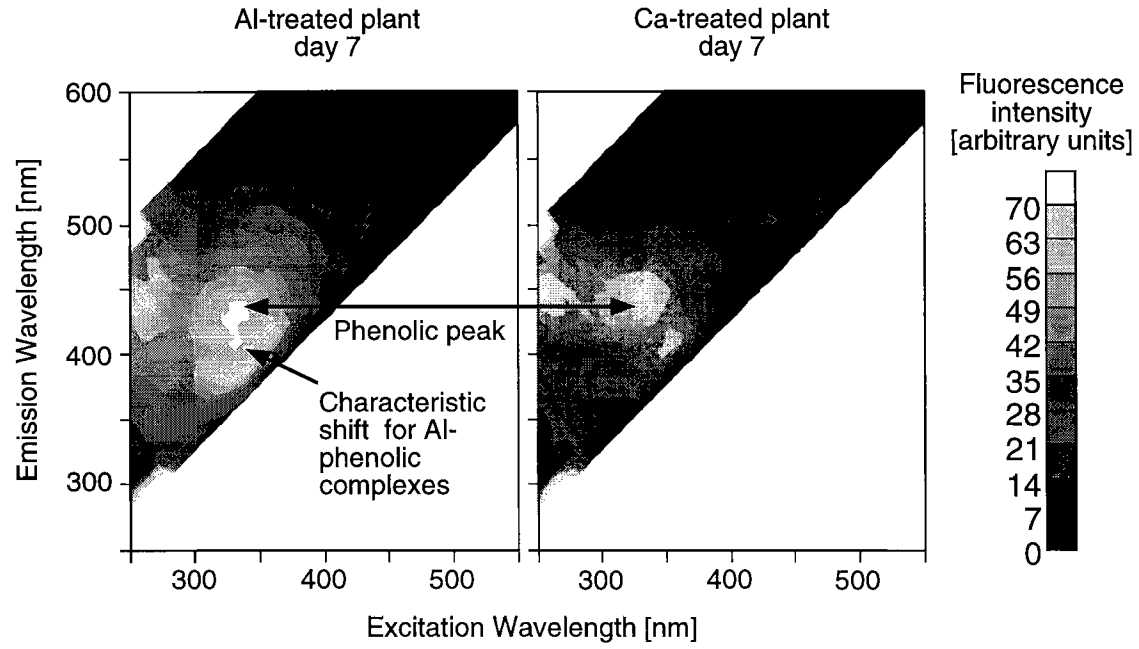

Figure 2.7. Fluorescence spectra of Norway spruce treatment solutions at the end of the experiment.

2.4.5. Characterisation of released C

Total carbohydrates represented between 25% and 45% of released C over all samples. The percentage of carbohydrates did not differ significantly between the treatments. Thus, the release of total carbohydrates per g root fresh weight followed a similar pattern as total C (table 2.3). For periods 1 and 2, carbohydrate release was significantly lower in Al treatments.

Concentrations of total phenolics amounted to 5 - 10% of released C in the first periods and 10 – 20% in the second and third periods. The percentage of phenolics was significantly higher for Al treated plants during the second period only. As with total C, the solution concentrations in the first periods of both treatments were highest, ranging between 6 and 14 µM, while they reached only 3-6 µM in the following periods. In the first period, the release of phenolics in the Al treatment was significantly lower than in the Ca treatment (table 2.3), while in the later periods there were no differences.

Total amino acids represented between 4 and 20 % of total C, if a mean number of 5 C atoms per molecule is assumed. Alkaline hydrolysis did not significantly increase amino acid concentrations in the samples. In the first and third periods, Ca treated plants tended to release more amino acids than did Al treated plants, but the difference was only slightly significant for hydrolysed samples of the first period. Organic acids analysis included oxalate, tartrate, malate, citrate, succinate, glutarate, glycolate, pyruvate, acetate and lactate. Taking into account the concentration procedure, the detection limit for these acids was in the range of 1-1.5 µM in solution and 0.1-0.2 µmol per g root dry weight. Except for traces of acetate in some samples of both treatments, all organic acids were below the detection limit. Compared with total C released by the plants, this is less than 2-5% for a single acid, depending on its number of C atoms per molecule.

2.4.6. Molecular weight of released C

A large portion of the dissolved C did not pass a 1kDa filter (table 2.3). In the first period, significantly more high molecular weight C was released by the Ca treated plants than by those treated with Al. During the experiment, the percentage of high molecular weight C decreased from 70 to 30 % in Ca treatments and from 45 to 25 % in Al treatments. In periods 2 and 3, differences between the treatments were statistically not significant.

A similar pattern was observed for high molecular weight phenolics. When compared with total phenolics, their percentage decreased from 60 to 30% in Ca treatments, from 30 to 20% in Al treatments. Differences between the treatments were significant in the first and second periods.

2.4.7. Fluorescence spectra

All samples used for fluorescence measurements showed a peak at an excitation wavelength of 330 nm and an emission wavelength of about 440 nm, which is characteristic for simple phenolic structures (Wolfbeis 1985, Blaser et al. 1999). If phenolic substances are complexed with Al, a second peak appears at the same excitation wavelength but a lower emission wavelength (about 410 nm) (Luster et al. 1996, Blaser et al. 1999). Such a peak can be clearly seen in the spectra of the Al treatment solutions as shown for one example (fig. 2.7).

2.5. Discussion

2.5.1. Processes at the root

The rapid removal of Al^{3+} ions from solution can be explained by exchange processes occurring at the root surface. Due to the substrate within which the plants were raised, their root exchange complex was almost saturated with nutrient cations at the beginning of the experiment. In the Al treatments, nutrient cations were easily displaced by the highly charged Al^{3+} ion with its higher affinity to the exchange sites. This effect was most pronounced when there was still little Al bound to the roots. As the Al saturation of the root exchange complex increased, there were less binding sites available which could release nutrient cations and remove Al^{3+} ions from solution. The agreement between the theoretical 1:1 exchange line and measured cation concentrations in solution is strong evidence that cation exchange is the main process removing Al^{3+} ions from solution.

These Al^{3+} ions were adsorbed to exchange sites on the root surface. In Ca treatments, these sites were occupied by Ca^{2+} ions as indicated by the fact that the equivalent sum of exchangeable Ca^{2+} + Al^{3+} did not differ between the treatments, whereas the equivalent ratio of adsorbed Ca/Al reached 11 (Ca treatment) and 0.6 (Al treatment).

The high percentage of NH_4Cl-extractable phenolics in Al treatments suggests that part of the exchangeable Al ions were not adsorbed as free Al^{3+} ions but as Al-phenolic complexes. If a simple complex of one phenolic unit and one Al^{3+} ion was assumed, there were sufficient phenolic ligands present in these extracts to bind to two thirds of the adsorbed Al. The sequential extraction results indicate that at the end of the Al treatment a large percentage of the Al adsorbed during the treatment is more strongly bound at the root in an acid soluble form. These results are in agreement with the findings of Dahlgren et al. (1991) who examined Al forms in *Abies amabilis* roots. They found only 12-17% of root Al to be exchangeable and discussed co-precipitation of Al with phosphate or oxalate within roots or on root surfaces. Our data do not suggest that oxalate is involved in the formation of an acid-soluble Al precipitate, as no oxalate could be found in the HCl extracts (data not shown). Formation of aluminium phosphates might occur to some extent as EDX microanalysis revealed an increase in P at the surface of roots in the Al treatments. However, as in HCl extracts of roots of both treatments P was found at levels near the detection limit (data not shown), aluminium phosphates, if present, did not account for more than 30% of the acid soluble Al and thus are not the major form of acid soluble Al.

Total phenolics in the HCl extract of Al treated roots accounted for only 6% of the Al equivalents found in this extract. Therefore, it must be concluded that the nature of the HCl extractable Al could not be elucidated completely.

The behaviour of K may be explained by its specific functions in plant cells. The cytoplasmic concentrations are generally very high and almost constant. Potassium easily leaks out of cells if membrane stability is compromised by either high proton influx (Sasaki et al. 1994) or insufficient Ca saturation (Mengel 1991). The absence of strong binding forms of K in plant cells (Mengel 1991) suggests that the HCl extractable K rather represents cytoplasmic K than strongly bound K. For Al treated roots, HCl extractable K was low, as most of the cytoplasmic K may already have leached across the plasmalemma membrane during the preceding NH_4Cl extraction. In Ca treated roots, membranes remained intact during the NH_4Cl extraction and K only leaked outside when high proton influx during HCl extraction caused depolarisation of the membrane.

2.5.2. X-ray microanalysis

The accumulation of Al in the cell walls of peripheral cortex cells as confirmed by the EDX microanalysis is in accordance with the results of Bauch and Schröder (1982), who investigated fine roots of healthy and diseased silver fir and Norway spruce trees and found Al mainly in cortex cell walls and only very low concentrations in cell walls of the xylem.

Accumulation of Al and decrease of Ca in cell walls has been shown by several authors (Godbold et al. 1988, Schröder et al. 1988, Kuhn et al. 1995, Godbold and Jentschke 1998). The decrease of Ca in cell walls of Al-treated plants is in accordance with the results of the NH_4Cl extraction, as cation exchange sites are mainly located on pectins, proteins and phospholipids in the cell wall (Horst 1995).

High X-ray net counts of K in Ca treated roots may be partly due to overlapping of the primary Ca peak (Ca Kα) and the secondary K peak (K Kβ, Lazof and Läuchli 1991). Additionally, it is possible that the very mobile K ion (Marschner 1995) was translocated from the shoot to the roots in order to compensate the dehydrating effects of high Ca concentrations (Bergmann 1993). Chloride present in the treatment solutions was taken up by the treated plants to reach a higher level than in the control as confirmed by EDX microanalysis. For reasons of charge balance, this should increase the pH of the treatment solutions if no equivalent amounts of cations are taken up. In accordance with this, in Ca treatments in which Cl concentrations in cell lumina were high, more HCl than in Al treatments had to be added during the experiment in order to keep the pH of the solution below 4.0 (data not shown).

2.5.3. Release of organic substances

Our experimental system very likely included rhizospheric microorganisms living at the root surface that were transferred to the treatment flask together with the roots. Therefore, this setup did not allow to calculate total C released by the roots, as part of it may be metabolised by microorganisms (Marschner 1995). However, it is well suited to characterise those organic substances that are not rapidly degraded. With respect to Al detoxification by complexation with organic ligands (Delhaize et al. 1993, Pellet et al. 1995, Zheng et al. 1998), only this fraction is considered efficient.

If Norway spruce followed the strategy to exclude Al by exudation of organic substances with strong complexing properties, either an enhancing effect of Al on

release of organic substances, or qualitative changes in their composition would be expected. In our experiment, Al treatment reduced total C release, and only during the second period the Al treated plants released a significantly higher percentage of phenolics than Ca treated plants. No other changes in composition were observed. The decrease in total C release could be the consequence of reduced C assimilation of Al treated spruce (Hentschel et al. 1993), or it could be due to changes in plasma membrane permeability. In various plant species, membrane permeability to electrolytes may increase by Al treatment (Calbo et al. 1997, Ishikawa and Wagatsuma 1998) and permeability to nonelectrolytes may decrease (Zhao et al. 1987, Parent et al. 1996), but the membrane itself is considered to stay intact although specific transport proteins may be blocked by Al (Kochian 1995).

Organic acids are suitable ligands that could effectively detoxify Al (Hue et al. 1986). However, our results support the finding of Eltrop (1993) that organic acid release by Norway spruce is generally very low. In contrast to the findings on maize (Pellet et al. 1995), wheat (Delhaize et al. 1993), and buckwheat (Zheng et al. 1998), where exudation of organic acids has been shown to be an effective detoxifying mechanism for Al, our results indicate that Norway spruce is not able to increase the release of organic acids to a level where effective detoxification of Al could occur. The conclusion, that organic acid exudation is not an important mechanism in alleviating Al toxicity in spruce, is indirectly supported by an investigation of organic acid concentrations in Norway spruce fine roots sampled in the humus layer and the upper mineral soil of a dystric cambisol (humus form: moder) in Germany (Nowotny et al. 1998). Upon acid irrigation, which very likely increased Al concentrations in the soil solution, citrate and malate concentrations in the roots decreased or remained constant, indicating that synthesis of these acids was not stimulated.

Beside organic acids, a role of polysaccharides and polyuronic acids in mucilage for Al tolerance has been proposed (Horst et al. 1982). However, excretion of carbohydrates and high molecular weight substances was not enhanced by Al treatment but reduced to the same degree as total C release, when compared with the results of the Ca treatments. About 10% of the released C can be attributed to phenolic substances, a group that can form stable complexes with Al^{3+} ions (Martell and Smith 1977). The fluorescence spectra of our solutions show Al^{3+} complexation by phenolic substances. Although during the second period phenolics represented a higher percentage of total C in Al

treatment solutions, Al treatment decreased the amount of phenolics released during the first period and had no effect on the amount in the later ones. Thus, complexation of Al^{3+} by phenolic substances exuded by Norway spruce roots cannot be regarded as an active protective mechanism. Furthermore, as total phenolics are only present at micromolar levels, only a minor part of the total Al in solution can be bound by these substances.

The low concentrations of free amino acids released do not suggest an important role for these compounds either. Amino acid release was of the same order of magnitude as release of phenolics. This is in good agreement with the results of Eltrop (1993) who found phenolics and amino acids at equal but low levels in the exudates of 5-month-old Norway spruce seedlings. Since hydrolysis did not increase amino acid yield, specific root exudation of polypeptides in response to Al stress as has been demonstrated in wheat (Basu et al. 1994, 1997) is unlikely to occur in Norway spruce.

2.5.4. Characterisation of released C

When summing up the percentage of analysed organic substances, at best 60 % of the released C could be identified. However, the analysis of carbohydrates and phenolic substances was done in terms of the low-molecular weight substances glucose and phenol, respectively. Considering the high content of high-molecular weight substances indicated by the ultrafiltration results, it is likely that the effective concentrations of released carbohydrate C and aromatic C were higher. Additionally, it should be noted that organic acids may constitute a significant portion of total C, although all single acids were below the detection limit. The sum of several single acids, each of them being present at concentrations below 2% of total C, could easily account for 10-20% of total C. This could not be checked for, since there is no method available to assess total organic acids. Generally, the results suggest that only minor changes of the composition of the released C occur while quantity is more clearly affected by Al treatment. This is further supported by the molar absorptivity data, which can be used as an indicator of aromaticity (Chin et al. 1994). In contrast to absolute absorptions, they did not differ significantly between the treatments in the remaining solutions of each period, which indicates that quality of released C is less affected by treatment than quantity.

2.5.5. Temporal changes in C release

The temporal pattern of C release was independent of treatment and very similar for all C fractions observed. The pattern might be partly influenced by the experimental design. The higher C loss and the lower percentage of phenolics in the first period might be attributed to a treatment shock, when plants were transferred from soil to hydroculture. Treatment solutions were single salt solutions and thus differed significantly from soil solution. As a consequence, root chemistry, which, at the beginning of the first period, was still influenced by the chemical conditions in the soil, had to be adjusted to the solution conditions. By contrast, in the following periods, the roots were pre-conditioned by the first treatment period. This explains why the results from the second and third periods for a given treatment differ much less from each other than they do from the results of the first period. The data from the second and third periods represent the C release which is characteristic of the given experimental conditions, while the first period must be considered to represent a transient state.

2.6. Conclusions

The results of this study imply that root exudation of organic substances with complexing properties does not contribute significantly to the relatively high Al tolerance of Norway spruce. Uptake of Al is impeded by immobilisation at the root surface and in the cell walls of epidermis and cortex. Complexation of Al with phenolic substances present at the root surface and precipitation as phosphate may play important roles in this immobilisation. If such a mechanism was to work over long periods of time, however, high turnover rates of the fine root system would be necessary (Vogt et al. 1987).

Chapter 3

Root exudation and chemistry of aluminium treated Norway spruce seedlings

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3.1. Abstract

Seedlings of Norway spruce (*Picea abies* [L.] Karst.), which had been grown under sterile conditions for three months, were treated for one week in a hydroculture system with either 500 µM $AlCl_3$ or 750 µM $CaCl_2$ solutions at pH 4. Organic acids were determined in extracts of ground root tissue. Oxalate (3.3 - 6.6 µmol (g root dry weight)$^{-1}$) was most abundant. Malate, citrate, formate, acetate and lactate concentrations ranged between 1 – 2 µmol (g root dry weight)$^{-1}$. The treatment solutions were analysed for organic substances and phosphate released from the roots. Total root exudation within a 2-day period ranged from 20 - 40 µmol C (g root weight)$^{-1}$. In root exudates, organic acids and total carbohydrates, total amino acids, and total phenolic substances were quantified. Citrate and malate, although present in water extracts of roots, were not detected in the root exudates. Phosphate was released from Ca treated plants. In Al treatments, there was indication of Al phosphate precipitation at the root surface. Oxalate and phenolics present in the exudates of Norway spruce seedlings are potential ligands that can form stable complexes with Al. However, concentrations of these substances in the treatment solutions were at a micromolar level. Their importance for Al detoxification under natural conditions is discussed.

Keywords: Al tolerance, hydroculture, organic acids, phenolics, phosphate, *Picea abies*

3.2. Introduction

As a consequence of soil acidification, pH of the soil solution can drop to values of 4 or lower. In acid soils, weathering of Al-bearing minerals can lead to high concentrations of dissolved Al. Free Al^{3+} ions are potentially toxic to plants as they can inhibit root growth and impair nutrient uptake (Delhaize and Ryan 1995, Kinraide 1991, Kochian 1995). Plants that grow on acid soils, therefore, must have strategies to cope with these conditions. Norway spruce (*Picea abies* [L.] Karst.) is a common conifer in Northern and Central Europe, and is found extensively on acid soils. This tree is known to tolerate concentrations of up to 0.3 mM Al^{3+} before growth is reduced. Lethal concentrations are even 30 times higher (Göransson and Eldhuset 1991). There is, however, little information on the mechanism of the Al resistance in this and other forest tree species.

Aluminium resistance mechanisms can be divided into internal and external mechanisms (Taylor 1991, Kochian 1995). Internal tolerance mechanisms detoxify Al in the symplasm, whereas external mechanisms either exclude Al from sensitive sites in the root (Taylor 1991) or, more generally, from root uptake. It is known that Al is mainly retained in the roots of Norway spruce and only very little is translocated to the shoot (Göransson and Eldhuset 1991, Hentschel et al. 1993). Dahlgren et al. (1991) discussed co-precipitation of Al with oxalate and phosphate as possible retention mechanisms of Al in fine roots of a Northwest American conifer stand dominated by *Abies amabilis* (Dougl.) Forbes and *Tsuga mertensiana* (Bong.) Carr. In the outer root compartments of Al treated 3-year-old Norway spruce, Al was immobilised in different chemical forms, with phenolics and phosphate as probable ligands (Heim et al. 1999, chapter 2 of this thesis).

