Manganese uptake, transport, and toxicity in two varieties of Douglas fir (*Pseudotsuga menziesii*) as affected by mycorrhizae: from the cellular to the organismic level

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Tanja Dučić

aus Užice, Serbien

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Referent: Professor Dr Andrea Polle

Korreferent: Professor Dr Dieter Heineke

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Submitted for the degree of Doctor of Mathematic- Natural Sciences

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by

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Summary

Manganese (Mn) is an essential nutrient element necessary for activation of a wide range of enzymes. To fulfil its metabolic functions Mn is only required at low concentrations. Plant availability of Mn depends on soil properties and on root exudates for Mn chelation or reduction. The availability of Mn increases with decreasing soil pH. In general, excess Mn causes disorder of normal plant metabolism. Therefore, Mn-homeostasis by plants must be strictly regulated.

Douglas fir (*Pseudotsuga menziesii*) has been cultivated in Europe for more than 100 years. Two main races can be distinguished: P. *menziesii* var. *viridis* (DFV) and *P. menziesii* var. *glauca* (DFG). DFG but not DFV showed symptoms of Mn toxicity in some field sites in Rheinland-Pfalz in Germany.

To investigate whether the two varieties of Douglas fir DFV and DFG differed in Mn metabolism, biomass partitioning, Mn concentrations, subcellular localisation and ^{54}Mn-transport were investigated. Total Mn uptake was three-times higher in DFG than in DFV. DFV retained more than 90 % of ^{54}Mn in roots, whereas more than 60 % was transported to the shoot in DFG. The epidermis was the most efficient Mn-barrier since DFV contained lower Mn concentrations in cortical cells and vacuoles of roots than DFG. In both varieties, xylem loading was restricted and phloem transport was low. DFV displayed higher biomass production and higher shoot/root ratios than DFG. These results clearly show that both varieties of Douglas fir differ significantly in Mn-uptake and allocation patterns rendering DFG more vulnerable to Mn toxicity.

To address seedling performance under conditions of Mn toxicity, plants biomass, Mn concentrations in different tissues and Mn subcellular localisation were determined. Shoot biomass was more affected in DFV and root biomass in DFG, respectively under Mn stress. X-ray microanalysis showed Mn accumulation in epidermal and cortical cells of both varieties after Mn treatment, suggesting that the root endodermis was a barrier for Mn to protect the vascular system and shoot from high Mn. In epidermis and cortical tissues precipitates were observed with extremely high Mn concentrations, which correlated with P and Ca.

To investigate the role of P, exposure of Douglas fir to excess Mn was also conducted under P-deficiency. P-limited DFV seedlings were less Mn-susceptible, than P-sufficed ones. In DFG, but not in DFV, the P concentration was kept at a constant level even under P

deficiency and root growth reduction by Mn was partially, probably due to increased Ca-uptake under these conditions.

Forest trees are normally associated with mycorrhizal fungi, which affect nutrient uptake and protect plants from stress. Mycorrhizae symbiosis is founded on the mutualistic exchange of C from the plant in return for P, N and other mineral nutrients from the fungus. To investigate the effect of Mn- excess on ectomycorrhizal seedlings of DFG and DFV with *Rhizopogon subareolatus*, the plant performance, elements concentrations in different tissues and Mn subcellular localisation were determined. Biomass production of both varieties was diminished by *Rhizopogon subareolatus*. Subcellular localisation of Mn in mycorrhizal root tips showed increased Mn accumulation in tissues closer to endodermis, but not in the vascular system. Ectomycorrhiza with *Rhizopogon subareolatus* may have a detrimental rather than a beneficial effects on Mn uptake and productivity of Douglas fir.

To investigate the performance of Douglas fir seedlings in natural soils, biomass production, nutrition and the abundance of ecto- and endomycorrhiza colonization of two-years-old Douglas fir DFV and DFG grown in two different soil types, were studied. Molecular identification of mycorrhiza based on DNA extraction of mycorrhizal roots showed that both soil- and variety-specific mycorrhizal communities were established on Douglas fir roots. *Rhizopogon vinicolor* was associated to variety *viridis*, while *Cadophora finlandia* was more frequently found on *glauca*. *Tomantella sublilacina* was specific for soil from Solling, whereas *Wilcoxina mikolae* for Unterlüss soil. Analyses of net assimilation of nitrogen, phosphorus and carbon in relation to mycorrhizal abundance indicated that nutrient uptake was positively correlated with degree of endomycorrhiza of Douglas fir. Solling soil generally showed better conditions for growth of both varieties and a higher degree of root ectomycorrhization.

Based on the above results, it is concluded that both varieties of Douglas fir differ significantly in Mn-uptake, transport, toxicity and allocation patterns. The data show that seedlings of DFG were more susceptible to Mn toxicity than DFV. Since Mn concentrations are increasing over the years, it is not still known how these two varieties will behave after long exposition to Mn- stress. The variety *viridis* showed considerably higher degree of ectomycorrhization during the first years of growth, being a finally reason for better development and biomass production. These results do not support the hypothesis that ectomycorrhiza protect against Mn excess. Overall the experience results with Dougals fir

seedlings support empirical data that DFV is more suitable for cultivation in Germany than DFG.

Key words: Heavy metals, manganese, phosphorus, transport, mycorrhiza, neophyte, *Pseudotsuga menziesii*.

Zusammenfassung

Aufnahme, Transport und Toxizität von Mangan in zwei Varietäten der Douglasie (*Pseudotsuga menziesii*) unter dem Einfluß von Mykorrhizen: von der zellulären bis zur organismischen Ebene

Mangan (Mn) ist ein essentielles Mikronährelement, welches für die Aktivierung zahlreicher zellulärer Abläufe wie zum Beispiel die Aktivierung von Enzymen notwendig ist. Um diese Aufgabe erfüllen zu können, bedarf es nur sehr niedriger Konzentrationen freien Mn in der Pflanze. Die Verfügbarkeit von Mn für Pflanzen hängt vor allem von den chemischen Bedingungen im Boden sowie den Fähigkeiten der Wurzeln, Mn aus der Rhizosphere aufzunehmen. Die Verfügbarkeit von Mangan steigt mit abnehmenden pH-Werten im Boden. Generell führt ein Überschuss von verfügbarem Mangan im Boden zu einer Beeinträchtigung des pflanzlichen Metabolismus und letztendlich sichtbaren Schädigungen in der Pflanze. Deshalb muss das Mn-Gleichgewicht in der Pflanze auf einem niedrigen Niveau reguliert werden.

Die Douglasie (*Pseudotsuga menziesii*) wird in Europa seit mehr als 100 Jahren angebaut. Zwei Varietäten werden in Europa verwendet: *P. menziesii* var. *viridis* (DFV) and *P. menziesii* var. *glauca* (DFG). Auf einigen Standorten in Rheinland-Pfalz konnten bei DFG Symptome von Mn-Toxizität festgestellt werden, während DFV unter gleichen Bedingungen keine derartigen Symptome zeigte. Das Ziel dieser Arbeit war es zu untersuchen, wo die Ursachen in der offensichtlich unterschiedlichen Mn-Sensitivität liegen.

Um zu untersuchen, wie sich die beiden Varietäten der Douglasie in ihrem Mn-Metabolismus unterscheiden wurden die ^{54}Mn- Aufnahme, Transport sowie die subzellulären ^{54}Mn -Verteilung untersucht.

Die Gesamtaufnahme von Mangan ist in DFG drei Mal höher als in DFV. Während in DFV mehr als 90 % des aufgenommenen Mangans in den Wurzeln akkumuliert werden, transportiert DFG mehr als 60 % des Mangans in den Spross. In beiden Varietäten wird Mangan vor allem in der die Epidermis zurückgehalten; gleichzeitig akkumulierte DFV geringere Mn-Konzentrationen in Cortexzellen und Vakuolen der Wurzeln.

In beiden Varietäten war die Beladung des Xylems mit Mangan eingeschränkt und es wurde ein geringer Phloemtransport beobachtet. Im Vergleich zu DFG entwickelte DFV eine

größere Biomasse und höheres Spross/Wurzel Verhältnis. Diese Ergebnisse zeigen eindeutig, dass sich beide Varietäten significant in Mn-Aufnahme und Verteilung unterscheiden und dies sich in einer höheren Mn-Sensitivtät von DFG manifestiert.

Um die Wirkung toxischer Mn-Konzentrationen auf die Keimlingsentwicklung zu untersuchen wurden die Biomasse, Mn-Konzentrationen in den unterschiedlichen Kompartimenten gemessen. Während in DFG die Wurzelbiomasse eine stärkere Reaktion auf Mn-Stress zeigte wurde in DFV das Sprosswachstum am stärksten gehemmt.

Mittels EDAX-Mikroanalyse konnte eine Mn-Akkumulation in Epidermis und Cortexzellen in beiden Varietäten nachgewiesen werden. Diese Ergebnisse lassen die Annahme zu, dass die Wurzelepidermis eine Barriere für Mangan darstellt um das vaskuläre System und den Spross vor zu hohen Mangan Konzentrationen zu schützen. In der Epidermis und in den Cortexzellen konnten Prezipitate mit hohen Mn-Konzentrationen nachgewiesen werden, die eng mit P und Ca in diesen Prezipitaten korrelierten

Um die Rolle von Phosphor (P) zu untersuchen, wurden Douglasienkeimlinge beider Provienzen mit toxischen Mn-Konzentrationen und unterschiedlichen P-Gehalten inkubiert. Unter P limitierenden Bedingungen waren DFV Keimlingen weniger anfällig gegenüber Mn als unter optimaler P Ernährung. In DFG aber nicht in DFV wurde die P Konzentration auch unter P-Mangel in der Pflanze konstant gehalten.

Waldbäume der gemäßigten Zonen sind normalerweise in den Wurzeln mit Pilzen assoziert. Diese so genannte Mykorrhiza ist essentiell für die Nährstoffversorgung des Baumes. Während der Baum N und P und anderen Nährelemente von dem pilzlichen Partner bezieht, erhält der Pilz Kohlenstoff aus der Photosynthese. Darüber hinaus übt Pilz auch eine protektive Wirkung gegenüber Stress auf den Wirt aus.

Um den Effekt der Mykorrhizierung auf Mn-Stress in der Douglasie zu untersuchen, wurden Keimlinge von DFG und DFV mit *Rhizopogon subareolatus* mykorrhiziert und das Pflanzenwachstum, Elementkonzentration in den unterschiedlichen Geweben sowie die subzelluläre Mn-Konzentration wurden bestimmt. Die Biomasseproduktion beider Varietäten war durch die Mykorrhizierung mit *Rhizopogon subareolatus* gehemmt.

Mykorrhizierte Wurzeln zeigten eine stärkere Mn-Akkumulation in Geweben in der Nähe der Endodermis aber nicht im vaskulären System. Die Die Ergebnisse deuten daraufhin, dass die Mykorrhizierung mit *Rhizopogon subareolatus* eher einen negativen Effekt auf die Mn-Aufnahme und Wachstum beider Provienzen hat.

Um die Entwicklung der Douglasie unter natürlichen Bodenbedingungen zu untersuchen, wurden Biomasse Produktion Nährstoffaufnahme und die Entwicklung der Mykorrhiza an den Wurzeln in zwei Jahre alten Jungpflanzen, welche in zwei unterschiedlichen Böden gewachsen sind, beobachtet. Die molekulare Identifikation der sich in den zwei Jahren entwickelten Mykorrhiza an beiden Varietäten in den unterschiedlichen Böden zeigte, dass sowohl der Boden wie auch die Varietät entscheidenden Einfluss auf die Ausbildung einer jeweils spezifischen Mykorrhiza-Gesellschaft hatte.

Während *Rhizopogon vinicolor* mit der Varietät *viridis* assoziert war, so konnte *Cadophora finlandia* als dominante Art in der Varietät *glauca* identifiziert werden. *Tomantella sublilacina* ist ein Pilz, der spezifisch für einen Boden aus dem Solling war während *Wilcoxina mikolae* als dominante Art in dem Boden aus dem Unterlüss identifiziert wurde. Der Boden aus dem Solling war generell besser geeignet für das Wachstum beider Varietäten und es wurde eine bessere Mykorrhizierung beobachtet. Nährelementuntersuchungen deuten daraufhin, dass die Nährelementaufnahme positive mit dem Vorhandensein der Endomykorrhiza der Douglasie korreliert ist.

Basierend auf diesen Ergebnissen kann geschlussfolgert werden, dass beide Varietäten der Douglasie sich signifikant in der Mn-Aufnahme, Transport, Toxizität sowie der Verteilung von Mn unterscheiden. Die Daten zeigen auch, dass DFG empfindlicher gegenüber Mn ist als DFV. Da die Mn-Konzentrationen in den Pflanzen einem jahreszeitlichen Rhythmus unterliegt ist es schwer zu untersuchen wie sich beide Varietäten über einen langen Zeitraum gegenüber erhöhten Mn-Konzentrationen verhalten.

Keimlinge der Varietät *viridis* zeigten einen erheblich höheren Mykorrhizierungsgrad während des ersten Jahres der Entwicklung was ein Grund für die besssere Entwicklung und die größere Biomasse im Vergleich zu DFG sein könnte. Auf der anderen Seite kann durch diese Ergebnisse die These nicht gestützt werden, dass eine Mykorrhizierung generell vor Mn-Toxizität schützt. Abschließend lassen die Ergebnisse den Schluss zu, dass DFV is eher geeignt für den Anbau in Deutschland da diese Varietät besser an die vorherrschenden Bedingungen angepasst zu sein scheint.

Schlüsselwörter: Schwermetalle, Mangan, Phosphor, Transport, Mykorrhiza, Neophyten, *Pseudotsuga menziesii*.

CHAPTER 1

Introduction

1.1 Manganese characteristics

Manganese (Mn) belongs to the group of heavy metals, i.e. elements with densities above 5 g cm^{-3} such as Cd, Cu, Fe, Zn, Ni, etc. Heavy metals occur normally in trace concentration but regionally at elevated concentrations in natural soils (Bergmann, 1992). Of particular concern is, however, soil pollution with heavy metals introduced by human activities. The United Nations and the International Union of Biological Sciences listed threshold values of heavy metal for plants (International Union of Biological Sciences, 1994). Soil concentrations of manganese are however not meaningful since the plant availability of this element depends strongly on soil pH. The availability of Mn increases as soil pH decreases. When the soil pH drops below 5.5, Mn toxicity may be evident, whereas above pH 6.5 deficiencies are more likely (Marschner, 1995). At lower pH the manganous Mn^{2+} is dominant and readily available to plants. At higher pH the manganic forms $Mn^{3,4-7+}$ dominate, which are less plant available (Morgan, 2000). Liming acid soils changes the availability of Mn by changing soil solution pH and the form of manganese. Soil acidification, e.g. by use of ammonia-based fertilisers, can result in manganese toxicity. Unlike highly toxic heavy metals such as cadmium or lead, which have no known function in plants, manganese is an essential nutrient necessary for normal growth and development of plants.

Mn even though essential for normal plant growth and development can be toxic when present in excess in the environment. For normal plant growth, maintenance of Mn homeostasis is important. Excess uptake of redox active elements, such as Mn, causes oxidative destruction (Soliman et al, 1995). Thus, its uptake, transport and distribution of Mn within the plant must be strongly controlled. Regulation includes precisely targeted transport at the macro-level of the tissue, but also at the micro-level of the cell and organelles. Membrane transport systems seem to play most important roles in metal trafficking (Luk et al., 2003a).

The fine control of cellular concentrations of transition metals needs to be strictly adjusted. Changes out of a range needed for sufficient nutrition can lead to toxic or deficiency effects. Mn is a transition metal involved in cellular redox reactions. Excess of Mn can induce a range of negative

effects including the production of reactive oxygen species via the Fenton reaction, the exchange of essential metal ions from active centres of enzymes or by binding to functional groups (carboxyl, phosphate or histidyl groups) (Elstner et al., 1988), causing visible symptoms like chlorosis, necrosis and growth inhibition (Marschner, 1995). Since Mn is an essential component of reaction centres of enzymes, deficiency will also lead to stress symptoms. The fine regulation can be achieved by controlling uptake and transport, or by sequestration and compartmentation.

The biochemical responses of higher plants to toxic doses of heavy metals are generally very complex and several defence strategies have been suggested. These include complexation of metal ions, reduced influx of metals and enhanced production of antioxidants that detoxify reactive oxidative species produced in response to toxic metals (Van Assche and Clisters, 1990; Radotić et al., 2000, Schützendübel and Polle, 2002).

1.1.1 Manganese metabolic function

Manganese is an essential micronutrient throughout all stages of plant development. It is important for vital plant functions. Mn is acting as a cofactor of various enzymes such as Mn–superoxide dismutase, Mn-peroxiadse, pyruvate carboxylase, and phosphoenolpyruvate carboxykinase. Therefore, the incorporation of Mn by cells is essential, particularly in photosynthesis, where Mn plays a critical role as an accumulator of positive charge equivalents in a reaction catalyzed in photosystem II (Marschner, 1995). Mn aids the biosynthesis of chlorophyll, riboflavin, carotene, and ascorbic acid, is involved in assimilation of nitrate and activates enzymes of fat biosynthesis (Marschner, 1995).

Plant species differ considerably in their normal or adequate Mn leaf concentrations (30–500 mg Mn kg^{-1} dry mass, Clarkson 1988) and in their susceptibility to Mn deficiency (Reuter et al., 1988; Marschner, 1995; Mengel and Kirkby, 2001). The critical deficiency range in fully expanded leaves is reached when Mn concentrations drop below 10 to 20 mg Mn kg^{-1} dry mass (Marschner, 1995). Mn^{2+} toxicity can be an important factor limiting plant growth, particularly in acidic, poorly drained soils (Horst, 1988). On the other hand, critical concentrations for toxicity can vary within a very wide range, depending on plant species and genotypes within species, and on environmental conditions, such as temperature and Si, Ca, Mg, or Fe nutritional status (Horst, 1988; Le Bot et al., 1990; Wang et al., 1992). Critical toxicity concentrations ranging from 200 to 5300 mg kg^{-1} dry mass have been reported by Edwards and Asher (1982). Since Mn is involved in oxygen radical

detoxification via Mn-SOD, in Mn-deficient plants, the cell metabolism cannot efficiently control excess formation of oxygen radicals and oxidative damage occurs (Tanaka et al., 1995; Yu et al., 1998; Yu and Rengel, 1999). Tight interactions of Mn-nutrition and antioxidant metabolism exist since cytosolic CuZn-SOD and mitochondrial Mn-SOD activities increase under conditions of Mn-excess as well as Mn-starvation (Shenker et al., 2004).

Manganese has several different chemical roles in biological systems. It is involved in scavenging of superoxide and hydrogen peroxide:

$Mn(II) + O_2^{\cdot-} + 2H^+ \rightarrow Mn(III) + H_2O_2$

$2Mn(III) + H_2O_2 \rightarrow 2Mn(II) + O_2 + 2H^+$

The exact mechanisms for catalytic scavenging of H_2O_2 are, however, not clear and thought to involve intermediate steps. Reaction intermediates, $O_2^{\cdot-}$ and $OH^{\bullet}$ have been observed in vitro (Stadtman et al., 1990).

Excess Mn results in apoplastic deposition of oxidized Mn and phenolics. There is evidence that peroxidases are involved in this reaction (Fecht-Christoffers et al., 2003). Mn also induces PR- and thaumatin-like proteins in the apoplast. However, it is still unclear whether these responses belong to the activation of protection against Mn or whether these typical defence reactions occur relatively unspecific due to Mn-induced H_2O_2-production and injury (Horst et al., 1999).

1.1.2 Manganese transport from soil to cell

In higher plants the analysis of transport and sequestration of transition metals is complex because of tissue- and cell-specific differences and organ-specific transport. The processes that are assumed to be influencing metal accumulation rates in plants are mobilization and uptake from the soil, compartmentation and sequestration within the root, efficiency of xylem loading and transport, distribution between metal sinks in the aerial parts, sequestration and storage in leaf cells (Clemens et al., 2002). At such levels of the transport within the plant, concentrations and affinities of metal chelators as well as the presence and selectivity of transporters may influence metal accumulation rates (Marschner, 1995; Clemens et al., 2002). The apoplast continuum in the root epidermis and cortex is readily permeable for solutes. In general, solutes have to be taken up into the root symplast to cross the endodermis before they can enter the xylem (Tester and Leigh, 2001). Following metal uptake into the root symplast, three processes determine the movement of metals from the root into the xylem: sequestration of metals inside root cells, symplastic transport into the stele and release

into the xylem (Clemens et al., 2002). The transport of ions into the xylem is generally a tightly controlled process mediated by membrane transport proteins (Gaymard et al., 1998).

The traditional idea is that manganese as a divalent ion can move freely in the xylem sap and is transported to leaves with the transpiration steam. Re-distribution is thought to be limited since Mn has been classified as phloem immobile (Loneragan, 1988). However, even during xylem transport Mn may not be present as a "free" ion but may form complexes with organic acids or phosphate (Rauser, 1999; Luk et al., 2003a). Furthermore, the phloem sap also contains high concentrations of Mn (Rengel, 1988). In Douglas fir the sieve cells may participate in Mn-deposition and transport since they contained higher Mn-concentrations than the xylem (Dučić et al., 2006). Radioactive labelling showed that Mn-uptake into leaves transport back to roots was found (Dučić et al., 2006).

1.1.3 Intracellular transport distribution and homeostasis of Mn

One important task of metabolism is to supply proteins with the correct metal cofactors needed for their activity and moreover to deliver these cofactors at the right time and to the right site of the target protein. At the same time possible toxic reactions of the metals have to be avoided. It is, therefore, crucial that among the many different metals accumulated by cells, only the correct ion is presented to the metalloproteins (Luk et al., 2003a). Thus, when metals enter a cell, they are delivered to one of several possible pathways, depending on physiological needs. These routes are usually are called "metal trafficking pathways".

The molecular basis for the transport of manganese across membranes in plant cells is poorly understood. There are several metal transporters which play role in Mn- trafficking, like IRT1, a member of the ZIP family (Korshunova et al., 1999), MtZIP3, MtZIP4 and MtZIP (Lopez-Millan et al., 2004), SMF1, a member of the Nramp family comprising divalent metal transporters (Cellier et al., 1995) (Fig.1). *S. cerevisiae* PHO84 is a well-known transporter for the high-affinity uptake of phosphate; recently, a role for this protein in manganese transport has been uncovered (Luk et al., 2003a; Luk et al., 2003b). It is quite possible that phosphate transporters also contribute to manganese uptake in other organisms, particularly under conditions of manganese toxicity. For more details about Mn- transporters see Appendix I.

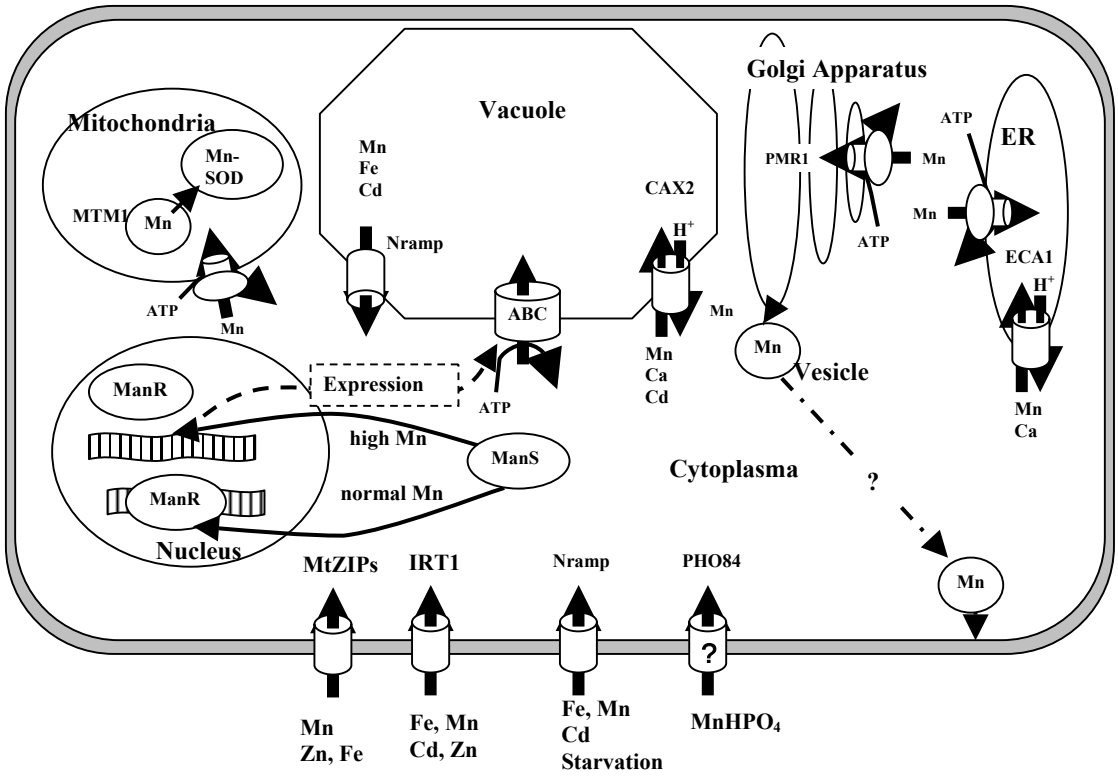

Figure 1. Hypothetic view of Mn-trafficking and cellular sensing. IRT1, Nramp and PHO84 are transporters putatively mediating Mn-uptake. PHO84 homolog in plants have not been detected yet. Inside the cell, ATPases pump Mn into subcellular compartments (ER = endoplasmatic reticulum, Golgi apparatus, and plastids). Vacuolar Mn-transport is achieved by CAX2, an H^+/Me^{2+}-antiporter. In mitochondria, MTM1 function as Mn-chaperone delivering Mn specifically to Mn-superoxide dismutases. MnS, a His-Kinase, acts as Mn sensor and suppresses activation of ABC transporter expression under normal conditions by repressing MnR (after Dučić and Polle, 2005). For further details, see text.

1.1.4 Manganese detoxifications

One way to prevent toxic effect of heavy metals is efflux. To facilitate manganese efflux from the cell, the metal is delivered into the Golgi apparatus and ultimately exported from the cell via secretory pathway vesicles that carry the metal to the cell surface (cf. Fig.1). P-type ATPase, known as PMR1 (transporters for both calcium and manganese) pump manganese into the secretory pathway (Rudolph et al., 1989; Durr et al., 1998).

Another way to prevent metal toxicity is compartmentation. Several transporters can potentially mediate transport of metals and compartmentation. These include the heavy metal ATPases (HMAs), the Nramps, the cation diffusion facilitator (CDF) family, the ZIP family, and the cation antiporters (Hall and Williams, 2003).

The main storage compartment for toxic compounds in plants is the vacuole (Vögeli-Lange and Wagner, 1990). In plants and fungi, vacuolar transporters help to remove potentially toxic cations from the cytosol. Metal/H^+ antiporters are involved in metal sequestration into the vacuole. About cation exchangers capable to transport Mn^{2+} into the vacuole and possible roles for ABC transporters in Mn^{2+} transport (Bartsevich and Pakrasi, 1995; Yamaguchi et al., 2002), as well His-kinase (ManS), which might sense the extracellular concentration of Mn^{2+} ions and lead to expression of operon for ABC transporter for Mn^{2+} see Appendix I and Figure1.

The intracellular trafficking of manganese in yeast is highly dependent on SMF2, another member of the Nramp family (West et al., 1992). MTM1 (manganese trafficking factor for mitochondrial SOD2) delivers manganese specifically to SOD2 and it is the only known Mn-chaperone to date (Luk and Cullota, 2001; Luk et al., 2003a, b). Similar proteins in plants have not yet been found.

The characterisation of keys steps involved in Mn uptake, transport and biochemical characterization of tolerance mechanisms is necessary to understand the fine balance of Mn in cellular and whole plant level.

1.2 Significance of manganese in Douglas fir (*Pseudotsuga menziesii*)

Douglas fir (*Pseudotsuga menziesii* (Mirbel) Franco) occurs naturally in North America. Because of its fast growth and good wood properties, it is also cultivated outside of its native range, in Europe now for almost 200 years. Worldwide, silviculture of Douglas fir is currently expanding (Herman and Smidt, 1999). Estimations predict that the proportion of Douglas fir stands will increase up to 10% of the total forest area in Germany (Kleinschmit, 1991; Knoerzer, 1999). To test the performance of exotic trees, various subspecies of Douglas fir have been planted in Germany, among them the two main varieties: *P. menziesii* var. *viridis* (DFV), the coastal type originating from the coastal regions of the mountains from British Columbia to California, and *P. menziesii* var. *glauca* (DFG), the interior type spreading along the Rocky mountains to the southwest of the USA to Mexico (Kleinschmit, 1974) (Fig. 2).

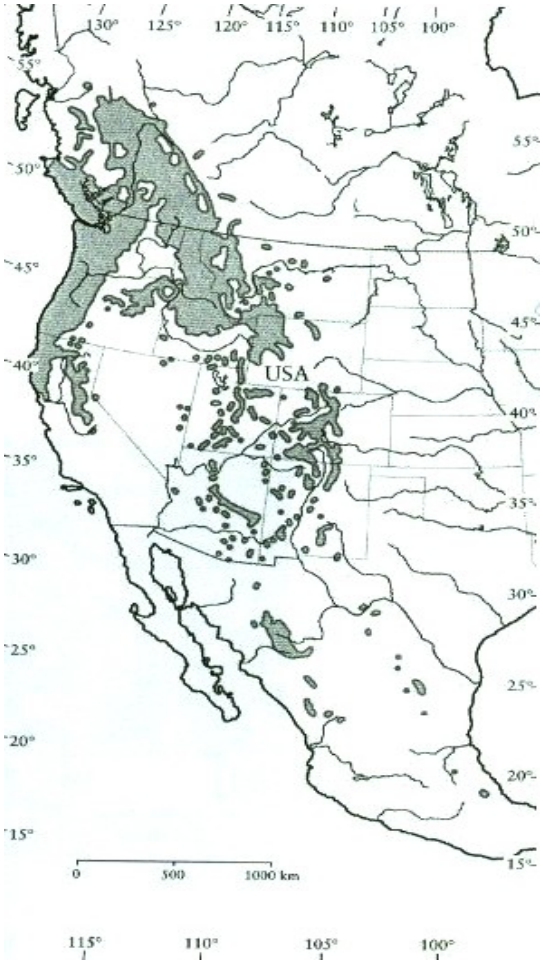

Figure 2. Natural habit of Douglas fir (*Pseudotsuga menziesii* (Mirbel) Franco) between Canada, USA and Mexico (after Schütt, 2000).

The varieties differ in both growth rate and size at maturity, DFV growing more rapidly and larger (Waring and Franklin, 1979). Usually old DFV trees can reach height around 75 m and DVG around 65 m (Waring and Franklin, 1979). DFG is more shade tolerant and has a more pronounced tap-root (Ernst, 1972). The coastal and interior varieties also differ in botanical and morphological characteristics. DFG contains higher oil contents, and has higher ability to withstand extremes of hot, cold and dry weather conditions (Sweet, 1965).

On the basis of quantitative traits such as growth rate, frost, and disease resistance the coastal variety (DFV) was found to be more appropriate for cultivation in Europe than the interior type (DFG) (Kleinschmit et al., 1974; Larsen, 1978; Liesebach and Stephan, 1995; Schober et al., 1983).

Although the performance of this introduced tree species was generally good, in some regions, e.g. in Rhineland-Palatinate (Rheinland-Pfalz, Germany) severe disease symptoms such as needle necrosis, defoliation as well as formation of dark slimes and bark necrosis were observed in

young stands of Douglas fir (20 to 40 years) (Schöne, 1992). Needles of injured trees contained excessive concentrations of manganese. Therefore, the decline of Douglas fir in some sites in Germany has been related to Mn toxicity (Schöne, 1992). By analysis of the genetic structures, the damaged stands in Rhineland-Palatinate were identified as belonging to the interior variety (DFG), whereas healthy stands were more closely related to the coastal variety (DFV) (Leinemann, 1996). Since healthy and damaged trees grew in close vicinity, it has been suspected that the two varieties differed in Mn-uptake rates (Schöne, 1992). It is unknown whether this might be a plant-inherent feature or modulated by tree-environmental interactions. Experiments to investigate the physiology of Mn-metabolism in the two subspecies of Douglas fir have not yet been conducted.

Because of the economic interest in Douglas fir silviculture, it is important to find out whether the two varieties differ in Mn-uptake and accumulation patterns or whether the more tolerant variety has a higher capacity for internal Mn-detoxification. A further possibility is that both species differ in mycorrhization and than this affects Mn-uptake and susceptibility.

1.3 Effect of mycorrhiza on Mn-metabolism in plants

More than 90% of land plants form symbiotic associations with mycorrhizal fungi (Devisser, 1995). In most tree species so-called ectomycorrhizae dominate but Douglas fir also forms theendo-type of mycorrhiza. Mycorrhizal fungi assisting plants with the uptake of phosphorus, nitrogen and other mineral nutrients from the soil are important for plant growth and development, especially under nutrient-limiting conditions (Harley and Smith, 1983; Bolan, 1991; Harrison and Vanbuuren, 1995; Hartley et al., 1997; Smith and Read, 1997). Mycorrhiza symbiosis is founded on the mutualistic exchange of C from the plant in return for P, N and other mineral nutrients from the fungus. It was found that influx of phosphorus in roots colonized by mycorrhizal fungi could be 3 to 5-times higher than in non-mycorrhizal roots (Smith and Read, 1997). The increased surface area of ectomycorrhizal root systems increases the efficiency of absorption and translocation of inorganic nitrogen to the root (France and Reid, 1983).

Mycorrhizal associations are related to several benefits to the host plant. Besides the improvement of the nutritional state, other benefits are also relevant, such as increasing plant resistance to pathogens (Hampp et al., 1999), to heavy metal stress (Leyval et al., 1997; Jentschke and Godbold, 2000; Schützendübel and Polle, 2002), or to drought stress (Davies et al., 1992;

Nilsen et al., 1998; Shi et al., 2002. The most studies addressed the influence of ectomycorrhizae in protection against heavy metals stress such as Cd, Cu, Pb, and Zn (Galli et al., 1994; Leyval et al., 1997; Jentschke and Godbold, 2000; Liu et al., 2000; Schützendübel and Polle, 2002), but not with respect to shield against excess of essential nutrients such as Mn.

Depending on the interactions between the mycorrhizal fungus, the host and the environment, the effects may as well be negative or deleterious to the host plant (Medeiros et al., 1995; Eltrop and Marschner, 1996; Cardoso et al., 2003; Corrêa et al., 2006). Growth depressions following mycorrhizal inoculation are generally attributed to the carbohydrate drain of the mycorrhizal fungus, while positive growth effects of mycorrhiza are thought to occur when the benefits of increased nutrient uptake exceed the carbon cost of the association (Schroeder and Janos, 2004; Thomson et al., 1994).

Plants react differently to ectomycorrhiza formation depending on their age, their initial nutritional status, and the amount of supplied nitrogen (Corrêa et al., 2006).

In northern Spain inoculation of Douglas fir with ectomycorrhizal fungi improved the field performance of seedlings (Pera et al., 1999). Total plant biomass of Douglas fir is usually significantly increased up to four times by the presence of any fungal inoculation compared with non-inoculated controls (Parladé and Álvarez, 1993). In Germany, positive effects on Douglas fir growth were reported in association with *Rhizopogon parksii* (Gross et al., 1980), and in the Netherlands Douglas fir was found in symbiosis with *R. viinicolor*-like ectomycorrhiza (Jansen and de Vries, 1989)

The particular roles of endo- and ectomycorrhizal fungi in nutrition and Mn-uptake have not been studied. In the most cases, manganese stress was investigated in crop plants with vesicular-arbuscular mycorrhiza (VAM) (Cardoso, 1985; Bethlenfalvay and Franson, 1989; Kothari et al., 1991; Posta *et al.*, 1994). It is still unclear whether the VAM type of mycorrhiza can help plants against Mn stress. In some cases VAM inhibited root dry mass production and increased the concentrations of Mn in shoots (Posta et al., 1994). As the availability of Mn to plants is influenced by oxidation and reduction processes (Marschner 1995), this negative effect of VAM was connected to lower reduction processes of Mn in rhizosphere (Posta et al., 1994).

In contrast to these negative effects, in several studies a lower uptake of Mn by mycorrhizal than non-mycorrhizal plants has been found (Pacovsky 1986; Arines and Vilarino, 1989; Kothari et al., 1990; Kothari et al., 1991).

Knowledge about the responses of ectomycorrhizal fungi to toxic metals is not only of importance for tree performance but, as the decrease of metal phytotoxicity by mycorrhizal fungi has been widely demonstrated (Jones and Hutchinson, 1986; Dixon and Buschena, 1988; Colpaert and Van Assche, 1993), another important aspect is the reclamation of polluted sites (Blaudez et al. 2000).

Objectives of the present thesis

The aim of this thesis was to investigate whether uptake, transport, distribution of Mn differed in two different varieties of Douglas fir (*Pseudotsuga menziesii* (Mirbel) Franco) var. *viridis* (DFV) and var. *glauca* (DFG). For this purpose the following hypothesis were tested:

- The two varieties DFV and DFG differ in Mn uptake and translocation under normal conditions.