There is little evidence that exclusion of Al from root uptake by formation of stable complexes with organic ligands in the soil solution has any importance for the Al resistance of trees. Such a reaction has been observed with Al resistant varieties of wheat (*Triticum aestivum* L.) (Delhaize et al. 1993), maize (*Zea mays* L.) (Pellet et al. 1995), and buckwheat (*Fagopyrum esculentum* Moench. cv Jianxi) (Zheng et al. 1998). In these plants, root exudation of organic acids was enhanced as a reaction to Al exposure. Information on tree root exudates is rare (Grayston et al. 1996). Organic acids have been identified in the root exudates released by seedlings of various pine species (Slankis et al. 1964, Smith 1969, Leyval and Berthelin 1993). However, Eltrop (1993)

found that the exudation of organic acids by Norway spruce seedlings in semi-hydroponic culture was below the detection limit of modern analytical tools. Results of a hydroponic experiment with non-sterile 3-year-old Norway spruce trees (Heim et al. 1999) were in good agreement with the latter finding. Organic acid release was detected in none of the treatments, and total organic C release was even lower for Al treated when compared to Ca treated plants. On the other hand, the formation of complexes between Al and released phenolic substances in the treatment solution could be shown qualitatively. There was no experimental evidence, however, for the release of the phenolic substances being an active reaction of the tree to the Al treatment.

Under the non-sterile conditions of the latter work, however, easily degradable compounds like organic acids could have been metabolised to a large degree by microorganisms (Von Wirén et al. 1993, Jones et al. 1996b), so that concentrations in hydroculture were too low to be detected. Under natural conditions, however, limited diffusion in the rhizosphere will lead to higher concentrations of exudates. There is also some controversy if metal complexes of these acids are as rapidly degraded as free acids (Bergsma and Konings 1983, Boudot et al. 1989, Jones 1998). Due to these uncertainties, an estimation of the potential exudation of easily degradable substances therefore requires axenic conditions. The efflux of negatively charged ions into the rhizosphere is favoured by the electrochemical gradient from cytosol to apoplast. Therefore a minimum baseline exudation of those anions that are present in the cytosol can be postulated (Jones 1998).

The aim of this paper was to determine the composition of root exudates from axenic Norway spruce seedlings in the presence of Al^{3+} as opposed to the presence of a nutrient cation (Ca^{2+}). Water soluble organic acids in roots, which are the source of organic acids in the rhizosphere, were included in the study. In addition, the fate of the added cations in roots was studied using electron-dispersive X-ray microanalysis (EDX).

3.3. Material and Methods

3.3.1. Plant material

Seeds of Norway spruce (*Picea abies* [L.] Karst.) from a common parent tree near Tägerwilen (canton of Thurgau, Switzerland, #846 of the seed collection of the Swiss Federal Institute for Forest, Snow and Landscape Research) were surface sterilised in

30 % H_2O_2 for 40 minutes and washed by four consecutive rinses with sterile deionised water. Seedlings were grown in sterilised mini-greenhouses. This system consisted of an autoclavable, sunlight-transparent plastic bag with a filter allowing gas exchange (Sun-Bag, Sigma), in which two beakers were placed, each filled with 600 ml of washed perlite and 300 ml of Melin-Norkrans nutrient solution (Marx 1969) without sucrose. The entire system was autoclaved before sowing out about 100 sterilised seeds into each beaker. The bags were closed and transferred to a climate chamber, and plants were grown for 13 weeks under the following conditions: temperature 20°C, relative humidity 50%, 16 h photoperiod. No visual contamination of fungal or bacterial origin was observed during the growth period.

3.3.2. Treatments

After pregrowth, plants were carefully removed from the perlite. A part of the seedlings was used directly for EDX measurements (untreated control). For the hydroculture treatments, 100 seedlings per treatment (average fresh weight per seedling: shoot 52.2 ± 5.5 mg, root 24.9 ± 4.7 mg) were transferred under sterile conditions to a comb-like support (Fig. 3.1).

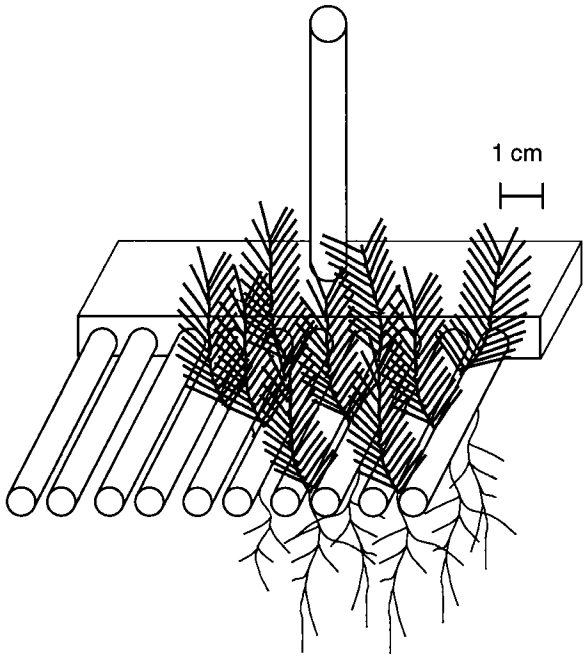

Figure 3.1. Supporting device for hydroculture treatments. 100 seedlings of Norway spruce were placed into the slits and the whole device was transferred to a rectangular covered glass container with treatment solution.

Until the transfer of the seedlings was finished, their root systems were immersed in a 10^{-4} M HCl solution to prevent them from drying and to wash away traces of the growing solution. The completely filled support was finally placed into an open glass container with 300 ml of treatment solution, in a way that the roots were immersed in the solution, while the shoots remained outside. The treatment solution was either 750 µM $CaCl_2$ or 500 µM $AlCl_3$, both set to pH 4.0 and sterile filtered before use. In both treatments, the pH was kept between 3.8 and 4.0 by a titroprocessor (Metrohm 670), and solutions were constantly aerated with 0.2 µm filtered air. After installation of the pH control and aeration devices, the glass container was covered with sunlight transparent material cut from Sun-Bags (Sigma). The treatments lasted for one week, which was divided into three periods of 2, 2, and 3 days. After each of these periods the treatment solutions were replaced by fresh ones. The purpose of this time pattern was to separate rapid effects caused by the treatment shock at the beginning of the experiment from longer-term plant reactions (Heim et al. 1999). The experiments were carried out in a growth chamber under the same conditions as mentioned above. Each treatment was repeated four times.

3.3.3. Solution sampling

Solution samples were taken three times a day during the first and second period and once a day during the third period. Twelve ml were sampled at each sampling date. The samples were filtered immediately through 0.45 µm syringe filters (Spartan 30 B, Schleicher & Schuell). The first 2 ml of the filtrate were discarded to exclude contamination originating from the filters. The rest was stored at –20°C until analysis. At the end of each treatment period the remaining solutions were filtered and stored in the same way.

3.3.4. Harvest and plant analysis

Upon harvest, root and shoot fresh weights were determined. The plant material of each replicate treatment (i.e. from 100 plants) was pooled and immediately frozen in liquid nitrogen and stored at –20°C until freeze-drying. About 70 mg of the lyophilised root material were ground to a fine powder. Twenty mg of this powder were extracted with 0.5 ml of hot water at 80°C for 20 min (Nowotny et al. 1998). Extraction was done in triplicate. After centrifugation, the supernatant was used for further analysis. The

residue was then extracted five times by shaking with 400 µl of 1mM Na_2EDTA for 30 min. Organic acids in these extracts were determined by capillary electrophoresis (CE) (BioFocus 3000, BioRad, 40 cm x 50 µm fused silica capillary). For the analysis of organic acids, 35 µl of 1mM Na_2EDTA were added to an equal sample volume in order to bind cations which otherwise would form complexes with the organic acids to be analysed and thus interfere in their determination. The buffer used for CE was 10 mM potassium hydrogen phthalate, 2.5% Waters OFM Anion-BT and 1mM EDTA, adjusted to pH 5.6 with 0.5 M LiOH. Preliminary tests had shown that the addition of EDTA to the buffer improved oxalate and citrate determination.

Root and shoot element contents were determined after digestion with 1 ml of 40 % HNO_3 and 40 µl of conc. HF in a microwave oven at 240°C and 120 bar (UltraClav, Microwave Laboratory Systems). Total element contents in the digests were measured with ICP-MS (Elan 6000, Perkin Elmer).

3.3.5. Solution analysis

On all treatment solution samples the following analyses were performed: cations were measured directly by the CE method of Göttlein and Blasek (1996), which uses a metol buffer. This method has been shown to determine the concentration of free Al^{3+} in soil solutions, while complexes between Al and organic ligands are not detected (Göttlein 1998). Dissolved organic carbon (DOC) was determined with a TOC-Analyser (Shimadzu TOC-500). UV absorption was measured at 280 nm with a UV-VIS spectrophotometer (Shimadzu UV-240).

On the solutions remaining at the end of each treatment period, additional analyses were performed. About 12 ml of the sample solution were filtered through an ultrafilter (1 kDa nominal molecular weight cut-off, Macrosep™ centrifugal concentrator, Pall Filtron, USA) at 2500 g for about 3 h. Retentate and filtrate were analysed for DOC and UV absorption at 280 nm was recorded.

A total of 120 ml was freeze-dried in PE bottles and first redissolved in 3 ml of Milli-Q water. In a second step, after removal of the concentrated solutions, an equal volume of 0.1 *M* HCl was added to these bottles in order to dissolve less soluble residues. In a third step, an equal volume of 1mM EDTA was used as solvent. In the first concentrate (water-soluble) the following analyses were performed: Total amino acids were determined before and after alkaline hydrolysis in 40x concentrated solutions using the

ninhydrin method (Allen 1981) with glycine as standard. Total carbohydrates were determined in 40x concentrated solutions by the phenol-sulfuric acid assay according to Chaplin (1994) using glucose as standard. Both methods were slightly modified to accommodate the small sample volumes. Total phenolics were analysed in 4x concentrated solutions with a colorimetric method according to Swain and Hillis (1959) using phenol as standard. In all three concentrates (water-, HCl- and EDTA-soluble) organic acids were determined by CE as described above. Prior to this analysis, cations and chloride were removed from the concentrated water and HCl solutions by passing the samples over a cation exchange resin saturated with Ag^+ ions.

3.3.6. Electron microscopy and X-ray microanalysis

Fine root samples were taken from plants treated in hydroculture and from untreated control plants harvested directly from the pregrowth pots. Lateral roots of approx. 5 mm length were mounted vertically in slits of supporting brass stubs using a cryo glue, frozen in liquid nitrogen and freeze-fractured with a rotating microtome at –90°C in the preparation chamber (Balzers SCU 020) of a cryo scanning electron microscope (Philips 515). For surface analyses, the samples were mounted horizontally with double-sided adhesive tape and frozen in liquid nitrogen. Elemental concentrations on the root surface, in cell walls and lumina of epidermis and cortex, and in the stele were assessed by energy-dispersive X-ray microanalysis (EDX) in a cryo scanning electron microscope (Brunner et al. 1996). The microscope was operated at an acceleration voltage of 20 kV with a beam current of 80 µA and a take-off angle of 15°. Working distance was 12 mm. All spectra were acquired for 120 s (live time) and a dead time of 20 %. Spot analysis was carried out with a maximum magnification of 10 000. Spectra were analysed using the Voyager software package, and results for individual elements are presented as background-corrected net counts. These net counts are semi-quantitative measures of concentration, but as analysis conditions and the etching process were not controlled rigorously and because of the problems of obtaining fully quantitative results from bulk-frozen hydrated samples (Van Steveninck and van Steveninck 1991), they were not converted to absolute concentrations. For surface analyses, three samples per treatment were analysed; on each sample four to six measurements were performed. For cross-fractures, four roots per treatment were analysed and three measurements per compartment were done.

3.3.7. Statistical analysis

In order to compare treatment effects on exudation in a given period, one-way ANOVA tests were performed using Data Desk 6.0.2 for Macintosh (Data Description Inc.). Data over all periods were analysed using Repeated Measures ANOVA in StatView 5.0 (SAS Inst. Inc.). The number of replicates per treatment was four in both analyses.

For the statistical analysis of organic acid and element content of roots an ANOVA procedure was chosen in Data Desk 6.0.2 for Macintosh that accounted for the nested design of three replicate chemical analyses within each of the four replicate treatments. For shoot nutrient content, the means of two replicate chemical analyses per sample were used in ANOVA, with n = 4 replicates per treatment.

3.4. Results

3.4.1. Treatment solutions

During the first 2-day-period, concentrations of free Al in the treatment solution, as measured by CE, decreased from the initial level of 500 µM to about 400 µM. In the following periods, measured Al concentrations remained constant at the level of 500 µM (table 3.1). Small amounts of Ca and Mg were released from the roots and detected in the Al treatment solutions, which had been prepared as single-salt solutions. The Ca and Mg concentrations, however, did not exceed 50 µM each during the first period and often were at the detection limit of 10 µM during the later periods (data not shown). Ammonium was always below the detection limit. The amount of K released from the root could not be determined, since K also leached from the pH electrode.

Table 3.1. Al^{3+} concentration in Al treatment solutions as measured by capillary electrophoresis. Values are mean concentrations ± SE in each of the three treatment periods of four replicate treatments and 4-7 sampling dates per period.

Period	Al^{3+} [µmol l^{-1}]
1	406 ± 7
2	521 ± 6
3	532 ± 10

3.4.2. Localisation of elements

In Al treated roots, Al was mainly found at the surface (table 3.2) and in cell walls (table 3.3). A low amount of Al also occurred in Ca treated roots and untreated control roots, where Al was evenly distributed over all compartments. Al was also detected in shoots of both treatments, but concentrations in Al treatments were not significantly higher than in Ca treatments. In Ca treatments, Ca was found mainly in the cell walls and at the surface. Net counts of Ca in Al treated plants were similar to those in untreated control plants. Ca ions in the treatment solution were taken up by the plants and transported to the shoots, where the Ca content reached 1.14 mg g^{-1}, although a large percentage remained in the roots (2.70 mg g^{-1}; table 3.4.).

At the surface and in epidermal cell walls, higher net counts of P were recorded in Al treated roots than in Ca treated roots and controls. In all other compartments, net counts of P in Al- and Ca-treated plants were lower than in the control plants. Total P content of Al treated roots was slightly higher than of Ca treated roots (table 3.4). Potassium was mainly present in cell lumina and less in cell walls of treated plants. In control plants, it was more evenly distributed between the compartments. A high number of K counts was recorded at the surface in both treatments, most probably due to leakage from the pH electrode and subsequent adsorption to the root surface. In control plants, K counts were much lower.

Table 3.2. X-Ray net counts of selected elements at the surface of fine roots of Norway spruce seedlings. Values are mean ± SD of 3 samples per treatment and 4-6 spectra per compartment.

	Net counts			
	Al	Ca	K	P
Ca treatment	802 ± 161	3346 ± 972	7872 ± 2460	636 ± 203
Al treatment	6723 ± 1877	920 ± 211	5491 ± 1779	1584 ± 358
untreated control	570 ± 286	539 ± 247	2728 ± 589	769 ± 290

Table 3.3. X-Ray net counts of selected elements in various cell types and compartments of freeze-fractured fine roots of Norway spruce seedlings. Values are mean ± SD of 4 samples per treatment and 3 spectra per compartment.

	Net counts			
	Al	Ca	K	P
Ca treatment				
Epidermal cell wall	395 ± 210	1556 ± 748	2020 ± 591	426 ± 138
Epidermal lumen	324 ± 151	557 ± 168	7978 ± 2490	983 ± 412
Cortical cell wall	297 ± 64	1304 ± 124	5037 ± 1068	1679 ± 537
Cortical lumen	330 ± 311	728 ± 424	3084 ± 2097	1223 ± 582
Stele	n.d.	696 ± 201	3751 ± 849	425 ± 191
Al treatment				
Epidermal cell wall	2563 ± 826	223 ± 49	1302 ± 321	1870 ± 746
Epidermal lumen	585 ± 272	382 ± 54	5182 ± 407	1367 ± 588
Cortical cell wall	1012 ± 287	593 ± 226	2063 ± 472	1398 ± 353
Cortical lumen	318 ± 262	492 ± 115	3287 ± 790	2386 ± 902
Stele	172 ± 57	132 ± 84	4826 ± 413	449 ± 143
untreated control				
Epidermal cell wall	277 ± 96	549 ± 258	2916 ± 994	745 ± 236
Epidermal lumen	272 ± 121	410 ± 108	4503 ± 530	5023 ± 1546
Cortical cell wall	358 ± 84	547 ±187	3648 ± 574	3147 ± 355
Cortical lumen	290 ± 243	438 ± 129	4225 ± 762	4700 ± 2099
Stele	131 ± 81	376 ± 92	7990 ± 1907	1362 ± 414

Table 3.4. Element content in shoots and roots of Norway spruce seedlings at the end of the hydroculture treatments (mean ± SE of four replicates per treatment). Differences between the treatments are significant at a level of ***: $p < 0.001$; **: $p < 0.01$; (*): $p < 0.1$; ns: not significant

	Element content [mg (g DW)$^{-1}$]				
	Al	Ca	K	Mg	P
Shoots					
Al treatment	0.19 ± 0.02	0.93 ± 0.03	9.18 ± 0.45	1.41 ± 0.05	5.17 ± 0.25
Ca treatment	0.13 ± 0.02	1.14 ± 0.03	9.68 ± 0.34	1.49 ± 0.07	5.40 ± 0.38
Significance level	ns	* *	ns	ns	ns
Roots					
Al treatment	2.53 ± 0.19	0.98 ± 0.06	4.70 ± 0.12	0.62 ± 0.02	3.34 ± 0.09
Ca treatment	0.59 ± 0.12	2.70 ± 0.04	4.71 ± 0.11	0.66 ± 0.05	2.89 ± 0.14
Significance level	* * *	* * *	ns	ns	(*)

3.4.3. Hot water extract of ground root tissue

The following organic acids were identified in the hot water extracts of Norway spruce roots: oxalate, formate, malate, citrate, acetate and lactate (table 3.5). Oxalate was the most abundant of these acids. Al treated roots contained significantly higher amounts (6.6 µmol (g DW)$^{-1}$) of hot water extractable oxalate than Ca treated roots (3.3 µmol (g DW)$^{-1}$). Total extractable oxalate in roots as determined by water and EDTA extractions, however, reached 12 µmol (g DW)$^{-1}$ in both treatments and did not differ significantly between the treatments (not shown). The hot-water soluble acetate concentration was also significantly higher in Al treated roots, while the levels of the other organic acids that were detected in the roots did not differ significantly between the treatments.

Table 3.5. Water extractable organic acids in Norway spruce roots (mean ± SE of four replicates per treatment and three extractions per replicate treatment). Water extractable tissue concentrations are different between the treatments at the following levels of significance: **: $p < 0.01$, *: $p < 0.05$, ns: not significant.

	μmol (g root DW)$^{-1}$					
	oxalic	formic	malic	citric	acetic	lactic
Al treatment	6.6 ± 0.6	1.7 ± 0.1	1.4 ± 0.2	1.2 ± 0.1	1.5 ± 0.1	1.9 ± 0.5
Ca treatment	3.3 ± 0.2	1.4 ± 0.1	1.4 ± 0.2	1.7 ± 0.3	1.1 ± 0.1	0.6 ± 0.1
Level of significance	**	ns	ns	ns	*	ns

3.4.4. Carbon release

Total C release on a root fresh weight basis varied considerably between the replicate treatments. No difference between the treatments could be observed. Table 3.6 gives an overview of the C fractions that could be identified. Although ultrafiltration through a 1 kDa filter indicated that low molecular weight compounds formed the major part of the C exuded by the plants, only between 10 and 47% (mean: 25%) of the dissolved organic C in our treatment solutions could be identified assuming an average number of C per molecule as indicated in table 3.6. Total carbohydrates formed the largest percentage of identified C, but these did not exceed 12%.

Four organic acids were identified in the solutions, including oxalate, formate, acetate and lactate. Citrate and malate, although present in the roots, were below detection limits throughout. The identified organic acids represented 4-10% of total C. Phenolics were below 5% and amino acids (after alkaline hydrolysis) contributed up to 7% to total C. Hydrolysis of solution samples increased the yield of amino acids in most samples, which indicated the presence of peptides in the solution. Aluminium treated roots tended to release more total amino acids than Ca treated roots in periods 2 and 3.

No differences between treatments were found for molar absorptivities at 280 nm.

When a Repeated Measures ANOVA was performed over all data, none of the C fractions showed a treatment effect. However, for all organic acids and for phenolics, significant effects of the treatment period on exudation were observed, which was not the case for total C, carbohydrates, amino acids, high molecular weight compounds and molar absorptivity.

Table 3.6. Release of organic substances by roots of Norway spruce seedlings during treatments in hydroculture (mean ± SE). Data were calculated from concentrations in the solution after each period. Influence of the factors period and treatment on exudation of a specific compound is significant at a level of ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, ns: not significant

	Ca treatment			Al treatment			*Significance level*	
	1st period Day 2	2nd period Day 4	3rd period Day 7	1st period Day 2	2nd period Day 4	3rd period Day 7	Period	Treatment
	µmol C (g root fresh weight)$^{-1}$							
Total carbon	43.6 ± 7.4	21.9 ± 3.1	30.0 ± 6.2	37.4 ± 13.5	24.9 ±7.2	24.5 ± 5.1	ns	ns
Total carbohydrates [a]	4.1 ± 1.1	1.7 ± 0.3	3.6 ± 1.6	1.4 ± 0.2	2.6 ± 1.1	2.2 ± 0.4	ns	ns
Total phenolics [b]	1.3 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	0.6 ± 0.1	1.1 ± 0.4	*	ns
Amino acids unhydrolysed [c]	1.3 ± 0.6	0.3 ± 0.1	0.3 ± 0.0	1.1 ± 0.6	0.6 ± 0.2	1.0 ± 0.3	ns	ns
Amino acids hydrolysed [c]	1.7 ± 0.6	0.3 ± 0.1	0.6 ± 0.1	1.6 ± 0.8	1.4 ± 0.5	1.6 ± 0.3	ns	ns
Oxalic acid	0.9 ± 0.2	0.2 ± 0.1	0.4 ± 0.2	0.9 ± 0.3	0.2 ± 0.0	0.1 ± 0.0	***	ns
Formic acid	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.4 ± 0.2	0.1 ± 0.0	0.2 ± 0.1	**	ns
Acetic acid	2.1 ± 0.5	0.6 ± 0.1	0.9 ± 0.3	2.0 ± 0.7	0.6 ± 0.2	0.9 ± 0.3	**	ns
Lactic acid	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	*	ns
HMW carbon [d]	13.5 ± 4.8	3.4 ± 0.7	4.2 ± 0.7	5.6 ± 1.8	4.4 ± 1.1	6.2 ± 1.8	ns	ns
	L (mol C)$^{-1}$ cm^{-1}							
Molar absorptivity	129 ± 2	108 ± 15	104 ± 19	128 ± 19	97 ± 12	100 ± 14	ns	ns

[a] C concentration calculated as 6x carbohydrate concentration
[b] C concentration calculated as 6x phenolics concentration
[c] C concentration calculated as 5x amino acid concentration
[d] high molecular weight (>1kDa)

3.4.5. Phosphate release

Figure 3.2 shows the amounts of phosphate released into the treatment solutions during each treatment period. In Ca treatments, the phosphate release reached 5.8 µmol (g root fresh weight)$^{-1}$ during the first period and 1- 1.5 µmol (g root fresh weight)$^{-1}$ during the later periods. In Al treatments, the amounts of phosphate found in solution were roughly one order of magnitude smaller.

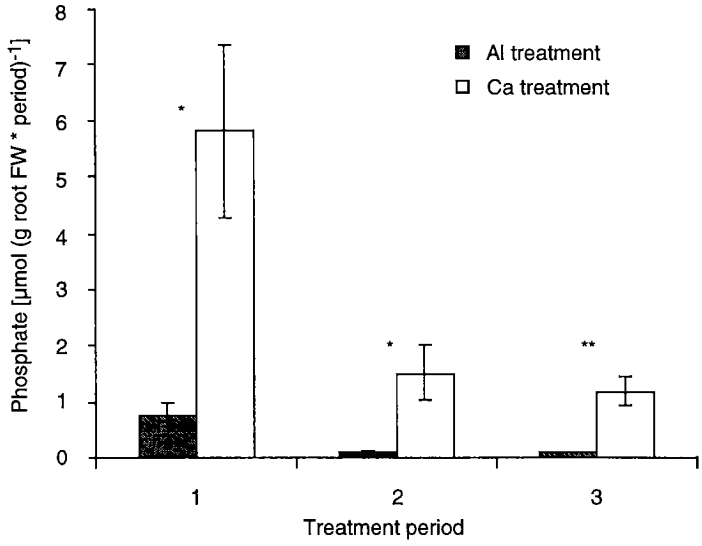

Figure 3.2. Phosphate released by Norway spruce roots into the treatment solution during the three treatment periods (mean ± SE of four replicates). Phosphate release in Ca and Al treatments in a given period is different at a significance level of *: $p < 0.05$; **: $p < 0.01$

3.5. Discussion

3.5.1. Experimental design

In this experiment, short term hydroculture treatments using simple salt solutions were conducted in order to observe the effect of Al^{3+} as opposed to the effect of the nutrient cation Ca^{2+} on root exudation and organic acid content of axenic Norway spruce roots. Single salt solutions were chosen in order to exclude interference from other ions (Grauer and Horst 1992, Grauer 1993). The Cl concentration was equal in both treatments (1.5 mM) in order to avoid Cl effects. As seedlings had been supplied with sufficient nutrients during the 3-months preculture period, it is assumed that the 1-week-treatment did not induce any nutrient deficiency.

3.5.2. Tissue concentrations of elements

Both Ca and Al, when added to the hydroculture solutions, accumulated mainly in roots. From the EDX data, it can be seen that the major sites of accumulation are the epidermal and cortical cell walls. Lazof et al. (1994, 1997) have pointed out that lateral resolution of EDX is not always sufficient to allow differentiation of cell walls and cell lumina. The resolution obtained with the present method is approximately 3 µm. Where the cell walls were thick enough, it was therefore possible to obtain cell wall spectra that excluded vacuolar and cytoplasmic contents. Accumulation of Al and Ca in cell walls is in agreement with the restricted transport of Ca^{2+} and Al^{3+} ions into the cytoplasm (Marschner 1995) and confirms our finding with 3-year-old Norway spruce roots (Heim et al. 1999), as well as earlier reports on Al distribution in Norway spruce roots (Bauch and Schröder 1982, Hodson and Wilkins 1991). Comparison with untreated control roots showed that Ca counts in roots were almost unaffected by Al treatment (table 3.3). This result is in contrast to the displacement of Ca by Al observed by several authors (Godbold et al. 1988, Cronan 1991, Kuhn et al. 1995, Godbold and Jentschke 1998). It may, however, be explained by binding of Al in a non-exchangeable form (Heim et al. 1999), e.g. precipitation of sparingly soluble Al phases. For Ca, in addition to binding to apoplastic pectate groups, precipitation with oxalate may be responsible for the storage of Ca in the apoplastic compartment (Fink 1991, 1992). However, added Ca was also transported to the shoots, as indicated by the higher shoot concentrations in the Ca treatments. Shoot Al in both treatments originated mainly from the perlite substrate used for pregrowth. The fact that the 1-week Al treatment did not significantly increase shoot Al concentrations emphasises the ability of roots to efficiently retain this element. This retention is not complete, however, which can be seen from the fact that during pregrowth a slow uptake of Al into shoots occurred.

3.5.3. Organic acids in the roots

An internal tolerance mechanism (Al chelation in the symplast) is not of the same importance in Norway spruce as in hydrangea (*Hydrangea macrophylla*) and buckwheat (Ma et al. 1997, 1998) because EDX analyses as well as reports by Bauch and Schröder (1982) and Hodson and Wilkins (1991) showed that accumulation of Al in Norway spruce roots is confined to the cell walls. Nevertheless, if small amounts of Al^{3+} ions below the detection limit of EDX enter the symplast, they will be chelated by the

organic acids present. The main objective of observing water extractable organic acids in Norway spruce roots, however, was to gain information on the pool of organic acids that is potentially available for exudation.

The higher water extractable root tissue concentrations of oxalate in Al treatments when compared with Ca treatments, did not indicate a higher production of this organic acid. It could be shown by five consecutive EDTA extractions that total amounts of extractable oxalate in the root tissue did not differ between the treatments. The low extractability of oxalate by water in Ca treatments can be attributed to the precipitation of Ca oxalate. We cannot decide whether this happened during extraction or whether Ca oxalate was also present in living roots. However, Ca precipitation has already been described by Fink (1992) for Norway spruce roots treated with Ca concentrations of 2.5 mM. The hot-water soluble amounts in the roots of two other organic acids that form strong complexes with Al, malate and citrate, were not affected by the treatments. Similar observations were made by Nowotny et al. (1998) who investigated the concentrations of these organic acids in Norway spruce fine roots sampled in the upper mineral soil of a dystric cambisol in Germany. Upon acid irrigation, which very likely increased Al concentrations in the soil solution, citrate and malate concentrations in these fine roots remained constant. The slightly higher amounts of hot-water soluble acetate in Al treated roots point to changes in metabolism, but as this organic acid anion only forms weak complexes with Al (Hue et al. 1986), a protective role of acetate is unlikely.

3.5.4. Carbon release

Seedlings of Norway spruce released a variety of C fractions of low molecular weight, including carbohydrates, amino acids, phenolic substances and organic acids. Except for organic acids, these substances have also been reported in earlier work on Norway spruce root exudates by Eltrop (1993) and our laboratory (Heim et al. 1999). Furthermore, various pine species and deciduous trees have been reported to release a wide range of these compounds (Grayston et al. 1996), which are known to be main constituents of root exudates (Marschner 1995). If based on root weight, the total amount of C found in solution was much higher for seedlings compared with an earlier experiment with 3-year-old Norway spruce (Heim et al. 1999). One possible explanation for this is the absence of microorganisms in the present experiment with

sterile seedlings, whereas the 3-year-old trees had been raised in soil and transfer of microorganisms to the hydroculture system had to be expected. It is known that microorganisms in the rhizosphere feed on root exudates (Lynch 1990). However, the amounts of identified easily degradable compounds like carbohydrates and amino acids were similar in exudates of seedlings and 3-year-old trees (Heim et al. 1999). Differences in root structure provide an additional explanation for the apparently higher total C release by Norway spruce seedlings. It is generally assumed that root exudation of C is higher in apical than in basal root zones (Uren and Reisenauer 1988). The basal root parts of the seedlings were light brown in colour and electron microscopy showed that cells were still intact. In contrast, part of the root system of the older trees was dark-coloured and electron microscopy showed lignification of the outer cell layers. It can therefore be assumed that a larger proportion of the root system contributed actively to C release in the seedlings than in older trees.

None of the various exudate fractions showed a treatment effect, which is in contrast to the well-documented stimulation of organic acid exudation observed for various crop plants as a reaction to high Al concentrations (Delhaize et al. 1993, Pellet et al. 1995, Zheng et al. 1998). This result confirms the conclusion of our study with 3-year-old Norway spruce trees that exudation of organic substances is not an active Al resistance mechanism in Norway spruce (Heim et al. 1999).

A temporal variability in exudation was detected for organic acid and phenolic fractions in the exudate but not for the other fractions. Especially the higher exudation in the first period probably is caused by the treatment shock, when plants were transferred to hydroculture.

Of the organic acids extracted from the ground root tissue, citrate and malate were not detected in the exudates, although their water extractable tissue concentration was as high as the tissue concentrations of acids that were detected in the exudates, viz acetate, formate and lactate.

There are two different possibilities to explain this observation. On the one hand, the organic acids in Norway spruce roots may be present in different compartments. From an electrochemical point of view, organic acid anions in the cytosol could diffuse passively through anion channels in the cell membrane. This is not the case for anions stored in the vacuole (Jones 1998). On the other hand, discrimination of ions could

occur at the cell membrane. Transport pathways across the cell membranes may be strictly selective (Tyerman 1992) and might not be available for all organic acid anions. Among the acids detected in the exudates, oxalate forms strong complexes with Al^{3+}, thus potentially reducing the concentration of toxic Al species (Hue et al. 1986). Complex formation between phenolic ligands exuded from roots and Al has been demonstrated for the 3-year-old Norway spruce trees (Heim et al. 1999). When present at the concentrations found in our treatment solution, both oxalate and phenolics could detoxify less than 2% of the Al^{3+} ions each, but in soils, limited diffusion may lead to higher concentrations at the rhizoplane and the amounts excreted may be physiologically relevant to the protection of the sensitive apical zone.

3.5.5. Phosphorus release

While P was released as orthophosphate in the Ca treatments, no significant P release was observed in the Al treatments. On the other hand, it could be demonstrated by EDX that P accumulates at the root surface of Al treated roots only. These two results are well compatible if we assume that phosphate release from the spruce roots occurred in both treatments but that in the presence of high Al concentrations, this phosphate precipitated with Al at the root surface and in the apoplast to form Al phosphate. This is further supported by the chemical analyses, where a higher P content was detected in Al treated roots than in Ca treated roots. Increased P net counts at the surface of Al treated roots were already observed in the previous experiment with 3-year-old trees (Heim et al. 1999). An analogous precipitation of Ca phosphate in Ca treatments, however, did not occur to a significant extent in our system due to the higher solubility of Ca phosphates in acid solutions (Mengel 1991).

Phosphate release in the presence of high Al concentrations has been observed in various crop species. In wheat, Pellet et al. (1997) found higher constitutive phosphate excretion in Al tolerant than in Al-sensitive cultivars. Phosphate release by tolerant maize cultivars even seems to be stimulated by Al (Pellet et al. 1995). In Al-resistant sugar beet (*Beta vulgaris* L.), Lindberg (1990) speculated about a metabolically dependent P efflux in the presence of Al.

Norway spruce appears to constitutively release phosphate from its roots under P sufficient conditions. In the presence of Al, Al phosphate then precipitates at the root surface, reducing the uptake of this toxic element. Most acidic soils, however, are P-

limited (Marschner 1991a). If Norway spruce relied exclusively on phosphate excretion to protect its roots from Al, the operation of this strategy would represent a substantial P drain on the tree. However, Norway spruce roots are frequently ectomycorrhizal under natural conditions, and this association is known to improve the P nutrition of the host tree, possibly providing phosphate for the excretion process.

3.6. Conclusions

The experiment with Norway spruce seedlings confirmed our conclusion from the experiment with non-sterile 3-year-old trees (Heim et al. 1999) that under hydroponic conditions exudation of organic substances is of little importance for the Al resistance of Norway spruce. Although micromolar amounts of oxalate, acetate, lactate, formate and phenolics were detected in the exudates of axenic Norway spruce seedlings, there is no specific stimulation of their exudation by Al. Comparison with root tissue concentrations of organic acids shows that malate and citrate are retained efficiently in the roots.

These findings do not completely rule out a role of chelating exudates in Al tolerance under soil conditions. At the apex, which is the main site of exudation (Uren and Reisenauer 1988, Marschner 1995), limited diffusion in soils can lead to higher concentrations of released ligands. Since the apex usually is the site most sensitive to Al toxicity (Kochian 1995), these ligands could be physiologically relevant there.

Precipitation of Al with phosphate released by Norway spruce roots under P-sufficient conditions represents another possibility for Al immobilisation that deserves further research.

Chapter 4

Effect of Al and inoculation with the ectomycorrhizal fungus *Hebeloma crustuliniforme* on growth, mineral nutrition and organic acid exudation of Norway spruce seedlings

4.1. Abstract

A growth experiment with Norway spruce (*Picea abies* [L.] Karst.) seedlings in perlite was performed in order to study the effect of Al and ectomycorrhizal inoculation on growth parameters, mineral nutrition as well as root content and exudation of organic acids. Of particular interest was the potential role of organic acid exudation in Al resistance of Norway spruce. Surface sterilised seeds of Norway spruce were sown into beakers filled with perlite and grown axenically for five months. Nutrients were added at the level found in the soil solution of an acidic forest site, and 0, 100 or 500 µM Al were added. The system was inoculated or not with the ectomycorrhizal fungus *Hebeloma crustuliniforme* (Bull.: St. Amans) Quél. Germination success was negatively affected by Al as was total biomass per beaker. A significant positive effect of inoculation on tree biomass production was observed, which compensated the negative Al effect at the high Al level. Although most of the Al was retained in roots, especially when inoculated, significant uptake of Al into shoots was observed at the high Al level, independent of inoculation. Phosphorus and Mg nutrition were negatively affected by Al. Phosphorus in plants originated mainly from the seed reserves, and Al caused P loss from non-inoculated seedlings. This loss was compensated by inoculation, which was the most obvious beneficial effect of the fungus on nutrition. Among the organic acids in roots, only formate and acetate were positively affected by Al. Oxalate was strongly reduced by inoculation, and succinate was produced mainly by the fungus, while malate, citrate and lactate were unaffected by treatments. In exudates, oxalate was dominant, while the other potential chelators malate and citrate were less abundant. No significant effect of Al on exudation of organic acids was observed. It is concluded that the presence of plant-available Al does not trigger organic acid release by Norway spruce or *Hebeloma crustuliniforme*. The efficiency of the constitutively released organic acids and the fungal inoculation in detoxifying Al is discussed.