  This was tested by using Douglas fir seedlings exposed to radioactive labelled ^{54}Mn in split-root systems. Biomass partitioning, Mn concentrations, ^{54}Mn-transport was investigated. Subcellular localisation of Mn was characterised by transmission electron microscopy coupled with X-ray microanalyses **(chapter 2).**
- The two main varieties vary in uptake and plant-internal distribution of Mn and other macro- and microelements under toxic conditions of Mn. The tolerant variety of Douglas fir expresses protective mechanism at the cellular level.

  To test this assumption plants growth, biomass partitioning, Mn and other elements concentrations were investigated. To investigate the subcellular Mn localisation transmission electron microscopy coupled with X-ray microanalysis was used (**chapter 3**).
- The colonisation with the ectomycorrhizal fungus *Rhizopogon subareolatus* influences Mn uptake and toxicity in the two varieties of Douglas fir.

  To test this point plant growth, biomass portioning and subcellular localisation of Mn were investigate under Mn stress and *Rhizopogon subareolatus* inoculation **(chapter 4).**
- The differences in Mn-metabolism in DFG and DFV mare are related to differences in the colonisation by mycorrhizae.

  To test this hypothesis we examined the pattern and type of mycorrhizae formed on DFV and DFG in natural soils taken from Unterlüss and Solling (Low Saxony, Germany) in relation to growth and mineral nutrition. We were interested in the question whether naturally present fungi would form variety-specific mycorrhizal communities on roots of DFV and DFG (**chapter 5**).

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Chapter 2

2.1. Uptake and translocation of manganese in seedlings of two varieties of Douglas fir (*Pseudotsuga menziesii* var. *viridis* and *glauca*)

2.1.1 Abstract

Douglas fir (*Pseudotsuga menziesii*) variety *glauca* (DFG) but not *viridis* (DFV) showed symptoms of Mn toxicity in some field sites. We hypothesised that these two varieties differed in manganese metabolism. To test this hypothesis, biomass partitioning, Mn concentrations, subcellular localisation and ^{54}Mn-transport were investigated.

Total Mn uptake was three-times higher in DFG than in DFV. DFV retained more than 90 % of ^{54}Mn in roots, whereas more than 60% was transported to the shoot in DFG.

The epidermis was probably the most efficient Mn-barrier since DFV contained lower Mn concentrations in cortical cells and vacuoles of roots than DFG.

In both varieties, xylem loading was restricted and phloem transport was low. Still, sieve cells contained high Mn-concentrations.

DFV displayed higher biomass production and higher shoot/root ratios than DFG. Our results clearly show that both varieties of Douglas fir differ significantly in Mn-uptake and allocation patterns rendering DFG more vulnerable to Mn toxicity.

2.1.2 Introduction

Douglas fir (*Pseudotsuga menzesii*) occurs naturally in North America. Because of its fast growth and good wood properties, it is also cultivated outside of its native range, in Europe now for almost 200 years. Worldwide, silviculture of Douglas fir is currently expanding (Herman and Smidt, 1999). Estimations predict that the proportion of Douglas fir stands will increase up to 10% of the total forest area in Germany (Kleinschmit, 1991; Knoerzer, 1999). To test the performance of exotic trees, various subspecies of Douglas fir have been planted in Germany, among them the two main varieties: P. *menziesii* var. *viridis* (DFV), the coastal type originating from the coastal regions of the mountains from British Columbia to California, and *P. menziesii* var. *glauca* (DFG), the interior type spreading along the Rocky mountains to the southwest of the USA to Mexico (Kleinschmit *et al.,* 1974). Based on quantitative traits such as growth rate, frost, and disease resistance the coastal variety (DFV) was found to be more appropriate for cultivation in Germany than the interior type (DFG) (Kleinschmit *et al.,* 1974, 1979; Larsen, 1978; 1981; Liesebach and Stephan, 1995; Schober *et al.,* 1983, 1984).

Although the performance of this introduced tree species was generally good, in some regions, e.g. in Rhineland-Palatinate (Rheinland-Pfalz, Germany) severe disease symptoms such as needle necrosis, defoliation as well as formation of dark slimes and bark necrosis were observed in young stands of Douglas fir (20 to 40 years) (Schöne, 1992). Needles of injured trees contained excessive concentrations of manganese. Therefore, the decline of Douglas fir in some sites in Germany has been related to Mn toxicity (Schöne, 1992). By analysis of the genetic structures, the damaged stands in Rhineland-Palatinate were identified as belonging to the interior variety (DFG), whereas healthy stands were more closely related to the coastal variety (DFV) (Leinemann, 1996). Since healthy and damaged trees grew in close vicinity, it has been suspected that the two varieties differed in Mn-uptake rates (Schöne, 1992). It is unknown whether this might be a plant-inherent feature or modulated by tree-environmental interactions. Experiments to investigate the physiology of Mn metabolism in the two subspecies of Douglas fir, have not yet been conducted.

Mn is an essential nutrient element necessary for activation of a wide range of enzymes and an indispensable constituent of the catalytic centre of enzymes such as

Mn-superoxide dismutase and the water splitting complex. To fulfil its metabolic functions, Mn is only necessary at low concentrations (20 µg per gram dry mass, Marschner, 1995). However, tissue concentrations of Mn may vary considerably. In their natural habitats, the coastal variety, DFV contained 100 – 800 and the interior type, DFG 200 - 2000 µg Mn g^{-1} dry mass, respectively (Baronius and Fiedler, 1996; Zasoski *et al.*, 1990).

Plant availability of Mn depends on soil properties and on root exudates for Mn chelation or reduction. The availability of Mn increases with decreasing soil pH (Marschner, 1986). In general, excess Mn causes chlorosis and necrosis, the appearance of brown, necrotic spots or small reddish purple spots and sometimes, dark root tips (Horst, 1988). High concentrations of Mn interfere with the absorption, translocation, and utilisation of other mineral elements such as Ca, Mg, Fe and P (Clark, 1982), stimulate phenolic metabolism (Brown *et al.*, 1984; Wissemeier and Horst, 1992), affect energy metabolism, respiration rates (Nable *et al.*, 1988), and cause oxidative stress (Del Rio *et al*., 1985; Panda *et al.*, 1986; Horst *et al*., 1999; Fecht *et al*., 2001). Therefore, Mn-uptake by plants is strictly regulated (Dučić and Polle, 2005).

To date, most studies concerning Mn uptake and translocation have been conducted with agronomically important plants or with unicellular model organisms like bacteria and yeast. In crops such as spinach, pea, bean, and various cereals race-specific differences in Mn toxicity have been reported (Horst, 1983, 1988; Graham, 1988; Foy *et al*., 1988; Rout *et al*., 2001). Much less is known about uptake, translocation and toxicity of Mn in forest trees and virtually nothing about varietal differences in Mn-metabolism. Since Douglas fir is of increasing silvicultural interest and field data point to differences in Mn-toxicity in different subspecies, the goal of this study was to find out whether DFG and DFV differ in Mn metabolism under non-toxic conditions. For this purpose, Douglas fir seedlings were exposed to radioactively labelled Mn. Uptake, transport, and allocation of Mn were investigated at the whole plant level. Furthermore, transmission electron microscopy coupled with X-ray microanalysis was used to characterise the subcellular Mn localisation.

2.1.3 Material and methods

2.1.3.1 The varietal origin of seed lots

Seeds of *Pseudotsuga menziesii* DFV and DFG were purchased from Niedersachsen Forstamt (Oerrel, Munster- Oerrel, Germany) and Sheffield's Seed Company (Locke, New York, USA), respectively. To evaluate the varietal origin of the two seed lots, genetic structures at the isozyme gene loci for 6-phosphogluconate-dehydrogenase (6-PGDH-A) were analyzed according to Leinemann (1996, 1998). Endosperm and embryo from each seed were extracted and separated by horizontal starch gel electrophoresis using a TRIS-citrate buffer, pH 7.4 (Leinemann, 1998). Activity staining was conducted according to Rothe (1994).

The zymograms of the two seed lots show strong differences in their banding patterns.

As expected the allele 6-PGDH-A_3 is dominant in *P. viridis* samples, whereas the *P. glauca* samples shows higher frequencies of homozygote varieties containing the allele 6-PGDH-A_6 (Leinemann, 1996). This result confirms the varietal origin of the two seed samples.

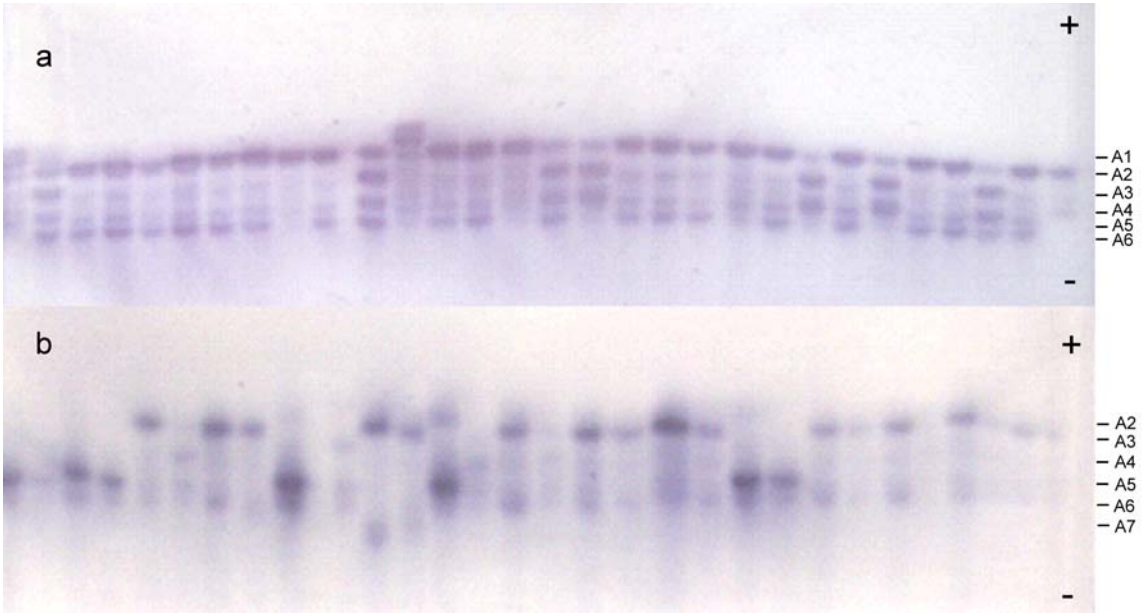

Figure 1. Zymograms of 6-phosphogluconate-dehydrogenase from seed endosperm and embryos of *Pseudotsuga menziesii viridis* (a) and *glauca* (b). Alleles from A1 to A7 are marked. Each pair of tracks shows the isozyme pattern from one endosperm and its corresponding embryo, respectively.

2.1.3.2 Plant material

Seeds of both varieties of Douglas fir were soaked in tap water for 7 days at 2°C and surface sterilised in 96% ethanol for 30s, in 0.2% $HgCl_2$ for 30s, and in 30% H_2O_2 for 45 min. Subsequently, the seeds were placed on sterile 1.5% (w/v) water-agar, pH 4.5 in Petri-dishes (d=140 mm), maintained for 7 days in darkness at 21°C and subsequently for 3 weeks with a day/night regime of 16 h/8 h (white light of 150 µmol m^{-2} s^{-1} photosynthetic photon flux, OSRAM L 18-W/21-840 (Lumlux Pluseco, Germany) at 23°C/21°C air temperature. After germination for seven days, the plants were transferred to hydroponic solutions. Aerated nutrient solution contained the following nutrient elements: 300 µM NH_4NO_3, 100 µM Na_2SO_4, 200 µM K_2SO_4, 60 µM $MgSO_4$, 130 µM $CaSO_4$, 30 µM KH_2PO_4, 10 µM $MnSO_4$, 92 µM $FeCl_3$, and 5 mL of a stock solution of micronutrients: 0.1545 g/L H_3BO_3, 0.012 g/L $NaMoO_4$, 0.0144 g/L $ZnSO_4$ and 0.0125 g/L $CuSO_4$, per litre of nutrient solution. The pH was adjusted to 4. The solution was changed every 3rd day.

2.1.3.3 Experimental set-up to determine Mn-transport

Young DFG and DFV plants were transferred into specially constructed exposure boxes (Fig. 2). The exposure boxes consisted of 4 separated small chambers (Fig. 2a).

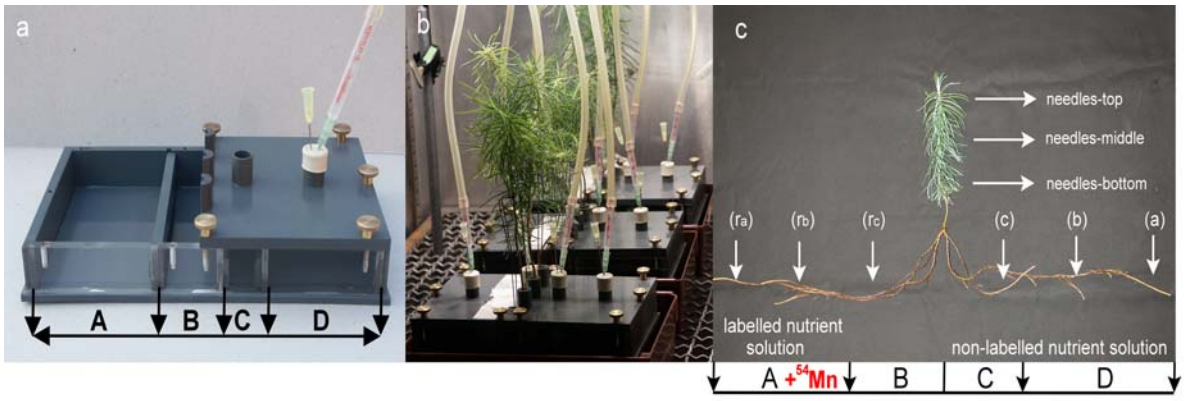

Figure 2. Experimental set-up for ^{54}Mn feeding of Douglas fir seedlings. (a) Exposure box with four chambers to expose split roots to ^{54}Mn in chamber A. (b) Exposure boxes containing plants. (c) Tissues taken for biomass, Mn, and ^{54}Mn analyses after harvest; i.e. needles from the top, middle and bottom, roots from the labelled side: tip-r_a, middle-r_b and upper part-r_c and roots from the non-labelled side: tip-a, middle-b and upper part-c.

Three seedlings were inserted in chamber B. The root system of each seedling was divided and spread into chamber A as well into chambers C and D. $^{54}MnCl_2$ (0.86 MBq or 0.75 MBq of ^{54}Mn in 0.5 M HCl in two experiments [PerkinElmer, Boston, USA]) was added to the nutrient solution in chamber A once at the beginning. The pH was adjusted to 4 by 0.5 M NaOH. The intermediate chambers B and C served to avoid spill over and contamination of the root parts in chamber D. The boxes were covered with a lid to avoid evaporation. The nutrient solution was aerated with syringes introduced into the solution through perforations in the lid. The level of the nutrient solution in each chamber was checked through a transparent side of the apparatus. Nutrient solution was supplied with a syringe as necessary and the added volume was recorded. The plants were maintained for 21 days in the exposure box under the following conditions: temperature 20°C, relative humidity 40% and white light 24h (200 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetically active photon flux, OSRAM, Powerstar HQI-T/D, Germany).

Needles from the bottom and upper of the crown were harvested every third day to determine radioactivity. After 21 days plants were separated into root, stem and needles, and these parts were further divided in three pieces: upper part, middle and bottom part (as indicated in Fig. 2c). Each experiment was conducted with six to nine individual seedlings in 3 replicate exposure systems. The experiments were repeated with DFG and DFV seedlings of the same age and of similar biomass.

Radioactivity was quantified in a γ-counter (Automatic gamma counter 1480 Wizard 3´´, Wallac, Turku, Finland) using 50 mg of dry mass of each tissue. Distribution of ^{54}Mn in whole plants was detected by autoradiography using a phosphor imaging plate scanner (Fuji BAS 1500) on imaging plates (BAS-III, Fuji) (Fuji, Stamford, CT, USA) with 85 minutes up to 21h exposure times.

2.1.3.4 Element analyses

Samples for element analyses (root, stem and needles) were dried to a constant weight and ashed at 170°C in 65% HNO_3 for 12h (Feldmann, 1974). Elements were determined by inductively coupled plasma- atomic emission spectroscopy (Spectro Analytical Instruments, Kleve, Germany).

2.1.3.5 Energy dispersive X-ray microanalyses (EDX)

Needle and root pieces taken 15 mm from the root tip from 1.5-months old seedlings were cut in several 2 mm and 10 mm long pieces, respectively, and were rapidly frozen in a mixture of propane:isopentane (2:1) cooled with liquid nitrogen to –196°C in a aluminium mesh. Samples were freeze-dried at –45°C for three days and stored at room teperature in a desiccator over silica gel. For transmission electron microscopy, freeze-dried samples were infiltrated with ether in a vacuum-pressure chamber and embedded in styrene-metacrylate using a technique specifically developed for analysis of diffusible elements (Fritz, 1989). 1 µm thick sections were cut dry by glass knives, mounted on adhesive–coated 100-mesh hexagonal grits, coated with carbon, and stored over silica gel. Details and testing of the method have been reported previously (Fritz, 1989; Fritz and Jentschke, 1994). The samples were analysed with a Philips EM 420 with the energy dispersive system EDAX DX-4 (EDAX Inc., Mahwah, NJ, USA). The accelerating voltage was 120 kV, the take off angle 25° and counting time 60 live seconds. Mn concentrations in cross-sections of roots and needles were analysed in cell walls and vacuoles of the following tissues: epidermis, needle mesophyll, cortex of roots, endodermis, xylem, and phloem. Nine replicates were analysed in each compartments in three different plants.

2.1.3.6 Statistical analyses

Data are means (± SD) of five to nine seedlings. Statistical analysis of the data was performed using student´s t-test or analysis of variance (ANOVA) followed by a multiple range test (LSD, Statgraphics 2.1, St. Louis, USA). Means were considered to be significantly different from each other, if the level of significance was $P \leq 0.05$.

2.1. 4 Results

2.1.4.1 Growth performance, biomass, Mn partitioning and subcellular distribution in Douglas fir var. viridis and var. glauca

Growth of DFV and DFG seedlings differed significantly resulting in 1335 ± 118 and 250 ± 4 mg dry mass per plant, respectively (P = 0.023), after five months culture in hydroponic solutions. DFV seedlings reached shoots lengths and biomass similar to those of 5-months-old DFG seedlings after only 3.5 months (Table 1). Biomass partitioning to the major plant compartments showed pronounced differences between the two varieties with DFV favouring above-ground and DFG below-ground growth (Table 1). Although DFG developed almost three-times longer and, thus, more root biomass than DFV (Table 1), the root system of DFV was more branched displaying many small lateral roots. The subspecies-related differences in above- and below-ground partitioning reported here for hydroponically grown plants were also found in Douglas fir seedlings cultivated in soil (data not shown).

The needle concentrations of Mn tended to be slightly elevated in DFG compared with DFV (Table 1) and were in the same range as those of field grown mature Douglas firs (Baronius and Fiedler, 1996).

Stem and root Mn concentrations did not differ between the two varieties (Table 1). The Mn content of whole seedlings of DFG was similar to that of DFV (Table 1). Because of the differences in biomass partitioning, both DFV and DFG had slightly different Mn allocation patterns with relative portions of 37%, 12%, and 50% Mn and of 43%, 8%, and 49% Mn in needles, stem and roots of DFG and DFV, respectively.

To find out whether the varieties differed in the subcellular distribution of Mn, we investigated cross sections of root tips employing energy dispersive X-ray microanalyses (Fig. 3a). Epidermal and cortex cell walls of DFG contained significantly higher concentrations of Mn than those of DFV (Fig. 3a). Furthermore the vacuolar Mn concentration in DFG epidermis cells was almost 8-times higher than in DFV. The relative enrichment of Mn in DFG compared with DFV decreased towards the endodermal barrier and was absent in the vascular system (Fig. 3a). In DFV maximum

Mn concentrations were found in epidermal cell walls. In all other locations analysed in DFV root cross sections, Mn was present at low concentrations displaying no obvious differences between cells types and subcellular compartments (Fig. 3a). In needles, significant subspecies-related differences n the localisation of Mn were not observed (Fig. 3b). But unlike in roots, both varieties showed differences with respect to the subcellular location of Mn with significantly higher concentrations in cell walls than in vacuoles (P = 0.025 and P = 0.010 for DFV and DFG respectively, Fig 3b). It is also remarkable that Mn concentrations in needle cell walls were generally higher than in root cell walls (Fig. 3), which might be due to the fact that Mn is considered a relatively phloem-immobile element (Loneragan, 1988) not easily re-translocated to other tissues. Bearing this in mind it was even more surprising to find very high Mn concentrations in sieve cells of the needle phloem of both varieties (Fig. 3b).

Table 1. Biomass, growth parameters, and manganese concentrations of *Pseudotsuga menziesii* var. *viridis* (DFV) and var. *glauca (DFG)*. DFV was harvested after 3.5 and DFG after 5 months. Data indicate means of n = 5-9 (± SE).

		DFV	DFG	*P*-value
Biomass (per plant)		Mean± SE	Mean± SE	
Needles	(mg)	129 ± 18	93 ± 12	0.319
Stem	(mg)	41 ± 3	29 ± 4	0.014
Root	(mg)	95 ± 14	128 ± 13	0.137
Whole plant	(mg)	265 ± 33	250 ±254	0.874
Growth morphology				
Ratio root/shoot		0.6 ± 0.3	1.0 ± 0.3	0.002
Root length	(mm)	134 ± 48	352 ± 97	0.002
Shoot height	(mm)	103 ± 18	91±14	0.348
Manganese concentrations				
Mn in needles	mg/kg^{-1}	130 ± 28	216 ± 28	0.068
Mn in stem	mg/kg^{-1}	131 ± 39	135 ± 30	0.963
Mn in root	mg/kg^{-1}	238 ± 25	178 ± 24	0.405
Mn content per whole plant	(µg)	646 ± 106	616 ± 84	0.855

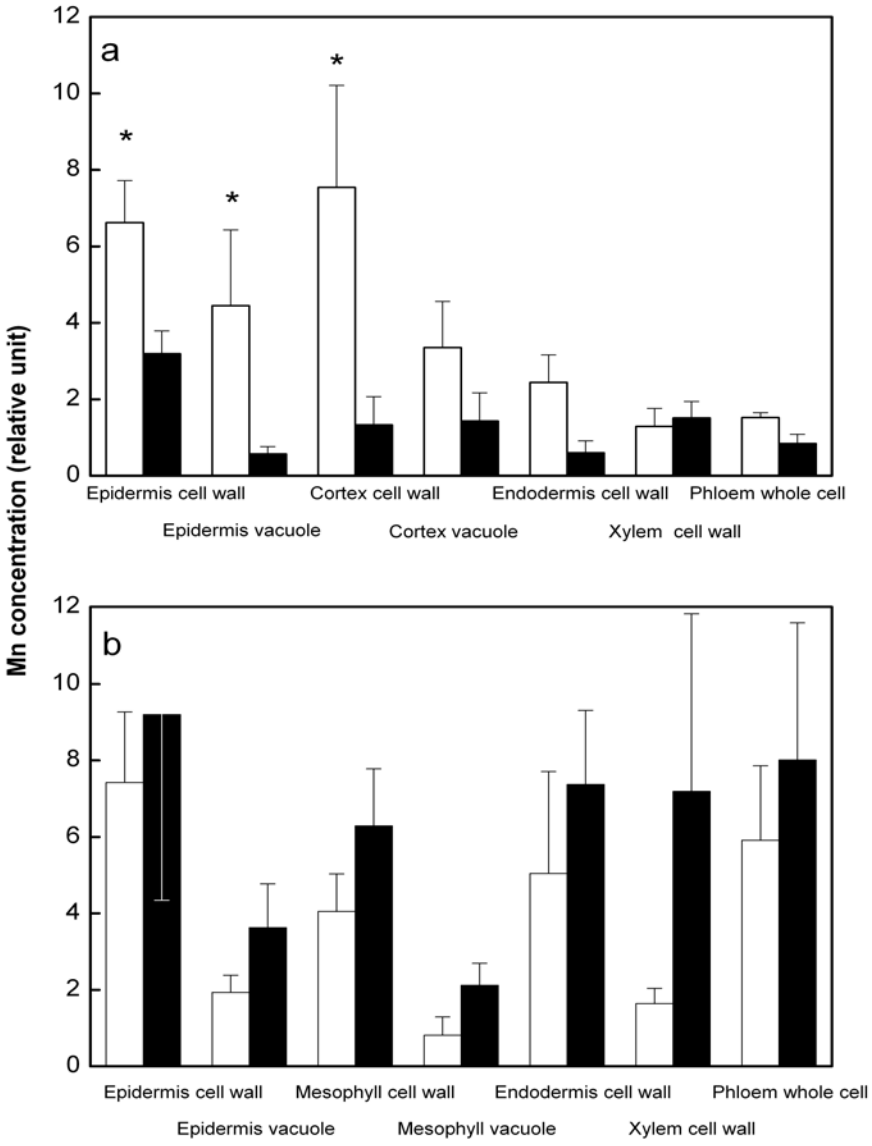

Figure 3. Spatial resolution of manganese concentrations in different cell types and subcellular compartments of roots (a) and needles (b) of *Pseudotsuga menziesii* var. *viridis* (DFV, black bars) and var. *glauca* (DFG, white bars). Cross sections were analysed by TEM-EDX. Bars indicate means of n=6-9 (± SE). * indicates $P \leq 0.05$.

This observation suggests that either Mn must be immobilised by unknown mechanisms in sieve cells or that Mn can be transported and circulated in both Douglas fir varieties.

2.1.4.2 Uptake, transport, and allocation of Mn in Douglas fir var. viridis and glauca

To find out whether the two varieties of Douglas fir show differences in Mn-uptake and transport, young seedlings were exposed to ^{54}Mn in the nutrient solution. A split-root system was used to investigate the possibility of re-translocation of Mn (Fig. 2). The kinetics of ^{54}Mn accumulation was determined in top, i.e., younger and bottom, i.e. older needles of DFG and DFV, respectively (Fig. 4 a, b).

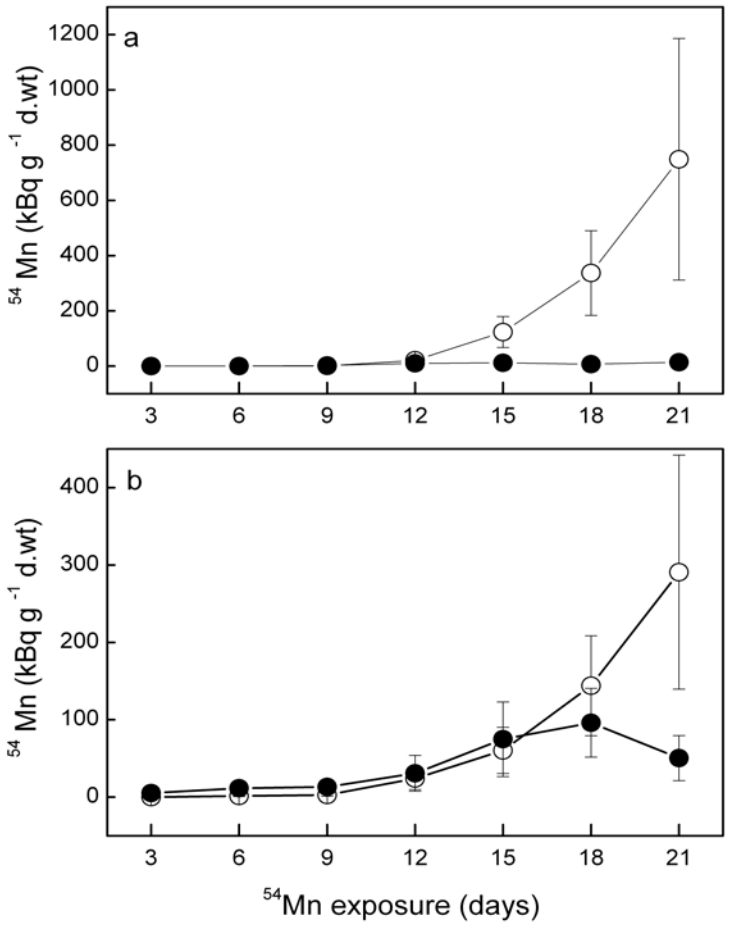

Figure 4. Time-dependent accumulation of ^{54}Mn in needles of *Pseudotsuga menziesii* var. *viridis* (DFV, black circles) and var. *glauca* (DFG, white circles). Radioactivity was applied to chamber A of the exposure box (see figure 2). Radioactivity was determined in top (a) and bottom needles (b) as indicated in fig. 2. n=5-9 (±SE). When no error bars are apparent, they were smaller than the symbols.

The accumulation rate of Mn in old needles of DFV was similar to that of DFG during the first two weeks of the experiment (Fig. 4b). Thereafter, no further accumulation of ^{54}Mn in older needles of DFV was found and after three weeks, the concentration of ^{54}Mn tended to decrease (Fig. 4b). In contrast to the restriction in Mn translocation to older needles in DFV, an accelerated increase in ^{54}Mn was observed in DFG after the initial lag-phase (Fig. 4a).

Varietal differences in Mn-transport were even more pronounced in young needles (Fig. 4a). After an initial lag-phase of about 10 days, young needles of DFG showed about two-times higher ^{54}Mn accumulation rates than old needles (Fig. 4 a, b). In contrast to DFG, transport of Mn to young needles was strongly suppressed in DFV (Fig. 4a) and even lower than to older needles (Fig. 4b). Since we observed that DFG consumed higher volumes of nutrient solution per plant than DFV (Table 2), despite less needle biomass (Table 1), we suspect that higher transpiration rates in DFG may have led to higher transport of Mn to the above-ground compartment.

Table 2. Consumption of nutrient solution during the whole experimental period (21 days) expressed per plant and expressed as daily consumption per needle biomass in *Pseudotsuga menziesii* var. *viridis* (DFV) and var. *glauca* (DFG). * indicates $P \leq 0.05$.

Consumption of nutrient solution	DFV	DFG
Total volume in 21 days (ml)	150 ± 10	211 ± 41*
Rate (ml g^{-1}·dry mass x day^{-1})	55	108

After three weeks, the seedlings were harvested to image Mn distribution at the whole plant level by autoradiography. Figure 5 displays typical examples of whole plants and their corresponding autoradiograms. DFV was characterised by more needle and less root biomass than DFG (Fig 5a, e, and Table 1) and showed strong ^{54}Mn accumulation in those root parts exposed to the labelled solution (Fig. 5b).

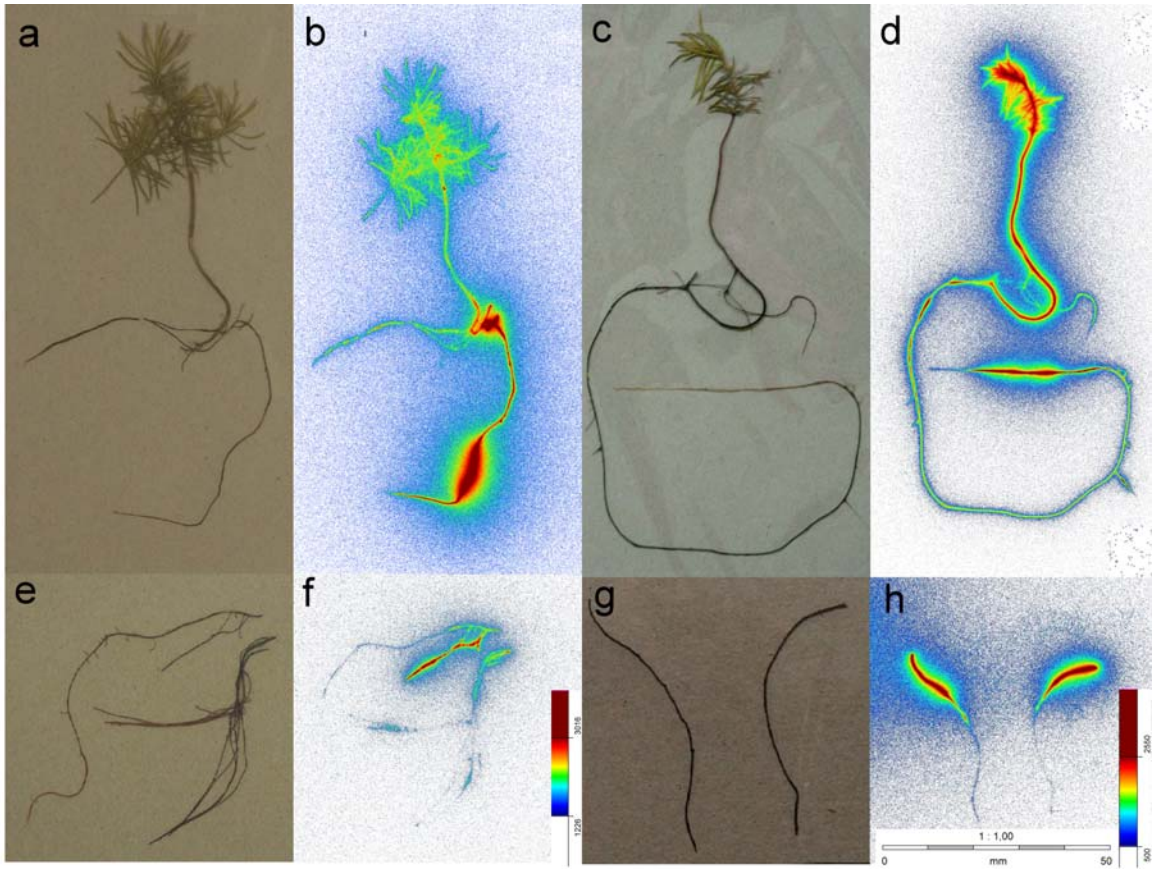

Figure 5. Photographs and autoradiograms of whole seedlings of *Pseudotsuga menziesii viridis* (DFV) and *glauca* (DFG) after exposure to ^{54}Mn in a split root system. Photographs of whole dry seedlings and their corresponding roots from the non-labelled chamber (a,e = DFV, c,g = DFG). Autoradiograms of whole dry seedlings and their corresponding roots from the non-labelled chamber (b,f = DFV, d,h = DFG). Colours from blue to red represent increasing intensities of ^{54}Mn-labelling.

DFV autoradiograms showed only little ^{54}Mn in stem and needles (Fig. 5b). In contrast to DFV, stem and needles of DFG contained strong ^{54}Mn activity (Fig. 5d). It appeared that Mn was retained at the bottom of needles and not transported to the needle tip (Fig. 5d). Roots of both DFG and DFV exposed to the non-labelled solution also displayed radioactivity (Fig. 5f, h). This suggests that apart from important differences in Mn allocation between both varieties, DFG and DFV might be able to re-translocate and probably exude Mn.

To quantify varietal differences in Mn-uptake and allocation, radioactivity was determined in different plant fractions as indicated in Fig. 2 and expressed on the basis of dry mass (Fig 6a) or as specific activity on the basis of the tissue concentration of Mn (Fig. 6b).

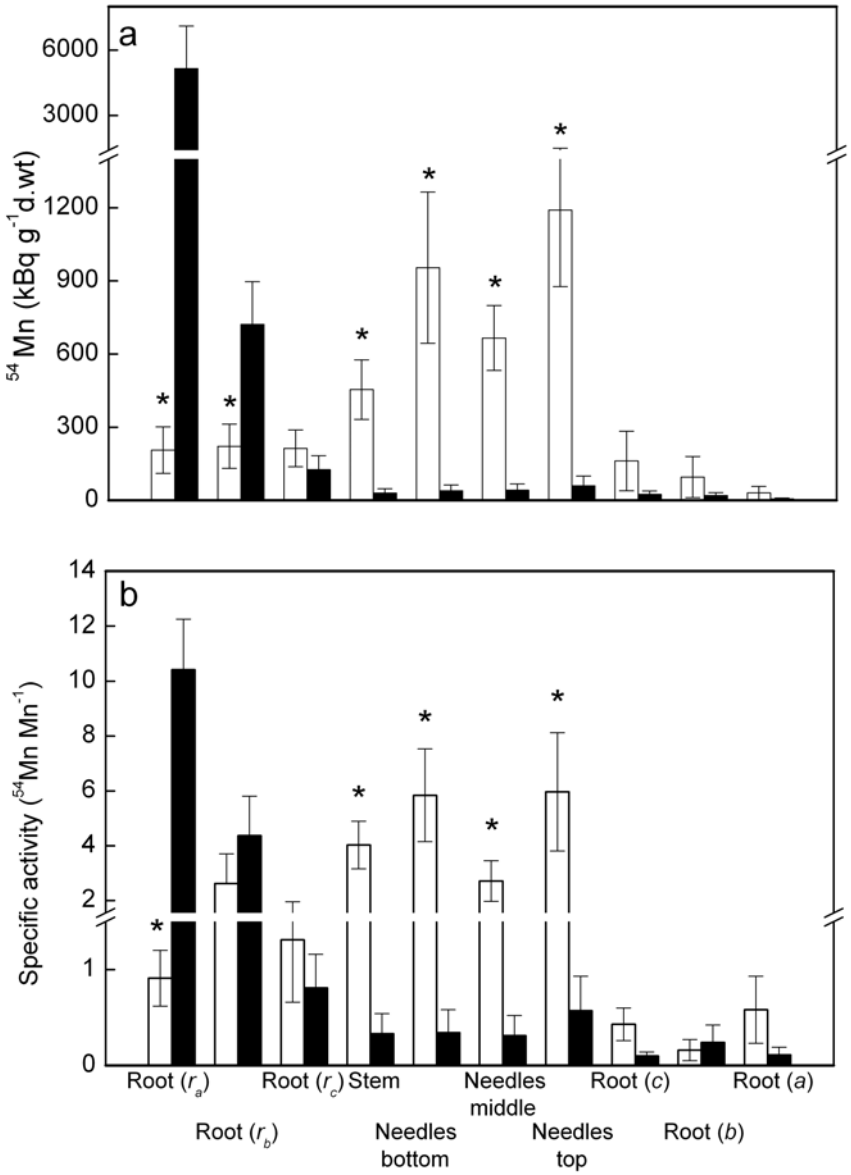

Figure 6. ^{54}Mn in different parts of seedlings of *Pseudotsuga menziesii* var. *viridis* (DFV, black bars) and var. *glauca* (DFG, white bars) after exposure to ^{54}Mn applied in a split root system. Bars indicate means on the basis of dry mass (a) and as specific activity on the basis of the Mn concentration of the same tissue (b). n=5-9 (±SE). * indicates $P \leq 0.05$. Denomination of tissues, see Fig. 2.