4.2. Introduction

Acid forest soils in Central Europe are characterised by low nutrient availability and the risk of Al toxicity to trees (Ulrich 1986). Toxic effects of Al on growth and mineral nutrition of Norway spruce have been reported frequently for laboratory experiments (Rost-Siebert 1983, 1985, Asp et al. 1988, Arovaara and Ilvesniemi 1990, Godbold 1991). However, these effects were observed only at Al concentrations above the values typically found in forest soils (Göransson and Eldhuset 1991, Henriksen et al. 1992). This suggests resistance of this species to naturally occurring Al concentrations. Among the mechanisms of adaptation of plants to acid soils, root-induced chemical changes in the rhizosphere are of major importance (Marschner 1991a). The exudation of organic substances, especially of chelating organic acids, by roots can increase nutrient availability (Uren and Reisenauer 1988, Jones 1998) and reduce the toxicity of metal ions by formation of stable complexes (Hue et al. 1986, Martell and Smith 1977, Martell et al. 1988). Detoxification of Al by organic acids was recently reviewed by Ma (2000). However, most of the work in this field has been done with crop plants. Only a few studies have investigated tree root exudates (Grayston et al. 1996) and the effect of Al on tree root exudation has not been studied extensively. In chapters 2 and 3, we examined root exudation of non-sterile and axenic Norway spruce (Picea abies [L.] Karst.) in the presence of toxic Al^{3+} ions under hydroponic conditions and found only little exudation of organic chelators. However, natural conditions differ from hydroculture in many ways. In view of Al resistance, the following features have important implications:

Firstly, it has been shown by several investigators (Barber and Gunn 1974, D'Arcy-Lameta 1982, Mozafar 1991), that exudation is increased by the mechanical resistance of a solid substrate compared with hydroponic conditions. This is believed to be a reason for the higher Al resistance of soybean in solid substrate than in solution culture (Horst et al. 1990). Additionally, the spatial restrictions in soil pores impede a rapid diffusion of exudates to the bulk soil. As a consequence, higher concentrations of organic acids have been predicted to exist in the rhizosphere than in bulk soil (Jones et al. 1996a, Darrah 1991, Jones 1998).

Secondly, natural stands of conifers in boreal forests are frequently ectomycorrhizal. It is generally accepted that this symbiosis improves mineral nutrition of the host tree, in particular with respect to N and P (Marschner 1995). Several investigators have found

additional beneficial effects of ectomycorrhizas. A "sheathing" (Wilkins 1991) or "filtering" (Turnau et al. 1996) effect of the fungal mycelium is proposed for the observed metal tolerance of some ectomycorrhizal vs. non-mycorrhizal trees. Metal tolerance correlates with the amount of fungal mycelium produced (Colpaert and van Assche 1992, 1993). However, ectomycorrhizas do not systematically alleviate metal toxicity in trees, but the effect depends on tree, metal and fungal strain (Godbold et al. 1998). A few studies have been conducted investigating Al detoxification by ectomycorrhizal fungi in forest trees. The fungus *Pisolithus tinctorius* Coker and Couch was effective in reducing Al uptake by pitch pine (*Pinus rigida* Mill.) and compensated growth reductions caused by 200 µM Al. In this system, inoculation considerably improved mineral nutrition during a 49–days treatment (Cumming and Weinstein 1990). *Paxillus involutus* Fr. was able to compensate Al-induced growth reductions in Norway spruce seedlings from a calcareous site, but it had no effect on growth of seedlings from an acidic site (Wilkins and Hodson 1989). Inoculation increased the immobilisation of Al in the roots of Norway spruce thereby improving its Al resistance and reducing uptake into the needles (Hentschel et al. 1993). By contrast, in ectomycorrhizal Norway spruce treated in sand culture with 0.8 mM $Al(NO_3)$, negative effects of Al on root growth, Mg uptake and chlorophyll content of needles occurred despite the inoculation with *Lactarius rufus* (Scop.) Fr. (Jentschke et al. 1991).

Thirdly, the factors exudation and ectomycorrhizal fungi cannot be considered separately, as fungi themselves have been shown to produce and exude organic acids (Lapeyrie et al. 1987, Devêvre et al. 1996). A recent hypothesis postulates that nutrient and Al dynamics in podzol soils under boreal forests are governed by mycorrhizal fungi which dissolve minerals by organic acid exudates and then transport elements to tree roots via their hyphal systems (Van Breemen et al. 2000a, 2000b).

The objective of this work was to study the effect of Al addition and mycorrhizal inoculation on growth, element content, and amounts of organic acids in roots and root exudates of Norway spruce seedlings grown in a solid substrate.

4.3. Materials and Methods

4.3.1. Culture system

The substrate used for growth was commercial horticultural perlite with a mean diameter of 5 mm, which was washed thoroughly with sulfuric acid and then rinsed with deionised water until pH was neutral and conductivity below 10 µS/cm (J. Colpaert, pers. comm.). The perlite contained 9.4 mg g^{-1} Al, 8.6 mg g^{-1} Na, 6.1 mg g^{-1} K, and 9 µg g^{-1} P as determined by digestion with 1 ml of 40 % HNO_3 and 40 µl of conc. HF in a microwave oven at 240°C and 120 bar (UltraClav, Microwave Laboratory Systems) and element analysis using ICP-AES (Optima 3000, Perkin Elmer).

A dry weight of 50 g perlite each was filled into 800 ml PP beakers (13.5 cm height, 9.5 cm diameter), resulting in a filling height of 8 cm. Four holes of 1 mm diameter were drilled into the bottom of the beaker, which allowed water circulation. Two of the beakers were placed into one sterilised sunlight-transparent plastic bag with a filter allowing gas exchange (Sunbag, Sigma). The unit consisting of bags and beakers filled with perlite was then sterilised by heating at 105 °C for at least two days, as autoclaving had proved to be impracticable, because it released millimolar concentrations of sodium from the perlite.

The growth solutions were synthetic nutrient solutions which reflected the typical ionic composition of an acidified forest site in Germany and contained 300 µM NH_4NO_3, 50 µM Na_2SO_4, 100 µM K_2SO_4, 30 µM KH_2PO_4, 60 µM $MgSO_4$, 130 µM $CaSO_4$, 5 µM $MnSO_4$, 5 µM $FeCl_3$, 5 µM H_3BO_3, 0.1 µMNa_2MoO_4, 0.1 µM $ZnSO_4$, 0.1 µM $CuSO_4$ (Jentschke et al. 1991). To the nutrient solutions, 0, 100 or 500 µmol/l $AlCl_3$ were added and the pH was adjusted to 4.0 using HCl. Solutions were sterilised by filtration through a 0.2 µm membrane.

Seeds of Norway spruce (*Picea abies* [L.] Karst.) were surface sterilised in 30% H_2O_2 for 40 minutes and rinsed four times with sterile deionized water. Inocula of *Hebeloma crustuliniforme* (Bull.: St. Amans) Quél. were prepared from cultures grown on MMN agar (Marx 1969).

Growth solution (250 ml per beaker), seeds (80 per beaker) and inocula (none or 8 per beaker) were added to the beakers in a sterile bench and the bags were closed and transferred to a climate chamber (conditions: 20°C, 50% humidity, 16 h photoperiod).

Nutrient solution was added only once when the experiment was set up in order to avoid contamination by later additions.

The experiment was set out in a 2 (inoculation) x 3 (Al level) factorial design with six replicate beakers each, giving a total of 36 beakers or 18 bags. Additionally, control beakers without seeds were prepared in duplicate for all treatments.

4.3.2. Fungal liquid culture

Pure fungal cultures were grown in liquid culture in autoclaved 250 ml Erlenmeyer flasks in the following solutions: MMN solution without agar, synthetic soil solution without Al (as above) and synthetic soil solution + 500 µM $AlCl_3$ (as above). Each treatment was done in triplicate. Six inocula were added per flask and the flasks were placed on an automatic shaker in the same climate chamber as the bags with plants. Fungi were grown for two months in this system.

4.3.3. Harvest

After five months of growth, the bags were opened and plants were harvested. The number of established seedlings, root and shoot fresh weight were recorded for all beakers. The number of seeds that had germinated, but failed to establish, was recorded separately and these individuals were not included in the analyses. For two replicate beakers per treatment the length of the longest root was measured for all seedlings. The plant material was pooled per beaker, frozen in liquid N_2 and lyophilised.

About 10 g of the perlite substrate were sampled from each beaker and stored at –20°C until analysis. The growth solutions remaining in the bags were pooled per treatment and stored at -20°C.

Liquid cultures of fungi were harvested after two months of growth. They were vacuum filtered over a 0.45 µm membrane filter, then frozen in liquid nitrogen and lyophilised.

4.3.4. Analysis

Lyophilised root and fungal material was ground in liquid nitrogen to a fine powder. Twenty mg of the powder were weighed into Eppendorf vials and extracted with 0.5 ml of hot water (80°C) for 25 minutes. After centrifugation at 25,000 g, the residue was extracted with 2 mM EDTA for 40 minutes to dissolve more strongly bound organic

acids (chapter 3). Hot-water extracts were diluted with an equal volume of 1mMNa_2-EDTA before analysis.

Analysis of organic acids and orthophosphate in the extracts was performed by capillary electrophoresis (BioFocus 3000, BioRad, Hercules CA) under the following conditions: 40 cm x 50 µm fused silica capillary, pressure injection (5 psi*s, 34 kPa*s), voltage 15 kV, phthalate buffer (10 mM potassiumhydrogenphthalate, 2.5% OFM Anion-BT (Waters), 1mMNa_2-EDTA, pH set to 5.6 with LiOH), detection wavelength 215 nm.

About 10g of moist perlite were crushed with a mortar and pestle. The resulting suspension was washed with deionized water into centrifuge vials and centrifuged at 20,000 g in a fixed-angle rotor for 10 minutes to yield the water extractable fraction. The residual perlite was then extracted with 20 ml of 0.01 M NaOH for 1 hour to give a NaOH-soluble fraction. This latter fraction was passed over a H^+-saturated cation exchange resin in order to remove Na^+ ions before analysis. Analysis for organic acids and orthophosphate in both fractions was done on CE under the same conditions as above except for injection, which was 8 psi*s (55 kPa*s). Total extracted organic acids and phosphate were calculated as the sum of these two fractions taking into account the carry-over of water-soluble ions into the NaOH extract. The difference between total extracted organic acids in treatments with plants and the respective controls without plants was considered to represent net exudation during the experimental period.

Growth solutions remaining at the end of the experiment were pooled per treatment and pH was measured and free Al^{3+} was determined using capillary electrophoresis (Göttlein 1998).

Shoot and root element contents were determined after digestion with 1 ml of 40 % HNO_3 and 40 µl of conc. HF in a microwave oven at 240°C and 120 bar (UltraClav, Microwave Laboratory Systems). Total element concentrations in the digests of roots were measured with ICP-MS (Elan 6000, Perkin Elmer), those of shoots with ICP-AES (Optima 3000, Perkin Elmer).

4.3.5. Determination of element content in newly germinated seedlings

Seeds of *Picea abies* from the same seed lot than those used for the growth experiments were surface-sterilised as above and germinated on water agar for 24 days. At that time they had shed the seed coat. For analysis, 200 seedlings were pooled. Dry weight was recorded and total seedlings without seed coat were ground and mineral element content

determined as mentioned above for shoots. The net amount of element provided by the seed to the newly germinated seedling was calculated by multiplication with dry biomass per seedling and found to be 0.1 µg Al and 47.2 µg P per seedling.

4.3.6. Statistical analysis

A 2-way ANOVA was performed on the data with inoculation and Al level as independent factors (Datadesk 6.1.1. for Macintosh). Treatment means were compared using Scheffé's post-hoc test at a significance level of 0.05. The mean P content in newly germinated seedlings was compared with the P content in 5-months-old seedlings both separately for each treatment (n=6) and for inoculated vs. non-inoculated roots (n=18, including all 3 Al levels) by a t-test. The same test was applied to compare the amount of phosphate that was added to the beakers with the nutrient solution with the amount of phosphate that was extracted from the substrate at the end of the experiment.

4.4. Results

4.4.1. Growth parameters

Growth parameters of the seedlings are summarised in table 4.1. Generally, there was no effect of inoculation on germination or establishment of seedlings, but the negative effect of Al on germination and number of established seedlings was highly significant. In inoculated beakers, already the low Al addition significantly reduced the number of established seedlings after five months, while this was observed only at the high Al addition for non-inoculated seedlings.

Values of root, shoot and total biomass are presented as dry weight per beaker and dry weight per seedling. Overall, inoculation had an increasing effect on shoot and total dry mass per beaker, but not on root dry mass. Al decreased root, shoot and total dry weights per beaker. The negative effect of Al on biomass formation per beaker was partly compensated by inoculation. At the high Al level, non-inoculated beakers produced significantly less total biomass than inoculated beakers, which was mainly due to reduced shoot growth of these plants. Accordingly, these plants had a higher root/shoot ratio than all of the other plants. On the basis of individual seedlings, a significant increase of root, shoot and total dry mass with increasing Al was observed.

Table 4.1. Norway spruce biomass formed during the experimental period. Root biomass in inoculated treatments includes fungal biomass associated with roots. Values are means of six replicate beakers per treatment. Values followed by the same lowercase letter within the same inoculation treatment are not significantly different. An uppercase A indicates that the value is significantly different from the corresponding value in the non-inoculated treatment ($p < 0.05$, Scheffé test). ANOVA: ns: not significant ($p > 0.05$), *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

	Number of seedlings [x]		Root	Biomass per beaker [mg DW]			Biomass per seedling [mg DW]			
	Germ.	Est.	length[xx]	Roots	Shoots	Total	Roots	Shoots	Total	Root/Shoot
Non-inoculated										
Al 0	47 a	28 a	5.4 a	231 a	609 a	841 a	8.2 a	21.7 a	30.0 a	0.38 a
Al 100	43 a	32 a	5.5 a	220 ab	713 a	933 a	7.2 a	23.4 a	30.6 a	0.31 b
Al 500	23 b	16 b	6.9 b	184 b	403 b	586 b	12.2 b	26.1 a	38.3 a	0.46 c
Inoculated										
Al 0	44 a	30 a	4.7 a	234 a	720 a	953 a	8.1 a	24.5 a	32.5 a	0.33 a
Al 100	36 ab	22 b A	4.8 a	192 b	638 ab	829 a	8.9 ab	29.4 ab	38.3 ab	0.30 a
Al 500	27 b	19 b	6.7 b	216 ab	598 b A	815 a A	11.8 b	32.2 b	44.0 b	0.37 a A
ANOVA										
Al	***	***	**	**	***	***	***	**	**	***
Inoculation	ns	ns	ns	ns	**	*	ns	**	*	**
Al x Inoculation	ns	*	ns	*	***	***	ns	ns	ns	ns

[x] Number of seedlings per beaker: Germ.: Total germinated seedlings; Est.: Seedlings successfully established after five months.

[xx] Mean length of the longest root for each plant

Root length was equally increased in Al treatments. At the high Al level, the length of the longest root was significantly higher than at the medium and low Al level. Fungal inoculation tended to reduce root length, especially in the Al 0 and Al 100 treatments, but the effect was only significant at a level of $p = 0.06$.

Root systems in all inoculated beakers were covered by fungal mycelium and almost all short roots were covered by a dense fungal mantle. No fungal mycelium was detected on non-inoculated roots. Short roots in non-inoculated treatments showed abundant root hairs.

4.4.2. Plant nutritional parameters

Phosphate in perlite

When the perlite was extracted sequentially with water and 0.01 M NaOH at the end of the experiment, both inoculated and non-inoculated beakers with seeds contained on average significantly more extractable phosphate (table 4.2) than had been added with the nutrient solution (7.5 µmol per beaker). This was not the case for control beakers without seeds. As variation was large, no statistical differences were detected between treatment means. However, the proportion of NaOH extractable phosphate tended to be larger in Al treatments.

Growth solution

At the end of the experiment, the pH value in the remaining solutions in the bags (only a few ml) had risen to values between 6 and 7 with no trends in all treatments including control beakers which contained only perlite. Accordingly, no free Al^{3+} could be detected in these solutions by capillary electrophoresis (< 20 µM).

Table 4.2. Phosphate extracted by sequential water and NaOH extraction from perlite at the end of the experiment (mean ± SE)

	Extractable phosphate (µmol beaker^{-1})			
	Water	0.01 M NaOH	Sum of both extractions	n[x]
Non-inoculated				
Al 0	41.5 ± 16.3	< 1	41.5 ± 16.3	6
Al 100	37.5 ± 10.2	7.3 ± 2.3	44.7 ± 9.8	6
Al 500	8.8 ± 4.0	17.9 ± 4.4	26.7 ± 8.4	6
Inoculated				
Al 0	20.2 ± 5.8	2.5 ± 1.1	22.7 ± 6.4	6
Al 100	29.2 ± 6.5	5.5 ± 1.6	34.7 ± 6.9	6
Al 500	18.5 ± 8.2	12.0 ± 5.3	30.4 ± 7.7	6
Control				
Al 0	< 1 – 2.8	<1 – 2.8	<1 – 5.6	2
Al 100	< 1	6.1 ± 3.6	6.1 ± 3.6	2
Al 500	< 1	2.9 ± 0.9	2.9 ± 0.9	2
Inoculated control				
Al 0	17.4 ± 1.3	< 1	17.4 ± 1.3	2
Al 100	< 1 – 2.1	< 1	< 1 – 2.1	2
Al 500	< 1	4.4 ± 0.8	4.4 ± 0.8	2

[x] Number of replicates

Elements in roots

Aluminium accumulated in roots of all plants (table 4.3). Tissue concentrations of Al in roots of the Al 500 treatments were 2-3 times higher than those at the Al 0 treatments. Inoculation with *Hebeloma crustuliniforme* significantly increased the accumulation of Al in roots. There was no significant effect of either factor on Ca concentrations in roots, while K, Mg and Na contents in root tissue were dependent on Al level but not on inoculation. Whereas K in roots increased with Al level, Mg and Na in roots were reduced by Al addition. The ratio of both Ca/Al and Mg/Al was negatively affected by both inoculation and Al addition. Phosphorus concentrations were highest in the Al 0 treatments of both inoculated and non-inoculated seedlings and were about 25% lower in Al 500 treatments. Mycorrhizal inoculation increased P content of roots. This effect

was most pronounced for the Al 500 treatments, where the negative effect of Al addition was compensated by the inoculation effect.

Elements in shoots

Plants grown at the high Al level showed significantly higher Al tissue concentrations in the shoots. Inoculation had no effect on shoot Al contents. Shoot tissue concentrations of Ca and K were positively affected by Al addition. This effect was more pronounced in inoculated beakers, although the effect of inoculation over all beakers was not significant. Magnesium in shoots was negatively affected by both inoculation and Al addition. The Al 500 treatments were characterised by significantly reduced Ca/Al and Mg/Al ratios in shoots. Shoot tissue concentrations of P were not affected by inoculation. However, a negative effect of Al on P in shoots was observed in both high and low Al treatments when the plants were inoculated, but only in high Al treatments when the plants were not inoculated.

When P in plants (sum of shoot and root) was calculated per seedling and compared to the amount of P found in newly germinated seedlings (table 4.4), then only inoculated treatments showed a significant net P uptake during the 5 months growth period, which, however, was only around 8% of total P on average. In non-inoculated Al 0 and Al 100 treatments, the amount of P in 5-months old seedlings was not significantly different from the amount found in newly germinated seedlings. The non-inoculated plants in the Al 500 treatment experienced a net loss of 15% of their initial P reserve. When calculated over all Al levels, non-inoculated seedlings showed net P loss during the growth period, while inoculated seedlings showed net P uptake.

Table 4.3. Root (a) and shoot (b) element content of Norway spruce seedlings grown for five months in perlite at low nutrient supply and three Al levels (mg (g DW)$^{-1}$). Values are means of 6 beakers. Means followed by the same letter within one element and inoculation level are not significantly different at $p < 0.05$. An uppercase A indicates a significant difference ($p < 0.05$) to the corresponding value of the non-inoculated plants.

(a)	Al	Ca	K	Mg	Na	P	Ca/Al	Mg/Al
Roots	mg (g DW)$^{-1}$						mol mol^{-1}	mol mol^{-1}
Non inoculated								
Al 0	0.35 a	1.17 a	1.74 a	0.53 a	1.54 a	1.21 a	2.7 a	2.0 a
Al 100	0.40 a	1.08 a	1.98 a	0.47 a	1.47 a	1.09 ab	1.8 ab	1.3 a
Al 500	0.93 b	1.31 a	2.53 a	0.41 a	0.74 b	0.87 b	1.0 b	0.5 b
Inoculated								
Al 0	0.44 a	1.07 a	1.88 a	0.54 a	1.43 a	1.40 a	1.7 a	1.4 a
Al 100	0.68 a	1.17 a	1.94 a	0.42 ab	1.33 a	1.14 b	1.3 a	0.8 ab
Al 500	1.28 b A	1.36 a	2.67 a	0.39 b	0.95 a	1.17 b A	0.7 a	0.3 b
ANOVA								
Al	***	ns	**	**	**	***	***	***
Inoculation	***	ns	ns	ns	ns	**	*	*
Al x Inoculation	ns	ns	ns	ns	ns	ns	ns	ns

Table 4.3 (continued)

(b)	Al	Ca	K	Mg	Na	P	Ca/Al	Mg/Al
Shoots	mg (g DW)$^{-1}$						mol mol^{-1}	mol mol^{-1}
Non inoculated								
Al 0	0.05 a	0.77 a	2.33 a	0.70 ab	0.25 a	1.65 a	11.3 ab	16.6 a
Al 100	0.04 a	0.92 a	2.80 a	0.75 a	0.18 a	1.69 a	15.5 a	20.9 a
Al 500	0.09 b	0.94 a	2.83 a	0.60 b	0.20 a	1.17 b	06.9 b	07.6 b
Inoculated								
Al 0	0.04 a	0.70 a	2.42 a	0.66 a	0.32 a	1.64 a	13.9 a	21.8 a
Al 100	0.05 a	0.85 ab	2.60 a	0.60 a A	0.24 ab	1.33 b A	13.2 a	16.0 a
Al 500	0.09 b	1.04 b	3.25 b	0.60 a	0.17 b	1.22 b	07.7 b	07.4 b
ANOVA								
Al	***	***	**	*	*	***	***	***
Inoculation	ns	ns	ns	*	ns	ns	ns	ns
Al x Inoculation	ns	ns	ns	ns	ns	*	ns	*

Table 4.4. Total P content in 5-month-old Norway spruce seedlings as percentage of P content in seeds (mean ± SE of six replicate beakers). Significance levels for ANOVA and t-test: ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, ns: not significant ($p > 0.05$)

	Total P in seedling (% of P in seeds)	*n*	Significant difference from 100% (t-test)
Non-inoculated			
Al 0	96.3 ± 3.1	6	ns
Al 100	99.5 ± 4.6	6	ns
Al 500	84.8 ± 4.1	6	*
Mean	93.5 ± 2.6	18	*
Inoculated			
Al 0	108.9 ± 2.3	6	*
Al 100	103.6 ± 5.4	6	ns
Al 500	111.2 ± 4.1	6	*
Mean	107.9 ± 2.4	18	**
ANOVA			
Al	ns		
Inoculation	***		
Al x Inoculation	*		

4.4.3. Organic acids in roots and fungi

In roots, the following organic acids were detected: oxalate, malate, citrate, formate, acetate, lactate and succinate (table 4.5). Oxalate was the most abundant acid. The sum of both extractions yielded an average concentration of 16 µmol $(g\ DW)^{-1}$ in roots of the non-inoculated treatments. Extraction of inoculated roots yielded significantly less oxalate, averaging 9 µmol $(g\ DW)^{-1}$. Malate and citrate ranged from 6 – 10 µmol $(g\ DW)^{-1}$. While malate was unaffected by treatments, citrate was reduced in inoculated Al 500 treatments compared with inoculated Al 100 treatments. In non-inoculated roots, no significant differences in citrate content were observed at the three Al levels. Formate ranged from 4 - 7 µmol $(g\ DW)^{-1}$ and acetate from 1 – 3 µmol $(g\ DW)^{-1}$. Tissue content of these two acids was positively affected by Al. The effect on formate was

much stronger than on acetate. Succinate was found in higher concentrations in some inoculated pots, but concentrations never exceeded 1 µmol (g DW)$^{-1}$. As the concentration in many samples was at the detection limit, no statistical treatment was possible. Lactate content was also below 1 µmol (g DW)$^{-1}$ throughout, and was unaffected by treatments.

Extractions of fungal mycelium grown in liquid MMN culture medium without roots showed that *Hebeloma crustuliniforme* does not contain detectable amounts of oxalate but more succinate than non-mycorrhizal Norway spruce roots (table 4.6). Malate and citrate content in fungal tissue was lower than in roots, whereas formate, acetate and lactate were 5 - 30 times higher. Growth of the fungus in the synthetic soil solution with or without Al did not produce enough biomass for extraction.

Table 4.5. Organic acids in roots of Norway spruce seedlings grown for five months in perlite at three levels of Al addition and inoculated or not with *Hebeloma crustuliniforme*. Values are means of six replicates per treatment. For the meaning of the statistics symbols, see table 4.1.

	Oxalate	Formate	Malate	Citrate	Succinate	Acetate	Lactate
	µmol (g DW)$^{-1}$						
Non-inoculated							
Al 0	14.9 a	4.4 a	7.7 a	8.4 a	0.1 [x]	1.4 a	0.3 a
Al 100	15.6 a	4.8 a	9.0 a	7.9 a	0.1	1.6 a	0.5 a
Al 500	17.9 a	6.2 b	9.1 a	8.9 a	0.1	2.2 a	0.5 a
Inoculated							
Al 0	9.0 a A	4.2 a	7.7 a	9.5 ab	0.3	1.4 a	0.4 a
Al 100	7.6 a A	5.7 b	8.6 a	10.0 a	0.7	2.3 a	0.5 a
Al 500	10.3 a A	5.8 b	7.3 a	6.6 b	<0.1	2.2 a	0.5 a
ANOVA							
Al	ns	***	ns	ns		*	ns
Inoculation	***	ns	ns	ns		ns	ns
Al x Inoculation	ns	ns	ns	*		ns	ns

[x] Succinate was below the detection limit in various samples and no statistical treatment was possible.

Table 4.6. Extractable organic acids in *Hebeloma crustuliniforme* grown in liquid MMN culture medium (µmol (g DW)$^{-1}$), range of two samples

	Tissue content (µmol (g DW)$^{-1}$)
Oxalate	< 0.4
Formate	22.6 - 35.8
Malate	1.2 - 1.8
Citrate	< 0.1
Succinate	2.1 – 3.7
Acetate	35.8 – 36.2
Lactate	9.9 –21.7

4.4.4. Organic acids in exudates

All of the acids found in roots could also be extracted from the perlite substrate at the end of the experiment. Formate, acetate and lactate were also extracted from control beakers in concentrations similar to those in beakers with seedlings. Thus, they cannot be interpreted as exudates. All other organic acids, however, have been released from roots or fungi during growth of the seedlings.

Variation among replicates was generally high, and no significant effects of Al or inoculation on extractable organic acids from perlite were observed (table 4.7). The only exception was noted for succinate, which was more abundant in inoculated beakers than in non-inoculated beakers in both Al treatments but not in controls. Although extractable succinate concentrations in roots were about 10 x lower than malate or citrate concentrations, it was found in the perlite at levels similar to or higher than these two acids.

Table 4.7. Net release of organic acids by Norway spruce seedlings grown for five months in perlite at three levels of Al addition and inoculated or not with *Hebeloma crustuliniforme*. Organic acids were extracted sequentially from perlite with deionized water and 0.01 M NaOH. The sum of both extractions is presented. Values are means of six replicates per treatment. For the meaning of the statistics symbols, see table 4.1.

	Oxalat	Malate	Citrate	Succinate
		µmol (g root DW)$^{-1}$		
Non-inoculated				
0	38.5 a	1.5 a	0.5 a	2.3 a
100	32.4 a	1.5 a	0.6 a	1.9 a
500	34.4 a	3.2 a	3.1 a	2.1 a
Inoculated				
0	13.7 a	0.7 a	1.4 a	2.5 a
100	36.9 a	1.8 a	2.0 a	6.0 b A
500	21.1a	2.6 a	1.0 a	4.7 ab A
ANOVA				
Al	ns	ns	ns	*
Inoculation	ns	ns	ns	***
Al x Inoculation	ns	ns	ns	*

4.5. Discussion

The pH increase of the growth solutions was most probably caused by slow dissolution of the perlite substrate, which buffered the proton activity in solution. The similar pH increase in control beakers without plants rules out the possibility that plant activity alone was responsible for these effects. The proton buffering had consequences on Al speciation as significant concentrations of Al^{3+} appear in solution only below pH 5 (Sposito 1996). However, although the proportion of total Al that was present as the toxic Al^{3+} ion must have decreased during the experiment, there were significant effects of different levels of Al on growth and nutrition parameters of the plants. Al uptake into shoots and roots increased at high Al addition, indicating that Al was available to the plants during growth, however with toxicity gradually reduced with time by the formation of less toxic species (Kinraide and Parker 1989, Kinraide 1991). The effects

observed under these conditions can be expected to be more pronounced under conditions of constant toxicity.

4.5.1. Plant growth and germination

The negative effect of aluminium on germination of seedlings is in contrast to the report by Henriksen et al. (1992) who found no effects of Al on germination but on seedling establishment in Norway spruce, which was in line with reports for white spruce (*Picea glauca* (Moench) Voss) (Nosko et al. 1987) and red spruce (*Picea rubens* Sarg.) (Scherbatskoy et al. 1987). About 70% of the germinated seedlings established successfully in our study, a somewhat lower success (60%) was observed in non-inoculated Al 0 treatments and in inoculated Al 100 treatments.

The germination success affected biomass yield per beaker in two opposing ways. On the one hand, total biomass per beaker increased with the number of established seedlings. On the other hand, the biomass per seedling decreased with the number of seedlings, probably as a result of competition for nutrients. This resulted in the observation that Al 500 treatments had the highest biomass per seedling. This must, however, not be interpreted as a positive effect of Al itself, but an indirect effect of reduced competition due to negative effects of Al on germination. In plants grown at high Al addition, inoculation compensated negative Al effects on biomass production. Thus, the inoculated Al 500 beakers produced similar biomass to non-inoculated Al 0 beakers, although they had a lower number of seedlings. Such a compensation has already been reported for *Paxillus involutus* as ectomycorrhizal symbionts of Norway spruce (Wilkins and Hodson 1989, Hentschel et al. 1993) as well as for *Pinus rigida* inoculated with *Pisolithus tinctorius* (Cumming and Weinstein 1990).

4.5.2. Element contents

Uptake of Al into the shoots of Norway spruce was observed in all plants. This confirms previous reports that Norway spruce is unable to exclude Al completely from the shoot, e.g. (Jentschke et al. 1991, Hentschel et al. 1993). There was also a baseline level of Al in shoots of the Al 0 treatments, indicating that the acid nutrient solution or root exudates dissolved Al from the perlite, making it available for plant uptake. This is consistent with the observation of Arp et al. (1989), who found that uptake of Al into needles of black spruce (*Picea mariana* [Mill.] B.S.P.) was determined by the level of

dithionite-extractable (i.e. mainly amorphous Al hydroxide) rather than water-extractable or exchangeable Al in the soil. Nevertheless, the significantly higher Al content in the shoots of the Al 500 treated plants indicates that Al added with the nutrient solution in these treatments caused additional Al uptake by the spruce seedlings. However, most of the aluminium accumulated in roots. This effect was stronger in inoculated than in non-inoculated roots. This observation is in line with observations by (Hodson and Wilkins 1991). Contamination of roots with perlite particles can not be responsible for Al accumulation in inoculated roots, as both Na and K, which are main constituents of perlite, do not equally accumulate in inoculated roots. Aluminium appears to accumulate in a non-toxic form, as growth was enhanced in inoculated plants. The positive effects of Al on Ca and K shoot and K root concentration are likely to be partly indirect effects of the reduced competition for nutrients in Al 500 beakers, where the number of seedlings was lower than in Al 0 treatments. However, as the increase in Al content was larger than the increase in Ca content, a smaller Ca/Al ratio resulted in these plants, which indicates that their nutritional status was worse. The negative effect of Al on Mg uptake in Norway spruce has already been observed by several authors (Jorns and Hecht-Buchholz 1985, Rost-Siebert 1985, Stienen and Bauch 1988) in hydroponic experiments. The same effect is also known for other species (e.g. Grimme 1983). Neither ectomycorrhizal symbiosis nor the reduced competition in Al 500 beakers were able to keep the Mg contents in shoots at the level of the Al 0 treatments.

4.5.3. Phosphorus balance

Phosphorus nutrition of the seedlings was determined by the following effects: First, net P uptake by the seedlings was zero or low. In all treatments, there was water-extractable, i. e. plant-available, phosphate present in the substrate. Thus, some P uptake by plants probably has occurred, but P was also lost from roots at about the same rate. Quantification of gross uptake and loss is not possible with the present data, but would require isotope studies. The low net P uptake suggests that P reserves in seeds can be sufficient to sustain plant growth for at least 5 months. In this respect, the lower shoot P content in non-inoculated Al 500 treatments as well as in inoculated Al 100 and Al 500 treatments, can be attributed partly to a dilution effect of P in shoot tissue as a

consequence of improved shoot growth (Hentschel et al. 1993), which itself was caused by the lower number of established seedlings in these treatments.

Second, Al addition caused loss of phosphate from non-inoculated seedlings below the initial amount in seeds. Aluminium might have increased leakiness of root cells. Al effects on cell wall permeability have been demonstrated by Zhao et al. (1987). If Al caused a damage to the plasma membrane, this would result most probably in a significant loss of mobile K^+ ions from the root cells. However, in the present experiment, root K content tended to increase with Al, which is consistent with the view that Al does not cause significant damage to plasma membranes (Kochian 1995). Thus, the P loss could be an active reaction of Norway spruce in order to precipitate toxic Al outside roots. A metabolically dependent P efflux in the presence of Al has been proposed by Lindberg (1990) to confer Al resistance in a sugar beet (*Beta vulgaris* L.) cultivar. In an earlier experiment with Norway spruce in hydroculture (chapter 3), precipitation of Al phosphate was observed at the root surface. In the present experiment, EDX analyses did not indicate P accumulation at the surface in Al treatments (not shown). However, an increase of NaOH-extractable phosphate in the substrate during plant growth indicated that Al phosphate precipitation occurred at the perlite surfaces instead.

Third, inoculation improved P nutrition of the roots. Improved P nutrition may include more efficient P uptake as well as lower P loss. Compared with other ions, mobility of phosphate in soil is low. Therefore, in non-mycorrhizal roots, P is usually taken up from a rather small soil volume close to the roots (Jungk 1991), resulting in a distinct P depletion zone. The extraradical fungal mycelium is able to exploit a larger substrate volume than roots (Smith and Read 1997), including small pores that are inaccessible to roots. In the present experiment, such pores existed in the perlite. Hyphal growth into these pores may have contributed to more efficient P uptake in inoculated seedlings. Additional P supply by ectomycorrhizas was shown to compensate negative effects of Al on P nutrition of pitch pine (Cumming and Weinstein 1990). In Norway spruce inoculated with *Paxillus involutus*, Hentschel et al. (1993) even found that the effect of mycorrhizal colonisation on P nutrition was stronger than the effect of Al. In the present experiment, the beneficial effect of mycorrhizal inoculation was restricted to the root P content. As the determination of total element content did not distinguish between fungal and root tissue, it is possible that the higher P content in mycorrhizal roots was

due to P storage in the fungus. Storage of P in the form of polyphosphates has been reported in various ectomycorrhizal fungi (Bücking and Heyser 1999) including *Hebeloma arenosa* (MacFall et al. 1992) and *Hebeloma crustuliniforme* (Martin et al. 1985). Our results indicate that in an early stage of seedling growth, the main beneficial effect of the fungus is to prevent or compensate P loss from seedling roots, while shoot P content is not positively affected by inoculation yet.

4.5.4. Organic acids

A positive Al effect on root organic acid content was detected for acetate and formate only, but not for malate, citrate or oxalate, which are known to be strong Al chelators (Hue et al. 1986). A positive Al effect on water-soluble acetate and a similar trend for formate in Norway spruce roots have already been observed in a previous hydroponic experiment (chapter 3). This indicates that Al affects metabolism of Norway spruce roots, but it appears unlikely that accumulation of these two acids is involved in Al resistance as they only form weak complexes with Al (Hue et al. 1986). In the substrate, formate, acetate and lactate were similar in all beakers with or without plants. Thus, an exudation of these acids cannot be inferred from our data. For oxalate, malate, citrate, and succinate, the amount of acid extracted from the perlite substrate will be referred to as exudates, as they were virtually absent from control beakers and thus originate from either roots or fungi. Generally, the variation between beakers of the same treatment was higher for organic acids in exudates than in roots. Oxalate and succinate showed similar trends in roots and in exudates, although statistically significant differences between treatments were only found for exuded succinate. Oxalate in roots and exudates was unaffected by Al level. Inoculation, however, had a strong negative effect on root content of oxalate. The same trend was observed in the exudate pool, although it is not significant due to the large variability.