The analysis of root segments from different parts of the exposure chamber and of stem and young, middle and older needles confirmed the differences of the Mn-distribution in DFG and DFV found by autoradiography: DFV root segments from the labelled solution contained 25-times higher ^{54}Mn concentrations than those of DFG (Fig. 6a).

In DFV, ^{54}Mn decreased strongly towards the stem and was maintained at low, relatively even levels in stem and needles. In contrast to this behaviour, the concentration of ^{54}Mn was relatively stable throughout the part of the root system of DFG that had access to the labelled solution. In stem and needles of DFG a sharp increase of ^{54}Mn concentrations occurred resulting in 22-times higher activities and 14-times higher specific activities than in DFV. In roots spread across the non-labelled solutions a decrease in activity towards the root tip was found (Fig. 6a). DFV roots from the non-labelled chambers also contained ^{54}Mn, however, at lower concentrations than those of DFG.

Analysis of whole plant uptake of ^{54}Mn revealed that DFV acquired 14.7 % and DFG 41 % of the total radioactivity supplied with the nutrient solution. In DFV about 90 % of the activity taken up by the plants was retained in the root system. Only a small, less than 7 % fraction of radioactivity was allocated to stem and needles (Fig. 7).

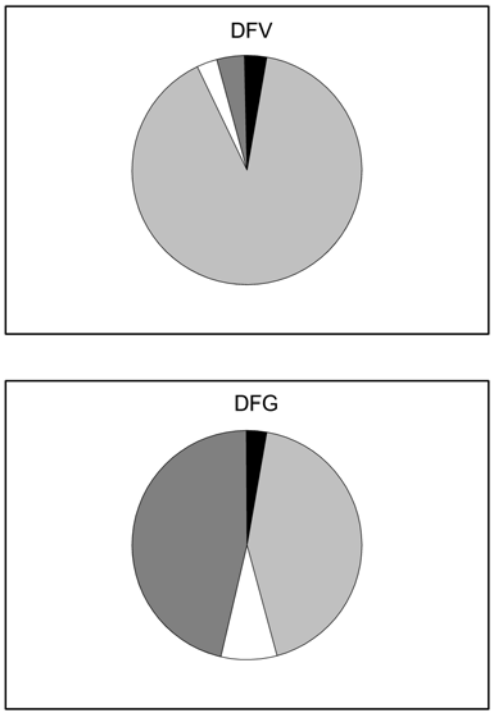

Figure 7. Allocation of radioactivity to different tissues of seedlings of *Pseudotsuga menziesii* var. *viridis* (DFV) and var. *glauca* (DFG) after exposure to ^{54}Mn applied in split root system. n=5-9. Pies indicate relative distribution of radioactivity in the following plant parts: roots from non-labelled solution (black), roots from labelled solution (grey), stem (white), and needles (hatched).

Under the same conditions, DFG retained about 43% of newly taken up Mn in roots and allocated about 54% to stems and needles. In both varieties fractions of about 3% of the total activity taken up by the plants were present in non-labelled root parts (Fig. 7).

At harvest, the nutrient solution in the non-labelled chamber D (Fig 2) contained 0.043% and 0.15% of the total radioactivity administered to DFV and DFG, respectively.

2.1. 5 Discussion

The most important result of this study is that uptake and translocation of Mn differed fundamentally in seedlings of DFG and DFV, the two main varieties of Douglas fir (Fig. 5, 6). Firstly, uptake rates were about 3-times higher in DFG than in DFV, despite similar plant size (Fig. 7, Table 1) and secondly, Mn was more readily transported to the shoot, in particular to the youngest needles in DFG than in DFV resulting in pronounced differences in Mn allocation patterns (Fig. 6, 7). To date, only few studies addressed Mn-uptake and translocation in tree species. Lin and co-workers (1995) applied ^{54}Mn and ^{65}Zn to the soil surface of balsam fir (*Abies balsamea*) seedlings and found the following ^{54}Mn distribution: 31% in roots, 31% in twigs, 26% in stems, and 12% in needles with a preferential allocation to younger needles. Our study on the two varieties of Douglas fir showed that whole plant allocation of the micro-nutrient Mn is a genetically determined trait, which differs not only between different species but also between closely related subspecies. Lin *et al*. (1995) also studied Zn translocation and observed that roots retained 86% of the total ^{65}Zn taken up indicating significant differences in the translocation of two the micro-nutrients Zn and Mn, respectively. Such differences in element mobility of different nutrients are known (Marschner, 1995). Our study shows that pronounced difference exist also for same element in different varieties of the same species.

Race-specific differences in Mn metabolism have mainly been investigated in agricultural crops in relation to Mn toxicity or tolerance (Horst, 1988; Rengel, 2000; Dučić and Polle, 2005). In the present study, the subject of Mn toxicity has not been addressed. However, the study was based on the observation that in some plantations DFG showed excessive Mn accumulation in needles as well as bark necrosis and slime

flow, leading to a decline of these stands (Schöne, 1992; Kaus and Wild, 1998). Similar symptoms have previously been observed in apple tree plantations and could by related to Mn-toxicity (Zeiger and Shelton, 1972). Since Douglas fir is an introduced species, planted because of its high productivity, the selection of suitable provenances is important. We have shown that the capacity for Mn uptake is higher in DFG than in DFV, probably also due to its more extended root system (Table 1). This may render DFG more prone to Mn-toxicity than DFV in acidic soils with high Mn availability. But it should be taken into account that under field conditions additional factors such as biotic interactions are important in controlling nutrient supply to the plant. Douglas fir forms symbioses with both arbuscular and ecto-mycorrhizal fungi (Cazares and Trappe, 1993; Parladé *et al.*, 1996), which modulate nutrient supply to the host. At least for agricultural plants, e.g., wheat and lettuce it has been shown that arbuscular mycorrhizal symbioses diminished Mn uptake (Azcon *et al.*, 2003; Ryan and Angus, 2003). To fully understand the consequences of the differences in Mn metabolism in the two Douglas fir varieties in different forest ecosystems, it will also be necessary to study the influence of symbiotic interactions on micro-nutrient supply.

Apart from additional effects of symbiotic fungi on Mn supply to the plant, uptake and transport of nutrient elements occur in the following steps: first from the rhizosphere into the root, then inside the root cortex towards the endodermis using apoplastic or symplastic pathways, loading into xylem, transport with the transpiration stream to the leaves, and re-circulation via the phloem. An intriguing question is, therefore, which of these steps was regulated in different ways in DFG and DFV. The steep gradient of radioactivity persisting in roots of DFV (Fig. 6) even after an extended feeding period together with lower total plant ^{54}Mn accumulation indicate that uptake into the roots is more restricted in DFV than in DFG and that Mn once taken up is less mobile in DFV than in DFG. Analysis of Mn at the subcellular level of DFV roots supported this view since only epidermal walls but not cortical, endodermal or xylem walls contained elevated Mn concentrations (Fig. 3). In contrast to DFV, high Mn concentrations in cortical walls as well as in the vacuoles of root cells indicated higher accessibility of Mn into roots of DFG.

In both varieties the endodermis appears to have important barrier functions since Mn transport to needles displayed a pronounced delay of almost 2 weeks. Assuming that Mn is freely mobile in the xylem as in other species (Rengel, 2000) and considering

the substantial consumption of nutrient solution during the experiment (Table 2), the delayed appearance of Mn in needles can only be explained by restricted xylem loading. Anatomical microanalysis indicated higher Mn concentrations in endodermal and parenchyma cell walls of DFV needles than in those of roots ($P \leq 0.04$, Fig. 3). This could be due to the fact that Mn once transported into the needles can hardly be remobilised and, thus, accumulated mainly in cell walls but also in vacuoles as shown here (Fig. 3). In mature conifers grown on calcareous soils with low Mn availability, Mn-translocation also appears to be a slow process. On these sites, young needles displayed severe symptoms of Mn-deficiency only in the first year after emergence. These symptoms disappeared after gradual accumulation of sufficient Mn over a time course of 2 to 3 years (Kreutzer, 1972; Polle *et al*., 1992).

A puzzling question is how DFG avoided overaccumulation of Mn and maintained its needle concentrations not excessively higher than those of DFV (Table 1), despite much higher uptake rates. Since DFG produced less above-ground biomass than DFV, "dilution" by growth was not the reason. Another possibility is re-circulation via the phloem. It is still a matter of discussion to which extent Mn can be re-circulated in plants. In an earlier publication (Epstein, 1971) suggested that Mn is mobile in the phloem but that the degree of mobility varies with plant species. For example, Mn added by spraying directly to oat shoots resulted in translocation to roots over an extended period of time (Boken, 1960). In other studies also employing agricultural crops, it was concluded that the movement of Mn in the phloem occurs only in physiologically insignificant amounts (Mengel and Kirkby, 1982; Nable and Loneragan, 1984a,b; Loneragan, 1988). Pearson and Rengel (1995) analysed Mn and Zn distribution during grain development of wheat and observed a pattern suggesting that Mn was supplied via the xylem and Zn via the phloem. More recently, Rengel (2000) concluded that manganese mobility in the phloem sap depends on the type and age of the plant part. Our result that roots of Douglas fir seedling in the non-labelled nutrient compartments contained low, but significant radioactivity indicates that phloem transport must have occurred but at slow rates (Fig. 5,6). Whether this transport is circulation in the strict sense of leaf-to-root transport (as defined by Marschner, *et al.* 1997), is not clear since radial exchange of Mn between xylem and phloem in the stem can not be excluded. The presence of radioactivity in compartment D, to which only non-labelled nutrient solution was added and which contained the root tips of the split

root system was small. Unspecific spillover cannot be completely excluded. However, this would have been expected to result in similar degrees of contamination for both varieties. Higher ^{54}Mn levels in DFG root tips in the non-labelled compartment than in those of DFV suggest that the radioactivity was at least to some extent due to transport towards the root tips and exudation or root leaching. If circulation took place at similar rates as root-to-shoot transport, the time course of the present experiment would have been too short to give already conclusive data. Recently, Krüger *et al*. (2002) identified a protein in the phloem of castor bean, which bound preferentially Fe but also Mn and was involved in Fe-transport in vivo. Since our data reveal high concentrations of Mn in the sieve cells (Fig. 3), it is tempting to speculate that Mn can be transported from the shoot back to the root employing specific proteins.

Although the major topics of this study were Mn-uptake and transport, it is also notable that both varieties of Douglas fir showed distinct differences in growth and biomass partitioning (Table 1). A yet unresolved question is whether the traits "root/shoot-ratio", respective "biomass" and "Mn-uptake" are independent or linked. The latter option is not unlikely, since root elongation, side root formation are regulated via auxin metabolism involving manganese stimulated auxin oxidases (Morgan *et al.*, 1986). Recently, *Arabidopsis* mutants of the metal transporter ilr2-1 have been identified, which constitute a link between auxin metabolism and Mn-homeostasis (Magidin *et al.*, 2003). Whether a modification of a single micro-nutrient transporter gene can have such profound effects on biomass production and partitioning as found in the two Douglas fir varieties is currently very speculative but deserves further attention as a potential functional marker for breeding purposes. Genetic structures at the marker gene locus 6-PGDH-A are very unlikely to be functionally related to the physiological differences discussed above, but prove strong differentiation between the two varieties at the level of single genes.

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Tanja Dučić, Ludger Leinemann, Reiner Finkeldey Andrea Polle· Uptake and Translocation of Manganese in Seedlings of Two Varieties of Douglas Fir (*Pseudotsuga menziesii* var. *viridis* and *glauca*).

CHAPTER 3

3.1. Manganese toxicity in seedlings of two varieties of Douglas fir (*Pseudotsuga menziesii* var. *viridis* and *glauca*) seedlings and phosphorus effect

3.1.1 Abstract

Manganese is an essential micro-nutrient in all organisms but may become toxic when present in excess. In an earlier investigation we found that Douglas fir (*Pseudotsuga menziesii*) variety *glauca* (DFG) and variety *viridis* (DFV) differed in manganese uptake, subcellular localisation, transport and allocation (Dučić et al., 2006). To address seedling performance under conditions of Mn toxicity, we determined biomass, Mn concentrations in different tissues and Mn subcellular localisation.

To investigate role of P, exposure of Douglas fir to excess Mn, was also conducted under P-deficiency. In DFG, but not in DFV, the P concentration was kept at a constant level even under P deficiency. P-limited DFV seedlings were less Mn-susceptible. In DFG root growth was partly reduced under P-limitations, probably due to increased Ca- uptake under these conditions.

Shoot biomass was strongly diminished in DFV than in DFG, whereas root biomass decreased more strongly in DFG, under Mn excess. X-ray microanalysis showed Mn accumulation in epidermal and cortical cells of both varieties after Mn treatment, suggesting the root endodermis was a barrier for Mn to protect the vascular system and shoot from high Mn. In these tissues precipitates were observed with extremely high Mn concentrations, which correlated with P and Ca.

3.1.2 Introduction

Mn is an essential nutrient element, required for enzymatic activities in each subcellular compartment, e.g. for carboxylases and phospatases in the cytosol, for glycosyl tranferases in the Golgi apparatus, for MnSOD in mitochondria and for chloroplastic proteins, forming a cluster of Mn atoms required as the catalytic centre for light-induced water oxidation in photosystem II (Marschner, 1995). Mn is, however, only necessary at low concentrations, around 20 μg per gram dry mass (Marschner, 1995), though its tissue concentrations may vary. Excessive concentrations of Mn in plant tissues can alter the activity of enzymes (Horst, 1988), interfer with the absorption, translocation, and utilisation of other mineral elements such as Ca, Mg, Fe, and P (Clark, 1982), and cause oxidative stress (Dučić and Polle, 2005). Soil acidification (pH < 5.5) enhances the solubility of Mn increasing its bioavailability (Marshner 1986). After aluminium, Mn is toxicity is probably the most important growth limiting factor in soils with low pH (Foy, 1984).

Manganese toxicity is a major factor limiting crop growth on acidic, poorly drained or steam-sterilized mineral soils. In herbaceous plants Mn tends to accumulate in shoots rather than roots leading to visible injury in leaves (Loneragan, 1988). The symptoms of Mn toxicity vary among plant species and between varieties within one species. In general, excess Mn causes chlorosis and necrosis, appearance of brown, necrotic spots or small reddish purple spots (Horst, 1988). Necrotic brown spots are the result of accumulation of oxidized Mn (Horiguchi, 1987) or due to precipitated Mn compounds, e.g. with phenolics (Horst, 1988, Wissemeier and Horst, 1992, Fecht-Christoffers et al. 2003). To date most studies on Mn-toxicity have been conducted in crops plants such spinach, pea, bean, and various cereals (Horst, 1983, 1988; Graham, 1988; Foy et al., 1988, Rout et al., 2001). Much less is known about Mn-toxicity in forest trees

White birch *Betula platyphylla japonica*) developed chlorotic leaves when grown in hydroponic culture at 50 mg Mn/l and showed brown speckles in the marginal and interveinal area of leaves at >1 mg Mn/l (Kitao et al., 2001). Excess Mn in soil caused accumulation in leaves of broad-leaf deciduous trees resulting in diminished photosynthetic rates (Kitao et al., 1997). In Douglas fir (*Pseudotsuga menziesii* (Mirbel) Franco) variety-specific differences in Mn-uptake exist (Dučić et al., 2006). In their natural habitats in North America, the needles of the coastal genotype, *Pseudotsuga menziesii* variety *viridis* (DVF) contained 100 – 800 and of those the interior type, variety *glauca* (DFG) 200 - 2000 μg Mn g^{-1} dry mass (Baronius und Fiedler 1996; Zasoski et al. 1990). In Germany, decline of Douglas fir in some stands in Rhineland-Palatinate was related to Mn

overaccumulation and was observing particular in trees belonging to the interior race (DFG), whereas healthy stands were formed by the coastal race (DFV) (Leinemann, 1996).

Since Douglas fir showed evidence for variety-dependent differences in Mn-tolerance, we supposed that DFG and DFV may differ in uptake and/or plant-internal distribution of Mn in the presence of Mn sufficient. Under normal conditions DFV retained Mn predominantely in roots, whereas DFG had a higher capacity to translocate Mn to leaves (Dučić at al., 2006). It is, therefore, tempting to speculate that these variety-specific differences will result in higher Mn-accumulation rates in DFG than DFV under Mn-stress. It is also possible that excessive Mn concentrations in plant tissues can modify absorption, translocation and utilisation of other mineral elements, such as P, Ca, Si, thereby causing injury (Le Mare, 1977; Clark, 1982). The present study aims at distinguishing between these possibilities. DFG and DFV were exposed to excess Mn in the present or absence of sufficient P- supply, and biomass, Mn-allocation, subcellular localisation and influence on other nutrients was studied.

3.1.3 Material and methods

3.1.3.1 Plant material and growth conditions

Seeds of *Pseudotsuga menziesii* DFV and DFG were purchased from Niedersachsen Forstamt (Oerrel, Munster- Oerrel, Germany) and Sheffield's Seed Company (Locke, New York, USA), respectively. The genotype origins of the two seed lots were confirmed by analysis of genetic structures (Dučić et al., 2006).

Seeds of both varieties of Douglas fir were soaked in tap water for 7 days at 2°C and surface sterilised in 96% ethanol for 30s, in 0.2% $HgCl_2$ for 30s, and in 30% H_2O_2 for 45 min. Subsequently, the seeds were placed on sterile 1.5% (w/v) water-agar, pH 4.5 in Petri-dishes (d=14 cm), maintained for 7 days in darkness at 21°C and subsequently for 3 weeks with a day/night regime of 16h/8h (white light of 150 µmol m^{-1} s^{-1} photosynthetic photon flux, OSRAM L 18-W/21-840 (Lumlux Pluseco, Germany) at 23°C/21°C air temperature. After germination, plants were transferred to hydroponic solutions. Aerated nutrient solution contained the following nutrient elements: 1.4 mM NH_4NO_3, 0.130 mM $CaSO_4$, 0.100 mM K_2SO_4, 0.160 mM $MgSO_4$, 0.35 mM $(NH_4)_2HPO_4$, 0.7 mM KCl, 0.030 mM KH_2PO_4, 5 µM $MnSO_4$, 10 µM Fe-EDTA; and micronutrients: 5 µM H_3BO_3, 0.03 µM $NaMoO_4$, 0.24 µM $CuSO_4$, 0.23 µM $ZnSO_4$ and 0.05 µM $NiSO_4$. The pH was adjusted to 5. The solution was changed every 3 days. After 2 weeks of

acclimation, the seedlings were treated with different concentrations of $MnSO_4$ up to 10 mM. After 2 weeks plants were sampled for analyses. In experiments with different phosphorus concentrations, plants were grown on 0.38 mM P (control) or 0.038 mM P for one week and afterwards exposed for two weeks to 5 µM Mn (control) or 5 mM Mn.

3.1.3.2 Root Length Measurements

Before Mn exposure started, roots were marked 5 mm behind the tip with water-resistant ink. Root lengths were measured daily under a binocular (Stemi SV 8, Zeiss, Oberkochen, Germany) with a measuring ocular (Zeiss CPL 10-fold). Measurements were performed on 15 plants per treatment and date.

3.1.3.3Element analyses

Samples for element analyses (root, stem and needles) were dried to a constant weight and ashed at 170°C in 65 % HNO_3 for 12h (Feldmann, 1974). Elements were determined by inductively coupled plasma- atomic emission spectroscopy (Spectro Analytical Instruments, Kleve, Germany) according to Heinrich et al. (1986).

3.1.3.4 Subcellular element localisation by electron dispersive X-ray microanalysis by transmission electron microscopy (EDX-TEM)

Needles and roots (1.5 cm from the root tip) were cut in several 2 mm and 5 mm long pieces, respectively, and rapidly frozen in a mixture of propane:isopentane (2:1) cooled with liquid nitrogen to –196°C in a aluminium mesh. Samples were freeze-dried at –45°C for three days and stored at room temperature in a desiccator over silica gel. For transmission electron microscopy, freeze-dried samples were infiltrated with ether in a vacuum-pressure chamber and embedded in styrene-metacrylate (Fritz, 1989). 1 µm thick cross-sections were cut dry by glass knives, mounted on adhesive–coated 100-mesh hexagonal grits, coated with carbon, and stored over silica gel. Details and testing of the method has been reported previously (Fritz, 1989; Fritz and Jentschke, 1994). The samples were analysed with a Philips EM 420 with the energy dispersive system EDAX DX-4 (EDAX Inc., Mahwah, NJ, USA). The accelerating voltage was 120kV, the take off angle 25° and counting time 60 live seconds. Element concentrations were analysed in cell walls and vacuoles

of the following tissues: epidermis, needle mesophyll, cortex of roots, endodermis, xylem, and phloem. Nine replicates were analysed in each compartments in three different plants.

3.1.3.5 Statistical analyses

Data are means (± SD) of 5 to 15 seedlings were treated as replicates in three separated growing systems in three experiments. Statistical analyses of the data were performed using analysis of variance (ANOVA test), (LSD, Statgraphics 2.1; StatPoint, Inc., St Louis, MO, USA). Differences at $P \leq 0.05$ were considered as significant.

3.1.4 Results

3.1.4.1 Growth performance, biomass and Mn partitioning in Douglas fir var. **viridis** *and* **glauca** *in the presence of increasing Mn concentrations*

To investigate Mn-sensitivity, Douglas fir seedlings were grown in hydroponic media supplemented with increasing Mn concentrations for two weeks. Root length was measured regularly (see appendix) and used to calculated growth (Fig. 1). Growth rates declined with increasing Mn concentration more strongly in DFG than in DFV, confirming that DFV is more tolerant to Mn than DFG (Fig.1). It was surprising that intermediate Mn concentrations stimulated root growth (Fig. 1). This was, however not caused by insufficient supply with Mn in controls since total plant biomass was not stimulated under these conditions (Fig. 2). These intermediate Mn treatments resulted in roots that were thinner and finer than those of control plants (data not shown).

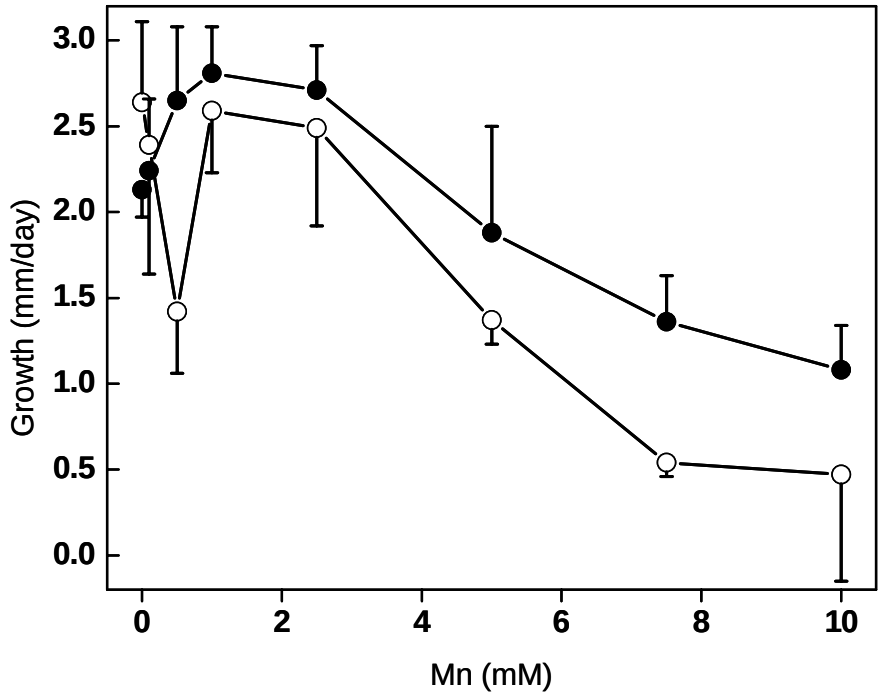

Figure 1. Root growth (mm/day) of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (DFV, closed circles) and variety *glauca* (DFG, open circles) in response to increasing Mn concentrations in the nutrient solution. Root length were determined 3-times a week. Data collected during the second week of exposure were used to determinate growth rates. n= 15 (±SD).

Enhancing Mn in the nutrient solution had stronger effects on the biomass of needles in DFV (-47%) than in DFG (-27%), while the inhibitory effect on roots was more pronounced in DFG (-44%) than DFV (-27%) (Fig. 2a).

Decreases in biomass (Fig. 2a) corresponded to increases in total plant Mn content (Fig. 2a). Exposure to 2.5mM Mn led to 30- and 37-fold increases in DFV and DFG, respectively (Fig. 2b). This Mn content was not further increased in shoots of plants grown with 5mM Mn, whereas further accumulation in root was found, i.e. 82- and 46-fold in DFV and DFG, respectively (Fig. 2b).

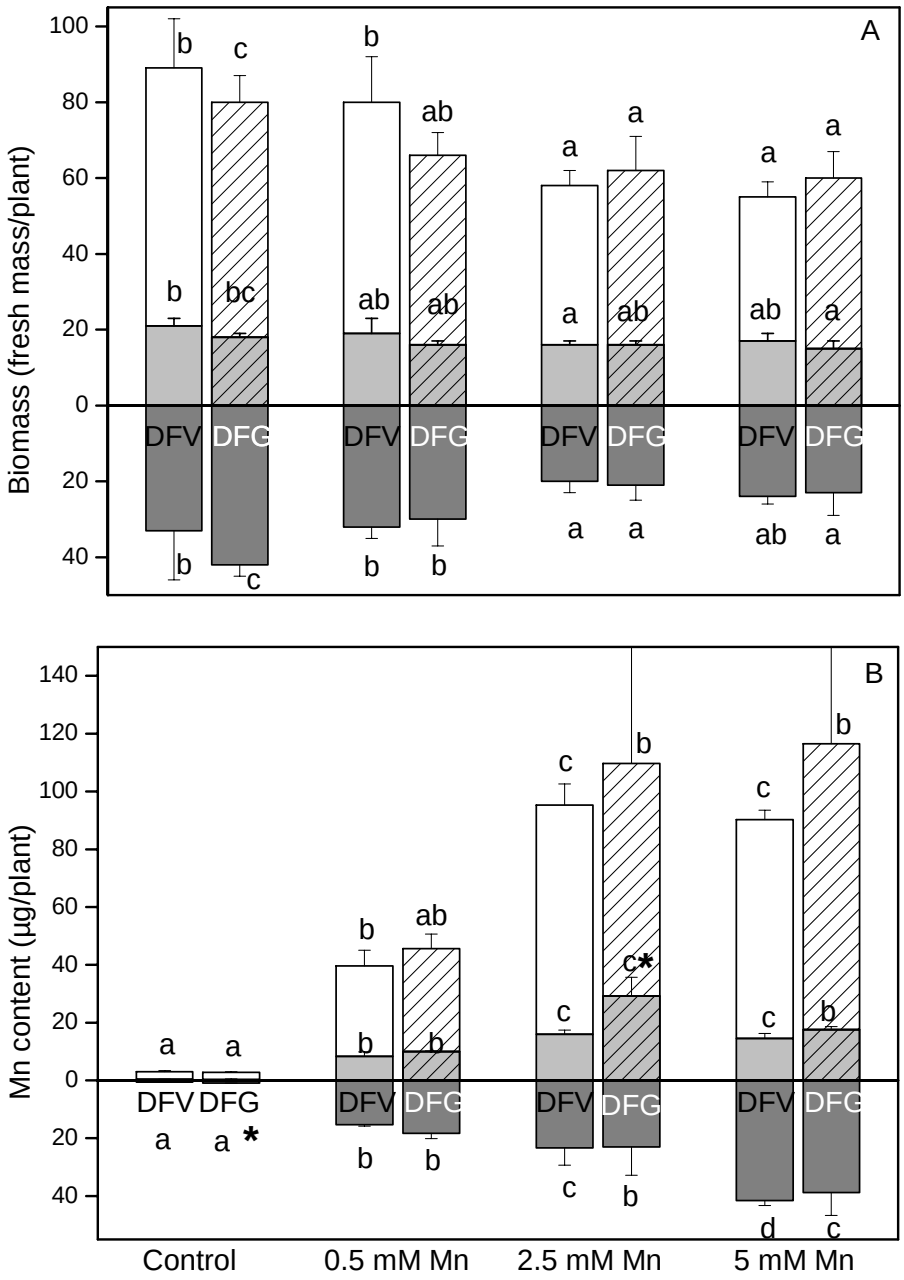

Figure 2. Biomass (mg fresh mass/plant) (A) and Mn content in plants (µg/plant) (B) of Douglas fir variety *viridis* and variety *glauca* exposed to increasing Mn concentrations in the nutrient solution. The bars indicate values for needles (white), stem (hatched) and root (grey), respectively after 2 weeks of exposure (±SD). Different letters indicate significant differences at $P \leq 0.05$ for needles, stems and roots separately. * indicates significant differences between DFV and DFG when $P \leq 0.05$.

Not only plant content, but also tissue concentrations increased with increasing Mn, especially in DFV roots (Table 1). This suggests that DFV may be able to tolerate intrinsically higher Mn concentrations than DFG.

Table 1. Concentrations of Mn (mg/g DW) in the needles, stems and roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after exposure to different Mn concentrations. n=3 (±SD).

Mn (mM) treatment	mg/gDW	viridis Mean	Mn SD	glauca Mean	SD	P-value
0.005	Needles	0.10 a	0.02	0.06 a	0.00	* 0.079
	Stem	0.04 a	0.00	0.03 a	0.02	0.605
	Root	0.06 a	0.01	0.07 a	0.01	0.124
0.5	Needles	1.09 b	0.20	1.21 ab	0.19	0.552
	Stem	1.09 b	0.12	1.28 b	0.13	0.193
	Root	1.45 a	0.05	1.38 b	0.06	0.315
2.5	Needles	3.61 c	0.02	2.32 bc	1.82	0.372
	Stem	2.24 c	0.12	3.26 c	0.72	0.118
	Root	3.39 b	0.98	2.44 c	0.74	0.334
5	Needles	3.78 c	0.34	3.43 c	0.90	0.63
	Stem	2.16 c	0.22	2.72 c	0.40	0.222
	Root	5.53 c	1.34	3.53 d	0.23	0.106

Since excess Mn has been reported to affect uptake of other nutrient elements (Le Mare, 1977; Clark, 1982), the concentrations of other macro- and microelements were also measured (appendix, Table 3). Increasing supply with Mn caused increases in phosphorus concentrations in needles ($P \leq 0.05$), but not in other tissues.

The concentrations of most analysed elements also increased in response to increasing Mn uptake, an exception was Mg, whose concentrations decreased in roots between 20 and 35% (appendix, Table 3).

3.1.4.2 Subcellular localisation of Mn

To investigate whether the ability of DFV to tolerate higher intrinsic Mn concentrations in roots was related to Mn detoxification in the vacuole or cell wall, the subcellular localisation of Mn was analysed by EDX-TEM. Specimen of root tips and of needles from control seedlings were compared with those of plants treated with 5mM Mn (Fig. 3, 4).

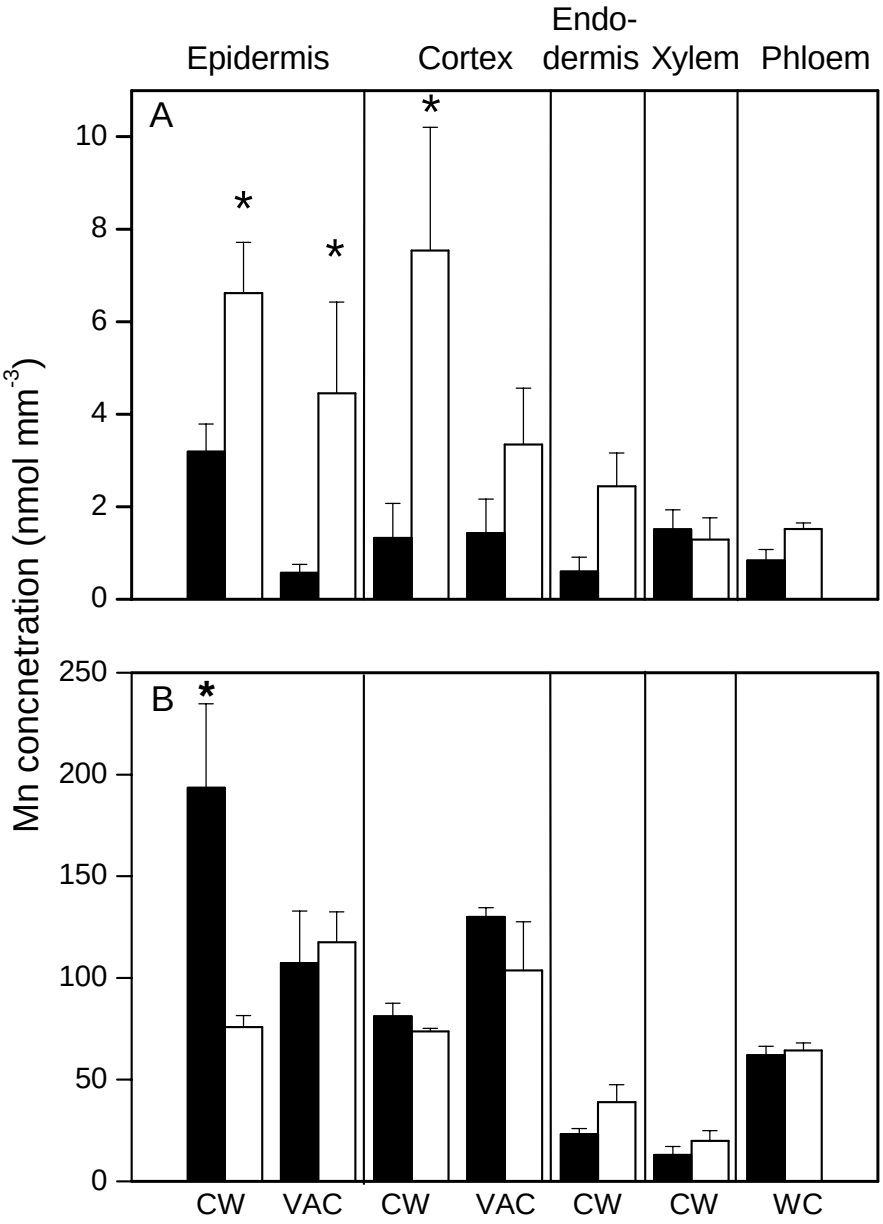

Figure 3. Subcellular localisation of Mn in different cell types of roots in control (A) and Mn-treated (B) seedlings of *Pseudotsuga menziesii* variety *viridis* (DFV- closed bars) and variety *glauca* (DFG- open bars). Cross-sections were analysed by EDX-TEM. The concentrations obtained by EDX refer to volume units of the embedded specimen. CW- cell wall, VAC- vacuole, WC- whole cell. Bars indicate means of n=6-9 (±SE). * indicates significant differences between DFV and DFG when $P \leq 0.05$.

In control plants the highest Mn concentrations in roots were observed in walls of the epidermal and cortex cells, especially in DFG (Fig. 3a). The vacuole of epidermis cells of DFG contained nearly 8-times higher Mn concentrations than those of DFV (Fig. 3a). The relative enrichment of Mn in control plants in DFG compared with DFV decreased towards the endodermal

barrier and was absent in the vascular system (Fig. 3a). In DFV maximum Mn concentrations were found in epidermal cell walls. In all other locations analysed in DFV root cross sections, Mn was present at low concentrations displaying no obvious differences between cells types and subcellular compartments (Fig. 3a).