The reduced oxalate content of inoculated roots can be explained by two phenomena. First, *Hebeloma crustuliniforme* does not appear to produce oxalate. This seems valid although the data on oxalate production had to be taken from the experiment with MMN nutrient solution as growth of fungi in the nutrient-poor synthetic soil solutions did not yield sufficient biomass, and the nutrient level might have affected oxalate production to a certain degree (Lapeyrie et al. 1987, Devêvre et al. 1996). Second, the oxalate reduction of 40-50% cannot be explained by the fungal contribution of oxalate-free

biomass alone. Münzenberger et al. (1990) reported, that in *Picea abies/Laccaria amethystea* 28% of the biomass were of fungal origin. However, this study specifically considered mycorrhizal short roots, while in the present study bulk root systems were analysed, where the proportion of fungal tissue is smaller. Ekblad et al. (1995) reported values close to 10% fungal biomass for whole mycorrhizal root systems of *Pinus sylvestris/Paxillus involutus* under various nutritional conditions. One possible explanation for the reduced oxalate accumulation in inoculated roots could be that the fungus uses oxalate as a carbon source.

In contrast to oxalate, more succinate was released in inoculated treatments than in non-inoculated treatments. This is in accordance with the succinate levels in inoculated and non-inoculated roots and can be attributed to the fungal succinate production. The Al-induced succinate exudation by fungi does not appear to be a resistance mechanism, as succinate forms only weak complexes with Al and therefore has a low Al detoxification potential (Hue et al. 1986).

Comparing the amounts of organic acids in roots and in exudates, there was an average of 2-5 times more oxalate in the perlite substrate than in roots at the end of the experiment. This ratio was even larger for succinate. It was approximately 10 for those samples which it could be calculated for. On the other hand, the amounts of malate and citrate extracted from the perlite typically were around 10-20% of the root concentrations. Given the findings from previous hydroculture experiments (chapter 3), where malate and citrate were not detected in the exudates, it can be speculated that such a small proportion could easily be explained by loss of these acids from sloughed-off cells, and that active exudation of malate and citrate from living cells was negligible. The fungus did not contribute significantly to malate or citrate exudation either. Extracts of pure fungal cultures grown in the liquid MMN culture medium showed that, in contrast to Norway spruce roots, *Hebeloma crustuliniforme* accumulated more succinate than malate and almost no citrate (table 4.1).

For oxalate, average net exudation rates over the entire experimental period can be estimated from the present data to be 1–3 nmol per seedling per day. Recalculation of data from an earlier experiment yields similar rates under hydroponic conditions, where the average oxalate exudation was approximately 2-3 nmol per seedling per day (chapter 3). In both experiments, the actual values will have varied strongly, as in hydroponics the exudation during the first two days was significantly enhanced and in

the present experiment, most of the exudates will have been produced in later growth stages when increasing photosynthetic carbon fixation provided more metabolites. Therefore, these average results are not contradictory to findings by other authors (Barber and Gunn 1974, D'Arcy-Lameta 1982, Mozafar 1991) that, under otherwise comparable conditions, root exudation was higher in solid substrates than in hydroponic culture.

In order to estimate if the amounts of organic acids found in perlite could chelate relevant amounts of Al and thus contribute to Al exclusion, the amount of Al that could be bound to these acids can be compared with the amount of Al found in roots within each beaker. If 1:1 complexes of Al with the organic acid anions are assumed, the amount of Al that can be chelated by oxalate outside the roots is similar to the amount of Al found in roots. On the one hand, this indicates that, theoretically, the size of the exuded pool was sufficiently large to chelate relevant amounts of Al. On the other hand, it shows that these amounts did not completely prevent Al adsorption to roots. Additionally, in natural soils, the rapid degradation of organic acids will prevent their accumulation and thus probably reduce their importance in Al detoxification (Jones 1998).

In summary, the small treatment effects on exuded organic acids were in contrast to the significant treatment effects on element content of the seedlings. In hydroponic experiments (chapter 3), Al had also failed to affect organic acid release by Norway spruce roots significantly. We conclude that also in solid substrate organic acid exudation in Norway spruce is not enhanced as a reaction to Al, and organic acids present in the rhizosphere are not capable of completely preventing negative effects of Al on plant nutrition. This is in contrast to the results of several authors who demonstrated efficient Al detoxification by organic acids in the root exudates of various crop species (Ryan et al. 1995, Pellet et al. 1995, 1997, Ma and Miyasaka 1998, Zheng et al. 1998). However, Al toxicity usually occurs in sensitive crop species already at concentrations of a few $\mu mol\ l^{-1}$, and in this concentration range exudation may be effective. By contrast, in acid forest soils, concentrations of Al may reach a few hundred $\mu mol\ l^{-1}$ and apparently other strategies of Al resistance than root exudation must be effective.

4.6. Conclusions

Under the conditions in our experiment, Al negatively affected germination of Norway spruce seedlings. In the presence of *Hebeloma crustuliniforme*, seedling establishment was affected by a lower Al level than in the absence of the fungus. During seedling growth, inoculation was able to compensate negative effects of Al on biomass production. This effect could not be explained by improved mineral nutrition of the shoots. However, inoculation prevented or compensated P loss from roots. Whether the increased accumulation of Al in inoculated roots contributes to Al resistance cannot be answered by now. Neither inoculation with *Hebeloma crustuliniforme* nor Al addition had significant effects on root exudation of organic acids by Norway spruce. Oxalate, as the most abundant organic acid in the exudate fraction, is capable to form strong Al complexes. However, oxalate exudation was not stimulated by Al, nor did the presence of oxalate in the substrate prevent an increased Al uptake and reduced Mg uptake at the high Al levels. Thus, for Norway spruce grown in solid substrate, a protective role of root exudates is unlikely.

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Chapter 5

Effect of Al and fungal inoculation on phenolic metabolites in Norway spruce roots

5.1. Abstract

Norway spruce (*Picea abies* [L.] Karst.) seedlings were grown for five months in an axenic system at three levels of Al addition and with or without inoculation with the ectomycorrhizal fungus *Hebeloma crustuliniforme* (Bull.: St. Amans) Quél. These experimental variations allowed investigation of Al and inoculation effects on root content and exudation of phenolic substances. In all roots, catechin, isorhapontin and astringin were major constituents. Various treatment effects were observed. For some substances, Al affected the response to inoculation. Thus, catechin which was unaffected by Al in non-inoculated roots, was reduced by inoculation at low Al levels, but not at the high Al level. Isorhapontin and cell-wall bound vanillin were reduced only in inoculated roots at the high Al level. For other substances, effects were caused by one of the factors only. Thus, astringin was unaffected by Al, but increased in inoculated roots. The root content of several minor compounds was positively affected by Al, but remained unaffected by inoculation. Exudation of total phenolics during five months was found to represent less than 1 % of the root content and was affected differently by Al in inoculated and in non-inoculated roots. The results suggest that Al disturbs the balance between mycorrhizal symbiosis and fungal parasitism. The fungistatic astringin, which remains unaffected by Al, might then be important for the regulation of hyphal colonisation. The Al induced increase of several substances may represent a defense reaction against Al. In contrast, the results suggest that root exudation of phenolics that can contribute to Al resistance by chelating Al in the rhizosphere or in the apoplast, is not induced by Al.

Keywords

Aluminium, astringin, catechin, chelation, conifer, ectomycorrhiza, Hebeloma crustuliniforme, isorhapontin, Norway spruce, phenolic metabolites, *Picea abies*, roots, stilbenes

5.2. Introduction

A wide range of chemical compounds in trees, as well as in other higher plants, are distinct from the intermediates and products of primary metabolism. These secondary metabolites serve a variety of functions, e.g. fruit and flower pigments, feeding deterrents, phytoalexins, antifungal agents or allelopathic agents (Harborne 1980). Many of these compounds are induced by biotic or abiotic stress (Dixon and Paiva 1995). During the last decades, it has become clear that phenolics are key components in plant defence mechanisms against pests and pathogens (Bennett and Wallsgrove 1994). In Norway spruce, infection with a pathogen fungus stimulated key enzymes of the secondary metabolism and enhanced the level of catechin near the site of infection (Brignolas et al. 1995). Stilbenes have also been demonstrated to possess high fungitoxicity (Woodward and Pearce 1988, Beyer et al. 1993). In roots, phenolics closely regulate ectomycorrhiza formation in coniferous forest trees by their fungitoxic properties (Münzenberger et al. 1990, 1995, Weiss et al. 1997, 1999). In addition to biotic factors, several abiotic stresses have been shown to induce phenolics in plant tissue. In aboveground plant parts, high light intensity and UV radiation induce formation of anthocyanins and flavones which are thought to attenuate the light reaching the photosynthetic cells (Dixon and Paiva 1995). In roots of legumes, low nitrogen levels induce flavonoids and isoflavonoids which function as *nod* gene inducers and chemoattractants for N_2-fixing symbionts (Graham 1991, Wojtaszek et al. 1993, Dixon and Paiva 1995). Iron deficiency can cause increased release of phenolic acids, e.g. caffeic acid, which are able to reduce Fe(III) and thus render it available for plant uptake (Olsen et al. 1981, Marschner et al. 1986, Marschner 1991b). Recently, Jung (2000) demonstrated that white lupin (*Lupinus albus* L.) responds to elevated Cu concentrations by exudation of phenolic substances and accumulation of genistein derivates in roots.

A major abiotic stress for plants growing on acid soils is the phytoavailability of toxic Al (Marschner 1991a). However, little information is available if Al similarly affects phenolic metabolism in plants as do other abiotic stresses mentioned above. McQuattie and Schier (1990) found accumulation of phenolics by microscopical observation of Al treated red spruce (*Picea rubens* Sarg.) root cells. Karolewski and Giertych (1994) studied total phenolic levels in Scots pine (*Pinus sylvestris* L.) after treatment with various metals. Levels of total phenolics increased in needles and decreased in roots of

Scots pine after Pb or Cd treatment, but were not significantly affected by Al. No information is available on the effect of Al on individual phenolic substances in conifer roots.

An important property of the many phenolic substances with an *o*-dihydroxy (catechol) binding site is their ability to chelate metals (Harborne 1980). This could be an efficient detoxification mechanism for Al as chelation strongly affects bioavailability of metals. The effectiveness of plant phenolic substances in chelating Al was demonstrated by Luster et al. (1996), who studied complex formation between Al and phenolic substances in aqueous leaf litter extracts. Recently, Malinowski and Beleski (1999) suggested that increased exudation of phenolic-like compounds from roots of endophyte-infected tall fescue (*Festuca arundinacea* Schreb.) may be involved in Al tolerance of this herbaceous species.

Compared with crop plants (Horst and Göppel 1986), many tree species withstand much higher Al concentrations in soil solution (Schaedle et al. 1989). Norway spruce (*Picea abies* [L.] Karst.) is a conifer that naturally occurs on acid soils in Northern and Central Europe and is known to tolerate Al concentrations up to 0.3 mM before root growth is affected (Göransson and Eldhuset 1991), which is about one order of magnitude higher than the value reported for an Al-tolerant wheat cultivar (Pellet et al. 1996). Results of a previous study (Heim et al. 1999) have shown that Al chelating phenolics are present in root exudates of Norway spruce and suggested that Al at exchange sites of the root apoplast is present partly as low-charged phenolic complex.

The aim of the present study was to investigate the effect of Al addition on root content and exudation of phenolic compounds in detail. As conifer roots are frequently ectomycorrhizal under natural conditions, which contributes to Al resistance of the host tree to varying degrees (Wilkins and Hodson 1989, Cumming and Weinstein 1990, Jentschke et al. 1991, Hentschel et al. 1993), both inoculated and non-inoculated root systems were studied. As mentioned above, ectomycorrhizal symbiosis itself affects the formation of phenolic compounds in roots. The data shall therefore be used to distinguish reactions caused by Al from those caused by fungal inoculation and to discover possible interactions of both factors.

5.3. Material and Methods

5.3.1. Culture system

Norway spruce (*Picea abies* [L.] Karst.) seedlings were grown for five months in an axenic system at three levels (0, 100, and 500 µM) of Al addition and with or without inoculation with the ectomycorrhizal fungus *Hebeloma crustuliniforme* (Bull.: St. Amans) Quél. The culture system is described in detail in chapter 4.3.1.

Pure fungal cultures were grown in liquid medium. Details are given in chapter 4.3.2.

5.3.2. Analysis

For each of the 36 beakers, lyophilised root and fungal material was ground in liquid nitrogen and 20 mg of the fine powder were weighed into 10-ml vials and extracted first for 1h with 4 ml, then twice with 2 ml 80% aqueous methanol for 10 min each (Münzenberger et al. 1995). Supernatants after centrifugation were combined and evaporated to dryness at 40°C under vacuum. The residue was redissolved in 0.5 ml of 50% aqueous methanol, centrifuged and the supernatant used for HPLC and total phenolics analyses (soluble phenolics fraction). Residual pellets of root material after methanol extraction were washed with 5 ml H_2O, 5 ml methanol (twice) and 1 ml acetone for 15 min each and the supernatants discarded. After drying (2h, 60°C), 1ml of 1 M NaOH was added to the residue and the samples were hydrolysed for 16 h at room temperature. After centrifugation, 500 µl of the supernatant were acidified with 50 µl of 85% H_3PO_4 and centrifuged again. The clear supernatant was used for HPLC and total phenolics analyses (cell wall bound fraction).

Total phenols were measured in the methanolic extracts after 200-fold dilution with 50% methanol using a colorimetric method according to (Swain and Hillis 1959). The volumes were modified as follows: 0.9 ml sample, 0.3 ml Folin-Denis reagent (Fluka, Buchs SG, Switzerland), 0.6 ml 20% Na_2CO_3. Absorption at 725 nm was read after at least 3 h reaction time using a UV/VIS spectrophotometer (Cary 50, Varian, Mulgrave, Australia). Catechin was used to prepare a standard curve for calibration and data are expressed as catechin equivalents. Samples of the cell wall bound fraction were diluted 20 times with deionised water and analysed accordingly. Calibration was done using ferulic acid standards prepared in the same matrix and data are expressed as ferulic acid equivalents.

HPLC analyses of individual phenolics were performed on a two-pump HPLC system (Pharmacia-LKB, Sweden); column: ET 250/8/4 Nucleosil 120-5 C18 (Macherey-Nagel, Düren, Germany). 20 µl of sample volume were injected by an autosampler (ISS-100, Perkin Elmer, Boston, USA). Elution system for soluble phenolics: linear gradient in 60 min from solvent A (1.5% (w/v) H_3PO_4 in H_2O) to 40% solvent B (H_2O/methanol/acetonitrile, 1/1/1, v/v/v), then to solvent B in 10 minutes, 100% solvent B for 20 min at a flow rate of 1 ml min^{-1}. Elution system for cell wall bound phenolics: 20 min from 25% solvent B to 60% solvent B. UV detection was carried out at 265 nm (soluble phenolics) or 320 nm (cell wall bound fraction). Separation of piceatannol and isorhapontin was achieved using the elution system of Turunen et al. (1999).

For identification of substances, the following standards were used: catechin, epicatechin, ferulic acid, vanillin (Fluka, Buchs SG, Switzerland), isorhapontin, astringin, picein (gifts from W. Heller, GSF Oberschleißheim, Germany), piceatannol (gift from A. Schützendübel, Inst. f. Forstbotanik, Univ. Göttingen, Germany). Para-hydroxyacetophenone was obtained by acid hydrolysis of picein (Strack et al. 1989). Substances were identified by comparison of retention times and confirmed by standard addition to samples. Peak areas were compared to external standards for quantification. Data for inoculated roots were not corrected for fungal biomass. Unidentified peaks were integrated as well and relative contents of the compounds were calculated based on the mean peak area in non-inoculated roots of the Al 0 treatment. On these relative values, the same statistical analyses were performed as on data of identified compounds. Perlite was extracted with water and 0.01 M NaOH as described in chapter 4.3.4. Additionally, some perlite particles adhering to roots at harvest were collected separately and considered to represent rhizosphere samples. They were pooled within treatments to obtain enough material for extraction. The same extractions were performed as with bulk substrate. In all extracts, total phenolics were analysed with the colorimetric method mentioned above using catechin as standard.

5.3.3. Statistical analysis

A two-way ANOVA was performed on all data with the factors inoculation and Al level and 6 replicates per treatment. Multiple comparison of treatment means was done using the LSD test at a significance level of 0.05. DataDesk 6.1.1. for Macintosh was used for all statistical analyses.

5.4. Results

Growth of the seedlings was good in all beakers and no visual symptoms of Al toxicity or nutrient deficiency were observed. Detailed effects of Al and inoculation on germination, root and shoot growth are given in chapter 4. Root systems in all inoculated beakers were covered by fungal mycelium and almost all short roots were covered by a dense fungal mantle. No fungal mycelium was detected on non-inoculated roots. Short roots in non-inoculated treatments showed abundant root hairs.

5.4.1. Phenolic substances in roots

Total soluble phenolics, as analysed by the colorimetric Folin-Denis method, slightly increased with Al addition ($p=0.077$), while fungal inoculation decreased total soluble phenolics (table 5.1). In non-inoculated roots, already the low Al addition resulted in a significant increase in total phenolics while this was not the case in inoculated roots. The individual substances showed patterns different from each other and from total phenolics. Figure 5.1 shows the HPLC trace of a typical non-mycorrhizal sample of the Al 0 treatment. The three major peaks were identified as the flavonoid catechin (1), and the two stilbene glucosides astringin (2) and isorhapontin (3). Together, these three substances constituted approximately 12-18% of the total soluble phenolic content of the roots. Their proportion increased with inoculation and decreased with Al addition.

Standards of picein (t_R ca. 24 min), p-hydroxy-acetophenone (t_R ca. 36 min), epicatechin (t_R ca. 42 min) and piceatannol (t_R ca 59 min) were also tested, but could not be assigned to any major peak. Piceatannol and isorhapontin coeluted with the method used, but were separated by the method of (Turunen et al. 1999). Thus, the peak was identified as isorhapontin. Relative quantification of unidentified peaks (denoted by capital letters in fig. 5.1) revealed that several of the minor compounds (B, C, E, F, G) were significantly increased by Al (table 5.2) while two compounds (A, F) were significantly reduced by inoculation. Substance D was not significantly affected by Al in non-inoculated roots, but was reduced in Al 0 and Al 100 treatments of inoculated roots.

In non-inoculated roots, absolute catechin levels were not affected by Al treatment (table 5.1). In inoculated roots, catechin was significantly reduced in the Al 100 treatment. Roots from the Al 0 treatment also tended to have lower catechin contents, whereas inoculated roots treated with Al 500 had catechin contents similar to non-inoculated roots. When expressed as a percentage of total phenolics, roots from the

inoculated Al 0 treatment had a significantly lower proportion of catechin than those of the corresponding non-inoculated treatment. Over all Al treatments, astringin content in roots was significantly enhanced in the presence of *Hebeloma crustuliniforme*, while the presence of Al had no significant effect. The ratio of astringin to total phenolics was significantly enhanced by inoculation, especially in Al 0 and Al 100 treatments. Isorhapontin content was reduced by Al addition in inoculated roots only. Also the proportion of isorhapontin in inoculated roots decreased significantly in the Al 500 treatment when compared to Al 0 and Al 100 treatments.

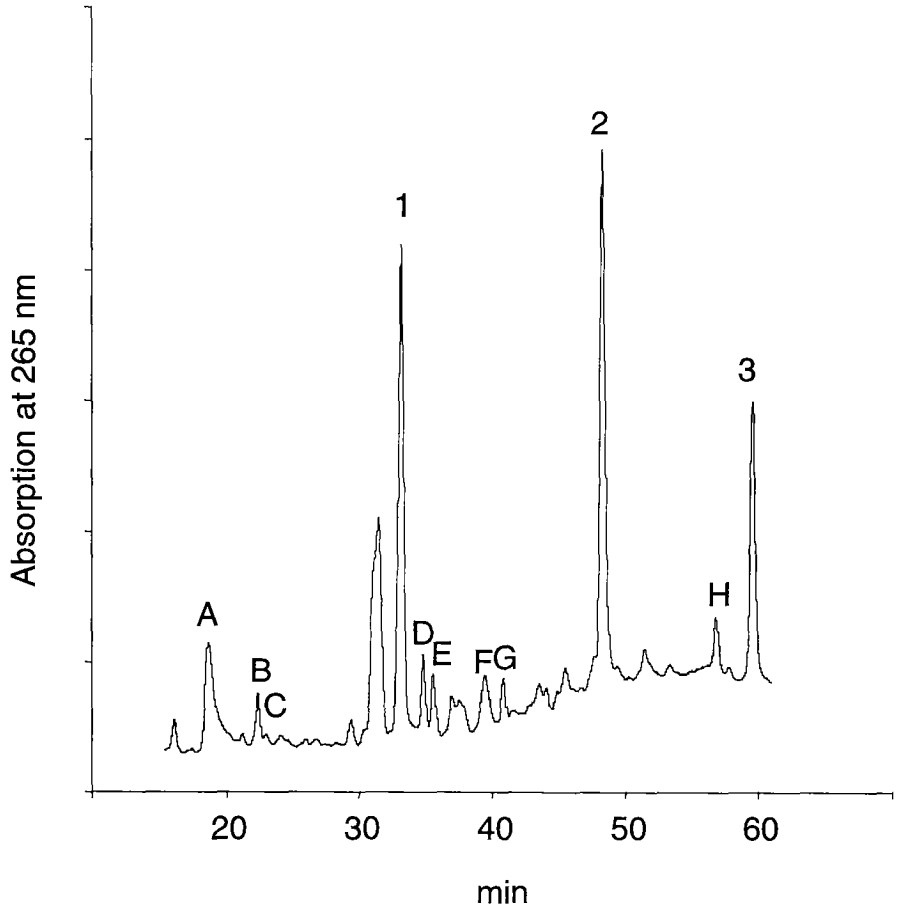

Figure 5.1. HPLC trace of a methanolic extract of non-inoculated Norway spruce roots (control without Al). Identified substances are (1) catechin, (2) astringin, (3) isorhapontin. Capital letters denote unidentified peaks that are quantified in table 5.2.

Table 5.1. Soluble phenolic substances in Norway spruce roots grown in perlite with 3 levels of Al addition and inoculated or not with *Hebeloma crustuliniforme*. Means of six replicates per treatment are presented. Values of inoculated roots were not corrected for fungal biomass. Values followed by the same letter within either inoculated or non-inoculated treatments are not significantly different at $p<0.05$ (LSD test). Values followed by a capital A are significantly from the corresponding value of the non-inoculated plants.

	Total [x]	Catechin	Astringin	Isorhapontin	Catechin	Astringin	Isorhapontin	Sum [xx]
Non-inoculated	mg (g DW)$^{-1}$				% of total			
Al 0	80.2 b	5.1 a	5.6 a	2.1 a	6.3 a	7.0 a	2.6 a	15.9 a
Al 100	106.0 a	5.9 a	5.4 a	1.9 a	5.6 ab	5.1 b	1.8 a	12.5 b
Al 500	94.3 ab	5.0 a	5.9 a	1.9 a	5.2 b	6.2 ab	2.1 a	13.5 ab
Inoculated								
Al 0	73.0 ab	3.8 ab	6.6 a	2.6 a	5.2 a A	9.3 a A	3.7 a	18.3 a
Al 100	68.3 b A	3.5 b A	5.9 a	2.2 ab	5.0 a	9.0 a A	3.4 a A	17.5 ab A
Al 500	88.7 a	5.1 a	6.8 a	1.4 b	5.8 a	7.8 a	1.6 b	15.2 b
				ANOVA p-values				
Al	0.077	0.449	0.291	0.053	0.492	0.078	0.015	0.008
Inoculation	0.003	0.004	0.033	0.696	0.283	<0.0001	0.046	0.0002
Al x Inoculation	0.032	0.035	0.858	0.159	0.064	0.12	0.039	0.138

[x] catechin equivalents
[xx] catechin + astringin + isorhapontin

Table 5.2. Relative amounts of selected unidentified metabolites in Norway spruce roots grown in perlite with 3 levels of Al addition and inoculated or not with *Hebeloma crustuliniforme*. Capital letters in the top line refer to the peaks in fig. 5.1. Means of six replicates per treatment are presented. Values of inoculated roots are not corrected for fungal biomass. For the meaning of the statistical symbols see table 5.1.

Peak	A	B	C	D	E	F	G	H
t_R [min] [x]	18.9 ±0.3	22.5 ±0.3	23.1 ±0.3	35.2 ±0.4	35.9 ±0.4	39.7 ±0.4	41.2 ±0.4	56.9 ±0.5
	Relative root content (%)							
Non-inoculated								
Al 0	100 a	100 a	100 ab	100 a	100 a	100 b	100 a	100 b
Al 100	85 ab	120 a	90 b	123 a	125 a	107 b	122 a	132 ab
Al 500	65 b	123 a	127 a	115 a	126 a	159 a	121 a	174 a
Inoculated								
Al 0	64 a A	98 ab	99 b	87 b	105 ab	78 a	94 b	120 a
Al 100	41a A	96 b	101 b	82 b A	92 b A	74 a	83 b	138 a
Al 500	64 a	122 a	149 a	132 a	137 a	112 a	142 a	162 a
	ANOVA p-values							
Al	0.099	0.034	0.003	0.029	0.036	0.012	0.043	0.073
Inoculation	0.002	0.206	0.311	0.179	0.533	0.017	0.499	0.832
Al x Inoculation	0.068	0.337	0.655	0.040	0.120	0.749	0.113	0.804

[x] retention time in HPLC

In the hydrolysate of the methanol-insoluble residues, vanillin and ferulic acid were detected and quantified (table 5.3). According to Münzenberger et al. (1990) and Weiss et al. (1999), this fraction will be referred to as cell-wall bound fraction. Together, vanillin and ferulic acid constituted no more than 3% of the total cell-wall bound phenolics. Further peaks were not resolved with the respective HPLC method but various substances coeluted within the first few minutes. No significant treatment effects on total cell-wall bound phenolics and ferulic acid were observed. Vanillin levels were unaffected by Al in non-inoculated roots, but were reduced in the Al 500 treatment in inoculated roots.

Only those pure fungal cultures grown in liquid MMN medium produced sufficient biomass for extraction. None of the substances extracted from roots of Norway spruce were found in extracts of these fungal cultures.

Table 5.3. NaOH-hydrolysable phenolic substances in Norway spruce roots grown in perlite with 3 levels of Al addition and inoculated or not with *Hebeloma crustuliniforme*. Means of six replicates per treatment are presented. Values of inoculated roots were not corrected for fungal biomass. For the meaning of the statistical symbols see table 5.1.

	Total [x]	Vanillin	Ferulic acid
	mg (g DW)$^{-1}$		
Non-inoculated			
Al 0	8.63 a	0.07 a	0.15 a
Al 100	9.44 a	0.08 a	0.16 a
Al 500	9.39 a	0.07 a	0.16 a
Inoculated			
Al 0	8.65 a	0.08 a	0.17 a
Al 100	9.36 a	0.06 ab	0.17 a
Al 500	9.47 a	0.04 b A	0.16 a
	ANOVA p-values		
Al	0.18	0.11	0.72
Inoculation	0.99	0.08	0.26
Al x Inoculation	0.99	0.11	0.36

[x] ferulic acid equivalents

5.4.2. Phenolic exudates

Table 5.4 shows total phenolics extractable from the growth substrate (perlite) after five months of plant growth. This fraction represents root exudates released during the growth period. While Al reduced phenolic exudation in non-inoculated plants, an opposite trend was observed in inoculated plants. Generally, the amount of exuded phenolics was low and all individual phenolics detected within roots were below the detection limit of the HPLC method in the exudates (3-8 µmol l^{-1} corresponding to 5-15 µmol (g root DW)$^{-1}$). Extraction of a few rhizosphere samples yielded on average about five times higher concentrations of total phenolics than extraction of bulk substrate (not shown). However, there was too little material available for systematic analyses.

Table 5.4. Phenolic root exudates as extracted from perlite after Norway spruce seedlings had grown for five months at 3 levels of Al addition and inoculated or not with *Hebeloma crustuliniforme*. Means of six replicates per treatment are presented. For the meaning of the statistical symbols see table 5.1.

	Total phenolics [x]	
	mg (g root DW)$^{-1}$	µg (g root DW*d)$^{-1}$
Non-inoculated		
Al 0	1.3 a	8.7
Al 100	0.6 ab	4.3
Al 500	0.5 b	3.4
Inoculated		
Al 0	0.6 a	4.2
Al 100	1.2 a	8.1
Al 500	1.3 a A	8.7
ANOVA	p	
Al	0.972	
Inoculation	0.269	
Al x Inoculation	0.012	

[x] catechin equivalents

5.5. Discussion

While the effects of fungal inoculation and mycorrhiza formation on phenolic metabolites in conifer roots have already received some attention (Münzenberger et al. 1990, 1995, Weiss et al. 1997, 1999), this is the first report on effects of Al on individual root phenolic substances in Norway spruce.

5.5.1. Soluble phenolics in roots and in the fungus

When comparing metabolite contents of inoculated and non-inoculated roots, a major uncertainty is the contribution of fungal and plant biomass to total root biomass. Münzenberger et al. (1990) reported that in mycorrhizas of Norway spruce with *Laccaria amethystea* (Bull.) Murr. the fungus contributed 28% to total biomass, as estimated by the concentration of a fungus-specific compound in mycorrhizas and pure fungal culture. Such a fungus-specific substance was, however, not detected in the present study with *Hebeloma crustuliniforme*. While the value of Münzenberger et al. (1990) was obtained using specifically mycorrhizas, Ekblad et al. (1995) used entire root systems of Scots pine ectomycorrhizal with *Paxillus involutus* (Fr.) Fr. and estimated the proportion of fungal biomass to be about 10%, a value which seems reasonable for the present system as well. As the fungus did not contribute measurable amounts of phenolic substances, the mere presence of fungal tissue could have decreased the measured phenolic content by a dilution effect without involving any physiological reaction. This effect would, in fact, suffice to explain the reduction in total soluble phenolics found in inoculated roots. However, when individual substances are considered, it is obvious that physiological reactions occur as well. This is indicated by the fact that several substances in inoculated roots are reduced more strongly than can be attributed to dilution, while others are enhanced. As the exact proportion of fungal biomass is not known, data were not corrected in this respect. Only those effects will be interpreted that are stronger than the presumable dilution effect.

The relatively small reduction of total soluble phenolics in the present study is in contrast to the finding of Münzenberger et al. (1995) in European larch (*Larix decidua* Mill.) who reported a strong general decrease of phenolic substances in mycorrhizas, but it is in accordance with the finding of the same authors that in *Picea abies/Laccaria amethystea* mycorrhizas only few individual substances were reduced (Münzenberger et al. 1990). It has also been shown that changes in the content of phenolic substances are

spatially limited to specific tissues within the mycorrhizas (Weiss et al. 1997, 1999). If such changes occurred they were not reflected on the whole root level.

5.5.2. Treatment effects on individual phenolic substances in roots

The individual compounds show varying effects of inoculation or Al addition, which can be grouped into several categories. The response of catechin is an example of the first type of reaction. This substance is not significantly affected by Al in non-inoculated roots, but it is reduced in inoculated roots in Al 0 and Al 100 treatments, but not in Al 500 treatments. The same reaction is also observed for substance D in table 5.2. Fungitoxicity has been shown to correlate with content of catechins (Alcubilla et al. 1987). Catechin levels in trees increase as a defence reaction to attack by pathogenic fungi (Brignolas et al. 1995, Evensen et al. 2000). By contrast, the association with ectomycorrhizal fungi is beneficial for the host tree, and accordingly, in mycorrhizas of Norway spruce, catechin was found to be reduced (Münzenberger et al. 1990). This reduction was interpreted by the authors as a contribution by the host to facilitate ectomycorrhizal symbiosis. The results of the present study for Al 0 and Al 100 treatments agree with this interpretation. However, they suggest that high Al addition interferes with the symbiosis in a way that the host tree fails to recognise the fungus as beneficial. On the other hand, although the catechol unit makes catechin a potent chelator of Al (Tang et al. 1992), the increase in catechin levels in Al 500 treated roots does not appear to be a specific plant response to Al, as it did not occur in non-inoculated roots.

A second type of reaction is similarly characterised by the absence of significant Al effects in non-inoculated roots, but in inoculated roots, the content of these substances (isorhapontin and call-wall bound vanillin) is strongly reduced by high Al addition. Both isorhapontin and cell-wall bound vanillin have been postulated to be involved in restricting hyphal growth to the outer cortex (Weiss et al. 1997, 1999). In accordance with this interpretation the proportion of isorhapontin is increased by inoculation. The observed reduction by the Al 500 treatment only could therefore be interpreted as an Al-induced imbalance of the mutualism-parasitism continuum (Johnson et al. 1997).

The other identified stilbene glycoside, astringin, although chemically closely related to isorhapontin, did not show this reduction, but was generally higher in inoculated roots, showing no significant Al effects. An increase in astringin was also reported by Weiss

et al. (1999) for *Picea abies/Russula ochroleuca* mycorrhizas compared with non-mycorrhizal roots, whereas Münzenberger et al. (1990) found no significant differences in astringin content between *Picea abies/Laccaria amethystea* mycorrhizas and non-mycorrhizal short roots. The two latter results are not necessarily contradictory as astringin could mainly accumulate in uncolonised parts of the root system, where it might be involved in restricting fungal growth to the mycorrhizal organs. As well as isorhapontin, astringin has been located in the pericycle, where these two fungitoxic substances (Woodward and Pearce 1988, Beyer et al. 1993) prevent possible fungal invasion of the stele (Weiss et al. 1999). The different effect of Al on these similar substances in inoculated roots suggests a risk-minimising strategy in Norway spruce. The existence of two similar substances ensures the proper functioning of hyphal growth regulation even if environmental conditions (in the present case: high Al) interfere with the production of one of the substances.

Several minor compounds show a fourth type of response to the treatments. These substances (B, C, E, F, G in table 5.2 – the same trend was also observed for H) increased in Al treatments both in non-inoculated and in inoculated roots. Only substance F was additionally reduced by inoculation, which compensated the Al effect. The other substances showed no obvious inoculation effects. Support for the observed Al effect comes in part from a study by Hamel et al. (1998), who reported that in wheat, Al up-regulates genes encoding, among others, phenylalanine-ammonia lyase (PAL), a key enzyme of secondary metabolism. However, from the absence of a general Al effect on all secondary metabolites and from the varying effects of inoculation on individual substances, it is obvious that regulation by both factors, Al and inoculation, also occurs at later stages of the biosynthetic pathway. The increase of individual phenolic substances as a response to Al treatment can be either a toxic effect or a resistance mechanism. Support for the latter interpretation comes from the fact that in leaves of the highly Al-tolerant tea plant (*Camilla sinensis*) Al is bound to catechins and phenolic acids (Nagata et al. 1992). In Norway spruce, a possible role for phenolic compounds in Al detoxification is indicated by our previous results (Heim et al. 1999). We hypothesised that a fraction of the root phenolics has a strong capacity to chelate metal ions in the apoplast. Chelation of Al in the symplast is unlikely to be important in Norway spruce, as previous studies agree that Al accumulates mainly in the root apoplast (Bauch and Schröder 1982, Hodson and Wilkins 1991, Heim et al. 1999).

The effects of Al are not a linear function of the amount of Al added. Rather, the patterns of root content for most substances suggest that a threshold value must be reached before reactions can be observed. No individual substance is significantly affected by the Al 100 treatment when compared to the respective Al 0 control. However, significant differences occur between Al 100 and Al 500 treatments for a number of substances, mainly in inoculated roots.

The absence of inoculation effects on cell-wall bound ferulic acid is in contrast to the observation by Münzenberger et al. (1990), who found a reduction of this compound in laboratory-grown mycorrhizas as well as in field samples of *Picea abies/Laccaria amethystea* associations. Cell wall bound phenolics may enhance the barrier effect of the cell wall against fungal pathogens (Keller et al. 1996) by decreasing cell wall digestibility to enzymes (Fry 1984, 1986, Beimen et al. 1992). Our data are in line with the interpretation that reduction of cell-wall bound ferulic acid occurs locally in order to facilitate establishment of the symbiosis (Münzenberger et al. 1990), but does not happen at the whole root system level.

5.5.3. Root exudates

Compared with the amounts found in roots, exudation of phenolics into the substrate was low. During the five-month experimental period, an amount equivalent to approximately 1 % of the soluble phenolics in plants was released into the substrate, corresponding to an average of 3.4-8.7 µg catechin equivalents per day and g dry weight. Recalculation of data from previous hydroculture experiments with Norway spruce (chapters 2 and 3) showed, that exudation of total phenolics in the latter was about 2-3 times higher. This can be attributed to differences in age, duration of the experiment or incomplete recovery of phenolic exudates from the perlite substrate in the present experiment. Especially accumulation of phenolics in the rhizosphere, as indicated by a few rhizosphere samples, probably led to an underestimation of phenolics exudation in the present experiment.