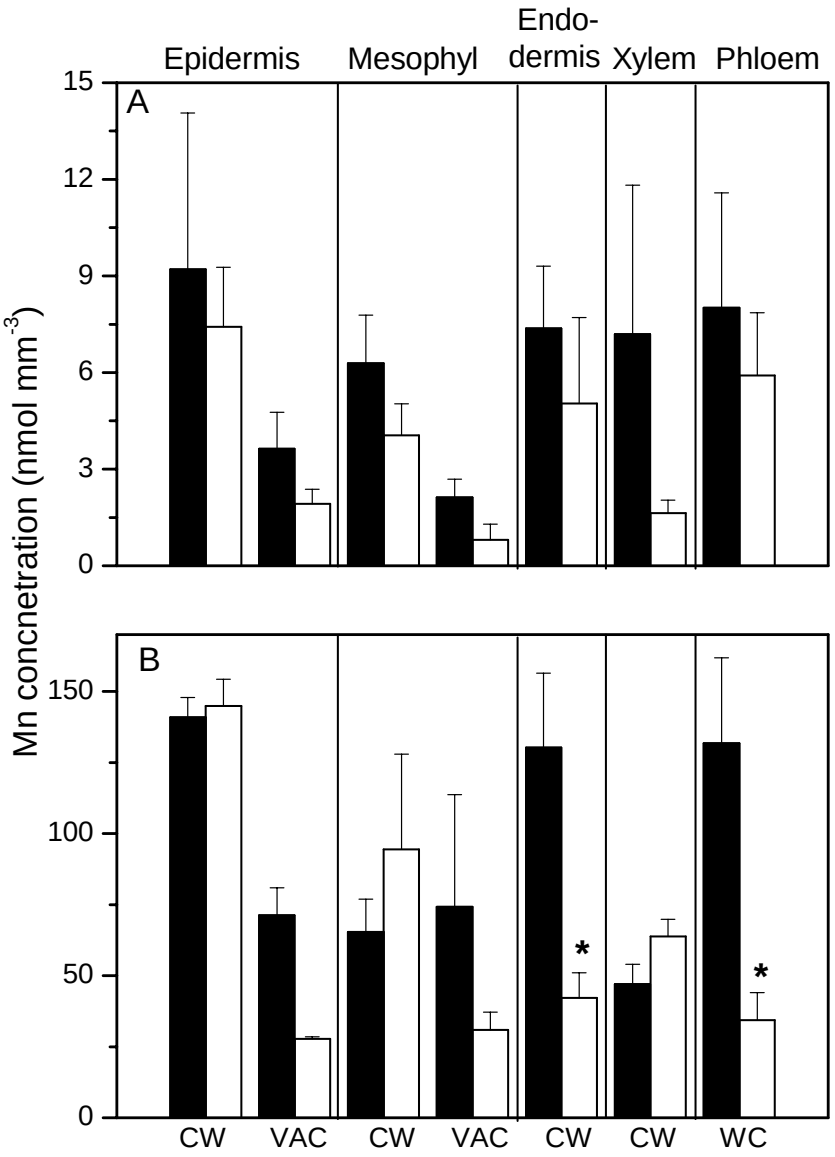

Figure 4. Subcellular localisation of Mn in different cell types of needles in control (A) and Mn-treated (B) seedlings of *Pseudotsuga menziesii* variety *viridis* (DFV- closed bars) and variety *glauca* (DFG- open bars). Cross-sections were analysed by EDX-TEM. The concentrations obtained by EDX refer to volume units of the embedded specimen. CW- cell wall, VAC- vacuole, WC- whole cell. Bars indicate means of n=6-9 (±SE). * indicates significant differences between DFV and DFG when $P \leq 0.05$.

In plants exposed to Mn stress it is evident that the Mn distribution changed throughout cross-sections of both varieties (Fig. 3b). All subcellular locations showed drastic increases in Mn

ranging from 10- to 180-fold. The highest Mn concentration was present in epidermis cell walls of DFV (Fig. 3b). The endodermis constituted an efficient barrier since Mn concentrations of 100 nmol·mm^{-3} Mn dropped to around20 nmol·mm^{-3} in the endodermis cells (Fig. 3b).

Excess of Mn also influenced the concentrations of other elements such as P, Ca, Mg, Si, S, K, Ca, Fe, and Zn in cell walls and vacuoles of roots (see appendix Table 5). In general increases in Si and S and decrease in Ca, Fe and Zn were found. P, Mg and K did not show consistent changes in the compartments analysed (appendix Table 5).

In contrast to roots, in needles of control plants, significant subspecies-related differences in the localisation of Mn were not observed (Fig. 4a). We found relatively high Mn concentrations in the phloem of both varieties (Fig. 4a). This observation suggests that Mn must be either immobilised by unknown mechanisms in sieve cells or that Mn can be transported and circulated in Douglas fir varieties as suggested Dučić et al. (2006).

In the presence of excess Mn concentration all compartments analysed displayed significantly higher Mn concentrations than those of control plants (Fig. 4b). The highest Mn accumulation occurred in the cell walls of the epidermis (Fig. 4b). The vascular system of both varieties showed accumulation of Mn, but the phloem of DFV contained significantly higher Mn concentration than those of DFG. It is important to note that the relative enrichment in Mn was higher in DFG than in DFV, ranging from 7 to 117-fold increases, whereas the latter showed only 6 to 19-fold increase. This is in contrast to roots, where the enrichment factors were 10 to30-fold and 15 to 186-fold in DFG and DFV, respectively. This points out differences in the higher capacity of DFV to retain Mn in the root system in comparison with DFG.

In addition to its localisation in specific subcellular compartments deposition of Mn was observed in black granules in the root epidermis and cortex of both genotypes (Fig. 5). These dark depositions were clearly detectable by electron microscopy and localised in the vacuole and in the cell wall (Fig. 5). The area of the “black bodies” of DFV was about 2.3-fold higher than these of DFG (Table 2). DFG contained 8-times more granules in DFV than in DFG. Although the Mn concentration in the granules was lower in DFV than in DFG, the total amount of Mn deposited by this way was about 3-times higher in cells of DFV that of DFG (Table 1). Precipitation of Mn in these granules took place take place in the epidermis and cortex of roots of both varieties of Douglas fir. The black bodies were not found in roots of control plants but occasionally in needles after Mn treatment. In needles, the “black bodies” however, did not contain prominent Mn concentrations (201 ± 25 and 72 ± 22 nmolmm^{-3} in DFG and DFV, respectively).

Table 2. Characteristics of Mn- granules in roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after expose to 5mM Mn. * indicates significant differences between DFV and DFG when $P \leq 0.05$.

"Black granules"	*viridis*		*glauca*		*P*-value
	Mean	SE	Mean	SE	
Area per Spot (μm^2)	0.084	0.027	0.037	0.005	0.137
Mn concentration per spot (relative unit)	715.5	176.6	1777.7	323.5	* 0.004
Spots number / cell	21.6	11.00	2.8	1.13	* 0.0002

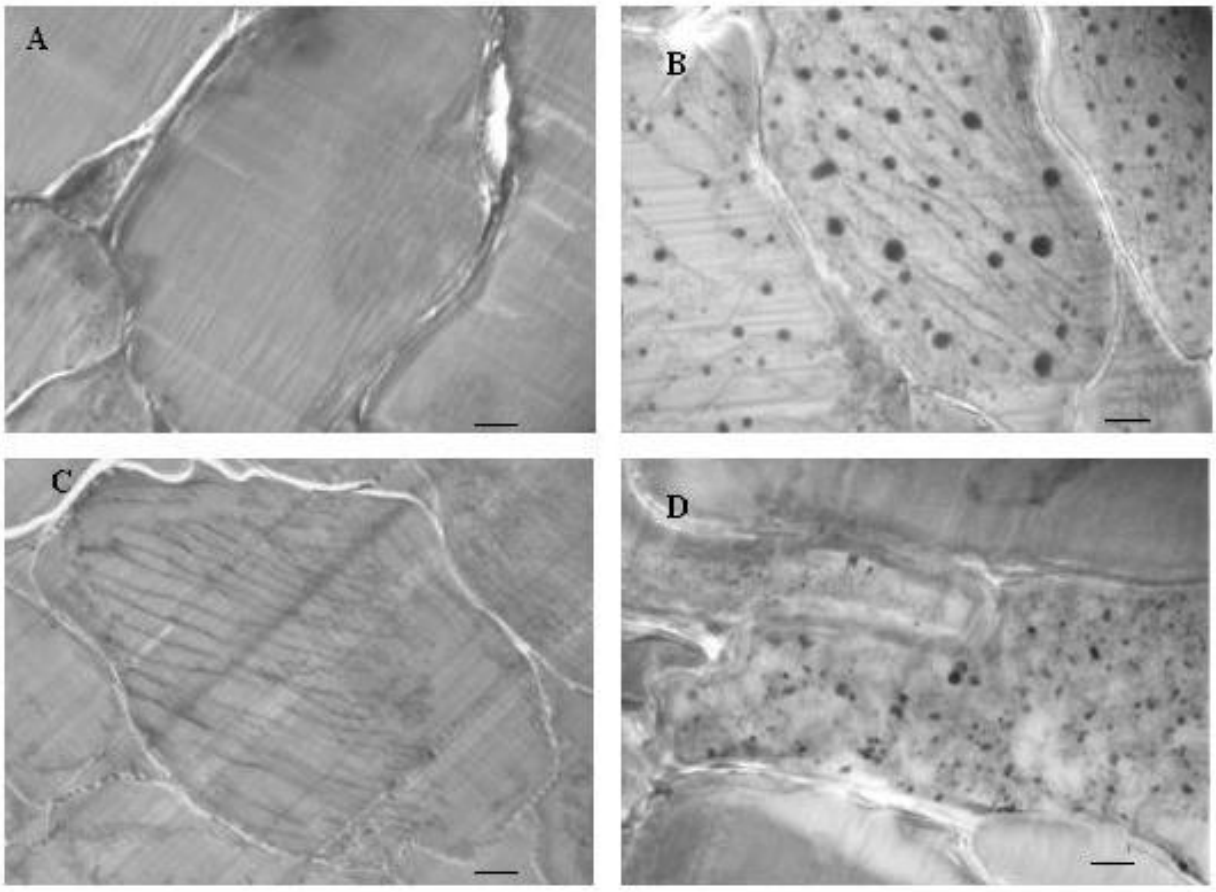

Figure 5. Transmission electron microscopy of cortex cells of controls and of Mn-treated seedlings of *Pseudotsuga menziesii* variety *viridis* (A, B, respectively) and variety *glauca* (C, D, respectively). Bar indicate 2µm.

Further EDX- analyses of the Mn-deposits in roots indicated the presence of other elements, especially of P and Ca. P constituted always a major fraction of ca. 85%. Correlation analysis

revealed showed a linear relationship of Ma with sum of P and Ca. In DFG 1 mol of Mn was deposited with 0.64 mol Ca+P and in DFV with 1 mol Ca+P (Fig. 7). These observations suggest that high Mn concentrations can be detoxified by precipitation together with P as the main component.

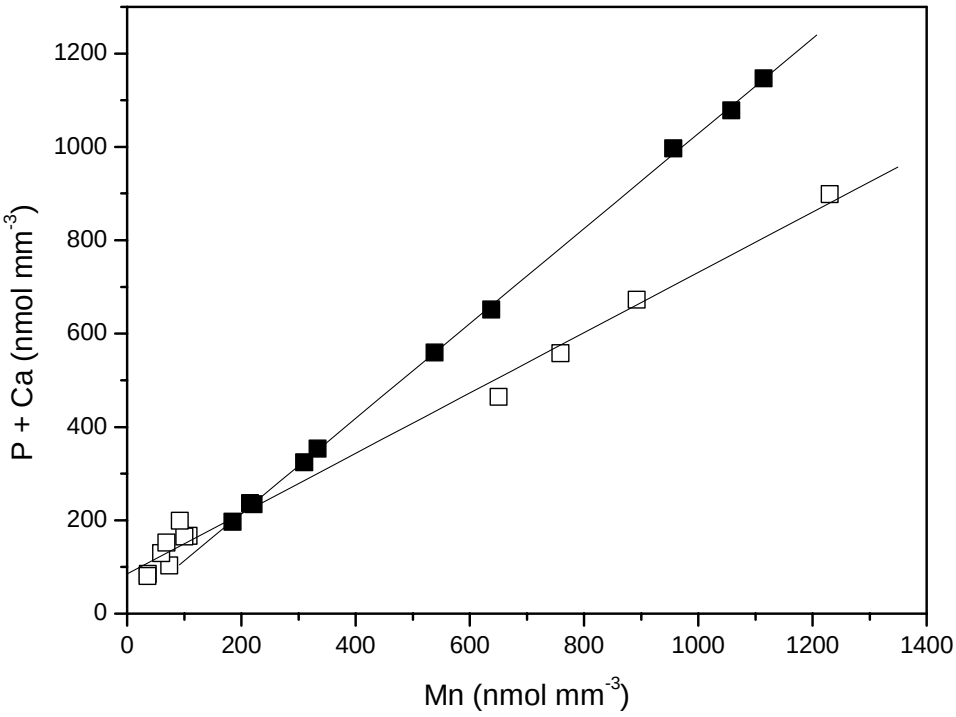

Figure 6. Correlation between Mn and P + Ca in granules found in roots of Mn-treated seedlings of *Pseudotsuga menziesii* variety *viridis* (DFV, closed squares) and variety *glauca* (DFG, open squares). Regression equations for DFV: y=11.3+1.0·x; R=0.9998, P<0.0001 and for DFG: y=85.2+0.6·x; R=0.9948, P<0.0001.

3.1.4.3 Phosphorus affects Mn toxicity

To test the influence of P nutrition on Mn accumulation and tolerance, both varieties of Douglas fir were subjected to mild P-deficiencng before exposure to Mn-stress. For this purpose plants were pregrown for 7 days in 0.038 mM P and afterwards exposed to 5 mM Mn for two weeks.

The 10-fold reduction of P concentration compared to control conditions caused reductions in root growth in both races of about 20 to 25% (Fig. 7). Mn exposure under normal P-nutrition

caused about 30% reduction in growth, but the combination of both, low P and high Mn, caused a recovery of growth in DFG and had not a additional negative effect in DFV (Fig. 3).

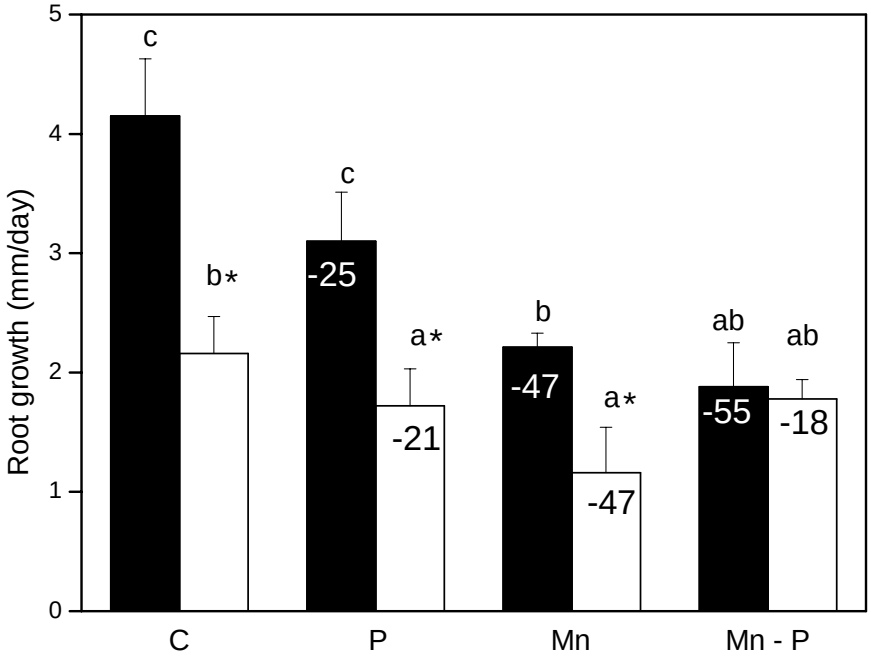

Figure7. Root growth (mm/day) of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (DFV, closed bars) and variety *glauca* (DFG, open bars) in response to 5mM Mn concentrations and 0.38 mM and 0.038 mM P concentrations in the nutrient solution. Root length were determined 3-times a week. Data collected during the second week of exposure were used to determinate growth rates. C-control plants, P-treatment with 0.038 mM P, Mn-treatment with 5 mM Mn, Mn-P - treatment with 5 mM Mn and 0.038 mM P. n= 15 (±SD). * indicates significant differences between DFV and DFG when $P \leq 0.05$.

The low concentration of P in the medium had negative effects on biomass of roots and needles in both varieties (Fig. 8). Normal concentrations of P and exposure to excess Mn had the strongest negative effect on the biomass of both varieties of Douglas fir. In the presence of diminished P and excess Mn plants showed lower decreases in biomass, especially in root biomass than those exposed to excess Mn in presence of sufficient P.

The P concentrations in different tissues of DFG were unaffected by the P concentration in the nutrient solution (Table 3). In contrast to DFG, reductions in P-supply caused diminished P concentrations in most tissues of DFV. Decreased supply with P caused higher accumulation of Ca in roots of DFG, but not in those of DFV (appendix Table 6).

Table 3. Concentrations of Mn and P (mg/g DW) in the needles, stems and roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after grown on 0.38 mM P (control) or 0.038 mM P (-P) for one week and afterwards exposed for two weeks to 5 µM Mn (control) or 5 mM Mn (+Mn). * indicates significant differences between DFV and DFG when $P \leq 0.05$.

		viridis		***glauca***		
Mn	**mg/g DW**					
		Mean	SD	Mean	SD	*P*-value
Control	Needles	0.13 a	0.02	0.10 a	0.02	0.158
	Stem	0.05 a	0.02	0.06 a	0.01	0.567
	Root	0.05 a	0.02	0.06 a	0.02	0.733
+ Mn	Needles	3.47 b	0.48	2.94 b	0.18	0.224
	Stem	3.51 b	1.33	3.83 b	1.55	0.836
	Root	4.61 b	0.58	3.71 b	0.49	0.171
- P	Needles	0.10 a	0.01	0.07 a	0.02	0.147
	Stem	0.04 a	0.01	0.05 a	0.01	0.205
	Root	0.04 a	0	0.06 a	0.01	* 0.020
- P + Mn	Needles	4.11 b	0.33	3.64 b	0.73	0.452
	Stem	2.84 b	0.46	3.45 b	0.47	0.264
	Root	5.36 b	0.46	4.87 c	0.77	0.481
		viridis		***glauca***		
P	**mg/g DW**					
		Mean	SD	Mean	SD	*P*-value
Control	Needles	7.26 b	1.35	6.69 a	0.3	0.592
	Stem	4.98 b	0.4	5.00 a	0.74	0.977
	Root	5.06 b	0.29	4.44 a	0.6	0.262
+ Mn	Needles	5.77 ab	0.97	6.53 a	0.66	0.413
	Stem	4.28 ab	0.79	6.46 a	1.65	0.167
	Root	4.04 a	0.37	4.46 a	0.31	0.279
- P	Needles	5.44 ab	0.39	6.45 a	0.29	* 0.042
	Stem	4.34 ab	0.32	5.23 a	0.36	0.060
	Root	3.59 a	0.37	3.82 a	0.03	0.712
- P + Mn	Needles	4.71 a	0.09	6.68 a	0.86	* 0.032
	Stem	3.47 a	0.29	5.39 a	0.73	* 0.026
	Root	3.93 a	0.02	4.96 a	0.66	0.092

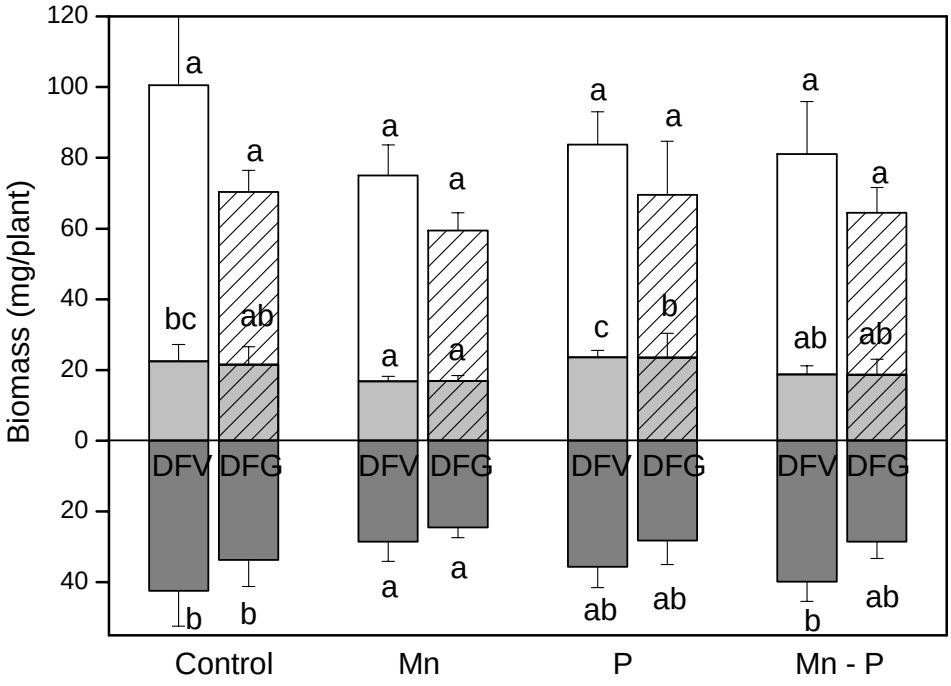

Figure 8. Biomass (mg fresh mass/plant) of Douglas fir variety *viridis* and variety *glauca* exposed to 5 mM Mn concentrations and 0.38 mM and 0.038 mM P concentrations in the nutrient solution. The bars indicate values for needles (white), stem (hatched) and root (grey), respectively after 2 weeks of exposure (±SD). P-treatment with 0.038 mM P, Mn-treatment with 5 mM Mn, Mn-P - treatment with 5 mM Mn and 0.038 mM P. Different letters indicate significant differences at $P \leq 0.05$ for needles, stems and roots separately.

3.1.5 Discussion

The main result of this study is that Mn translocation differed in two varieties of Douglas fir under Mn stress (Fig. 3 and 4). Mn concentrations were increased by exposure to excess Mn without significant differences of both varieties. The more tolerant DFV showed a more effective deposition of Mn in roots in particular in granules (Fig. 5). However, compared with crops and other plants, extremely high Mn concentrations were necessary to inhibit root growth in short- term experiments.

St Clair and Lynch (2005) compared conifers and deciduous foliage and found that in deciduous plants Mn was hyperaccumulated to concentrations more than twice as high as those found in evergreen needles of pine or spruce. Foliar Mn concentrations in our experiment were in range from 60-4000 µg g DW^{-1}; according to Timmer (1991) evergreen species contain Mn concentrations between 100 to 5000 µg g DW^{-1}. Generally, Douglas fir in forest ecosystems reached Mn concentrations from 100 to 2000 µg g DW^{-1} (Baronius und Fiedler 1996; Zasoski et al.

1990). This suggests that Douglas fir tolerance to Mn may be more than just a function of relatively low foliar Mn accumulation rates. Plant- internal allocation processes seem to ply a role, since we found that Mn was more readily transported to the shoots in more sensitive variety DFG than in DFV (Dučić et al., 2006).

In the present study we examined whether the mechanisms of tolerance to Mn were associated with difference in subcellular compartmentation the following mechanism of tolearnce have been suggested. In tolerant crop species and genotypes: chemical modification of Mn in the apoplast (Wissemeier and Horst 1992), compartmentation of Mn in nonphotosynthetic tissues and organelles (Gonzalez and Lynch 1999) and more efficient antioxidant systems (Gonzalez et al. 1998). Our analyses by transmission electron microscopy coupled with X-ray microanalysis showed that at the subcellular level enrichment of Mn in DFG was higher in needles tissues, while in DFV Mn was stored in preferentially in roots (Fig. 3, 4).

Earlier publications about the subcellular Mn deposition pointed to Mn localisation in the vacuoles of the epidermis of root tips (Memon et al., 1981; Horst, 1988; Pfeffer et al., 1986; Quiquampoix et. al, 1993; Gonza´lez and Lynch, 1999). Our experiments show that the dominant deposition site after exposure to excess Mn was in precipitates ("black bodies") in epidermis and cortex (Fig. 5, Table 1). Even though the highest deposition was found up to endodermis barrier, Mn appeared also in cells of the central cylinder. Such Mn granules, where Mn is effectively immobilised with P and Ca, may allow strong accumulation of Mn and may contribute to tolerance to Mn. In the variety DFV, which one is supposed to be more tolerant, we found such depositions more frequently (Table 1). Correlation analysis showed significant dependencies between Mn and P and Ca in these structures. We assumed that this was important for an effective immobilisation of Mn, which would allow strong accumulation, and retention of Mn in the root syste. Mn complexation with phosphate stabilized free Mn^{2+} in a very low nanomolar concentration range because of the high affinity of phosphate for Mn and its marked tendency to form insoluble complexes (Kihn et al. 1988; Farcasanu et al., 1995).

In South-western Germany where Douglas fir where exhibited and Mn high levels and internal bark necrosis and growth anomalies, this species showed also high concentrations of N, Ca and Zn, but deficiencies in P, K and Mg (Schöne, 1991). Manganese concentrations increased steeply with tree age, while the levels of phosphorus and potassium subsided to deficiency range (Schöne, 1992).

Horst and Marschner (1978) reported that exposure to excess Mn inhibited the translocation Ca into the shoot apex. In our experiments Ca concentrations in DFG increased with decreasing of P more than with increasing of Mn concentration (Table 3, 5). Le Mare (1977) found that plants are less sensitive to Mn toxicity when the concentration of Ca is high. Increasing Ca supply depressed Mn uptake but did not alter optimum foliar Mn concentration in *Pinus* (Kavvadias and Miller, 1999). This effect is probably due to competition of divalent cations, especially chemically similar ones like Mn, Ca, Mg (Amberger, 1972), to binding groups in the cell wall (e.g. carbonyl groups) and in the next step transport into the cell (Haysen, 1980; Wang et al., 1992). It was shown that COO^-/COOH ratio was higher in a Mn-sensitive tobacco genotype than in a sensitive and that this results in stronger interaction of with cell wall structure with Mn (Wang et al., 1992). This shows that chemical characteristics of surface cell walls play important roles in plant Mn uptake and may explain the high accumulation of Mn in the root epidermis of the Mn-tolerant variety (Fig. 3).

It was shown on the basis of ^{31}P NMR investigations that Mn^{2+} passes into the vacuole of *Acer pseudoplatanus*, while exhibiting only very minor accumulation in the cytoplasm (Roby et al., 1988). With time, gradual leakage of phosphate from the vacuole to the cytoplasm was observed along with an increase in glucose-6-phosphate (Roby et al., 1988).

High levels of P enhanced Mn uptake by sorghum and accentuated Mn toxicity at low Mn levels (Kuo and Mikkelson, 1981). In contrast, it was found that P inhibits Mn absorption in acid soils and encouraged it under normal conditions (Palaniyandy and Smith, 1979) Treatment with 5 mM Mn of potato microplants reduced ^{32}P concentration 2 to 3-times (Sharker et al., 2004). Apparently, under very excessive Mn supply P uptake is restricted.

We found that growth with low P caused Mn accumulation in roots and shoots in both varieties in comparison with control (Table 4). After low P treatment combined with different Mn concentrations, accumulation of Mn was more affected in DFG. However, DFG kept its P concentration at a more constant level than DFV. In both varieties less P in the medium led to higher Mn accumulation in root and shoot in comparison with control plants (Tab 4). One possibility is increased transport of Mn under P- limited and Mn surplus conditions. In *Sacharomyces cerevisiae* Pho84, a high activity inorganic phosphate transporter, is also an Mn-transporter (Jensen et al., 2003). This transporter plays a role predominantely under Mn toxicity and appears to be functioning like a low affinity metal transporter (Jensen et al. 2003). Such transport system has not yet been identified in plants.

The evolution of plants in environments where phosphate availability is low in the rhizosphere has led to numerous adaptations required for the survival of plants (Raghothama, 1999). These include the capacity to enhance P uptake during nutrient deficiency (McPharlin and Bieleski, 1987; Furihata et al., 1992). This increase has been correlated with an increased number of high-affinity phosphate transporters assembled in the plasma membrane (Drew and Saker, 1984; Shimogawara and Usuda, 1995; Muchhal and Raghothama, 1999). Since DFG could keep P concentrations more constant under P- deficiency, this means that this variety has better adaptations to poor environmental conditions, what is not case with DFV, naturally adjusted to grow in rich soils.

One of the consequences of Mn deposition in leaves is photosynthetic sensitivity (Clair and Lynch, 2004). More pronounced translocation of Mn to needles of DFG together with light could lead to higher production of free radicals, and thus higher sensitivity of DFG than of DFV. It was shown in common bean that excess Mn was implicated in oxidative stress under high light (Gonzalez et al., 1998).

The present study demonstrates that in the presence of excess Mn two main varieties of Douglas fir differ in tolerance. Needles injury was not observed but significant decreases in biomass. A possible mechanism of tolerance of DFV under toxic concentration of Mn is retention of Mn in roots in complexes with P and Ca (Fig. 6). The reasons for more pronounced abilities of DFV to keep Mn in roots, are not known, but retention in roots and protecting of shoots is one of the general mechanisms of tolerance to Mn.

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3.1.7 Appendixes

Table 1. Fresh mass, dry mass and water content of needles in mg, stems and roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* expose to different Mn concentrations. n=5 (±SD). Different letters indicate significant differences at $P \leq 0.05$.

viridis	**Control**		**5mM**		**7.5mM**		**10mM**	
Fresh weight	mean	SD	mean	SD	mean	SD	mean	SD
Needles	106.9 b	10.3	77.3 a	16.6	67.1 a	11.5	76.2 a	12.3
Stem	27.1 a	5.8	25.2 a	3.4	24.6 a	5.1	25.4 a	2.1
Root	38.1 b	11.6	23.2 a	6.8	19.4 a	5.1	24.2 a	3.8
Whole plant	172.1 b	19.4	125.6 a	24.8	113.8 a	22.0	125.7 a	16.7
Dry weight								
Needles	24.2 b	3.4	19.3 a	4.4	18.7 a	3.3	22.2 ab	3.0
Stem	5.5 a	1.1	5.0 a	0.6	4.8 a	1.5	5.1 a	0.9
Root	7.3 b	2.1	4.7 a	0.7	4.1 a	1.2	4.5 a	1.0
Whole plant	37.0 b	5.1	29.0 a	5.1	28.6 a	5.7	31.7 ab	4.6
Water content								
Needles	82.6 b	7.0	58.0 a	12.4	48.3 a	8.6	54.0 a	9.8
Stem	21.6 a	4.7	20.2 a	3.1	19.8 a	4.1	20.2 a	1.7
Root	30.9 b	9.6	18.5 a	6.0	15.3 a	3.9	19.7 a	2.8
Whole plant	135.1 b	14.4	96.6 a	19.9	85.2 a	16.5	94.0 a	12.7
glauca	**Control**		**5mM**		**7.5mM**		**10mM**	
Fresh weight	mg mean	SD	mean	SD	mean	SD	mean	SD
Needles	61.5 c	10.3	54.8 bc	17.3	35.8 a	7.0	41.3 ab	4.1
Stem	19.9 a	2.6	22.2 a	5.7	16.7 a	2.2	21.8 a	1.8
Root	20.6 a	7.7	25.3 a	6.0	19.2 a	4.9	20.2 a	7.0
Whole plant	102.0 b	15.7	102.2 b	26.6	71.7 a	10.3	83.3 ab	10.7
Dry weight								
Needles	15.4 ab	1.2	15.5 ab	4.0	12.0 a	1.5	15.8 b	3.6
Stem	4.1 b	0.7	3.7 ab	1.3	2.5 b	0.4	3.3 ab	0.6
Root	4.8 b	1.0	4.2 b	1.0	2.7 a	0.8	4.0 b	1.1
Whole plant	24.2 b	2.0	23.5 b	6.2	17.2 a	2.3	23.1 b	4.9
Water content								
Needles	46.1 b	9.6	39.2 b	14.2	23.9 a	5.7	25.5 a	4.5
Stem	15.8 a	1.9	18.5 a	4.5	14.2 a	1.9	18.5 a	1.3
Root	15.8 a	6.9	21.1 a	5.1	16.5 a	4.2	16.2 a	5.9
Whole plant	77.8 b	14.5	78.8 b	21.1	54.6 a	8.1	60.2 ab	7.2

Table 2. Fresh mass, dry mass and water content of needles, stems and roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* expose to different Mn concentrations . n=5 (±SD).Different letters indicate significant differences at $P \leq 0.05$.

viridis	**Control**		**0.1mM mn**		**0.5mM Mn**		**1mM Mn**		**2.5mM Mn**		**5mM Mn**	
Fresh weight	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Needles	68.3 b	12.6	59.3 b	9.8	60.7 b	12.1	46.6 a	8.1	41.6 a	3.9	38.0 a	4.4
Stem	20.7 b	2.1	18.3 ab	3.1	19.0 ab	3.5	17.0 ab	3.1	16.3 a	1.4	17.1 ab	2.4
Root	32.6 b	13.2	34.5 b	10.4	32.4 b	3.4	26.6 ab	6.5	19.5 a	3.1	23.9 ab	2.0
Whole plant	121.5 b	30.1	112.2 b	17.8	112.0 b	18.5	90.3 a	12.5	77.4 a	3.1	79.0 a	5.9
Dry weight												
Needles	16.6 c	3.5	14.5 bc	3.4	14.3 abc	3.1	11.9 ab	2.1	11.5 ab	1.3	9.9 a	0.9
Stem	4.6 b	0.6	3.6 a	0.4	3.8 ab	0.9	3.9 ab	0.4	3.5 a	0.4	3.3 a	0.6
Root	5.2 bc	1.4	5.6 c	1.0	5.2 bc	0.6	4.4 abc	1.6	3.7 ab	0.3	3.5 a	0.9
Whole plant	26.4 c	5.8	22.9 bc	4.8	23.3 bc	4.7	21.3 abc	3.7	18.7 ab	2.1	16.8 a	2.5
Water content												
Needles	51.7 b	9.1	44.9 b	6.4	46.4 b	9.0	34.8 a	6.0	30.1 a	2.6	28.0 a	3.5
Stem	16.1c	1.6	14.8 ab	2.7	15.1 bc	2.7	13.1 ab	2.6	12.8 a	1.0	13.8 ab	1.8
Root	27.4b	11.8	28.9 b	9.4	27.2 b	2.8	22.3 ab	4.8	15.9 a	2.8	20.4 ab	1.0
Whole plant	95.2 c	24.3	84.8 bc	16.2	88.7 c	13.9	66.2 ab	10.4	58.8 b	1.6	62.2 ab	4.5
glauca	**Control**		**0.1mM mn**		**0.5mM Mn**		**1mM Mn**		**2.5mM Mn**		**5mM Mn**	
Fresh weight	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Needles	61.9 c	6.8	59.9 bc	7.0	50.1 ab	5.9	54.4 abc	8.2	46.1 a	9.2	45.4 a	6.6
Stem	18.2 bc	1.4	17.9 bc	1.7	16.1 ab	1.3	18.5 c	1.5	16.1 ab	1.5	14.7 a	2.1
Root	42.2 c	2.7	38.4 c	6.3	30.1 b	6.6	39.7 c	2.5	21.4 a	3.7	22.5 a	6.2
Whole plant	122.3 b	6.6	116.2 b	14.0	96.3 a	13.9	112.7 b	8.7	83.5 a	14.2	82.6 a	13.4
Dry weight												
Needles	17.0 b	2.4	14.7 ab	1.6	13.3 b	1.8	15.0 ab	2.2	12.7 b	2.7	13.1 b	2.2
Stem	4.5 b	0.4	3.9 ab	0.4	3.7 a	0.5	4.1 ab	0.5	4.0 ab	0.6	3.3 a	0.3
Root	6.4 b	0.7	5.2 ab	0.7	5.7 ab	1.2	5.5 ab	0.3	4.4 a	0.9	4.9 a	1.4
Whole plant	27.9 c	3.2	23.8 abc	2.7	22.7 ab	3.5	24.5 bc	2.2	21.2 ab	4.3	19.3 a	4.6
Water content												
Needles	44.8 b	4.4	45.2 b	5.4	36.8 a	4.1	39.4 ab	6.1	33.3 a	6.5	32.3 a	4.4
Stem	13.7 bc	1.0	14.0 c	1.3	12.3 b	0.8	14.4 c	1.1	12.0 b	0.9	11.4 a	1.8
Root	35.8 c	2.1	33.1 c	5.6	24.4 b	5.4	34.3 c	2.2	17.0 a	2.8	17.7 a	4.7
Whole plant	94.3 b	4.0	92.4 b	11.6	73.6 a	11.0	88.1 b	6.8	62.4 a	10.3	63.3 a	9.8

Table 3. Elements concentrations (mg/gDW) in the needles, stems and roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after exposure to different Mn concentrations. Mn*- Mn concentration in nutrient solution (mM). n=3 (±SD). Different letters indicate significant differences at $P \leq 0.05$.