Despite a large variability of phenolics exudation among replicates, the results suggest that inoculated roots react differently to Al than do non-inoculated roots. The reduced exudation in non-inoculated Al 500 treatments is consistent with findings from a previous hydroponic experiment (Heim et al. 1999), where phenolics exudation by Norway spruce roots was reduced by Al treatment. By contrast, exudation by inoculated

roots tended to increase with Al addition and was significantly higher than in non-inoculated roots at the high Al level. Similarly, in tall fescue, endophytic fungi increased phenolic exudation and might thus contribute to Al tolerance of this species (Malinowski et al. 1998, Malinowski and Belesky 1999). In the present experiment, the apparently reduced exudation of phenolics in non-inoculated Al 500 treated roots could be attributed to formation and re-adsorption of Al-phenolic complexes in the root apoplast (Heim et al. 1999). Fungal colonisation of the apoplast might reduce the re-adsorption of these complexes so that higher exudation into the substrate is observed.

5.6. Conclusions

The first study on effects of Al on individual phenolic substances in inoculated and non-inoculated roots of Norway spruce demonstrated that Al modifies the root reaction to inoculation. In inoculated roots, the effects of Al on substances such as catechin, isorhapontin or cell-wall bound vanillin, which are important for the maintenance of the balance between mutualism and parasitism, suggest that the functioning of the ectomycorrhizal symbiosis can be disturbed by the presence of Al. It can be hypothesised that the parallel existence of two closely related stilbenes, of which astringin is unaffected by Al, represents a strategy to ensure the regulatory function in case of abiotic stresses.

Aluminium also enhances the root content of several minor substances. This effect is mostly independent of inoculation and suggests that these substances could be involved in an active Al resistance mechanism, presumably detoxifying Al by chelation in the apoplast. Further research will be needed in order to determine if these individual compounds are actually capable of conveying Al resistance. Although exudation of phenolic substances into the substrate is low and not significantly enhanced by Al, the release of phenolic metabolites may contribute to the detoxification of Al by the formation of Al-phenolic complexes in the rhizosphere or in the apoplast.

Chapter 6
General discussion

The experiments conducted within this study and presented in chapters 2 – 5 will be referred to as follows. The first experiment, where 3-year-old Norway spruce trees were treated in a non-sterile hydroponic system (chapter 2) is referred to as non-sterile hydroculture. The second experiment (chapter 3) where Norway spruce seedlings were treated in a hydroponic system under sterile conditions is referred to as sterile hydroculture. The third experiment, where Norway spruce seedlings were grown for five months under sterile conditions in perlite substrate (chapters 4 and 5), is referred to as perlite culture.

6.1. The limitations of interpreting exudation data

As mentioned in the introduction, the mere presence of a substance in the rhizosphere or the treatment solution (if hydroculture is concerned) does not give any information on the mechanism by which the substance came there. Exudation data presented in this thesis always represent the sum of all possible mechanisms by which a substance could be lost from roots. The chemical reactions of root exudates in the rhizosphere (chelation of metals, precipitation) are independent of these mechanisms, and thus, the experiments presented here allow characterisation of the chelating potential of root exudates. On a physiological level, such exudation patterns should only be considered indicators of possible reactions. If a physiological response to a stress factor was to be inferred, it would be necessary to know if the release of a substance is actively controlled by the plant.

6.2. Is organic acid production and root exudation responsible for Al tolerance of Norway spruce?

Organic acids are exuded by Norway spruce roots as was found in the experiments with sterile seedlings. Of the most widespread organic acids in natural soils, Hue et al. (1986) determined malate, citrate and oxalate to be those which most efficiently chelate and detoxify aluminium ions. These acids are present in roots of Norway spruce at tissue contents of several μmol g^{-1}. While in sterile hydroculture, oxalate is the only one of

these three acids that is released in measurable quantities into solution, in the perlite culture all three acids could be extracted from the perlite after the experiment, but oxalate was by far dominant. The presence of this acid in the rhizosphere of Norway spruce certainly contributes to lowering the activity of the toxic Al^{3+} species. From the data presented in chapter 4, it appears that in solid substrate, the amount of oxalate released over a time span of 5 months could be of some importance to the detoxification of Al. On average, the amount of Al that could be detoxified by the oxalate present in the substrate was similar to or higher than the amount of Al bound to roots.

However, the higher amounts of oxalate found in the perlite culture are likely to represent no more than an accumulation of this acid over the entire growth period. Under natural conditions, such an accumulation is strongly dependent on the rate of degradation by soil microorganisms. This rate, itself, is affected by metals bound to the exudates (Morel et al. 1991), as well as by the type of ligand and metal:ligand ratio (Boudot et al. 1989). For example, complexation with Al decreased the biodegradation rate of citric acid but not that of a synthetic fulvic acid (Boudot et al. 1989). The absence of measurable quantities of organic acids in the non-sterile hydroculture (chapter 2) indicated that Al-organic acid complexes in solution can be degraded microbially at a relevant rate. This might be different for complexes adsorbed to soil particles. Therefore, the efficiency of oxalate released by Norway spruce in detoxifying Al by chelation will strongly depend on the degradability of the complex that is formed.

If exudation rates are estimated for the two systems, then the average oxalate exudation was approximately 2-3 nmol per seedling per day in sterile hydroculture, and 1–3 nmol per seedling per day in perlite culture. The actual values will have varied strongly, as in hydroculture the exudation during the first two days was significantly enhanced and in the perlite culture experiment, exudation will have increased with increasing photosynthetic carbon fixation by the growing seedling. If exudation of chelators was an equally efficient Al detoxification mechanism in Norway spruce as it appears to be in tolerant crop (e.g. wheat) cultivars, then the release of these compounds in spruce would have to be higher than it is in the latter, as much higher Al^{3+} concentrations are tolerated by spruce than by wheat and have to be detoxified. Comparison of estimated exudation rates with data for citrate and malate exudation in maize (Pellet et al. 1995) and for malate exudation in wheat (Delhaize et al. 1993) shows, however, that oxalate exudation by Norway spruce is, on average, about an order of magnitude lower.

Therefore, the amounts of oxalate released by Norway spruce can be considered relevant for Al detoxification only if exudation is localised to small, sensitive regions with a high exudation rate (e.g. root apices). The presence of an ectomycorrhizal fungus can further decrease production and exudation of oxalate by roots, as shown for *Hebeloma crustuliniforme* in chapter 4. However, such an effect probably will depend strongly on the fungal species and strain.

Furthermore, in none of the experiments, the release of organic acids was enhanced by the presence of Al, nor were oxalate, malate or citrate content in roots affected. If organic acid exudation was an active reaction to an Al stimulus, this would have been expected. In Al resistant crop plants, such an Al stimulation of exudation was observed by several authors (Pellet et al. 1995, Ryan et al. 1995, Pellet et al. 1997, Ma and Miyasaka 1998, Zheng et al. 1998).

In conclusion, constitutive release of oxalate from Norway spruce roots might contribute to Al resistance of this species at regions of low microbial activity and high exudation rate, e.g. apices (Bowen and Rovira 1991, Marschner 1995). However, it cannot be considered the only Al resistance mechanism in Norway spruce.

6.3. Root exudation of phosphate and precipitation of Al phosphate – an Al tolerance mechanism in Norway spruce?

In sterile hydroculture, with P nutrient status of Norway spruce being sufficient, it was observed that Norway spruce released phosphate from its roots. In $CaCl_2$ solution, this resulted in increased phosphate concentrations in solution, while in $AlCl_3$ solution, there were indications for precipitation of Al phosphate at the root surface. It could not be determined if phosphate release was higher in either treatment.

Under growth conditions in the perlite culture, loss of P from non-inoculated seedlings was suggested by calculating the P balance for the seedlings, and was enhanced significantly at the high Al addition. The large amounts of phosphate that were extracted from perlite after the experiment indicated that released P precipitated at or adsorbed to the perlite surface.

In conclusion, the release of phosphate by non-mycorrhizal tree roots reduces the Al activity in solution by precipitation of Al phosphate at the root or soil surfaces and thus can impart Al resistance to Norway spruce. This release is constitutive when P nutrient

status is good, but it seems to be induced by Al under the nutritional conditions of the perlite culture.

From inoculated roots, no net P loss occurs. This suggests that the fungus is capable of either preventing P loss from roots or of compensating P loss by increased uptake. In the first case, phosphate release as an Al resistance mechanism would not work in the presence of mycorrhizal fungi. However, if the fungus compensated P loss, this would suggest that mycorrhizal symbiosis allows phosphate exudation, as a measure to reduce external Al activity, to be maintained for longer periods.

In perlite culture, phosphate and oxalate exudation by non-inoculated roots were of similar magnitude in high Al treatments, while in the control, oxalate exudation was higher than net phosphate exudation. Under sterile hydroponic conditions, where P nutrition was better during pregrowth, phosphate exudation dominated oxalate exudation. Exudation of phosphate therefore appears to depend much more on environmental conditions than does oxalate exudation and thus its contribution to the detoxification of Al varies accordingly.

6.4. Effects of ectomycorrhizal symbiosis with *Hebeloma crustuliniforme* on nutritional parameters of Norway spruce

The role of mycorrhizas in metal tolerance of plants has been a matter of discussion for many years now. Due to the wide variety of fungal species, there is no general answer to the question if the presence of mycorrhizas protects plant roots from toxic effects of high metal concentrations. Ectomycorrhizal symbionts are assumed to improve Al tolerance of their host plant either by providing additional nutrients when an imbalance has been caused by Al or by immobilisation of the toxic ion in the fungal tissue. The results of the present study indicate that mineral nutrition of Norway spruce is not generally improved by the fungus *Hebeloma crustuliniforme*, with the important exception of P nutrition, which is discussed in chapter 6.3. The absence of a general effect is in contrast to the results found by Hentschel et al. (1993) for an association of *Paxillus involutus* and Norway spruce in sand culture. On the other hand, Wilkins and Hodson (1989), who worked with *Paxillus involutus* and two provenances of Norway spruce in perlite, found no systematic effects of fungal inoculation on needle Ca and Mg contents. They note that the strain of *P. involutus* used in their experiment did not form mycorrhizas, although the fungus was associated with the root. In the present study,

electron microscopy revealed that, despite the presence of a dense hyphal mantle, Hartig net formation was not accomplished in all roots. Therefore the possibility must be considered that substrate affects the efficiency of mycorrhizal fungi in supplying nutrients to the host plant.

On the other hand, the immobilisation of Al by *Hebeloma crustuliniforme* represents a possibly beneficial effect. Inoculated roots of Norway spruce accumulated more Al than did non-inoculated roots. Nevertheless, transport of Al to the shoot was not impeded by mycorrhizas. The increased Al accumulation in roots therefore does not go along with decreased Al uptake into the shoot. The indirect effect of the ectomycorrhizal fungus by improvement of P nutrition has been discussed in the preceding chapter (6.3.).

6.5. Al and secondary metabolism in Norway spruce

Bulk analysis of roots showed that the root content of some phenolic substances is positively affected by Al (chapter 5). Any conclusions on their role in Al detoxification, however, must be drawn with caution, as neither the exact localisation of these compounds nor the causal relationships that lead to their accumulation are known. However, the results presented in chapter 2 suggest two possibilities how phenolic ligands can be involved in detoxifying Al.

First, a fraction of the exudates is released into the rhizosphere. Complex formation between these compounds and Al could be demonstrated by fluorescence spectroscopy. Such reactions can serve to reduce the activity of toxic Al^{3+} species in the rhizosphere soil solution. In the bulk substrate of the perlite culture, however, less phenolics were found than in solutions of both hydroponic experiments. Strong adsorption of phenolic exudates to the substrate could have led to an underestimation of exudation in this system. Additionally, diffusion in a porous system is by orders of magnitude smaller than in solutions (Jungk 1991). Therefore, increased concentrations of root-derived substances can build up in the rhizosphere, because they do not diffuse away rapidly. Such an accumulation was indicated by the higher content of extractable phenolics in the rhizosphere samples of the perlite culture. Under soil conditions, this accumulation will increase the efficiency of phenolics for Al detoxification near the root surface. Nevertheless, compared with oxalate and phosphate, the exudation of phenolics is about an order of magnitude smaller, when expressed as catechin units capable of forming 1:1 complexes with Al. Rhizosphere models (Tinker and Nye 2000) could be used in order

to estimate concentrations in the rhizosphere and resulting Al speciation from data on exudation rates, degradation rates and diffusion coefficients of individual substances.
Second, chelation of Al by phenolics occurs also in the apoplast. Data in chapter 2 suggest that the exchangeable Al fraction in Norway spruce roots is, at least partly, associated with phenolics. The adsorption capacity for Al of a given number of negatively charged binding sites in the cell walls will greatly increase if low-charged Al–phenolic complexes are bound instead of Al^{3+}. In addition, when adsorbed as a low-charged complex, Al is less toxic, as increasing evidence is found that only Al species with a charge greater than 2 exhibit toxic effects (Kinraide 1991, Kochian 1995). Thus, phenolics can contribute to immobilisation of non-toxic Al in the apoplast of roots.

6.6. The importance of provenances in Al resistance

In agricultural sciences, it is general practice to use tolerant and sensitive cultivars of a species for resistance experiments. The same could theoretically be imagined for various provenances of a tree species. However, the slow growth of trees and Norway spruce in particular, makes screening of various provenances for Al tolerance extremely time-consuming. In the present work, the progeny of two different trees was used in the experiments. While the 3-year-old trees (chapter 2) were progeny from a parent tree in Bremgarten AG, all seedlings used in the experiments (chapters 3 - 5) were grown from seeds of another parent tree in Tägerwilen TG. This choice was only determined by the availability of the plant material and was not based on any experiments on Al resistance of these populations.
It can be imagined that differences in Al resistance exist between different provenances of Norway spruce. During the last Ice Age, there were two main refuges for Norway spruce in Europe, one in the present day region of Moscow, the other in Southeastern Europe. Genetic differences developed during this time can be found between Central European and Scandinavian provenances today (Lagercrantz and Ryman 1990). Geburek and Scholz (1989) studied the effect of Al on growth parameters of 18 different provenances of Norway spruce across Europe. In the presence of 6.2 mM Al, mean growth reduction varied only from 40% to 60% between provenances, with most Central European provenances tending to be more tolerant than Scandinavian or Russian provenances. However, as differences were small, these authors concluded that differentiation into edaphic ecotypes varying in Al resistance is not very marked in

Picea abies. A similar degree of variability between provenances was found by Makkonen-Spiecker (1985), who studied Al effects on growth and nutritional parameters of four German *Picea abies* provenances. Wilkins and Hodson (1989) found different effects of Al only on shoot growth but not on root growth of two German provenances of Norway spruce. In this experiment, inoculation with the ectomycorrhizal fungus *Paxillus involutus* Fr. significantly improved Al resistance of the more sensitive provenance only.

It can be concluded that, in general, the reaction of Norway spruce to Al does not seem to depend strongly on the provenance. Even the somewhat more sensitive provenances tolerate Al levels that are by 1-2 orders of magnitude higher than in the case of tolerant wheat cultivars used by Pellet et al. (1996) or Delhaize et al. (1993). Therefore, the results of this thesis are likely to apply to a wide range of provenances. This might not be the case, however, for the effect of mycorrhizal inoculation, which is not only dependent on provenance of the host tree, but also on fungal species and strain (Godbold et al. 1998).

6.7. Other Al effects on metabolism and regulation of ectomycorrhizal symbiosis

Some of the Al effects observed are unlikely to be involved in Al resistance of Norway spruce, but indicate further effects of Al on cell metabolism. One example is the increase of acetate in roots both in sterile hydroculture and in perlite culture. Acetate itself forms only weak complexes with Al (Hue et al. 1986) and therefore it is unlikely, that accumulation of acetate in roots is of direct significance for Al detoxification. As acetyl units are formed by a number of different reactions in plant cell metabolism (Stryer 1981), it is probable, that the accumulation of acetate is due to the interference of Al with such a metabolic pathway. The Al induced increase in formate content of roots in perlite culture may be caused by similar mechanisms.

For ectomycorrhizal root systems, there are indications that Al disturbs the regulation of the symbiosis. The example of catechin illustrates such a case. In non-inoculated roots, catechin content is independent of Al addition. In inoculated roots, levels of fungistatic catechin are reduced in order to facilitate establishment of the symbiosis (Münzenberger et al. 1990). The strong increase in catechin at the high Al level suggests a pathogen defence mechanism, which could have been caused by the failure to recognise the beneficial fungus.

The formation of substances that restrict fungal growth to the mycorrhizal organs (isorhapontin and cell-wall bound vanillin) appears to be impaired by Al. However, it seems that the tree can maintain anti-parasitic defence nonetheless, as it possesses further fungistatic substances, e.g. astringin, that are unaffected by Al under the conditions investigated in the present work.

Chapter 7

Final conclusions and implications for further research

The present study has demonstrated that exudation of organic chelators by Norway spruce roots is not an active reaction to Al. In organic acid metabolism, no Al-induced production of chelating acids was observed. The constitutive release of oxalate as the main chelating organic acid in exudates, however, may affect Al speciation and toxicity in the rhizosphere. Further research should attempt to gain information on the site of oxalate exudation and on the distribution of microbial populations along Norway spruce roots. Ectomycorrhizal fungi affect oxalate production and subsequent exudation by roots. The possible range of such effects should be determined by inoculation with different fungi.

In secondary metabolism, Al increased production of several phenolic compounds. Their chemical identity and ability to detoxify Al remain to be investigated. Given the low concentration of phenolics in exudates, a possible detoxification of Al by phenolics can be assumed to occur principally in the apoplast. Further research could focus on a better identification of the phenolic ligands involved in this mechanism.

Aluminium also modifies the reactions in secondary metabolism to an ectomycorrhizal fungus. The results suggest that Al stress threatens the symbiotic balance between host tree and fungus but the tree is still able to prevent the fungus from becoming parasitic under the experimental conditions in this work.

In addition to organic exudates, exudation of inorganic phosphate occurs in non-mycorrhizal Norway spruce to various extents. This phenomenon contributes to immobilisation of Al by precipitation of Al phosphate. On the other hand, the loss of P must be regarded as critical for plants growing on acid soils, where P nutrition is often limited. However, mycorrhizal roots do not show net P loss. Isotope studies could be used to clarify if the fungus mainly prevents or compensates P loss. Furthermore, information would be desirable on the amounts of P released by adult trees under natural conditions and how this process affects the P nutritional status.

In order to gain a better insight into rhizosphere processes, the exudation data provided by this study could be used in a next step to model accumulation patterns in the rhizosphere and Al speciation.

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