	Mn*	mg/gDW	P		S		Na		K		Ca		Mg		Fe	
	(mM)		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
viridis	0.005	Needles	5.49 a *	0.52	1.67	0.25	0.29	0.09	7.76	1.11	0.88	0.02	1.93	0.09	0.06	0.01
		Stem	4.28 a	0.21	2.3	0.32	1.32	0.26	6.67	1.61	1.55	0.21	0.88	0.05	0.12	0.01
		Root	4.06 a	0.39	2.31	0.34	1.5	0.19	10.43	1.47	1.17	0.14	0.73	0.04	0.8	0.18
	0.5	Needles	6.19 ab	0.4	1.88	0.16	0.38	0.1	7.88	0.21	1.02	0.08	1.78	0.2	0.07	0.01
		Stem	4.39 a	0.56	2.44	0.56	1.42	0.15	6.55	0.21	1.17	0.07	0.81	0.21	0.14	0.05
		Root	4.31 a	0.2	2.22	0.32	1.9	0.15	12.71	0.51	1.72	0.9	0.59	0.01	0.43	0.06
	2.5	Needles	6.65 ab	0.79	2.34	0.34	0.37	0.14	9.47	0.64	1.04	0.22	1.93	0.18	0.07	0.01
		Stem	5.18 a	0.74	3.15	0.41	1.22	0.25	7.81	0.68	0.83	0.08	0.59	0.11	0.21	0.05
		Root	4.56 a	0.65	3.16	0.16	1.5	0.25	12.15	1.5	1.21	0.08	0.46	0.08	1.47	0.47
	5	Needles	6.95 b *	0.72	2.55	0.14	0.35	0.07	7.86	0.7	1.13	0.29	2.06	0.19	0.07	0
		Stem	4.72 a	0.22	2.75	0.36	1.93	0.44	6.26	0.74	1.85	0.35	0.59	0.06	0.21	0.12
		Root	4.52 a	0.47	3.33	0.15	1.82	0.18	10.54	0.42	8.51	2.91	0.52	0.03	0.61	0.05
glauca	0.005	Needles	4.21 a	0.32	1.36	0.21	0.28	0.05	6.06	0.56	0.83	0.12	1.53	0.13	0.1	0.03
		Stem	2.84 a	2	1.39	0.99	0.9	0.03	3.85	2.52	1.47	0.03	0.69	0.46	0.13	0.03
		Root	3.77 a	0.18	1.84	0.12	0.78	0.06	8.36	0.09	1.34	0.09	0.78	0.05	1.06	0.05
	0.5	Needles	5.86 b	0.49	1.77	0.3	0.3	0.11	8.17	1.71	0.85	0.02	1.98	0.05	0.07	0.01
		Stem	4.74 a	0.44	2.57	0.28	1.06	0.14	7.17	1.22	1.16	0.25	0.75	0.06	0.21	0.07
		Root	4.07 a	0.29	2.28	0.19	1.42	0.07	11.8	0.52	1.53	0.44	0.7	0.06	0.46	0.06
	2.5	Needles	5.92 b	0.75	1.92	0.43	0.32	0.07	5.95	2.4	0.73	0.09	1.78	0.13	0.07	0.01
		Stem	5.00 a	0.22	3.79	0.41	1.08	0.07	5.69	1.49	1.21	0.21	0.78	0.13	0.17	0.05
		Root	4.11 a	0.63	2.34	0.25	1.29	0.14	9.67	2.33	0.97	0.08	0.5	0.06	0.68	0.09
	5	Needles	4.99 ab	0.46	2.02	0.35	0.3	0.08	5.71	2.43	0.65	0.05	1.51	0.15	0.08	0.03
		Stem	4.25 a	0.32	2.61	0.43	0.84	0.07	6.64	0.92	1.21	0.01	0.49	0.08	0.3	0.06
		Root	3.75 a	0.19	2.26	0.15	1.39	0.2	10	1.27	1.35	0.33	0.49	0.02	0.58	0.01

Table 4. Plants biomass: fresh weight, dry weight and water content of needles, stems and roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after grown on 3.8mM P (control) or 0.38mM P for one week and afterwards exposed for two weeks to 5µM Mn (control) or 5mM Mn. n=5 (±SD). Different letters indicate significant differences at $P \leq 0.05$.

viridis	**Control**		**5mM Mn**		**-P**		**- P +Mn**	
Fresh weight	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Needles	78.0 a	19.6	58.2 a	8.6	60.1 a	9.3	62.3 a	14.8
Stem	22.5 bc	4.7	16.8 a	1.4	23.6 c	1.9	18.8 ab	2.4
Root	42.4 b	10.0	28.5 a	5.6	35.6 ab	5.9	39.9 b	5.5
Whole plant	142.9 b	34.2	103.5 a	14.7	119.3 ab	16.0	121.0 ab	22.9
Dry weight								
Needles	19.4 a	3.9	16.6 a	2.5	15.2 a	2.1	18.3 a	4.7
Stem	4.4 a	0.5	4.5 a	0.3	5.0 a	1.0	4.6 a	0.8
Root	7.0 b	1.2	5.1 a	1.0	5.7 ab	1.3	6.3 ab	0.7
Whole plant	30.9 a	6.0	26.3 a	3.9	25.9 a	3.7	29.2 a	6.7
Water content								
Needles	58.6 b	15.9	41.6 a	6.1	44.9 ab	7.3	44.0 ab	10.5
Stem	18.1 bc	4.5	12.2 a	1.4	18.6 c	1.4	14.2 ab	1.6
Root	35.4 b	8.9	23.4 a	4.8	29.9 ab	4.7	33.6 b	5.0
Whole plant	112.0 b	28.4	77.2 a	10.8	93.4 ab	12.5	91.8 ab	17.0
glauca								
Fresh weight								
Needles	48.9 a	6.0	42.5 a	5.1	46.0 a	15.2	45.9 a	7.1
Stem	21.5 ab	5.1	16.9 a	1.5	23.5 b	6.9	18.6 ab	4.5
Root	33.7 b	7.5	24.5 a	2.9	28.2 ab	6.8	28.5 ab	4.8
Whole plant	104.1 a	14.5	83.9 a	5.5	97.7 a	24.2	93.0 a	12.1
Dry weight								
Needles	16.0 a	2.1	14.8 a	1.4	16.3 a	3.3	14.9 a	2.5
Stem	5.8 a	1.2	5.1 a	0.3	5.8 a	2.2	5.5 a	1.9
Root	6.9 a	1.7	5.1 a	0.6	5.4 a	1.7	5.1 a	1.1
Whole plant	28.7 a	4.5	25.0 a	1.2	27.5 a	6.7	25.5 a	3.8
Water content								
Needles	32.9 a	3.8	27.7 a	3.7	29.7 a	11.9	31.0 a	4.6
Stem	15.7 ab	3.9	11.8 a	1.1	17.7 b	4.7	13.1 ab	2.6
Root	26.8 b	5.7	19.4 a	2.4	22.8 ab	5.1	23.4 ab	3.8
Whole plant	75.4 b	10.6	58.9 a	5.1	70.2 ab	19.3	67.5 ab	8.5

Table 5. Total content Mn and P in the needles, stems and roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after exposure to different grown on 0.38mM P (control) or 0.038mM P (-P) for one week and afterwards exposed for two weeks to 5µM Mn (control) or 5mM Mn (+Mn) different letters indicate significant differences at $P \leq 0.05$

viridis					Whole plant						Whole plant		
		µg Mn			**µg Mn**			**mg P**			**mg P**		
treatment		Mean	SD	*P*-value	Mean	SD	*P*-value	Mean	SD	*P*-value	Mean	SD	*P*-value
	Needles	2.990 a	0.635	* 0.025	**3.627 a**	**0.841**	0.103	0.163 b	0.037	0.117	**0.2271 b**	**0.0414**	0.156
Control	Stem	0.251 a	0.089	0.422				0.024 a	0.004	0.33			
	Root	0.386 a	0.136	0.561				0.040 b	0.004	0.355			
	Needles	62.704 b	6.421	* 0.017	**106.340 b**	**6.512**	* 0.0405	0.104 a	0.012	0.981	**0.1482 a**	**0.0173**	0.383
+ Mn	Stem	16.688 b	7.566	0.689				0.020 a	0.005	0.157			
	Root	26.948 b	3.122	* 0.010				0.024 a	0.002	0.11			
	Needles	1.583 a	0.273	0.264	**2.023 a**	**0.344**	0.845	0.085 a	0.01	0.064	**0.1289 a**	**0.0192**	0.057
- P	Stem	0.200 a	0.069	0.089				0.021 a	0.007	0.063			
	Root	0.239 a	0.06	* 0.016				0.023 a	0.007	0.995			
	Needles	88.533 c	4.561	0.085	**140.871 b**	**13.184**		0.102 a	0.012	0.545	**0.1470 a**	**0.0178**	0.275
- P + Mn	Stem	13.810 b	3.861	0.561			* 0.0474	0.017 a	0.003	0.202			
	Root	38.527 c	7.47	0.207				0.028 a	0.003	0.9			
glauca													
	Needles	1.642 a	0.231		**2.055 a**	**0.915**		0.116 a	0.017		**0.1801 a**	**0.0213**	
Control	Stem	0.345 a	0.074					0.028 a	0.005				
	Root	0.440 a	0.158					0.036 b	0.006				
	Needles	46.916 b	2.552		**84.788 b**	**10.635**		0.104 a	0.009		**0.1586 a**	**0.006**	
+ Mn	Stem	20.051 b	11.439					0.033 a	0.011				
	Root	17.821 b	1.376					0.022 a	0.001				
	Needles	1.195 a	0.403		**1.955 a**	**0.474**		0.107 a	0.011		**0.1669 a**	**0.0158**	
- P	Stem	0.367 a	0.108					0.036 a	0.008				
	Root	0.393 a	0.043					0.023 a	0.004				
	Needles	60.576 b	20.719		**105.449 b**	**26.963**		0.109 a	0.014		**0.1637 a**	**0.0144**	
- P + Mn	Stem	16.649 b	6.712					0.026a	0.01				
	Root	28.224 c	9.27					0.029 ab	0.01				

Table 6. Elements concentrations (mg/g DW) in the needles, stems and roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after grown on 0.38mM P (control) or 0.038mM P (-P) for one week and afterwards exposed for two weeks to 5µM Mn (control) or 5mM Mn (+Mn).

viridis	**mg/gDW**	**S**		**K**		**Ca**		**Mg**		**Fe**	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	Needles	1.91	0.44	10.93	1.54	0.91	0.18	1.61		0.05	0.01
	Stem	2.51	0.55	7.22	0.98	1.45	0.38	0.83	0.03	0.25	0.02
	Root	1.95	0.41	11.44	1.08	0.9	0.06	0.64	0.02	0.74	0.18
+ Mn	Needles	1.84	0.2	6.93	0.34	0.5	0.07	1.23	0.16	0.04	0
	Stem	3.34	1.02	6.4	1.11	1.28	0.13	0.5	0.12	0.31	0.09
	Root	2.27	0.08	10.07	1.31	0.53	0.08	0.31	0.02	0.7	0.17
- P	Needles	1.8	0.21	7.32	1.15	0.63	0.03	1.72	0.1	0.08	0.02
	Stem	2.55	0.16	4.8	1.09	1.71	0.27	0.66	0.07	0.35	0.19
	Root	2.12	0.16	10.41	1.2	0.84	0.11	0.6	0.04	0.76	0.16
- P + Mn	Needles	2.27	0.49	6.36	1.39	0.59	0	1.15	0.08	0.05	0
	Stem	2.92	0.79	5.36	1.13	1.38	0.71	0.36	0.06	0.19	0.08
	Root	2.61	0.08	8.82	1.45	0.82	0.06	0.34	0.02	0.82	0.24
glauca											
Control	Needles	1.96	0.18	9.14	1.75	0.53	0.11	1.93	0.13	0.1	0.02
	Stem	2.6	0.42	6.2	0.44	0.93	0.13	1	0.13	0.38	0.05
	Root	2.21	0.31	9.77	1.29	0.88	0.1	0.68	0.08	0.81	0.21
+ Mn	Needles	2.22	0.14	7.4	0.92	0.63	0.07	1.74	0.08	0.06	0
	Stem	4.93	1.33	7.06	1	0.47	0.09	0.59	0.07	0.32	0.11
	Root	3.14	0.52	8.12	0.68	0.93	0.04	0.48	0.02	0.98	0.32
- P	Needles	2.03	0.31	7.18	1.05	0.73	0.12	2.02	0.11	0.1	0.02
	Stem	3.02	0.46	4.74	0.67	0.91	0.04	1.12	0.32	0.41	0.04
	Root	2.67	0.09	6.76	1.22	1.75	0.13	0.83	0.02	1.26	0.29
- P + Mn	Needles	2.63	0.6	7.34	1.88	0.87	0.06	1.66	0.23	0.08	0.03
	Stem	3.87	0.92	6.58	1.49	0.52	0.16	0.56	0.11	0.25	0.11
	Root	3.21	0.49	10.63	1.73	1.92	0.54	0.6	0.04	1	0.3

Table 7. Change in element concentrations in different subcellular compartments Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* in roots in response to excess Mn (5mM). The ratio was calculate as concentrations in Mn-exposed / concentrations in control plants. n=6-9.

	RATIO: TREATMENT / CONTROL								
viridis root	Mn	P	Mg	Si	S	K	Ca	Fe	Zn
Epidermis CW	60.72	1.50	1.03	4.22	2.04	0.75	0.34	0.45	0.53
Epidermis vacuole	186.54	0.63	2.56	2.96	6.16	1.08	1.75	3.76	1.18
Cortex CW	60.81	0.88	1.28	6.15	2.36	1.41	1.03	0.24	0.81
Cortex vacuole	91.15	1.76	1.26	2.09	1.93	0.38	0.00	0.43	0.26
Endodermis CW	102.88	2.20	1.31	7.82	2.31	2.44	0.22	3.03	1.30
Xylem	15.43	0.87	0.61	7.49	2.29	1.40	0.17	0.94	0.47
Phloem whole cells	15.46	1.13	0.54	4.56	1.17	1.04	0.24	1.10	0.97
glauca root									
Epidermis CW	11.46	0.73	0.60	1.66	0.92	1.06	0.21	0.46	0.62
Epidermis vacuole	26.45	0.44	0.81	1.11	0.70	2.73	0.10	0.53	1.00
Cortex CW	9.77	3.46	0.75	2.67	1.61	1.76	0.27	0.12	0.86
Cortex vacuole	31.02	0.75	1.21	1.69	0.77	4.11	0.46	0.37	0.70
Endodermis CW	26.43	1.51	0.26	2.22	1.11	1.49	0.20	0.53	0.79
Xylem	30.17	3.50	0.44	2.04	3.32	1.81	0.27	0.62	2.37
Phloem whole cells	13.09	0.91	0.42	1.51	1.41	0.93	0.20	0.05	2.73
viridis needles									
Epidermis CW	8.69	1.13	0.34	2.19	1.34	1.53	0.62	0.64	0.92
Epidermis vacuole	19.88	2.24	1.68	2.65	0.44	1.13	1.01	1.13	1.44
Mesophyll CW	10.41	1.85	0.40	2.90	1.20	1.31	0.67	1.17	0.82
Mesophyll vacuole	11.42	1.82	1.72	2.00	1.83	3.93	6.53	1.13	3.41
Endodermis CW	17.66	1.56	0.48	2.83	2.09	1.37	1.05	0.31	0.92
Xylem CW	6.54	0.44	0.24	1.46	1.72	0.81	0.35	1.20	0.53
Phloem whole cells	16.45	2.79	0.79	3.00	8.93	1.48	0.47	1.45	1.36
glauca needles									
Epidermis CW	19.54	4.48	0.42	0.97	2.11	1.68	0.41	1.34	1.34
Epidermis vacuole	103.80	2.52	3.91	4.25	6.53	0.13	10.28	0.15	5.71
Mesophyll CW	6.87	2.14	3.70	4.12	4.06	0.35	1.30	8.76	6.51
Mesophyll vacuole	116.60	3.25	5.53	1.26	1.98	14.30	5.70	1.39	1.39
Endodermis CW	8.36	2.06	0.67	0.57	1.98	2.95	0.29	1.19	2.24
Xylem CW	38.92	3.98	1.70	0.44	2.02	8.44	0.94	0.85	2.39
Phloem whole cells	6.82	2.15	2.26	0.63	5.11	6.52	0.23	3.18	2.26

CHAPTER 4

4.1 The influence of the ectomycorrhizal fungus *Rhizopogon subareolatus* on the performance and manganese sensitivity of two varieties of Douglas fir (*Pseudotsuga menziesii* var. *viridis* and var. *glauca*)

4.1.1 Abstract

The goal of this study was to investigate the effect of ectomycorrhiza *Rhizopogon subareolatus* on plant productivity and Mn uptake and subcellular compartmentation in roots of two varieties of Douglas fir (*Pseudotsuga menziesii)* variety *glauca* (DFG) and variety *viridis* (DFV). We hypothesized that the observed differences in the performance of Mn metabolism in DFG and DFV (see chapters 2 and 3) may be influenced by the degree of mycorrhization. We expected that Mn-tolerance is differentially affected by symbiosis with an ectomycorrhizal fungi.

After germination both of the plant variety were precultured for 5 months in hydroponics and inoculated with *Rhizopogon subareolatus* for 10 months. Half of mycorrhizal and non-inoculated plants were fed with 10 mM Mn for 5 months. Ectomycorrhiza establishment was monitored 10 months after the inoculation. Biomass increment, root morphology and element concentrations were measured. Our results show that plants reacted to ectomycorrhiza inoculation with decreases in biomass production. Mn concentration in needles of *viridis* was decreased in mycorrhizal plants. Subcellular localisation of Mn in root tips after inoculation with *Rhizopogon subareolatus* showed higher Mn concentration towards endodermis, but in the vascular system Mn concentration did not differ after different treatments. We concluded that ectomycorrhiza *Rhizopogon subareolatus* formation may have a detrimental rather than a beneficial effect on biomass productivity and Mn protection in young Douglas fir seedlings.

4.1.2 Introduction

Mycorrhizal associations provide several benefits to the host plant. Besides the improvement of the nutritional state, other positive effects such as increases in plant resistance to pathogens (Hampp et al. 1999), in heavy metal tolerance (Leyval et al., 1997, Jentschke and Godbold, 2000, Schützendübel and Polle 2002), and in drought resistance (Davies et el., 1992; Nilsen et al., 1998; Shi et al., 2002) are relevant. Nevertheless, depending on the interactions between the fungal species, the host, and the environment, the effects may as well be negative or deleterious to the host plant (Medeiros et al., 1995; Eltrop and Marschner, 1996; Nogueira and Cardoso, 2003; Corrêa et al., 2006). Corrêa and co-workers (2006) showed that plants react differently to ectomycorrhiza formation depending on their age, their initial nutritional status, and the amount of nitrogen supplied.

In the view of this, it was shown previously that vesicular- arbuscular mycorrhiza (VAM) effectiveness varied in soybean from beneficial to adverse or indifferent according to the type of fungi, soil type and stage of plant development (Nogueira and Cardoso, 2003). Nogueira and Cardoso (2003) found that mycorrhizae alter Mn availability in the soil, and that this affects the Mn concentration in the plants. In the ineffective interaction between plant and fungus, mycorrhiza may increase the expression of Mn toxicity symptoms in the host plant. In the effective interaction, in spite of increased Mn availability in the soil and increased Mn concentration in plants, no Mn toxicity symptoms were detected in the plants (Nogueira and Cardoso, 2003).

The availability of Mn to plants is governed by oxidation and reduction processes (Marschner 1995). To date amelioration of manganese (Mn) stress has mainly been investigated in plant-VA mycorrhiza systems (Cardoso, 1985; Bethlenfalvay and Franson, 1989; Kothari et al., 1991; Posta et al., 1994). Posta and co-workers (1994) found that VAM colonisation slightly decreases root dry mass production of maize and inhibited accumulation of Mn in shoots above of control levels. In the rhizosphere soil, they found that mycorrhizal treatment decreased not only the number of Mn reducing microorganisms, but also increased Mn-solubilizing root exudates. In several studies a lower uptake of Mn by mycorrhizal than by nonmycorrhizal plants has been found (Pacovsky 1986; Arines et al. 1989; Kothari et al. 1990; 1991).

Responses of ectomycorrhizal fungi to toxic metals are of importance for the reclamation of polluted sites and their influence on plant growth and productivity (Blaudez et al. 2000). The decrease of metal phytotoxicity by mycorrhizal fungi has been widely demonstrated (Jones and Hutchinson, 1986; Dixon and Buschena, 1988; Colpaert and Van Assche, 1993). Many studies have been published to address the role of ectomycorrhiza in protection against heavy metal stress such Cd, Cu, Pb, Zn and other metals (Galli et al., 19994; Leyval et al., 1997, Jentschke and Godbold, 2000, Liu et al., 2000 Schützendübel and Polle 2002). However, very little is known about effect of ectomycorrhiza on plants grown with Mn excess.

Rhizopogon spp. are common ectomycorrhiza-building species that are often dominant on Douglas fir seedlings grown in disturbed forest soils (Molina and Trappe, 1994). They show strong host specificity with Douglas fir (*P. menziesii* var. *viridis*) (Molina and Trappe, 1982) and increase Douglas fir resistance against drought stress (Parke et al., 1983). Growth stimulation of Douglas fir viridis seedlings inoculated with Rhizopogon vinicolor and Rhizopogon parksii Smith was observed (Castellano, 1996). Rhizopogon subareolatus had positive effect on rooting of Douglas fir viridis, improved plant growth, and stimulated height growth during the first 3 years of seedlings establishment (Parladé et al., 1999, Pera et al., 1999). Nothing is known about the effect of Rhizopogon subareolatus on the Douglas fir variety glauca, which appeared to suffer from Mn-toxicity in stands in Rheinland-Pfalz (Schöne, 1992). Linnemann (1960) found that the two varieties of Douglas fir viridis and glauca, respectively in field studies differed in their degree of mycorrhization, and that the variety glauca showed a lower capability to build up mycorrhization than viridis.

We have previously shown that the two main varieties *viridis* (DFV) and *glauca* (DFG) differed in Mn-transport. DFG allocated Mn more readily to the shoot than DFV (Dučić et al., 2006, see also Chapter 2 and Chapter 3). However it is not known whether mycorrhiza can modify Mn-uptake and decrease Mn-toxicity. The aim of the present study was to determine Mn uptake and subcellular compartmentation in roots of two varieties of Douglas fir, which were infected with ectomycorrhizal fungus *Rhizopogon subareolatus*. We hypothesized that the observed differences in the performance of Mn metabolism in DFG and DFV (see Chapters 2 and 5) were influenced by the degree of mycorrhization. We supposed that ectomycorrhiza protect against Mn-toxicity.

4.1.3 Material and methods

4.1.3.1 Plant material

Seeds of *Pseudotsuga menziesii (*var. *glauca* and *viridis*) were obtained from Niedersachsen Forstamt (Oerrel, Munster- Oerrel, Germany) and Sheffield's Seed Company (Locke, New York, USA), respectively. The racial origin of seed lots was confirmed by isozyme analysis (Dučić et al., 2006).

Seeds of Douglas fir were soaked in tap water for 7 days at 2°C and surface sterilised in 96% ethanol for 30s, in 0.2% $HgCl_2$ for 30s, and in 30% H_2O_2 for 45 min. Subsequently, the seeds were placed on sterile 1.5% (w/v) water-agar, pH 4.5 in Petri-dishes (d=14 cm), maintained for 7 days in dark at 21°C and subsequently for 3 weeks with a day/night regime of 16h/8h (white light of 150 µmol m^{-1} s^{-1} photosynthetic photon flux, OSRAM L 18-W/21-840 (Lumlux Pluseco, Germany) at 23°C/21°C air temperature. After germination (seven days), the plants were transferred to hydroponic solutions. Aerated nutrient solution contained the following nutrient elements: 1.4 mM NH_4NO_3, 0.130 mM $CaSO_4$, 0.100 mM K_2SO_4, 0.160 mM $MgSO_4$, 0.35 mM $(NH_4)_2HPO_4$, 0.7 mM KCl, 0.030 mM KH_2PO_4, 5 µM $MnSO_4$, 10 µM Fe-EDTA; and micronutrients: 5 µM H_3BO_3, 0.03 µM $NaMoO_4$, 0.24 µM $CuSO_4$, 0.23 µM $ZnSO_4$ and 0.05 µM $NiSO_4$ (modified Ingestad and Lund, 1986). The pH was adjusted to 5. The solution was changed every 3 days. After 4 months the seedlings were transferred into 200ml Erlenmeyer flasks with peat: vermiculite medium (1:5). Forty plants of each variety were inoculated with *Rhizopogon subareolatus* and further forty were kept under the same conditions as controls. Pure cultures with *Rhizopogon subareolatus* strain 302 (Catalonia, Spain) were prepared in peat: vermiculite mixture (Massicotte et al., 1994) containing modified Melin-Norkrans fungal medium (½ MMN, Molina and Palmer, 1982) with 2.5 g/L glucose. 100 mL vermiculite (grade 3), 20 mL sieved peat and 70 mL ½ MMN medium were mixed. Seven disks (d=1cm) cut of colonies of young, well-growing fungi were gently buried in the substrate 7 days before planting Douglas fir seedlings. The flasks were closed and kept under sterile conditions under white light 150 µmol $s^{-1}m^{-2}$ and a temperature of 21ºC. The plants were maintained for 2 months in these conditions and then planted individually in 650ml tubes (d=5 cm, h=41 cm) which contained double sterilised peat:sand (corn size 0.5-2 mm) mixture (50:50). Ten disks of young fungus *Rhizopogon subareolatus* were placed at the roots. The growth systems were kept in a green house at a temperature of

22 ± 2°C and air humidity of 40%, with day/night regime of 16 h/8 h, achieved by additional light of 150 µmol m^{-1} s^{-1}photosynthetic photon flux. The plants were watered with 10 ml distilled water twice a week and once per week with 10ml plant nutrient solution described above. After 2 months of acclimatisation, half of each plants variety, with and without mycorrhiza was treated with 10 ml 10 mM $MnSO_4$ two times per week and 10 mL distilled water once per week. Plant heights were documented weekly during six months of the experiment and Mn-exposure.

4.1.3.2 Ectomycorrhiza observation and element analysis

At the end of the experiment, plants were harvested and one set was used to determine the degree of ectomycorrhiza by counting 100 root tips randomly chosen from each plant. Typical mycorrhiza were photographed under a binocular with 12x magnification (Zeiss, Germany).

Another set of plants was used for recording fresh and dry biomass. Plants were separated into their compartments (needles, stem, fine and coarse roots), dried at 60°C for 48 h, weighed and chemically analysed. Total element concentrations were measured using ICP-AES (Spectro Analytic Instruments, Kleve, Germany) after ashing in 65% concentrated HNO_3 at 170°C for 12 h (Heinrichs et al., 1986).

4.1.3.3 Subcellular element localisation by electron dispersive X-ray microanalysis by transmission electron microscopy (EDX-TEM)

A set of three plants was harvested for EDX analyses. Root pieces taken 15 mm from the root tip were cut in 5-mm long pieces, and were rapidly frozen in a mixture of propane–isopentane (2:1) cooled with liquid nitrogen to -196°C in a aluminium mesh. Samples were freeze-dried at -45°C for 3 days and stored at room temperature in a desiccator over silica gel. For transmission electron microscopy, freeze-dried samples were infiltrated with ether in a vacuum-pressure chamber and embedded in styrene-methacrylate using a technique specifically developed for analysis of diffusible elements (Fritz, 1989). 1-µm thick sections were cut using glass knives, mounted on adhesive-coated 100-mesh hexagonal grits, coated with carbon and stored over silica gel. Details and testing of the method have been reported previously (Fritz, 1989; Fritz and Jentschke, 1994). The samples were analysed with a Philips

EM 420 with the energy dispersive system EDAX DX-4 (EDAX Inc., Mahwah, NJ, USA). The accelerating voltage was 120 kV, the take-off angle 25° and counting time 30 live seconds. The Mn concentrations in cross-sections of roots were analysed in cell walls and vacuoles of the following tissues: epidermis, cortex and central cylinder of roots. In the root tips the vascular system was yet not well developed. Therefore the whole vascular system was analysed, without distinguishing between xylem and phloem elements. Six to nine measurements were done in each compartment in three different plants.

4.1.3.4 Anatomical analysis of mycorrhiza

Fine root tips without root-hairs were observed under a binocular (Zeiss, Germany) and photographed. Samples of fine roots were cut and fixed in a solution of FAE (7% formaldehyde, 18% ethanol, and 19.2% of acetic acid), for a minimum of 7 days. Samples were dehydrated, by soaking subsequently in 70, 80, 90, 96% ethanol for 15 min, 100% ethanol two times for 30 min, a mixture of 50% ethanol and 50% acetone (30 min) and two times in 100% acetone for 30 min. Samples were embedded in styrene-metacrylate (49%-49%) and 2% dibenzoyl peroxide starting with acetone (30%) and styrene-metacrylate (70%) for 12h and two times in the styrene-metacrylate mixture for another 12h and 24h. Then the samples from the styrene-metacrylate mixture were embedded in gelatine-capsules and polymerised for 14 days (minimum 1 week) at 30°C. Afterwards the samples were prepared for 1µm cutting with an autocut microtome (Reichner and Jung, Austria). The cross sections were mounted on gelatinated microscope slides, stretched in chloroform steam and stained with 0.1% Toluidine-Blue in 0.1% di-sodium tetra-borate for 3-5 minutes. Photographs were taken through the microscope (Zeiss, Germany).

4.1.3.5 Root morphology measurements

After washing with deionised water the roots were scanned with a calibrated colour optical scanner with a lighting system optimized for roots (STD1600+ System). Data were analysed with the *WinRhizo* software (Régent Instruments Inc., Québec, Canada) for calculation of the cumulative length, surface area and volume of the complete roots. Volume calculations were based on the TIFF files after the root scanning.

4.1.3.6 Statistical analyses

Data are means (± SD) of five to nine seedlings. Statistical analysis of the data was performed using analysis of variance (ANOVA) followed by a multiple range test (LSD, Statgraphics 2.1; StatPoint, Inc., St Louis, MO, USA). Means were considered to be significantly different from each other, if the level of significance was $P \leq 0.05$.

4.1.4 Results

4.1.4.1 Growth performance, biomass and Mn partitioning in non- and mycorrhizal Douglas fir var. **viridis** *and* **glauca** *in the presence of Mn excess concentrations*

Shoot growth of both varieties Douglas fir, mycorrhized and non-mycorrhized seedlings before and during exposure to Mn is shown in figure 1. DFV showed a better growth performance than DFG with about two times higher shoot increment (Fig. 1a). DFV infected with *Rhizopogon subareolatus* showed the best growth, higher than shoot growth of non-infected control plants. Mn-exposure suppressed slightly height growth of DFV (Fig. 1a). DFG showed the best shoot growth in the absence of any treatment (Fig. 1b). Mycorrhized DFG seedlings displayed diminished growth. The Mn effect was very similar in DFG plants with and without mycorrhiza (Fig. 1b).

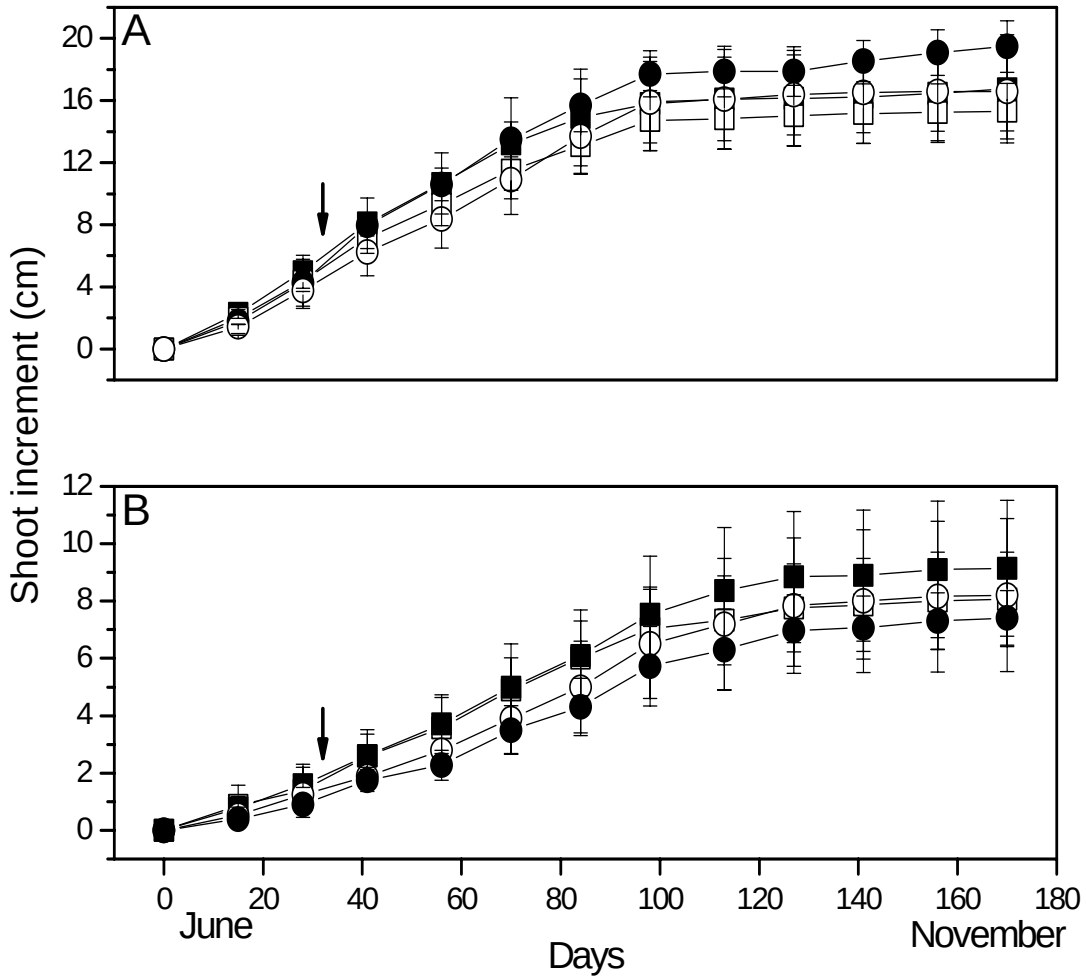

Figure 1. Shoot growth of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (A) and variety *glauca* (B). The arrow indicates beginning of the treatment with 10mM Mn (closed squares – control and open squares - treatment). Treatment started with 9-months old plants, which had been inoculated with *Rhizopogon subareolatus* for 6 months. Plants inoculated with *Rhizopogon subareolatus* are indicated as: closed circles- control and open circles- treatment with Mn. n=10 (±SE).

At harvest, Mn- exposed and mycorrhizal seedlings showed differences in plant development (Fig. 2). In DFV the root system was not strongly affected by ectomycorrhiza, while Mn- treatment reduced roots. In DFG root system was affected by mycorrhization. Mn-treatment resulted in lighter green colour of needles of both varieties of Douglas fir (Fig. 2).

Figure 2. Seedlings of 14-months-old Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (A) and variety *glauca* (B). Plants had been inoculated with *Rhizopogon subareolatus* for 10 months and treated with 10mM Mn for 5 months.

We found no positive effect of mycorrhiza on plant performance (Fig. 3). Biomass of both varieties was diminished in plants inoculated with *Rhizopogon subareolatus* by 24 and 58% in DFV and DFG, respectively. Mn-treatment caused decreases in plant biomass by12 and 17% in DFV and DFG, respectively (Fig. 3). Biomass of fine roots and needless of DFV and DFG were significantly reduced in mycorrhized plants in comparison with control seedlings. Mycorrhized seedlings treated with Mn had the lowest biomass (Fig. 3). Differences in dry mass are shown in appendix Table 1.

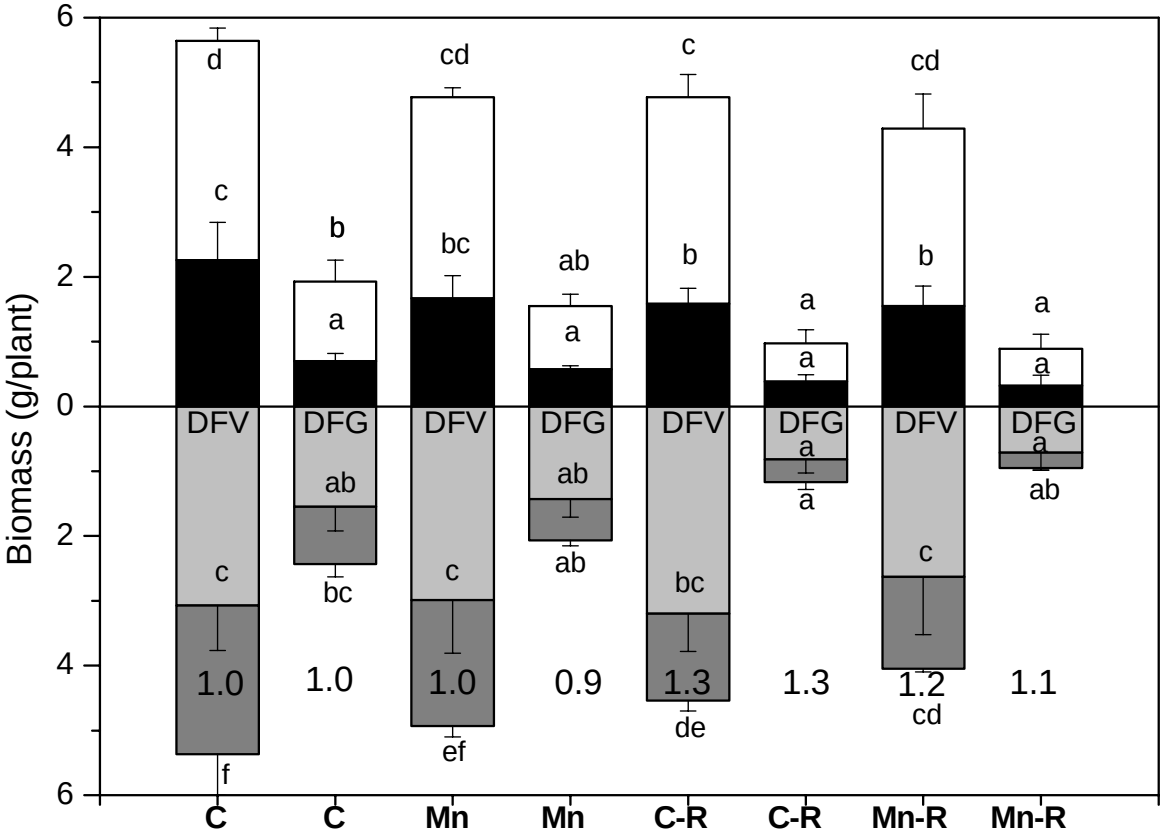

Figure 3. Biomass of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (DFV) and variety *glauca* (DFG) after treatment with 10mM manganese and after inoculation with *Rhizopogon subareolatus*. C indicate control plants, Mn- plants treated with 10 mM Mn, C-R indicate plants inoculated with *Rhizopogon subareolatus*, Mn-R show seedlings inoculated with *R. subareolatus* treated with Mn. Bars indicate means for fresh mass of fine roots (dark grey), coarse roots (grey), stem (black) and needles (white). Numbers indicate root/shoot ratio. n=3-4 (±SD). Different letters show significant differences with P≤0.05 for needles, stem and roots separately.

Root/shoot ratio was comparable between varieties and not affected by Mn-exposure, indicating that aboveground biomass production was not more suppressed than below-ground

biomass production (see appendix Table 1). Mycorrhized plants generally displayed higher root/shoot ratio than non-mycorrhized plants (Fig. 3).

Because of the negative effect of *Rhizopogon subareolatus* on plants performance, the morphology and anatomy of mycorrhizal root tips was analysed (Fig. 4). All analysed samples, showed the same morphotype, which was indicated as *Rhizopogon* spp. according to Molina and Trappe (1994) and Parladé et al. (1995). Cross-sections of root tips showed that a typical mycorrhiza with a Hartig net was developed (Fig. 4).

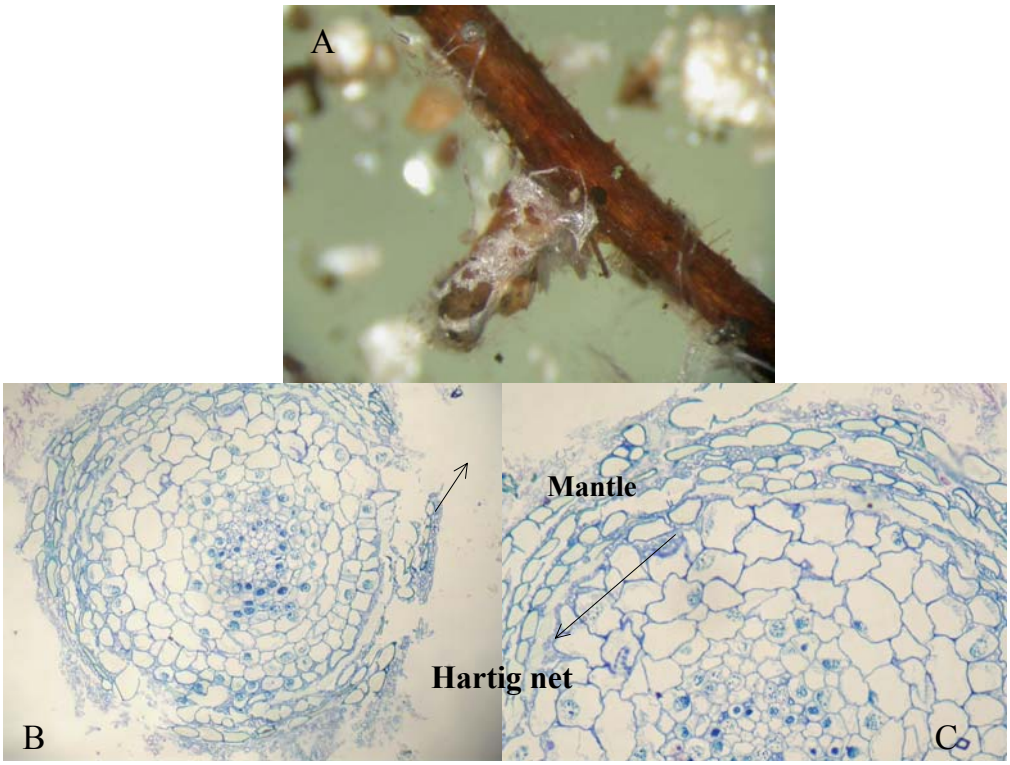

Figure 4. Mycorrhizal root tip of Douglas fir with *Rhizopogon subareolatus* fungus in pure culture system (A) and cross sections of mycorrhizal root tip with magnification 200 (B) and 400 (C).

Analyses of mycorrhiza abundance showed that the highest degree of the mycorrhization was found in inoculated DFV and that this decreased significantly after Mn treatment (Table 1). Since the experiment was not conducted under sterile conditions, control plants showed low percentage of mycorrhization. DFG roots were very poorly colonised with *Rhizopogon subareolatus*, but significantly more than control plants. Overall, DFV roots were almost 4- fold more strongly colonised than those of DFG (Table 1).

Table 1. Percentage of mycorrhizal root tips of Douglas fir (*Pseudotsuga menziesii)* variety *viridis* and variety *glauca* after inoculation with *Rhizopogon subareolatus* for 10 months and after 5 months of exposure to 10mM Mn. n=4 (±SD). Different letters show significant differences when $P \leq 0.05$.

	viridis		*glauca*	
Treatment	Mean	SD	Mean	SD
Control	8 b	1	1 a	1
+ Mn	6 ab	3	3 ab	2
+Rhizopogon	32 d	4	8 b	3
+ Mn + Rhizopogon	16 c	5	7 ab	2

Despite the observed differences in the degree of mycorrhization, there was only a trend, which showed decrease of root length in the presence of *Rhizopogon subareolatus* (Tab 2). Significant differences were found between the two varieties for total root length, average diameter, and root volume. Mycorrhiza had a significant effect on average diameter and total root volume. Mn- treatment affected only the root diameter (Table 2).

To investigate whether mycorrhiza and Mn-stress affected the nutrient status of Douglas fir, element analyses were conducted (see appendix Table2 for full info). Mn concentrations increased in roots of mycorrhizal DFG seedlings. After Mn-treatment roots and needles of DFV contained significantly higher Mn concentrations than those of DFG (Table 3). In DFG needles Mn concentration increased on the similar way like in root of mycorrhizal seedlings after Mn-treatment.

Table 2. Root morphology: total length, diameter average and total root volume of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (DFV) and variety *glauca* (DFG) after treatment with 10mM manganese and after inoculation with Rhizopogon subareolatus. n=4 (±SD) *P*-values of the ANOVAs show differences between varieties, mycorrhiza and Mn treatment and their interactions.

			Total Length(cm)	Average Diameter(cm)	Root Volume(cm^3)
viridis	DFV control	Mean	2021	3.10	5.74
		SD	311	0.30	2.67
	DFV +Mn	Mean	1475	2.20	6.01
		SD	182	0.10	0.42
	DFV +Rhizopogon	Mean	1526	2.00	5.16
		SD	444	0.10	1.19
	DFV +Rhizopogon + Mn	Mean	1504	2.00	5.38
		SD	433	0.50	1.28
glauca	DFG control	Mean	642	2.00	2.71
		SD	185	0.20	0.43
	DFG +Mn	Mean	430	0.90	1.69
		SD	133	0.40	0.66
	DFG +Rhizopogon	Mean	455	1.40	1.71
		SD	150	0.10	0.48
	DFG +Rhizopogon + Mn	Mean	468	1.40	1.72
		SD	72	0.10	0.19
	P-value	variety	0.000	0.000	0.000
		mycorrhiza	0.097	0.002	0.003
		Mn-treatment	0.469	0.041	0.351
	interaction	var. x myc.	0.946	0.385	0.417
		var. x Mn-treat.	0.218	0.361	0.236
		myc. x Mn-treat.	0.496	0.019	0.219
		all factors	0.264	0.242	0.153

The phosphorus concentrations in roots were significantly higher in DFG than in DFV in all treatments (Table 3). Mycorrhization of DFV had significant positive effect on P-concentration of root and needles in comparison to control non-mycorrhized plants (Table 3). Phosphorus concentrations in DFG were influenced by mycorrhization. Multifactoral analysis

showed significant differences in interaction of variety and Mn-treatment on Mn-concentrations of needles (Table 3).

Table 3. Concentrations of Mn and P in roots and needles of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after treatment with 10mM manganese and after inoculation with *Rhizopogon subareolatus*. n=3(±SD). Different letters show significant differences with P≤0.05. *P*-values of the ANOVAs show differences between varieties, mycorrhiza and Mn treatment and their interactions.

			Root		Needles	
			Mn	P	Mn	P
viridis	Control	Mean	0.21 a	1.73 a	0.99 a	6.36 a
		SD	0.13	1.23	0.14	1.2
	+Mn	Mean	2.58 d	2.75 ab	6.93 c	6.99 ab
		SD	0.46	0.15	1.6	1.54
	+ Rhizopogon	Mean	0.23 a	4.37 bc	0.78 a	10.97 c
		SD	0.09	0.27	0.13	0.83
	+Rhizop +Mn	Mean	2.10 d	5.45 cd	5.67 c	10.61 bc
		SD	0.3	0.68	1.4	1.26
glauca	Control	Mean	0.46 a	8.11 df	1.50 a	10.64 bc
		SD	0.07	1.35	0.4	1.68
	+Mn	Mean	1.15 bc	7.32 de	2.53 ab	6.92 ab
		SD	0.24	1	0.98	1.73
	+ Rhizopogon	Mean	0.61 ab	9.63 f	1.06 a	9.82 abc
		SD	0.04	1.11	0.07	2.07
	+Rhizop +Mn	Mean	1.28 c	8.41 df	3.55 b	10.33 bc
		SD	0.55	1.59	0.76	3.31
P-value	variety		0.292	0.463	0.005	0.000
	mycorrhiza		0.014	0.010	0.618	0.002
	Mn-treatment		0.022	0.438	0.000	0.970
interaction	var. x myc.		0.145	0.145	0.261	0.209
	var. x Mn-treat.		0.987	0.359	0.001	0.066
	myc. x Mn-treat.		0.067	0.391	0.821	0.858
	all factors		0.661	0.176	0.176	0.819

To find out whether the varieties differed in the subcellular distribution of Mn, we investigated cross-sections of root tips employing energy dispersive X-ray microanalyses (Fig. 5). Control plants of both varieties showed a similar spatial distribution of Mn in all tissues.

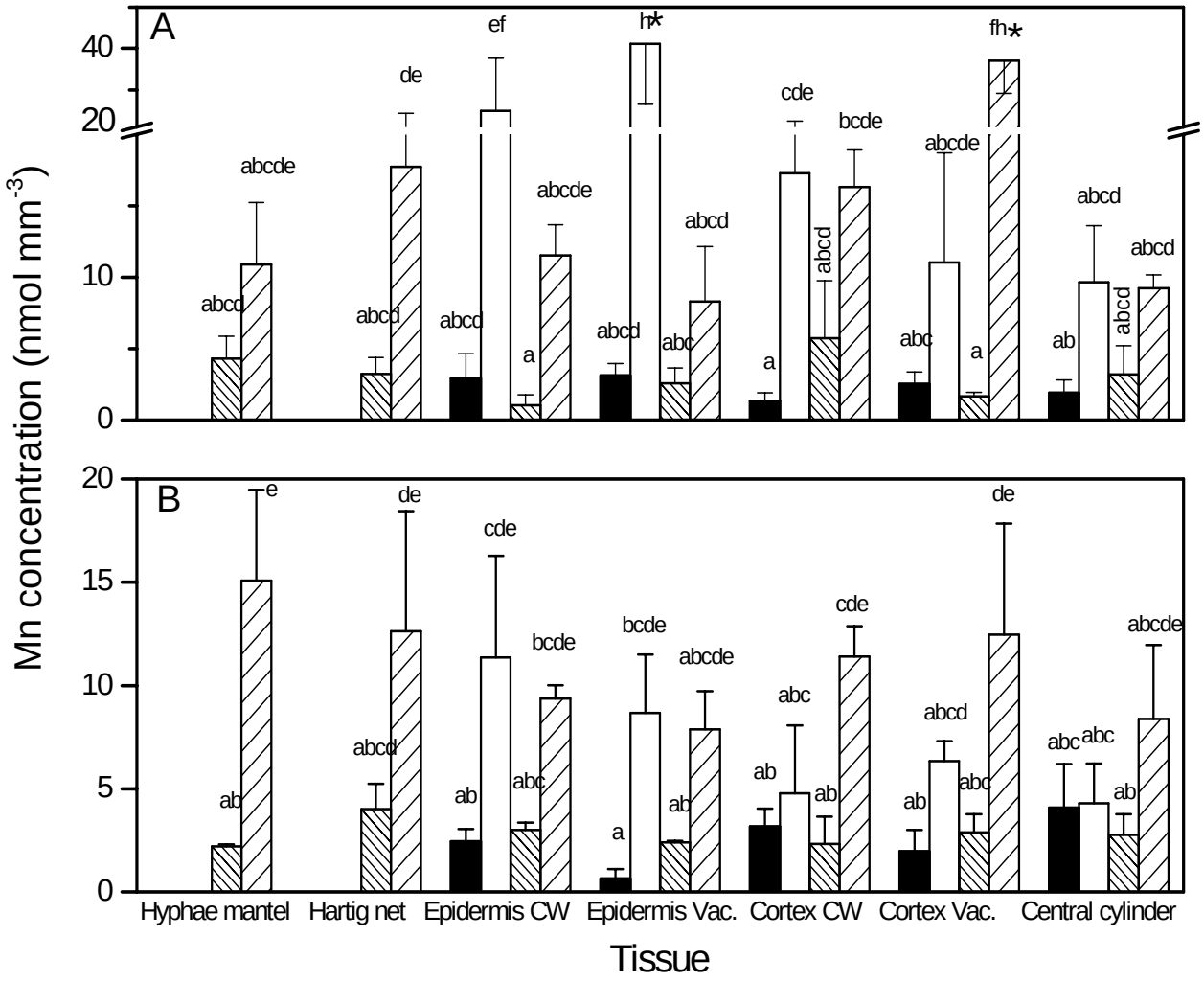

Figure 5. Manganese compartmentation in root tips after treatment with 10mM manganese and after inoculation with Rhizopogon subareolatus in Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (A) and variety *glauca* (B) – control (white bars), Mn treatment (black), mycorrhiza plant (right hatched) and Mn treatment in mycorrhiza plants (left hatched). Cross-sections were analysed by TEM-EDX. The concentrations obtained by EDX refer to volume units of the embedded specimen n=18-27 (±SE). Different letters show significant differences when P≤0.05. Stars indicate significant differences between two varieties when P≤0.05.

After Mn treatment cell walls and vacuoles of the epidermis and cortex of DFV contained 2-4-times higher concentrations of Mn than those of DFG and showed significant differences (Fig. 5). Inoculation with *Rhizopogon* did not affect the Mn concentrations in root tissues in comparison to non-inoculated control roots and also did not differ between the two varieties. Differences between the varieties were observed however, after Mn treatment of mycorrhizal plants. The hyphal mantel and Hartig net contained similar concentrations of Mn, but cortex cell walls and vacuoles contained almost two-times higher Mn concentrations in

DFV than in DFG (Fig. 5). The relative enrichment of Mn in DFV compared with DFG increased towards the endodermal barrier, but was absent in the vascular system of both varieties (Fig. 5). Subcellular concentrations of other elements are given in appendix (Table 3).

4.1.5 Discussion

4.1.5.1 Effect of mycorrhiza on Douglas fir performance

The main result of this study was that the ectomycorrhiza- forming fungus *Rhizopogon subareolatus* did not protect Douglas fir seedlings against Mn toxicity. We found that *Rhizopogon subareolatus* had a detrimental rather than a beneficial effect on plants productivity and development (Figs. 1 to 3). Even though height growth was positively affected by mycorrhization in DFV, total biomass was diminished in both varieties of Douglas fir in comparison with non-inoculated control seedlings (Fig. 3).

Growth depressions by mycorrhizal inoculation are generally attributed to the carbohydrate drain to the mycorrhizal fungus, while positive growth effects of mycorrhiza are thought to occur when the benefits of increased nutrient uptake exceed the carbon cost of the association (Schroeder and Janos, 2004; Thomson et al., 1994). Mycorrhiza establishment leads to an increase in the demand for carbohydrates for fungal maintenance and growth (Dosskey et al., 1990; Hampp et al., 1999; Reid et al., 1983). At least for *P. tinctorius* it was shown that this fungus acts as a strong C sink during the early stages of the symbiosis (Cairney and Chambers, 1997).

Generally it was found that ectomycorrhiza symbiosis enhances plant growth (Burgess et al., 1994; Lu et al., 1998). However, a number of studies have found no differences in growth between ectomycorrhizal and non-mycorrhizal plants (Baˆ et al., 1999; Thomson et al., 1994) and cases of reduced growth have also been reported (Colpaert et al., 1992; Eltrop and Marschner, 1996). It was shown that plants react differently to ectomycorrhiza formation depending on their age, their initial nutritional status, and the amount of supplied nitrogen (Corrêa et al., 2005).

Our study shows that in first year of growth in symbiosis with *Rhizopogon subareolatus* had negative effect on biomass, growth or root development of Douglas fir

(Figs. 1 to 3, Tables 1 to 3). According to Corrêa et al. (2005) this could be an initial effect, and does not exclude that mycorrhiza formation may have some beneficial influence on the host plant at later stage.

Teste and co-workers (2004) showed that inoculation with mycorrhizal fungi treatment of Douglas fir var. *glauca* did not increase survival and growth of 2-year- old seedlings. In another study young DFV inoculated with *Laccaria bicolor* had the same or lower fresh weigh than non-mycorrhized plants (Kamminga-van Wijk et al., 1992). The growth reduction observed here was not caused by nutrient deficiencies since concentrations of P were significantly increased in fine roots and needles of mycorrhized DFV seedlings (Table 3).

4.1.5.2 Mn stress

In both varieties of Douglas fir Mn- stress had negative effects on plant growth, biomass and root development (Figs. 1 to 3 and Tabs. 1, 3). In DFV the Mn concentrations in tissues were increased 3 to 4-times in DFV after Mn-treatment in comparison with DVG (Table 3). In contrast, biomass was even more diminished after Mn-stress in mycorrhizal plants than plants not inoculated with *Rhizopogon* (Fig. 3). This difference was the most pronounced in DFV evident from significantly decreased fine roots (Fig. 3).

It was shown previously that the effectiveness of a vesicular-arbuscular mycorrhiza (VAM) varied in soybean from beneficial to adverse responses according to the type of fungus, soil and stage of plant development (Nogueira and Cardoso, 2003). The latter authors found that mycorrhiza altered Mn availability in the soil, and that this affected the Mn concentration in the plants. In ineffective interactions between plant and fungus mycorrhiza may thus, increase the manifestation of Mn toxicity symptoms in the host plant. In the effective interaction, in spite of increased Mn availability in the soil and increased Mn concentration in plants, no Mn toxicity symptoms were detected in plants (Nogueira and Cardoso, 2003).

In root tips we found that subcellular localisation of Mn was not considerably affected by mycorrhiza (Fig. 5). In DFV, Mn concentrations increased towards vascular tissues (Fig. 5a). We may speculate that mycorrhiza brings Mn from root surface and epidermis deeper into tissues, because maximum of Mn concentrations after Mn treatment in non-mycorrhizal roots were the highest in epidermis vacuole (Fig. 5a). The cortex vacuole seems to be a

deposition site of Mn in mycorrhized DFV after Mn treatment, because it contained significantly higher Mn concentrations than other compartments (Fig. 5a). It even seems that mycorrhization facilitated Mn access of the cortex but not of the vascular system. In central cylinder Mn concentration did not significantly differ after different treatments (Fig 5a). In DFG mycorrhization had more negative effect on growth than in DFV (Fig. 3) but did not significantly change Mn-compartmentation in root tissues (Fig 5b).

This study shows that the performance of DFG was negatively affected after inoculation with *Rhizopogon subareolatus*. Earlier investigations demonstrated positive effects of mycorrhization of Douglas fir with *Rhizopogon subareolatus* (Parladé et al., 1999; Pera et al., 1999). We found that mycorrhized DFV seedlings showed mild stimulation in growth (Fig. 1), and decreased trend of Mn-concentration in needles (Table 3), and significantly higher P –concentrations in roots and needles (Fig. 3), but biomass of fine roots was significantly diminished in comparison with Mn-treated non-mycorrhized plants (Fig. 3). Mycorrhizal DFG seedlings did not show any beneficial effect. Biomass of needles and fine roots were significantly diminished in mycorrhizal DFG seedlings in comparison with control. Generally, we conclude that *Rhizopogon subareolatus* had no positive effects on plant biomass production and no protective effect against Mn- stress in young seedlings of Douglas fir var. *glauca* and in var. *viridis*.

4.1.6 References

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4.1.7 Appendixes

Table 1. Biomass (fresh (FW) and dry weight (DW)) of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after treatment with 10 mM manganese and after inoculation with *Rhizopogon subareolatus*. n=3-4 (±SD).

		Needles		Stem		Fine roots		Coarse roots		Whole pant	
viridis		FW	DW	FW	DW	FW	DW	FW	DW	FW	DW
Control	Mean	3.38	0.95	2.26	0.81	2.30	0.45	3.07	0.79	11.01	3.01
	SD	0.20	0.05	0.58	0.21	0.63	0.09	0.70	0.19	2.44	0.61
+ Mn	Mean	3.10	0.89	1.67	0.64	1.94	0.41	2.99	0.80	9.70	2.74
	SD	0.15	0.05	0.35	0.13	0.17	0.07	0.82	0.14	0.64	0.04
+ Rhizopogon	Mean	3.19	0.93	1.58	0.62	1.34	0.28	3.20	0.86	9.32	2.69
	SD	0.35	0.10	0.24	0.04	0.16	0.00	0.58	0.19	0.65	0.26
+ Rhizop+Mn	Mean	2.74	0.80	1.55	0.54	1.42	0.30	2.63	0.78	8.34	2.42
	SD	0.53	0.17	0.31	0.11	0.05	0.04	0.89	0.27	2.00	0.69
glauca											
Control	Mean	1.23	0.39	0.70	0.26	0.88	0.16	1.55	0.40	4.37	1.21
	SD	0.33	0.10	0.12	0.04	0.20	0.04	0.37	0.07	0.84	0.19
+ Mn	Mean	0.98	0.34	0.57	0.23	0.64	0.10	1.43	0.35	3.62	1.01
	SD	0.18	0.06	0.06	0.03	0.08	0.01	0.28	0.08	0.60	0.20
+ Rhizopogon	Mean	0.59	0.19	0.38	0.15	0.35	0.05	0.82	0.21	2.14	0.59
	SD	0.21	0.06	0.11	0.05	0.11	0.01	0.21	0.07	0.68	0.21
+ Rhizop+Mn	Mean	0.57	0.19	0.32	0.12	0.24	0.04	0.71	0.18	1.84	0.52
	SD	0.22	0.07	0.16	0.06	0.03	0.01	0.28	0.08	0.82	0.27

Table 2. Element concentrations in roots and needles in Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after inoculation with *Rhizopogon subareolatus* and treatment with 10mM manganese n=3 (±SD).

	Root	**mg/g DW**	**Ca**	**S**	**K**	**Mg**	**Fe**	**P**	**Mn**
viridis	Control	Mean	1.49	1.33	7.24	0.76	0.25	1.73	0.21
		SD	0.95	0.81	4.38	0.50	0.17	1.23	0.13
	+Mn	Mean	3.25	1.65	10.05	2.09	0.64	2.75	2.58
		SD	0.90	0.11	0.74	0.36	0.16	0.15	0.46
	+ Rhizopogon	Mean	1.91	4.38	10.60	1.26	0.49	4.37	0.23
		SD	0.17	0.50	1.13	0.08	0.13	0.27	0.09
	Rhizop +Mn	Mean	1.97	3.04	10.18	1.20	0.37	5.45	2.10
		SD	0.21	0.43	0.30	0.28	0.04	0.68	0.30
glauca	Control	Mean	2.58	2.18	12.57	1.86	0.58	8.11	0.46
		SD	0.19	0.45	4.39	0.67	0.18	1.35	0.07
	+Mn	Mean	4.01	1.61	12.39	2.25	0.90	7.32	1.15
		SD	1.43	0.19	1.77	1.07	0.32	1.00	0.24
	+ Rhizopogon	Mean	1.60	3.62	13.22	1.34	0.69	9.63	0.61
		SD	0.14	1.61	3.29	0.60	0.36	1.11	0.04
	Rhizop +Mn	Mean	1.96	2.05	11.25	1.82	0.82	8.41	1.28
		SD	0.34	0.37	2.69	0.84	0.38	1.59	0.55
	Needles	**mg/g DW**	**Ca**	**S**	**K**	**Mg**	**Fe**	**P**	**Mn**
viridis	Control	Mean	3.21	2.90	13.46	2.78	0.05	6.36	0.99
		SD	0.17	0.37	0.59	0.61	0.01	1.20	0.14
	+Mn	Mean	4.34	1.99	13.90	3.54	0.05	6.99	6.93
		SD	0.42	0.39	0.88	0.26	0.00	1.54	1.60
	+ Rhizopogon	Mean	3.05	8.66	14.36	3.72	0.03	10.97	0.78
		SD	0.80	0.96	2.14	0.32	0.00	0.83	0.13
	Rhizop +Mn	Mean	4.14	7.72	14.92	4.67	0.04	10.61	5.67
		SD	0.72	0.78	2.68	0.76	0.01	1.26	1.40
glauca	Control	Mean	2.95	1.86	9.03	3.16	0.04	10.64	1.50
		SD	0.46	0.35	0.48	0.71	0.00	1.68	0.40
	+Mn	Mean	2.79	1.67	9.06	3.21	0.05	6.92	2.53
		SD	0.45	0.16	1.51	0.47	0.01	1.73	0.98
	+ Rhizopogon	Mean	2.58	3.37	8.68	2.70	0.04	9.82	1.06
		SD	0.24	0.80	1.02	0.20	0.00	2.07	0.07
	Rhizop +Mn	Mean	3.39	3.70	10.53	3.81	0.04	10.33	3.55
		SD	0.14	0.33	1.97	0.58	0.00	3.31	0.76

Table 3. Element concentrations in compartments of root tips after treatment with 10mM manganese and after inoculation with *Rhizopogon subareolatus* in Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (DFV) and variety *glauca* (DFG). Cross-sections were analysed by transmission electron microscopy (TEM) coupled with electron dispersive X-ray microanalyses (EDX). The concentrations obtained by EDX refer to volume units of the embedded specimen (nmol/mm³). n=18-27 (±SE).

		Mg	±SE	Si	±SE	P	±SE	S	±SE	Cl	±SE	K	±SE	Ca	±SE	Mn	±SE	Fe	±SE
DFV Control	Epidermis CW	26.6	2.0	25.2	7.0	7.0	1.4	18.1	1.3	46.4	8.8	17.6	8.5	87.6	25.4	2.9	1.7	4.8	1.4
	Epidermis vacuole	21.5	4.0	27.8	1.7	34.6	2.7	28.3	2.2	174.5	87.3	216.5	100.2	74.7	30.6	3.1	0.8	4.5	1.2
	Cortex CW	10.5	2.4	15.0	2.4	94.7	48.5	16.7	1.9	65.4	24.3	154.6	47.0	5.5	2.8	1.4	0.6	4.6	1.9
	Cortex vacuole	15.9	3.7	21.1	8.4	50.2	16.0	65.2	39.4	67.1	38.6	188.4	44.0	9.3	4.3	2.5	0.8	6.3	0.8
	Central cilinder	7.8	2.2	18.6	7.3	9.0	3.1	8.3	3.2	24.7	17.2	36.1	27.2	8.7	2.5	1.9	0.9	3.2	1.3
DFV + Mn	Epidermis CW	19.1	7.8	21.2	5.7	40.6	21.1	42.2	12.4	28.9	7.0	36.0	22.3	105.4	43.9	24.9	12.8	5.8	2.5
	Epidermis vacuole	17.4	7.0	21.4	13.3	93.2	30.7	42.5	18.7	42.1	34.5	71.7	44.5	58.0	53.3	41.1	14.5	3.7	1.9
	Cortex CW	18.2	6.9	15.7	8.1	58.7	31.1	54.6	24.1	45.0	17.8	174.3	96.0	83.7	75.1	17.3	5.1	7.6	5.3
	Cortex vacuole	6.0	1.7	13.6	6.3	70.9	36.4	32.4	6.7	11.0	6.1	96.2	28.6	2.4	1.8	11.1	7.7	2.9	0.9
	Central cilinder	11.9	3.1	15.0	8.0	34.4	16.0	34.9	12.5	22.2	9.7	111.0	52.6	3.5	1.7	9.6	4.0	1.9	0.4
DFV + Rhizopogon.	Hyphae mantel	37.6	3.1	42.9	6.7	81.9	8.6	57.8	6.5	34.2	5.6	27.3	20.5	113.9	28.5	4.3	1.6	16.3	7.0
	Hartig net	30.1	8.2	39.1	8.0	34.5	6.4	44.9	4.8	29.1	15.6	14.9	6.3	149.7	57.0	3.2	1.2	9.8	3.3
	Epidermis CW	23.9	11.9	41.6	27.1	10.7	2.3	19.6	5.8	32.1	13.5	28.3	20.6	58.1	35.7	1.1	0.7	6.4	2.4
	Epidermis vacuole	21.4	13.7	25.6	16.0	70.6	27.9	38.1	7.8	34.7	24.5	91.3	87.2	41.4	19.2	2.6	1.1	16.6	7.4
	Cortex CW	17.2	7.4	24.7	11.9	39.2	18.0	32.1	1.9	29.3	11.1	126.4	82.6	35.0	14.8	5.7	4.0	5.4	2.2
	Cortex vacuole	6.6	3.7	15.6	9.4	87.6	54.8	40.0	11.1	12.6	7.0	103.2	59.4	7.8	0.8	1.7	0.3	6.5	2.5
	Central cilinder	6.6	3.4	22.0	8.3	26.2	23.5	13.8	2.9	13.1	7.0	57.0	28.3	5.9	1.9	3.2	2.0	5.1	2.0
DFV + Rhizo. + Mn	Hyphae mantel	19.9	7.6	30.0	7.6	40.7	9.5	35.7	3.8	30.0	2.6	10.3	1.5	87.0	12.8	10.9	4.4	13.6	3.7
	Hartig net	36.0	8.1	32.5	10.5	53.9	21.9	38.0	5.0	34.6	5.2	12.5	3.5	148.7	17.2	17.8	6.6	31.8	20.2
	Epidermis CW	29.7	10.7	25.6	4.7	15.9	5.4	24.5	7.3	66.0	30.9	20.1	10.2	145.5	48.6	11.5	2.1	14.3	1.7
	Epidermis vacuole	17.9	5.6	27.2	5.4	26.9	10.2	25.9	7.4	16.6	4.0	22.8	18.1	39.4	13.3	8.3	3.8	5.8	0.9
	Cortex CW	24.3	8.8	23.2	6.8	73.1	34.8	24.3	10.3	70.1	6.0	90.9	31.8	85.1	26.9	16.3	2.6	17.2	11.0
	Cortex vacuole	8.2	2.2	10.5	0.8	123.0	4.9	14.9	8.9	10.8	1.2	132.1	2.1	0.3	0.3	37.1	7.9	2.7	0.6
	Central cilinder	12.3	7.0	16.5	8.5	24.0	4.4	35.1	19.4	34.3	19.4	121.0	66.0	6.7	2.3	9.2	1.0	4.2	2.2

Table 3. Continuing.

		Mg	±SE	Si	±SE	P	±SE	S	±SE	Cl	±SE	K	±SE	Ca	±SE	Mn	±SE	Fe	±SE
DFG Control	Epidermis CW	31.4	7.8	37.8	17.8	30.0	17.2	19.0	1.8	50.2	12.9	15.2	3.0	177.1	36.0	2.5	0.6	8.0	2.4
	Epidermis vacuole	19.6	9.0	25.8	11.2	57.3	47.3	13.9	7.5	14.3	3.5	7.2	2.6	97.6	37.6	0.7	0.4	5.8	2.2
	Cortex CW	20.4	6.9	21.2	6.5	138.0	100.2	18.1	3.9	59.5	28.1	64.7	30.0	102.8	36.7	3.2	0.8	6.4	1.2
	Cortex vacuole	15.7	9.5	12.6	1.5	164.9	39.2	3.5	1.8	37.1	32.6	110.7	42.8	6.1	3.6	2.0	1.0	2.3	0.4
	Central cilinder	17.3	2.5	16.2	4.2	45.7	13.7	19.2	3.0	26.6	8.7	79.0	16.8	10.9	3.7	4.1	2.1	2.6	0.2
DFG + Mn	Epidermis CW	18.4	4.9	19.9	2.6	19.3	8.8	23.1	6.3	86.6	31.2	38.9	16.1	197.1	79.2	11.4	4.9	25.3	17.7
	Epidermis vacuole	21.9	7.1	11.0	0.8	151.0	74.2	49.9	26.4	255.5	234.9	338.7	333.2	136.8	59.1	8.7	2.9	14.1	8.6
	Cortex CW	17.2	4.4	14.7	3.8	38.1	30.0	34.3	7.5	77.2	25.8	110.8	80.5	86.3	44.3	4.8	3.3	4.2	1.1
	Cortex vacuole	11.7	2.9	11.0	2.3	127.5	56.6	56.0	34.3	21.0	6.7	102.6	82.2	37.0	12.2	6.3	1.0	5.6	1.0
	Central cilinder	9.1	0.8	42.4	24.4	22.9	3.7	26.7	4.1	21.2	11.0	17.2	9.7	52.6	15.6	4.3	1.9	9.5	5.9
DFG + Rhizopogon.	Hyphae mantel	28.8	3.7	38.9	20.1	66.6	35.5	37.7	3.6	59.8	15.9	98.2	58.4	96.5	72.6	2.2	0.1	8.2	3.0
	Hartig net	37.0	6.4	26.8	7.9	78.1	44.2	47.1	5.9	54.3	4.8	115.3	72.2	120.7	91.0	4.0	1.2	8.7	2.7
	Epidermis CW	34.9	0.2	29.3	0.3	124.8	22.7	22.0	1.6	97.7	3.4	126.5	82.9	148.9	26.7	3.0	0.3	4.6	1.4
	Epidermis vacuole	24.1	4.1	22.2	4.1	69.5	51.1	29.1	8.8	31.5	13.8	106.3	79.5	43.6	28.1	2.4	0.1	5.8	0.3
	Cortex CW	22.7	12.4	28.4	9.0	11.2	6.8	17.6	10.0	62.5	39.6	23.5	13.8	130.2	69.6	2.3	1.3	6.7	3.2
	Cortex vacuole	24.5	12.2	20.5	0.1	343.2	137.8	14.7	12.0	46.4	36.5	138.4	24.4	60.3	49.3	2.9	0.9	4.0	1.9
	Central cilinder	16.3	2.0	20.5	4.8	228.1	114.1	17.6	14.4	11.7	9.6	99.4	41.6	3.8	3.1	2.8	1.0	3.5	0.0
DFG+ Rhizo. + Mn	Hyphae mantel	29.2	11.8	30.4	6.3	63.9	29.3	44.5	15.2	72.0	21.0	19.1	12.2	159.6	47.9	15.1	4.4	15.6	4.7
	Hartig net	26.1	11.9	31.6	5.0	50.5	22.2	42.7	18.0	40.5	13.3	26.7	19.6	159.7	106.4	12.6	5.8	16.0	7.8
	Epidermis CW	16.5	3.1	28.3	6.2	11.7	2.7	22.0	3.0	63.1	15.8	13.9	4.1	134.4	11.2	9.4	0.7	7.7	1.0
	Epidermis vacuole	26.8	8.2	44.7	14.6	100.8	64.1	42.5	13.0	33.2	14.7	7.5	2.5	113.4	42.3	7.9	1.9	10.3	1.8
	Cortex CW	17.8	4.5	32.1	5.7	16.3	4.2	23.7	4.7	61.4	28.8	24.1	8.7	151.1	14.6	11.4	1.5	7.9	2.0
	Cortex vacuole	29.0	11.7	28.1	7.5	121.9	104.3	45.0	14.0	20.0	8.4	76.7	33.9	37.5	11.3	12.4	5.4	4.6	1.7
	Central cilinder	23.6	10.4	17.4	4.2	92.2	18.6	74.3	26.6	9.0	2.6	69.3	27.9	20.4	18.6	8.4	3.6	4.3	1.1

CHAPTER 5

5.1. Analysing of mycorrhizal communities of Douglas fir (*Pseudotsuga menziesii* var. *viridis* and var. *glauca*) and their influence on nutrition in different soils

5.1.1 Abstract

The objective of this study was to determine the abundance of ecto- and endomycorrhiza colonization of two varieties of Douglas fir (*Pseudotsuga menziesii* (Mirbel) Franco) variety *viridis* and variety *glauca* grown in two different soil types. The soils, a nutrient-rich (Solling) and a poor one (Unterlüss) were taken from sites stocked with pine but recommended for future Douglas fir cultivation. The soil pH was either kept at normal conditions or was slightly increased by liming. Seedlings germinated under sterile conditions and precultured in hydroponics were grown in Solling and Unterlüss- soils for two growth seasons.

Molecular identification of mycorrhiza based on DNA extraction of mycorrhizal roots showed that both soil- and variety-specific mycorrhizal communities were established on Douglas fir roots. *Rhizopogon vinicolor* was associated to variety *viridis*, while *Cadophora finlandia, Sebacinaceous, Tricholoma* sp. and uncultured *Tuber sp.* were more frequently found on *glauca*. Mycorrhizal basidiomycote isolate T01, *Tricholoma* sp., *Sebacinaceous* and group of unknown mycorrhizal fungi were specific for soil from Solling, whereas *Wilcoxina mikolae* and some of uncultured ascomycete for Unterlüss soil. Analyses of net assimilation of nitrogen, phosphorus and carbon in relation to mycorrhizal abundance indicated that nutrient uptake was positively correlated with degree of endomycorrhiza of Douglas fir. Solling soil generally showed better conditions for growth of both varieties and a higher degree of root ectomycorrhization.

Analyses of nitrogen and phosphorus uptake in relation to assimilation and mycorrhizal abundance indicated that nutrient – carbon - ratio was positively corelated with degree of endomycorrhization and not to ectomycorrhization. The degree of ectomycorrhization, but not species number, was positive correlated with biomass production.

5.1.2 Introduction

In more than 90% of land plants, especially trees, symbiotic associations are formed with mycorrhizal fungi (Visser, 1995). Mycorrhizal fungi are assisting plants with the uptake of phosphorus, nitrogen and other mineral nutrients from the soil and, thus, are important for plant growth and development, especially under nutrient-limiting conditions (Harley and Smith, 1983; Bolan, 1991; Harrison and Buuren, 1995; Hartley et al., 1997; Smith and Read, 1997). The efficiency of absorption and translocation of inorganic nitrogen to the root is achieved by an increase of surface area formed by hyphae connected to the root systems (France and Reid, 1983). Mycorrhizae symbiosis is founded on the mutualistic exchange of C from the plant in return for phosphorus (P), nitrogen (N) and other mineral nutrients from the fungus. It was found that influx of P in roots colonized by mycorrhizal fungi can be 3- to 5-times higher than in nonmycorrhizal roots (Smith and Read, 1997). Beside this function for plant nutrition, mycorrhizae play important role in stress tolerance (Leyval et al., 1997; Jentschke and Godbold, 2000; Schützendübel and Polle 2002).

In Europe silviculture with Douglas fir (*Pseudotsuga menziesii*) is expanding because of fast growth and good wood quality of this species. However, Douglas fir is an exotic species, which will have to establish mycorrhizae associations with non-native mycorrhizal flora in new surrounding. The significance of mycorrhiza for Douglas fir culture has been demonstrated since total plant biomass was significantly increased in the presence of mycorrhizal inoculation up to four times the mass obtained with uninoculated controls (Parladé and Álvarez, 1993). Pera and co-workers (1999) found that inoculation with ectomycorrhizal fungi improved the field performance of Douglas fir seedlings in northern Spain. Douglas fir can form symbiosis with both ecto- and

endomycorrhiza species. It is of great interest to study roles of these different symbioses for P and N nutrition in different Douglas fir varieties.

Two varieties of Douglas fir, frequently used for silvicultural purposes are *viridis* (DFV) and *glauca* (DFG). It has been reported that DFG showed lower ability to form mycorrhiza associations than DFV (Linnemann, 1960). One assumption was that the observed differences in the performance of mature field-grown DFG and DFV, such as growth or resistance to pathogens, may –at least partly- be related to differences in the degree of mycorrhization and/or fungal species composition.

Another factor affecting the performance of trees is soil pH. The two varieties of Douglas fir differ in Mn- mobility (Dučić et al., 2006), which may become a problem at low pH. The pH of non-calcareous forest soils in generally decreased considerably during recent decades (Hallbäcken and Tamm, 1986; Falkengren- Grerup, 1987; Eriksson, Karltun and Lundmark, 1992). Liming has been proposed as a method to counteract further acidification. Liming primarily raises the pH of the litter layer and first O and A soil horizonts, where has been suggested that activity of the mycorrhizal fungi are the most important (Abuzinadah et al., 1986).

The main aim of the present work was to investigate the extent and type of the mycorrhizal colonisation and the fungal community structure on fine roots of the two varieties of Douglas fir (*Pseudotsuga menziesii*) planted into different soils. In addition the soil pH was affected by liming.

We hypothesized that the abundance of mycorrhiza differs between the Douglas fir varieties DFV and DFG and that this would affect biomass production. An important question was whether mycorrhiza would enable nutrient homeostasis in poor soil and protect against uptake of excess Mn.

5.1.3 Material and methods

5.1.3.1 Plant material

Seeds of *Pseudotsuga menziesii (*var. *glauca* and *viridis*) were purchased from Niedersachsen Forstamt (Oerrel, Munster- Oerrel, Germany) and Sheffield's Seed Company (Locke, New York, USA), respectively. The racial origin of the seed lots was confirmed by isozyme analyses (Dučić et al., 2006).

Seeds of Douglas fir were soaked in tap water for 7 days at 2°C and surface sterilised in 96% ethanol for 30s, in 0.2% $HgCl_2$ for 30s, and in 30% H_2O_2 for 45 min. Subsequently, the seeds were placed on sterile 1.5% (w/v) water-agar, pH 4.5 in Petri-dishes (d=14 cm), maintained for 7 days in darkness at 21°C and subsequently for 3 weeks with a day/night regime of 16h/8h (white light of 150 µmol m^{-1} s^{-1} photosynthetic photon flux, OSRAM L 18-W/21-840 (Lumlux Pluseco, Germany) at 23°C/21°C air temperature. After germination (seven days), the plants were transferred to hydroponic solutions. Aerated nutrient solution contained the following nutrient elements: 1.4 mM NH_4NO_3, 0.130 mM $CaSO_4$, 0.100 mM K_2SO_4, 0.160 mM $MgSO_4$, 0.35 mM $(NH_4)_2HPO_4$, 0.7 mM KCl, 0.030 mM KH_2PO_4, 5 µM $MnSO_4$, 10 µM Fe-EDTA; and micronutrients: 5 µM H_3BO_3, 0.03 µM $NaMoO_4$, 0.24 µM $CuSO_4$, 0.23 µM $ZnSO_4$ and 0.05 µM $NiSO_4$. The pH was adjusted to 5. The solution was changed every 3 days. After 2 months of acclimation, the seedlings were transferred into containers with different types of the soils.

5.1.3.2 Types of the soils and site descriptions

Soils of two Scots pine stands from Solling and Unterlüss in Lower Saxony, Germany were chosen for experiment. Both sites have been chosen by Lower Saxony´s forest department to increase the area of forest stands with Douglas fir and to reduce pinewood stands. These soils are characterised by strong acidification with a base saturation of less than 10 % in the soil profile from 5 cm down to 100 cm depth.

The both soils have been classified as a well-drained dystric Cambisol with a moder-type O-horizon (FAO, 1998). Table 1 gives information about origin and general characteristics of soils from Solling and Unterlüss.

Table 1. General information about the forest areas of Solling and Unterlüss and climatic conditions.

Forest area	Stand	Geographical location	Altitude (m)	Stand age (years)	Air Temp. (°C)	Annual Precipitation (l m^{-2} yr^{-1})	Soil texture		
							Clay (%)	Silt (%)	Sand (%)
Solling	Pine	51°34‘N,9°40‘E	270	108	7.5	900	14.8	46.4	38.8
Unterlüss	Pine	52°50‘N,10°16‘E	110	59	8.4	837	3.6	15.5	80.9

In order to derive the amount of $CaCO_3$ for increasing soil pH to a desirable degree, the soil „Base Neutralisation Capacity“ (BNC) was determined according Berhold et al. (2006) and liming was performed according Table 2.

Table 2. pH of the soils from Solling and Unterlüss measured in two year seasons. n=6 ±SD.

		May 2004		September 2005	
Soil	Treatment	pH (KCl)	SD	pH (KCl)	SD
Solling	non-treated	3.46	0.05	3.67	0.04
Solling	limed	4.87	0.07	4.58	0.11
Unterlüss	non-treated	4.23	0.05	4.36	0.04
Unterlüss	limed	4.42	0.02	4.52	0.08

Cylinders 70 cm long with a perforated bottom were filled with untreated and limed soils from the two sites (Solling/Unterlüß; untreated/limed). Each cylinder was planted with 3 seedlings of *P. menziesii* var. *glauca* and var. *viridis*, respectively. 40 cylinders were prepared per treatment. To achieve near-natural growth circumstances for the plants in the pots, pots were filled with material from mineral soil first and subsequently with humus (5cm on the top). This simulated the natural situation with regard to element content, pH, and soil properties of the sites. Table 3 shows the concentrations of elements in the organic and mineral layers of the soils from Solling and Unterlüss.

Table 3. Element concentrations (mg/g dry weight) in the soils from Solling and Unterlüss, measured in Spetember 2005. n =6 ±SD.

Organic layer	N	P	S	K	Ca	Mg	Mn	Fe	Zn	Cu
Solling	8.930	0.569	1.137	3.157	3.161	1.451	0.620	10.158	0.105	0.017
SD	1.530	0.061	0.199	0.288	0.531	0.149	0.128	0.776	0.023	0.002
Solling limed	8.302	0.533	1.054	3.267	3.005	1.402	0.593	10.407	0.173	0.017
SD	1.968	0.081	0.234	0.369	0.463	0.114	0.188	0.841	0.130	0.002
Unterlüss	3.529	0.194	0.409	0.552	0.643	0.309	0.031	4.198	0.108	0.006
SD	1.038	0.041	0.130	0.052	0.152	0.037	0.004	0.551	0.093	0.001
Unterlüss limed	3.847	0.192	0.401	0.522	0.750	0.295	0.031	3.787	0.099	0.005
SD	1.611	0.026	0.085	0.039	0.129	0.027	0.004	0.349	0.054	0.001
Mineral layer										
Solling	0.545	0.197	0.127	5.354	0.919	2.022	0.347	14.076	0.030	0.009
SD	0.054	0.007	0.015	0.333	0.054	0.129	0.045	0.729	0.002	0.001
Solling limed	0.581	0.201	0.110	5.034	1.988	1.955	0.385	13.641	0.031	0.008
SD	0.094	0.006	0.015	0.349	0.130	0.161	0.042	0.810	0.003	0.001
Unterlüss	0.215	0.091	0.036	0.846	0.378	0.546	0.042	5.227	0.010	0.002
SD	0.150	0.007	0.021	0.045	0.031	0.032	0.004	0.261	0.001	0.000
Unterlüss limed	0.241	0.099	0.025	0.775	0.505	0.495	0.045	5.184	0.012	0.002
SD	0.185	0.009	0.025	0.089	0.036	0.063	0.007	0.366	0.002	0.000

5.1.3.3 Plant growth and chemical analysis

Two-months-old seedlings were planted into containers on the 17th May 2004 and maintained for two growing seasons under ambient conditions (Table 4). The plants were watered as necessary.

Table 4. Mean air temperature, precipitation and humidity in years 2004 and 2005 and in period from April to September.

Year		Mean air temperature (°C)	Precipitation ($l\ m^{-2}$)	% Air humidity
	2004	9.1	716.3	76.4
	2005	9.4	666.5	77.0
2004	April- September	14.2	405.6	72.4
2005	April- September	14.5	333.5	72.7

During the growing season plant heights was documented monthly during the two years-vegetation period. On the 13th December 2004 and 9th September 2005 plants were harvested, separated into needles, stem, fine and main roots, dried at 60°C for 48h, weighed and used for chemical analyses. In September 2005 another set of plants was used for root- and mycorrhiza-morphology determinations and third set of plants was used for DNA isolation and mycorrhiza detection.

The total C and N contents of the forest floor and mineral soils were analysed by dry combustion with a C/N analyser (Vario Elementar Analysensyteme, Hanau, Germany). Total element concentrations of P, S, K, Ca and Mg were measured using ICP-AES (Spectro Analytic Instruments, Kleve, Germany) after pressure digestion of samples in 65% HNO_3 for 12 h (Heinrichs et al., 1986). Soil samples (2.5g sieved material) were percolated with 1M NH_4Cl and exchangeable cations (Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Al^{3+}) were analyzed by AAS 300 AA (Varian Inc., Darmstadt, Germany). The pH-values were measured with a digital pH-meter (WTW GmbH, Wesel, Germany) in 0.1 M KCl. N and P uptake was calculated as N (total 2005) – N (total 2004).

To analyse root architecture, roots were washed with deionised water and scanned with calibrated colour optical scanner with lighting system optimized for roots (STD1600+ System). Data were analysed with the *WinRhizo* software (Régent Instruments Inc., Québec, Canada). The *WinRhizo* software calculated the cumulative

length, surface area and volume of complete roots for defined diameter classes (<0.2 mm- fine roots, and >2 mm coarse roots) as well as the average diameter (average of the all roots), surface area (calculate on the basis of the TIFF file after the root scanning) and root density calculated by relating the total root length to the soil volume of 9.77 dm^3 per column.

5.1.3.4 Endomycorrhiza observations

The observations of endomycorrhizae are based on staining with trypan-blue in lacto-phenol in host roots (Phillips and Hayman, 1970). Roots were first treated in hot 2.5% KOH on 90°C for 60 min, which removes the host cytoplasm and then the nuclei. After 3- times washing in demineralised water, roots were additionally treated with 3% H_2O_2 and 3% NH_4OH for 30 min. Afterwards decolourised roots were washed and stained in lacto-phenol for 2 min on object-glass under a microscope. The percentage of the endomycorrhiza was counted under microscope at a magnification of 10x40 folds. Three slides each with five to six randomly selected stained roots (approximately 1 cm long root) were prepared from each individual tree sample. Stained roots are counted by the gridline intersection method (Giovannetti and Mosse 1980), where roots were randomly disperse under microscope with grid lines objective and follow all horizontal and vertical lines intersects with mycorrhiza were counted. A total of 100 intersects per sampling site were examined with a compound microscope (Axioplan, Zeiss, Germany) recording the presence or absence endomycorrhiza.

5.1.3.5 Ectomycorrhiza observation

Directly after harvest the degree of ectomycorrhiza was determined and photographs were taken by a binocular.The 100 root tips randomly chosen from each plant were counted and the percentage of ectomycorrhiza was calculated.

5.1.3.6 Identification of mycorrhiza community composition

Root tips were pooled and frozen at -80°C up to isolation. After grinding root tips in liquid nitrogen, 0.1 g material was used according to the manufacturer's instructions for DNA kit for plants and fungi (DNeasy, Qiagen, Hilden, Germany).

Extracts were diluted 1:50 before addition to the PCR mix. The PCR reaction consisted of: PCR buffer (100 mM Tris, pH 8.8, 500 mM KCl, 1% Triton-X 100), containing 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 200 nM of primers ITS1 and ITS4, 0.04U Taq-polymerase, and 1 µl of template DNA. The PCR reaction was performed by following thermocycling program: 94°C for 5 min (1 cycle); 94°C for 1 min, 55°C 1 min, 72°C 1 min (35 cycles), followed by 72°C 10 min and stored at 4°C. Amplified products of the expected size were ligated into the pGEM-T vector (Promega, Madison, WI, USA). Ligation mixtures were transformed into electro-competent Escherichia coli Top 10 F' (Invitrogen, Groningen, Netherland) and transformants were verified by standard procedures. Plasmids were prepared and used as starting material for DNA sequencing using the dideoxy chain-termination method, employing the BigDye Terminator Kit (Applied Biosystems).

From each sample all white colonies were taken and prepared for sequencing. To check for the size and presence of the insert, PCR was done with ITS1 and ITS4 (White et al., 1990), and clones with inserts of expected size were sequenced. All clone sequences were compared to sequences present in GenBank using the Blast program at the NCBI homepage (http://www.ncbi.nlm.nih.gov/) to get a putative identification. Analised sequences were grouped according to similarity by alignments using Clustal W (software package Bioedit).

5.1.3.7 Statistical analyses

Data for plant growth and biomass are means (± SD) of 10 and 5 seedlings, respectively. Four plants were harvested for mycorrhiza observation, another three replicates for DNA isolation and three for root scanning. Statistical analyses of the data were performed using analysis of variance (ANOVA test), (program Statgraphics 2.1). Differences of $P \leq 0.05$ were considered as significant.

5.1.4 Results

5.1.4.1 Plant performance

At the beginning of the experiment young seedlings of DFG after preculture in hydroponics had produced higher biomass than DFV (Table5). After planting considerable differences were found between the growth patterns of the two different varieties of Douglas fir as well in different types of the soils (Fig. 1 a, b). Both varieties had the highest growth increment in Solling soil, and lowest Unterlüss limed soil (Fig. 1 a, b). Differences between limed and non-limed soils were generally small. However, DFV showed significantly larger shoot growth than DFG (Fig. 1 a, b).

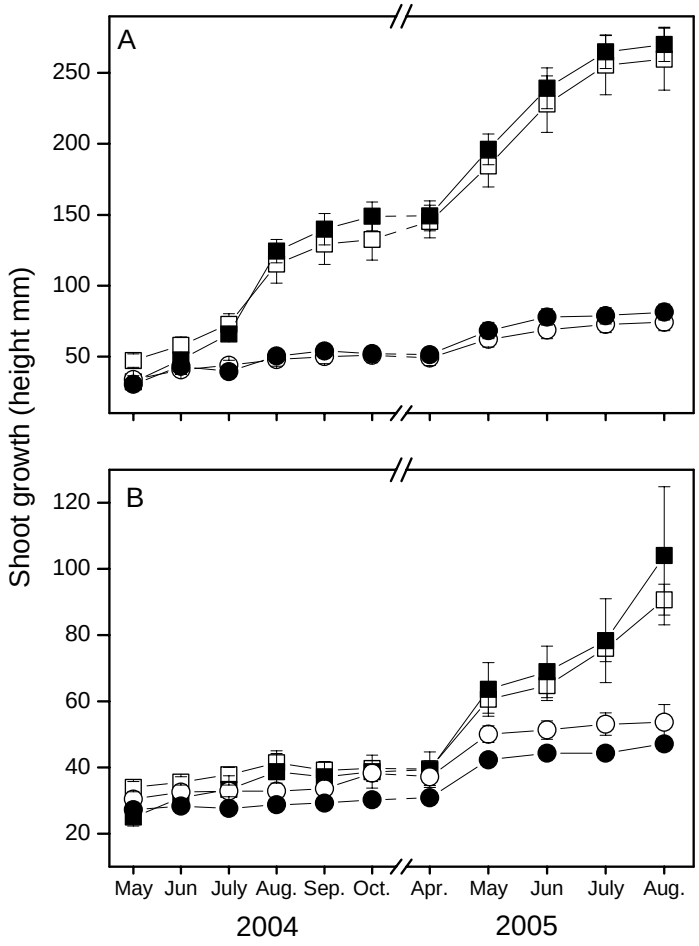

Figure1. Shoot growth Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (A) and variety *glauca* (B) during two years in non-treated soil from Solling (closed square marks), limed soil from Solling (open square marks), in non-treated soil from Unterlüss (closed circle marks), and limed soil from Unterlüss (open circle marks). n=10 (±SE).

Larger shoot growth corresponded also to larger biomass production in DFV in Solling soil (Table 5). After two seasons the dry mass of DFV from Solling soil was 5–9 times higher than that of DFG (Table 5).

Table 5. Total biomass (dry weight) of Douglas fir (*Pseudotsuga menziesii*) seedlings variety *viridis* and variety *glauca* during two years seasons 2004-2005. n=3-6 (±SD).

Dry weight		May-04		Dec-04		Sep-05	
(mg/plant)		Mean	SD	Mean	SD	Mean	SD
viridis	Solling	15.0 a	1.7	472.7 c	213.6	4561.2 b	2942.6
	Solling limed			288.0 abc	212.6	5443.3 b	1710.9
	Unterlüss			408.0 bc	171.1	1302.3 a	309.4
	Unterlüss limed			200.7 ab	144	2342.7 a	545.9
glauca	Solling	30.3 a	18.1	77.7 a	18.8	887.8 a	481.9
	Solling limed			118.7 a	26.1	1068.0 a	587.3
	Unterlüss			241.0 abc	85.1	791.2 a	100.1
	Unterlüss limed			125.0 a	2.6	489.7 a	397.5

When seedlings of both: DFG and DFV, were grown on Unterlüss soil no significant differences in plant biomass were found (Fig. 2a, b). In general root/shoot ratios are higher in seedlings grown on Unterlüss soil than those of seedlings in Solling soil (Fig. 2).

A pronounced difference between varieties was found only for plants grown on non-treated Unterlüss soils, where DFV had the highest root/shoot ratio. Fine root biomass of DFV was 7-10 fold higher in Solling soil in comparison with Unterlüss, depending on liming (app Table 3). Liming stimulated fine root production in Solling soil (app Table 3).

Root morphology of DFG was not affected by the experimental treatment (Table 6) but total length and surface area were generally higher in the poor Unterlüss soil than in nutrient rich Solling soil. DFV developed almost double root system in the rich soil than in poor soil (Table 6). Liming positively affected root development, especially in DFV grown in Solling soil.

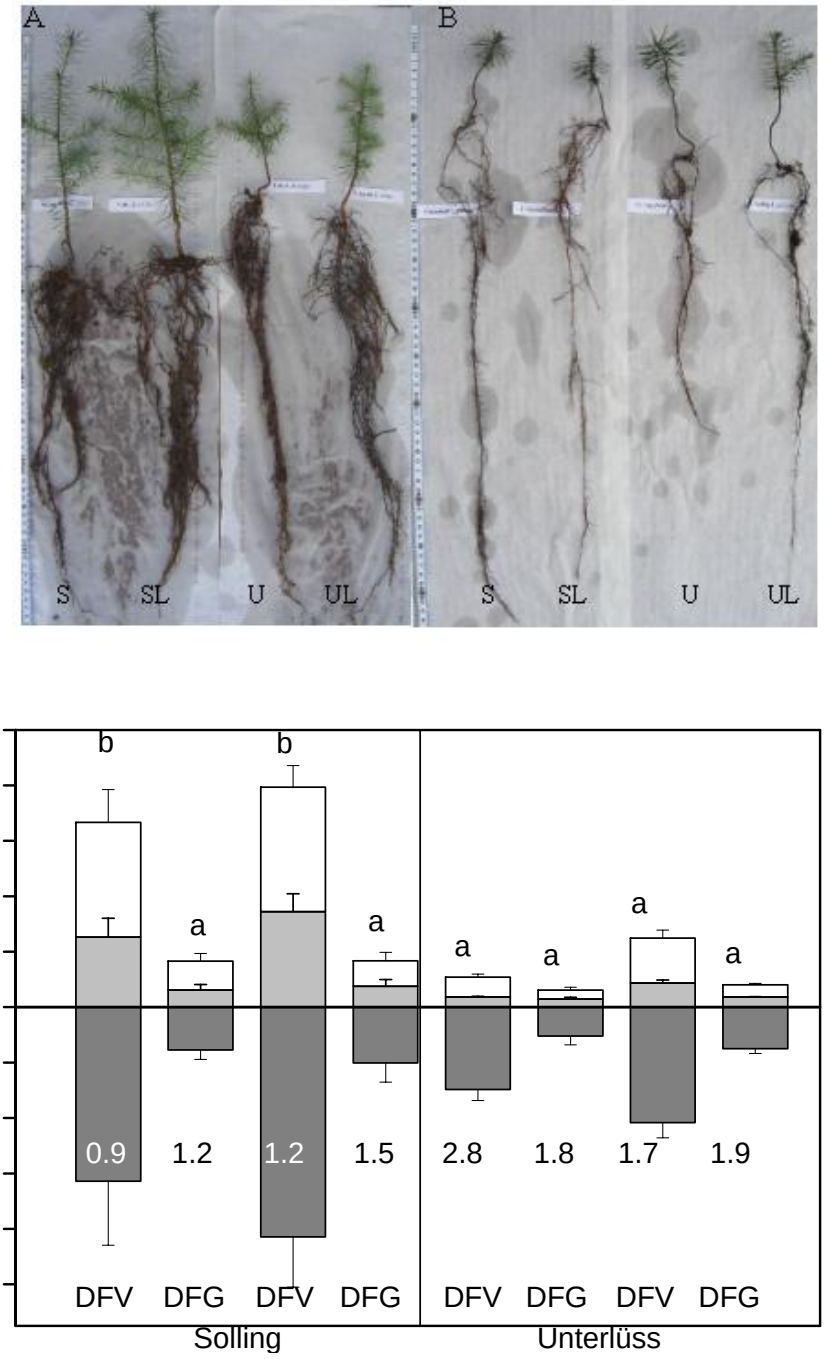

Figure 2. Two-years-old Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (A) and variety *glauca* (B) growing in two different soil types Solling (S) and Unterlüss (U) with different pH-values, after liming (L) **(a)**. Biomass of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after two growing season in two different soil types Solling and Unterlüss with different pH- values, after liming. Bars indicate means of fresh mass of needles (white), stem (hatched) and root (grey), respectively Numbers show root/shoot ratio based on fresh weight. n=5 (±SD) **(b)**.

Table 6. Root morphology: total length, surface area, diameter average, total length per volume and total root volume of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (DFV)and variety *glauca* (DFG) after 2 growing season on different types of soils Solling and Unterlüss. n=4 (±SE).

		Total Length (cm)	Surface Area (cm^2)	Diameter Average (cm)	Total Length per Soil Volume (m/m^3)	Total Root Volume (dm^3)
DFV Solling	Mean	1569 bc	400 c	0.27 b	1605.6 bc	8.10 b
	± SE	177	46	0.02	181.1	0.96
DFV Solling limed	Mean	2594 c	746 d	0.46 c	2654.5 c	17.13 c
	± SE	584	161	0.06	597.6	3.55
DFG Solling	Mean	298 a	62 a	0.07 a	305.0 a	1.01 a
	± SE	59	11	0	60.4	0.17
DFG Solling limed	Mean	333 a	72 a	0.09 a	340.8 a	1.23 a
	± SE	45	9	0.02	46.0	0.15
DFV Unterlüss	Mean	813 ab	182 ab	0.12 a	832.0 ab	3.25 ab
	± SE	244	54	0.03	249.7	0.95
DFV Unterlüss limed	Mean	1134 ab	252 ab	0.19 ab	1160.5 ab	4.46 ab
	± SE	342	75	0.03	350.0	1.3
DFG Unterlüss	Mean	344 a	72 a	0.07 a	352.0 a	1.19 a
	± SE	35	8	0	35.8	0.15
DFG Unterlüss limed	Mean	440 a	86 a	0.12 a	450.3 a	1.35 a
	± SE	61	12	0.02	62.4	0.2

5.1.4.2 Characterisation of mycorrhizal abundance and community composition

Soil origin and Douglas fir varieties strongly influenced mycorrhiza community. Ectomycorrhiza showed significant differences in abundance on roots in the two soils and in the two varieties (Table 7). The highest degree of ectomycorrhization was in non-limed Solling soil, and DFV had almost 3-times higher mycorrhization rates than DFG (Table 7). In limed soils ectomycorrhiza decreased 1.4-fold (Table 7). In Unterlüss soil, degree of ectomycorrhiza was very low in both varieties (0-1%) (Table7).

Table 7. Percentage of ecto- and endomycorrhiza in roots of Douglas fir (Pseudotsuga menziesii) seedlings variety viridis (DFV) and variety glauca (DFG), respectively. The roots were analyses after 2 growing seasons in soil of Solling and Unterlüss.

	% ectomycorrhiza		% endomycorrhiza	
	mean	SD	mean	SD
DFV Solling	33 e	8	5 a	4
DFV Solling limed	23 d	8	10 ab	7
DFV Unterlüss	1 ab	1	15 bc	11
DFV Unterlüss limed	0 a	0	14 abc	7
DFG Solling	13 c	2	21 c	11
DFG Solling limed	8 bc	0	19 bc	11
DFG Unterlüss	1 ab	0	14 abc	2
DFG Unterlüss limed	0 a	0	15 bc	6

Analysis of endomycorrhization patterns showed the opposite trend, i.e., DFG roots were more colonised in Solling soil than DFV and in Unterlüss soil the degree of colonisation was comparable (Table 7).

Typical ectomycorrhizal root tips from different soils, associated with DFV and DFG are shown in Figure 4. According to visible inspection the types of mycorrhiza were obviously different with respect to soil and variety. Comparison with published images suggests that DFV built mycorrhiza with *Rhizopogon vinicolor* (Agerer, 1987-2002, Massicotte et al., 1993, Molina and Trappe, 1994) and DFG with *Tuber maculatum* (Parladé et al., 1996) or *Wilcoxina mikolae* (Agerer, 1987-2002; Massicotte *et al*., 1993, Molina and Trappe, 1994) (Fig. 4a, c) and DFG with *Tuber maculatum* (Parladé *et al*., 1996) (Fig. 4d).

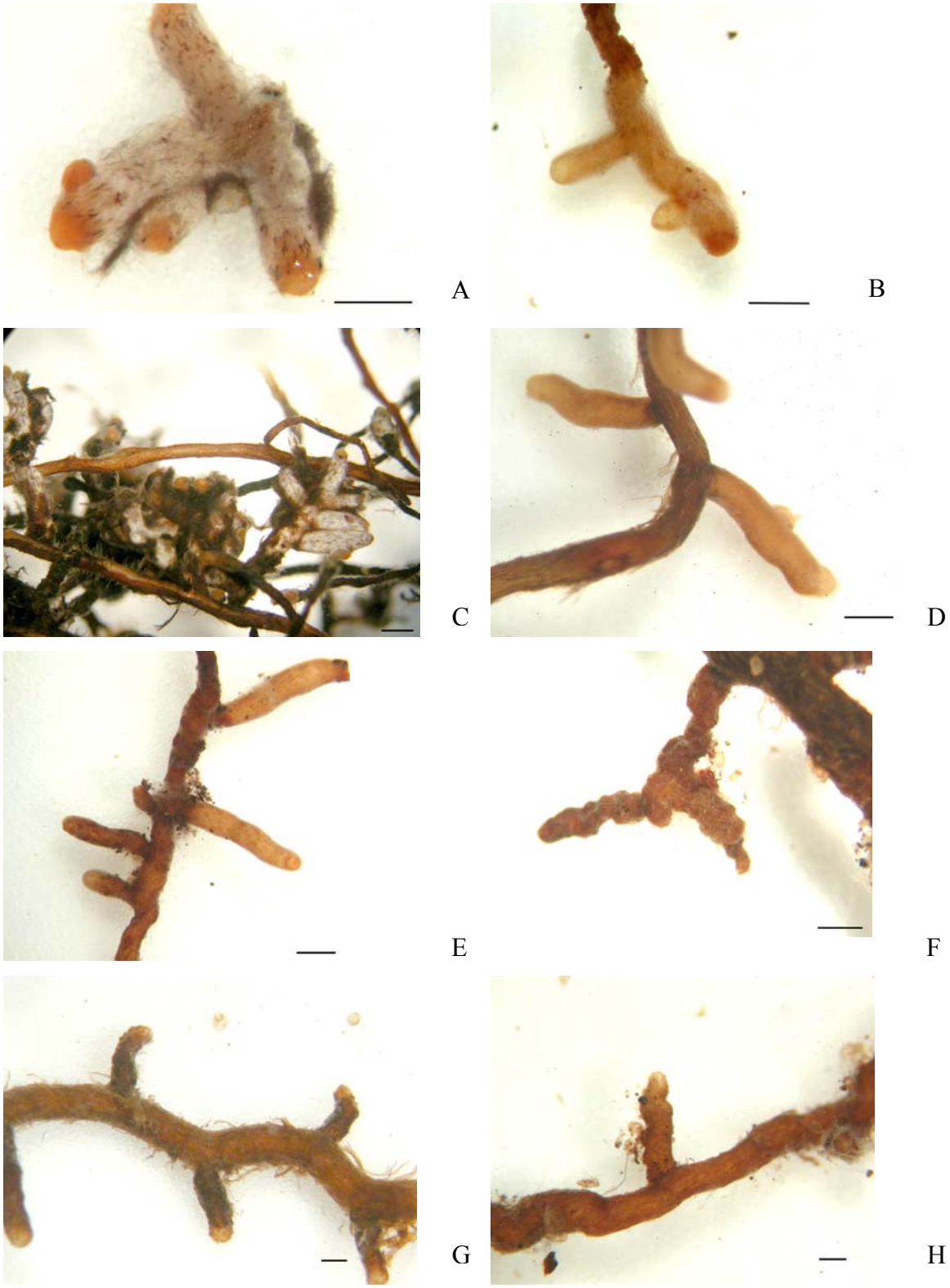

Figure 4. Typical ectomycorrhiza found on roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (DFV) and variety *glauca* (DFG). Solling soil- A: DFV, B: DFG; Solling limed soil- C: DFV, D: DFG; Unterlüss- E: DFV, F: DFG, Unterlüss limed soil- G: DFV, H: DFG. Bar indicate 1 mm.

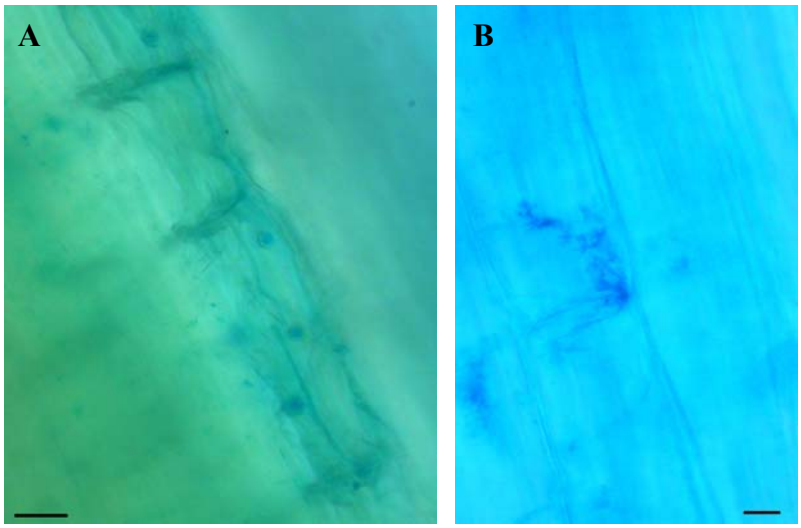

Figure 5. Typical endomycorrhiza (vesicular-arbuscular mycorrhiza) found in roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (DFV) and variety *glauca* (DFG). Vesicular endomycorrhiza type on DFV (A) and arbuscular endomycorrhiza type on DFG (B). Bar indicate 10 µm.

Figure 5 shows typical endomycorrhizae found in both varieties of Douglas fir, i.e. a typical vesicular endomycorrhiza (Fig. 5a), and a typical arbuscular endomycorrhiza (Fig. 5b). Arbuscular endomycorrhiza were found in roots from all treatments.

To identify ectomycorrhizal community we employed molecular methods using the ITS1 and ITS4 primers. Based on sequence similarity, the fungal sequences were sorted (Table 8). In a total of 153 analysed clones, 29 showed 99% nucleotide homology with published fungal sequences, indicating that identification was achieved at the space level. 103 clones showed similarity between 95 and 98% (identification on genus level) and 21 clones displayed homology <95% with published sequences (identification at family or ordinal level) (Table 8). It was possible to distinguish fungi typical for one type of soil or with preferences for one type Douglas fir variety. We found that *Rhizopogon vinicolor* was connected to the variety DFV, while *Cadophora finlandia Sebacinaceous, Tricholoma* sp. and uncultured *Tuber sp.* were more associated with DFG. On the other hand mycorrhizal basidiomycote isolate T01, *Tricholoma* sp., *Sebacinaceous* and group of unknown mycorrhizal fungi were specifically found in roots from plants in soil from Solling, whereas *Wilcoxina mikolae* and some of uncultured ascomycete in soil from Unterlüss. Between 26 all represented fungi species, 20 are able to build ectomycorrhiza (Table 8).

Table 8. Sequences of mycorrhizal roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* growing 2 season on different types of soils: Unterlüss (U) and Solling (S) without and with liming (l), by homology searching using blast in ***NBCI Sequence*** data base and by comparing sequences. Colours indicate % of the identity: hatched- 90-95%, grey- 95-99%, and dark grey- 99%, respectively. Every number in colour field represents number of analysed clones. Every number in colour field represents number of analysed clones. Plants replicates n=3.

Fungi group	**Identity**	EM	Blast match	DFV				DFG			
				S	S L	U	U L	S	S l	U	U L
Ascomycetes	*Hymenoscyphus ericae*		AJ430179	1							
			AJ308337					1			
		+	AJ430149				2	1	3		2
	Phialophora finlandia	+	AF486119				2				5
	Phialocephala fortinii		AY078131	1		2	1				1
	Uncultured ascomycete clone NS126	+	DQ069004	2				1			
	grass root mycorrhizal sp. PPO-2	+	AY599236				1				
	Uncultured mycorrhizal ascomycete	+	AB211141						1		
	Ascomycete sp. olrim884		AY805635				1				
	Tuber	+	AY634113						2		
	Cadophora finlandia	+	DQ069045						2		4
Basidiomycetes	*Tricholoma sp.*	+	AY254876					1	1		
	Sebacinaceous ectomycorrhiza	+	AY093436					7			
			AY093438					6			
	Russula puiggarii	+	AY667425			1					
	Heterochaete hirneoloides		AF291283			1					
	Wilcoxina mikolae	+	AY219841			1	11			4	6
	Tomentella sublilacina	+	AJ889976	2	1	1		3	1		
	Helotiaceae isolate AS2		DQ093762				1				
	mycorrhizal basidiomycote isolate T01	+	AB089818	18	3				3		
	Uncultured Atheliaceae		AM181408	1							
	Tylospora asterophora	+	AF052558			3			1	1	1
	Rhizopogon vinicolor	+	AF263933	1	7	2					
	Laccaria lacata	+	AJ699073		4	4				14	
	Uncultured basidiomycote		AY969895		1						
Unknown fungi	Uncultured ectomycorrhizal fungus clone 986/7	+	DQ233900	1							
	Mycorrhizal fungal sp. pkc21	+	AY394902		1						
	Uncultured ectomycorrhizal fungus clone 879/18	+	DQ233886					2			
	Uncultured fungus isolate SM19B1		DQ093748						2		
	% of analysed clones			87	84	75	78	75	80	86	86
	Number of species			8	6	8	7	6	9	3	6

From total 26 different species, 8 were found in associations with DFV and 9 with DFG (Table 8). Liming influenced the increase number of species in case of DFG. Only three different species was found in case of DFG from Unterlüss soil (Table 3). The abundance of fungal species extracted from roots of non-mycorrhizal or weakly colonised plants was surprising, and indicates that the inoculum is present but cannot be established under certain soil conditions or with some hosts.

5.1.4.3 Role of mycorrhiza and soil for nutrition of Douglas fir

To determine whole plant nutrition, element concentrations were determined in needles, stem and roots (appendix Table 5). Needle concentrations of N, P and K as major nutrients, which may be modulated by mycorrhization, are given in Table 9. The concentration of Mn was also considered because of the special interest in Mn-toxicity in Douglas fir (Schöne, 1992). DFG had significantly higher concentrations of all these elements, especially in Solling soil (Table 9), despite much lower growth rates (Fig. 1).

Table 9. Element concentrations in needles of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* (DFV) and variety *glauca* (DFG) after 2 growing seasonon soils from Solling and Unterlüss. n=6 (±SD).

Plant and soil	mg/gDW	N	P	K	Mn
DFV Solling	Average	9.33 a	0.47 a	4.43 cd	1.98 c
	± SD	1.48	0.08	0.69	0.13
DFV Solling limed	Average	11.21 a	0.85 c	5.41 de	1.15 b
	± SD	2.46	0.16	0.53	0.38
DFG Solling	Average	18.71 c	1.29 d	8.39 f	2.29 c
	± SD	2.52	0.16	1.99	0.65
DFG Solling limed	Average	21.38 c	1.22 d	6.10 e	1.02 b
	± SD	2.05	0.33	1.00	0.98
DFV Unterlüss	Average	7.76 a	0.77 bc	2.36 a	0.10 a
	± SD	3.01	0.25	0.87	0.01
DFV Unterlüss limed	Average	15.08 b	0.60 ab	3.21 ab	0.21 a
	± SD	3.59	0.01	0.79	0.05
DFG Unterlüss	Average	19.12 c	0.99 c	4.17 cd	0.15 a
	± SD	1.42	0.15	1.14	0.03
DFG Unterlüss limed	Average	20.72 c	1.30 d	3.98 bc	0.20 a
	± SD	3.47	0.07	0.72	0.02

Generally, Mn concentrations were 5 to 11-fold lower in needles of seedlings from Unterlüss soil than in those from Solling soil. After liming the Mn concentrations in seedlings grown in Solling soil decreased significantly (Table 9). Mn concentrations in the needles of the seedlings from Unterlüss soil were significantly lower than in those in Solling soil, but without any effect of plant variety or liming.

Correlation analysis between the mycorrhization rate and root length showed significant relationships with ectomycorrhiza (Fig. 3a and appendix Table 4). On the other hand, the degree of endomycorrhiza positively correlated with shoot/root ratio (Fig. 3b and appendix Table 4). To find out whether relations existed between biomass production and mycorrhization, net primary production of the second year was correlated with mycorrhizal rate (Fig. 6a and appendix Table 6).

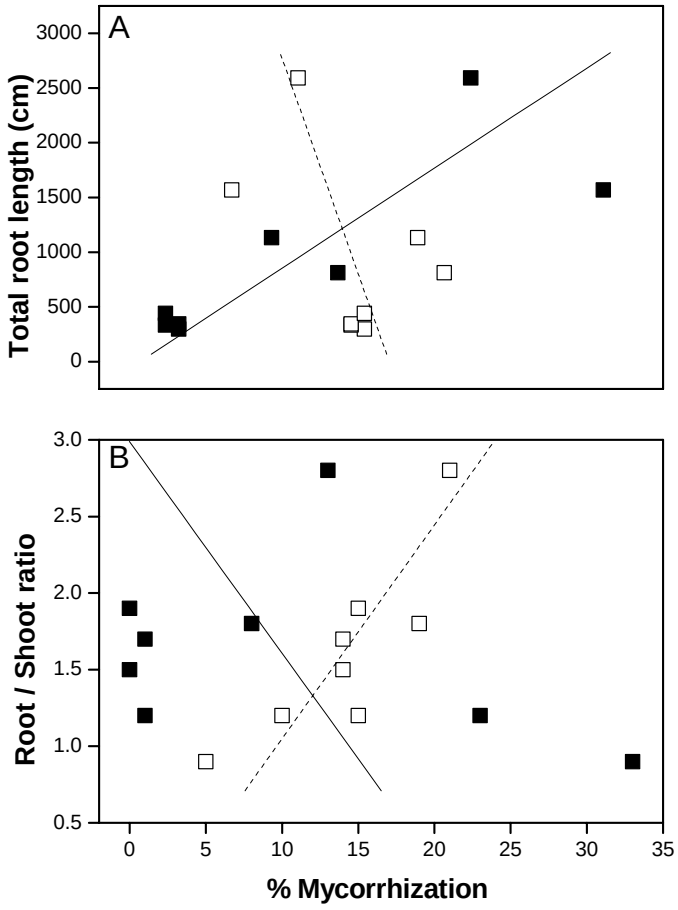

Figure 3. Correlation of root morphology A: total root length and B: root/shoot ratio with the abundance of ectomycorrhiza (closed squares) and endomycorrhiza (open squares) in seedlings of *Pseudotsuga menziesii*, grown for 2 seasons in two different soils, Solling and Unterlüss.

A significant positive correlation was found between net primary production and the degree of ectomycorrhiza, and a negative trend with endomycorrhization degree (Fig. 6 and Appendix Table 6). Ratios N/C and P/C were positively correlated with the degree of endomycorrhization. Mn concentration in needles correlate with abundance of ectomycorrhiza (Fig. 6 and Appendix Table 6).

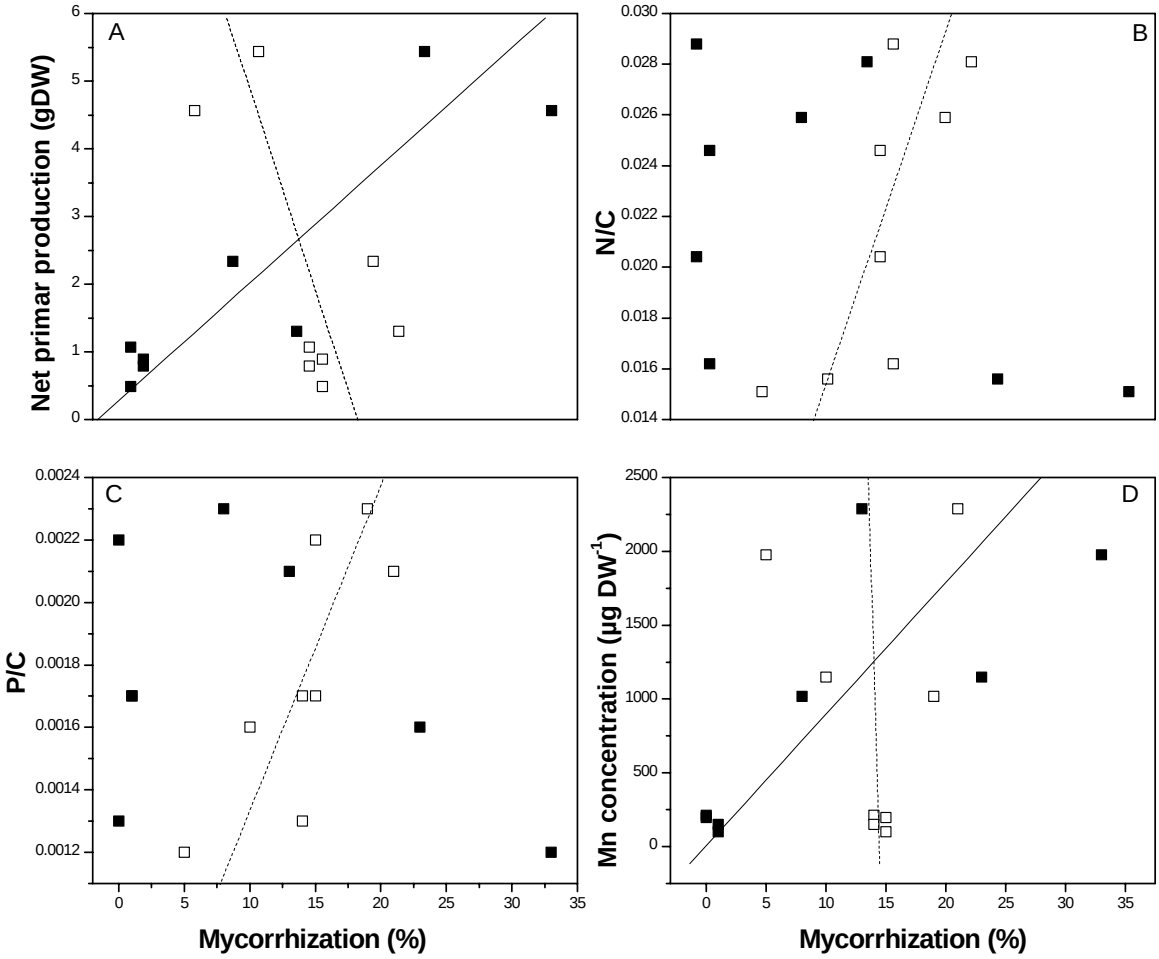

Figure 6. Correlation between plant net primary production (A), the nitrogen assimilation- ratio N/C- (B), phosphorus assimilation- P/C (C) and Mn concentrations in needles (D), with the abundance of ectomycorrhiza (closed squares) and endomycorrhiza (open squares).

5.1.5 Discussion

5.1.5.1 Interaction between plant performances, soil and mycorrhiza

Studies on the distribution of mycorrhizal fungi and identity are important to understand the plant-fungus-soil interaction. This work was carried out to determine the colonization of Douglas fir with mycorrhizal fungi in natural forests soils with different pH.

Abundance of ectomycorrhiza was positively correlated with plant biomass production and root development (Tabs. 5, 6) suggesting a stimulating influence of the mycorrhization. Root colonization by mycorrhizae fungi is a dynamic process influenced by the placement and density of their propagation in soil, soil properties such as soil type and fertility, climatic factors such as temperature and moisture, host factors such as species and root longevity and the fungal species (Amijee et al., 1989; Smith and Read, 1997). A possible reason for low percentage of root colonization with endomycorrhiza in Solling soil and with ectomycorrhiza in Unterlüss soil could be the competition by the presence of both fungal types on Douglas fir roots. However, this would only be a reason if a rich soil favoured ecto- and a poor soil endomycorrhiza formation. Lapeyrie and Chilvers (1985) and Founoune et al. (2002) found that in roots of *Acacia* and *Eucalyptus* spp. both fungal symbionts could coexist without competition. However, this area has only received little attention to date.

5.1.5.2 Mycorrhiza community

To our knowledge this is first analysis of the mycorrhizal community composition on two varieties of Douglas fir. The most of identified mycorrhiza species have been reported before to form symbiosis with Douglas fir or with pine (Brunus et al., 1995; Parladé et al., 1999a; Horton et al., 2005; De Roman et al., 2005). *Rhizopogon vinicolor* was often found to form symbiosis with Douglas fir (Castellano and Trappe, 1985, Massicotte et al., 1999, Parladé et al., 1999b). Here we show that this fungus was specifically interacted with DFV and not with DFG. *Cadophora finlandia* was more abundant on DFG, and this, fungus together with *Hymenoscyphus ericae* were found to

build mycorrhiza with *Pinus sylvestris* in Scotland (Villarreal-Ruiz et al., 2004). Apparently, typical pine associated mycorrhiza species are able to build mycorrhiza with Douglas fir (Table 8).

Laccaria laccata is often found in symbiosis with Douglas fir (Mortier et al., 1988, Duponnois and Garbaye, 1991, Frey-Klett et al. 1997) and occurred here on DFV grown in limed Solling soil. *Tricholoma* and *Sebacionoid* have been reported in association with Douglas fir from Coast Range of Oregon (Horton et al., 2005).

Tomentella sublilacina, which was specific for rich soil from Solling, was also found as a symbiont of Douglas fir in natural habits in USA (Cline et al., 2005). Both, *Tomentella sublilacina*. and *Russula* were also found in natural pine forest as the most abundant colonizers of bishop pine (Brunus et al., 1995).

Wilcoxina mikolae identified in roots of both varieties of Douglas fir in soil from Unterlüss, is also typical for pine wood species (Baar et al., 1998). *Wilcoxina* was found also as an associate to Douglas fir from British Columbia (Hagerman and Durall, 2004). *Tuber maculatum* associated with Douglas fir was found in north Spain (Parladé et al., 1996). *Lactarius deliciosus*, *Rhizopogon* spp., and *Suillus luteus* showed the highest host specificity in tests with *P. menziesii* seedlings in pure culture syntheses experiments (Parladé et al., 1995). Douglas fir was able to form ectomycorrhizae with 23 fungal isolates from 18 species collected in northern Spain (Parladé et al., 1995).

In forests dominated by Douglas fir in southern Oregon (western North America), over 200 morphologically distinct ectomycorrhiza were recorded in 198 soil samples taken over an area of about 2.1 ha (Luoma et al., 1997).

Ectomycorrhizae, which dominate in young stands, are usually present in low numbers in older stands (Visser and Danielson, 1990). Some of the species from the genera *Rhizopogon, Lactarius, Russula, Sebacinoid or Suillus* are dominant in different age-classes of Douglas fir stands, as well as on different sites, e.g. natural habit of habits of Douglas fir in Oregon (Horton et al., 2005), Vancouver Island (Goodman and Trofymow, 1998) or as an introduced species in Spain (Pera et al., 1999; Parladé et al., 1995). Seedlings planted in forests with minimal soil disturbance tend to form mycorrhizae with the dominant ectomycorrhizal fungi (Danielson and Pruden, 1990; Simard et al., 1997). The plant competition for soil resources may be influenced by common mycorrhizal

networks that distribute soil nutrients (Allen 1984, Simard et al., 1997, Horton et al., 2005).

Differences in ectomycorrhizal fungal abundance and species composition were found among successional Douglas fir age-classes (Smith et al., 2003). Comparison of communities of ectomycorrhizal fungi in old-growth, mature stands of Douglas fir on Vancouver Island showed that total richness was around 100 species, and the most dominant species were *Rhizopogon vinicolor*, an unidentified *Piloderma*-like species, *Lactarius rubrilacteus*, and *Piloderma fallax* (Goodman and Trofymov, 1997, Outerbridge and Trofymov, 2004). *Rhizopogon vinicolor* was also found also in the present study but only in symbiosis with DFV (Table 8).

5.1.5.3 Nutrient effect

In relation to nutrient assimilation, we found significant positive correlations of the degree of ectomycorrhiza with C. Higher biomass production also caused higher uptake of N, P and K (appendix, Table 5). In contrast to ectomycorrhiza, endomycorrhiza showed correlations with the N/C and P/C ratios, but not with net assimilation of individual elements (Fig. 6).

The nutrient uptake of trees dominating temperate forest ecosystems is strongly influenced by symbiosis with mycorrhizal fungi (Harley and Smith, 1983). The beneficial effects of mycorrhizae on plant growth have often been related to the increase in the uptake of immobile nutrients, especially N and P, and in other directions towards to fungi C-compounds move (Gray et al, 1995; Smith and Read, 1997). We found increased root production in DFV, which might have been the reason for higher degree of ectomycorrhiza (Table 6, 7). DFG had generally slower growth than DFV (Figs. 1, 2), and the lower percentage of ectomycorrhiza was a probably reason for lower uptake of elements like N and P (Appendix Table 5). Fogel and Hunt (1983) investigated contributions of mycorrhizae and soil fungi to nutrient cycling. They found in a Douglas fir ecosystem that returns of N, P, and K to soil by mycorrhizae was four to five times higher than that of roots. This mutual role of mycorrhiza needs to be taken into consideration. Furthermore, the more than 2000 mycorrhizal species associated with Douglas fir (Trappe, 1977), may differ in carbon demand and ability to absorb nutrients (Jones and Last, 1991). For

example carbon-sink stimulation in Douglas fir seedlings after inoculation with *Rhizopogon vinicolor* increased rate of photosynthesis, while *Hebeloma crustuliniforme* and *Laccaria laccata* had no effect (Dosskey et al., 1990).

Mycorrhiza also plays roles in protection from heavy metals (Jentschke and Godbold, 2000, Schützendübel and Polle 2002). Mn is an essential nutrient, but may become toxic on acidic soils (Foy, 1984). In Solling soil, Mn is the second the highest exchangeable cation after Al (Berthold et al., 2006). The concentration of Mn differs in the needles of two varieties of Douglas fir (Table 9). Mn was significantly higher concentrated in DFV, than in DFG in Solling soil (Table 9). Rich soil with low pH increases Mn-uptake. Interesting relations are found between Mn plant content and degree of both type of mycorrhization- a significant positive correlation with ectomycorrhiza and significant negative with endomycorrhiza (Fig. 6, Appendix Table 6). Samuel (1926) noted that the roots of oats grown in soils with low levels of available Mn had more vesicular – arbuscular mycorrhiza (VAM). McGee (1987) found that VAM ameliorated the reduction of plant growth by Mn, even though mycorrhizal development was decreased. We found a negative trend between Mn concentrations in seedlings and the degree of endomycorrhiza, and significantly positive correlation between ectomycorrhiza and Mn concentration (Fig. 6 D and appendix Table 6).

Figure 7. Fruit body of *Laccaria laccata* found in soil from Solling and Unterlüss.

The effect of liming positively affected biomass development as well absorption of N and P. Andersson et al. (1997) found that ^{15}N uptake decreased after lime treatment in both, non- and mycorrhized *Pinus sylvestris* plants. Our experiment showed higher total N uptake in both soils planted with DFV (Appendix Table 5).

Limed soils apparently show better conditions for *Laccaria laccata*, which was connected to DFV (Table 8). Fruit bodies of *Laccaria laccata* (Fig. 7) were also found in pots planted with DFG. *Sebacinaceous* species was detected in non-limed Solling soil with DFG.

The success of the introduction of exotic conifers, such as Douglas fir, for reforestation depends of their compatibility with native mycorrhizal fungi (Trappe, 1977; Marx, 1980; Álvarez et al, 1993). We found that Douglas fir made more abundant ectomycorrhiza in the rich soil and endomycorrhiza in the poor soil. Usual rates of ectomycorrhiza colonisation on Douglas fir were in a range from 30 to 45% in green house studies and ranged in field from around 45 to 60% (Teste et al., 2004).

It has been suggested that the diversity of plants at a site is influenced by the diversity of mycorrhizal fungi in the soil (van der Heijden. et al, 1998). The present results shows that endo- and ectomycorrhiza colonization was differed in two varieties of Douglas fir and in different soils and that these conditions led to specific fungi communities on roots. We suggest that mycorrhiza directly influence plant biomass production. This is important in forest management and introduction of neophyte like Douglas fir.

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5.1.7 Appendixes

Table 1. Plant biomass (fresh (FW) and dry weight (DW)) of two months old Douglas fir (*Pseudotsuga menziesii*) seedlings, variety *viridis* and variety *glauca*. n=3 (±SD).

Plant biomass (mg)		FW		DW	
May 2004		**Mean**	**SD**	**Mean**	**SD**
	root	15.67	6.51	2.33	1.53
viridis	stem	13.67	2.08	2.33	0.58
	needles	38.33	10.21	10.33	2.08
	root	20.67	2.31	2.00	1.00
glauca	stem	23.33	7.51	6.00	4.00
	needles	51.33	13.84	22.33	8.88

Table 2. Plant biomass (fresh (FW) and dry weight (DW)) of nine months old Douglas fir (*Pseudotsuga menziesii*) seedlings, variety *viridis* and variety *glauca* grown on soils from Solling and Unterlüss. n=3 (±SD).

Plant biomass (g) December 2004		FW		DW	
		Mean	**SD**	**Mean**	**SD**
Solling *viridis*	Root	0.959	0.605	0.244	0.124
	Stem	0.184	0.090	0.082	0.040
	Needles	0.388	0.198	0.147	0.077
Solling limed *viridis*	Root	0.538	0.437	0.126	0.098
	Stem	0.156	0.106	0.065	0.044
	Needles	0.260	0.193	0.097	0.072
Unterlüss *viridis*	Root	0.783	0.298	0.207	0.072
	Stem	0.138	0.035	0.061	0.016
	Needles	0.349	0.228	0.140	0.085
Unterlüss limed *viridis*	Root	0.314	0.218	0.074	0.049
	Stem	0.105	0.042	0.048	0.023
	Needles	0.190	0.195	0.078	0.079
Solling *glauca*	Root	0.091	0.050	0.026	0.016
	Stem	0.068	0.013	0.026	0.007
	Needles	0.072	0.022	0.026	0.008
Solling limed *glauca*	Root	0.162	0.055	0.040	0.012
	Stem	0.086	0.017	0.039	0.007
	Needles	0.104	0.023	0.040	0.010
Unterlüss *glauca*	Root	0.550	0.253	0.146	0.063
	Stem	0.091	0.009	0.039	0.003
	Needles	0.129	0.059	0.056	0.024
Unterlüss limed *glauca*	Root	0.264	0.014	0.065	0.002
	Stem	0.070	0.016	0.029	0.006
	Needles	0.058	0.006	0.031	0.003

Table 3. Plant biomass (fresh and dry weight) of one and half years old Douglas fir (*Pseudotsuga menziesii*) seedlings, variety *viridis* and variety *glauca* grown on soils from Solling and Unterlüss. n=6 (±SD).

Plant biomass (g)		FW		DW	
September 2005		**Mean**	**SD**	**Mean**	**SD**
Solling *viridis*	Fine roots	2.73	2.40	0.61	0.50
	Coarse roots	3.55	2.76	1.14	0.85
	Stem	2.53	1.51	1.07	0.67
	Needles 1-year old	2.08	1.15	0.85	0.52
	Needles 2-year old	2.05	1.51	0.88	0.70
Solling limed *viridis*	Fine roots	4.18	2.57	0.95	0.57
	Coarse roots	4.10	1.47	1.35	0.43
	Stem	3.44	1.44	1.47	0.63
	Needles 1-year old	1.97	0.88	0.72	0.32
	Needles 2-year old	2.53	0.87	0.95	0.33
Unterlüss *viridis*	Fine roots	1.77	0.49	0.43	0.12
	Coarse roots	1.20	0.41	0.39	0.12
	Stem	0.36	0.08	0.16	0.03
	Needles 1-year old	0.30	0.18	0.13	0.07
	Needles 2-year old	0.43	0.06	0.19	0.03
Unterlüss limed *viridis*	Fine roots	2.13	0.69	0.53	0.15
	Coarse roots	2.04	0.54	0.69	0.18
	Stem	0.87	0.24	0.38	0.11
	Needles 1-year old	0.62	0.33	0.28	0.15
	Needles 2-year old	1.00	0.32	0.46	0.15
Solling *glauca*	Fine roots	0.40	0.21	0.08	0.04
	Coarse roots	1.16	0.56	0.30	0.15
	Stem	0.61	0.44	0.20	0.15
	Needles 1-year old	0.56	0.28	0.19	0.11
	Needles 2-year old	0.50	0.32	0.11	0.09
Solling limed *glauca*	Fine roots	0.40	0.30	0.07	0.05
	Coarse roots	1.61	1.24	0.41	0.30
	Stem	0.75	0.53	0.26	0.14
	Needles 1-year old	0.43	0.27	0.16	0.10
	Needles 2-year old	0.49	0.39	0.17	0.13
Unterlüss *glauca*	Fine roots	0.46	0.30	0.13	0.09
	Coarse roots	0.58	0.39	0.18	0.13
	Stem	0.29	0.13	0.13	0.05
	Needles 1-year old	0.06	0.02	0.03	0.01
	Needles 2-year old	0.26	0.22	0.12	0.09
Unterlüss limed *glauca*	Fine roots	0.65	0.26	0.18	0.05
	Coarse roots	0.84	0.15	0.26	0.05
	Stem	0.36	0.03	0.15	0.01
	Needles 1-year old	0.09	0.03	0.04	0.01
	Needles 2-year old	0.36	0.09	0.16	0.03

Table 4. Linear Regression between abundance of ecto and endo- mycorrhiza and root total length, and root/shoot ratio based on fresh weight of plants after 1.5 years growing of Douglas fir (*Pseudotsuga menziesii*on soils from Solling and Unterlüss. Regression equation y=a+b·x.

Linear Regression for mycorrhiza and root morphology								
	% Ectomycorrhiza				% Endomycorrhiza			
	a	**b**	**R**	***P***	**a**	**b**	**R**	***P***
Total Length (cm)	-1.94	0.01	0.82	0.01	16.87	0.00	-0.48	0.24
Root/Shoot ratio	21.66	-7.25	-0.34	0.41	2.47	7.17	0.84	0.01

Table 5. Total content of C, N, P and Mn (mg) in the needles, stems fine- and coarse- roots of Douglas fir (*Pseudotsuga menziesii*) variety *viridis* and variety *glauca* after 1.5 years of growing on different types of soils Solling and Unterlüss. n=6 (±SD).

Seedlings	Soil	Plant tissue		C (mg)		N (mg)		P (mg)		Mn (mg)	
				Solling	Unterlüss	Solling	Unterlüss	Solling	Unterlüss	Solling	Unterlüss
viridis	without liming	Needles	Mean	811.9 b	199.8 a	15.6 cd	3.9 a	0.8 c	0.3 a	3.41 b	0.04 a
			± SE	239.1	42.7	4.2	0.7	0.2	0.1	1.02	0.01
		Stem	Mean	500.0 b	74.8 a	3.2 b	0.4 a	0.5 b	0.1 a	0.62 b	0.01 a
			± SE	143.4	6.2	0.8	0.1	0.2	0	0.17	0.00
		Fine roots	Mean	260.3 cd	182.3 bc	6.8 cd	4.0 abc	0.5 b	0.5 bc	0.31 b	0.01 a
			± SE	93	23.3	2.3	0.6	0.2	0.1	0.12	0.00
		Coarse roots	Mean	514.0 cd	175.0 ab	6.3 bc	2.2 a	0.7 bc	0.3 a	0.48 c	0.01 a
			± SE	167.8	27.4	2	0.3	0.2	0.1	0.16	0.00
		Whole plant	Mean	2086.1 b	631.8 a	31.9 cd	10.5 ab	2.5 b	1.1 a	4.82 b	0.06 a
			± SE	597.9	46.9	8.7	1	0.8	0.2	1.41	0.01
	limed soil	Needles	Mean	793.8 b	361.0 a	17.9 d	11.4 bc	1.4 d	0.4 ab	2.13 b	0.15 a
			± SE	129.2	47	2.8	2	0.2	0.1	0.55	0.02
		Stem	Mean	710.4 b	181.1 a	5.8 c	1.9 ab	0.9 c	0.2 a	0.53 b	0.03 a
			± SE	137.5	23.5	1.6	0.3	0.2	0	0.21	0.00
		Fine roots	Mean	356.0 d	217.8 c	8.7 d	5.1 bc	0.9 d	0.5 bc	0.21 b	0.02 a
			± SE	78.4	27.4	2	0.8	0.2	0.1	0.03	0.00
		Coarse roots	Mean	646.3 d	314.8 bc	7.0 c	3.9 ab	0.9 c	0.4 ab	0.30 b	0.03 a
			± SE	106.4	35.8	1.2	0.5	0.2	0	0.11	0.00
		Whole plant	Mean	2506.5 b	1074.7 a	39.5 d	22.2 bc	4.1 c	1.4 ab	3.18 b	0.23 a
			± SE	385.1	113	6.6	2.9	0.6	0.2	0.74	0.03
glauca	without liming	Needles	Mean	148.3 a	71.5 a	5.7 ab	2.9 a	0.4 a	0.1 a	0.69 a	0.02 a
			± SE	38	20.8	1.6	0.8	0.1	0	0.20	0.01
		Stem	Mean	96.7 a	87.6 a	2.1 ab	1.8 ab	0.2 a	0.2 a	0.12 a	0.01 a
			± SE	31.2	27.5	0.7	0.6	0.1	0.1	0.04	0.00
		Fine roots	Mean	32.6 a	44.2 ab	1.5 a	1.3 a	0.1 a	0.1 a	0.05 a	0.00 a
			± SE	7.7	14.2	0.4	0.4	0	0	0.01	0.00
		Coarse roots	Mean	138.8 ab	80.4 a	2.7 a	1.4 a	0.3 a	0.1 a	0.13 ab	0.01 a
			± SE	32	27.6	0.6	0.4	0.1	0	0.03	0.00
		Whole plant	Mean	416.4 a	283.7 a	12.1 ab	7.4 a	1.0 a	0.6 a	1.00 a	0.04 a
			± SE	101.8	75.3	2.9	1.9	0.2	0.2	0.27	0.01
	limed soil	Needles	Mean	160.4 a	99.7 a	6.6 ab	4.2 a	0.4 ab	0.3 a	0.50 a	0.04 a
			± SE	49.1	7	1.8	0.3	0.1	0	0.29	0.00
		Stem	Mean	122.9 a	72.2 a	2.6 b	1.9 ab	0.3 ab	0.2 a	0.06 a	0.01 a
			± SE	31.6	2.3	0.5	0.2	0.1	0	0.03	0.00
		Fine roots	Mean	33.4 ab	59.4 ab	1.4 ab	1.8 ab	0.1 a	0.2 ab	0.02 a	0.01 a
			± SE	9.9	8.6	0.4	0.2	0	0	0.01	0.00
		Coarse roots	Mean	191.0 ab	113.5 ab	3.1 a	2.4 a	0.4 ab	0.2 a	0.11 ab	0.01 a
			± SE	64	10.1	1	0.3	0.2	0	0.04	0.00
		Whole plant	Mean	496.5 a	344.8 a	13.2 ab	10.3 ab	1.2 a	0.9 a	0.69 a	0.06 a
			± SE	125.7	20.5	3.2	0.7	0.3	0.1	0.36	0.00

Table 6. Linear Regression between net element assimilation (C, N, P, K, Mn) and net primary production (NPP) in 1.5 years old seedlings of Douglas fir (*Pseudotsuga menziesii*) grown on soils from Solling and Unterlüss. and abundance of ecto and endo- mycorrhiza n=8.

Linear regression for mycorrhiza and element assimilation								
	Ectomycorrhiza				Endomycorrhiza			
	a	**b**	**R**	***P***	**a**	**b**	**R**	***P***
NPP	-2.60	5.91	0.90	0.00	17.77	-1.73	-0.65	0.08
N/C ratio	34.07	-1107.77	-0.51	0.19	-0.22	656.85	0.76	0.03
P/C ratio	30.77	-11855.45	-0.39	0.34	-2.82	9611.65	0.79	0.02
Mn	-0.07	0.01	0.79	0.02	14.44	0.00	-0.06	0.88

APPENDIX I

I 1. Manganese intracellular transport, distribution and homeostasis

*From**: Manganese and copper toxicity and detoxification in plants. Dučić T, Polle A** Brazilian Journal of Plant Physiology **2005.** 172: 115-122.*

One important task of cell metabolism is to supply proteins with correct metal cofactors needed for their activity and moreover to deliver these cofactors at the right time and to the right site of the target protein. At the same time it is required to avoid possible toxic reactions of the metals. It is, therefore, crucial that among the many different metals accumulated by cells, only the correct ion is presented to the metalloproteins (Luk et al., 2003a). Thus, when metals enter a cell, they are delivered to one of several possible pathways, depending on physiological needs. These routes are usually are called ‘‘metal trafficking pathways’’.

The molecular basis for the transport of manganese across membranes in plant cells is poorly understood. IRT1, a member of the ZIP family, identified in *Arabidopsis thaliana* is a broad-range metal ion transporter. It can complement a mutant *Saccharomyces cerevisiae* strain defective in high-affinity manganese uptake (smf1 Δ) (Korshunova et al., 1999). The IRT1 protein has previously been identified as an iron transporter, but later it was demonstrated that IRT1, when expressed in yeast, can transport manganese as well. This manganese uptake activity was inhibited by cadmium, iron(II) and zinc, suggesting that IRT1 can transport these metals. However, IRT1 did not complement a copper uptake-deficient yeast mutant (ctr1), implying that this transporter is not involved in the uptake of copper in plant cells (Korshunova et al., 1999) (see Introduction Fig.1).

Recently, Lopez-Millan and colleagues (2004) identified new metal transporters in the legume *Medicago truncatula* with have high similarities to the ZIP family (MtZIP). Six proteins, predicted from cDNA sequences, all contained eight transmembrane domains and the highly conserved ZIP signature motif which functions as metal transporter. When

MtZIPs were transformed into appropriate metal-uptake defective yeast mutants and grown on metal-limited media, MtZIP4 and MtZIP7 proteins restored yeast growth on Mn-limited media, whereas other complemented growth on Zn and Fe-limited media. In Mn-deficient plants, the transcript levels of MtZIP3 and MtZIP4 were down-regulated. MtZIP5 expression was up-regulated under Mn-limiting conditions in leaves, but in roots appeared to be down-regulated under Mn-deficient and toxicity conditions. The expression of MtZIP6 and MtZIP7 was unaffected by the metal supply. Future work will show the role of these proteins in the regulation/execution of plant metal homeostasis (Lopez-Millan et al., 2004).

One of the transporter for manganese in cells is SMF1, a member of the Nramp family comprising divalent metal transporters (Cellier et al., 1995). Since SMF1 seems to be a high affinity transporter activated under manganese starvation, under normal physiological conditions other high-affinity transporter(s) must be operating (Luk et al., 2003a; Luk et al., 2003b). *S. cerevisiae* PHO84 is a well-known transporter for the high-affinity uptake of phosphate; recently, a role for this protein in manganese transport has been uncovered (Luk et al., 2003a; Luk et al., 2003b). Yeast cells lacking PHO84 exhibited resistance to manganese toxicity accumulating only low Mn-levels. It is quite possible that phosphate transporters also contribute to manganese uptake in other organisms, particularly under conditions of manganese toxicity. Luk and co-workers (2003 a, b) hypothesized that in yeast PHO84 can transport phosphate in the form of $MnHPO_4$ (see Introduction Fig.1). Our own data show a tight correlation between phosphor and manganese in “black bodies” which are formed in roots of Douglas fir under Mn-stress (see Chapter 3).

One way to prevent toxic effect of heavy metals is efflux. To facilitate manganese efflux from the cell, the metal is delivered into the Golgi apparatus and ultimately exported from the cell via secretory pathway vesicles that carry the metal to the cell surface. P-type ATPase, known as PMR1 (transporters for both calcium and manganese) pump manganese into the secretory pathway (Rudolph et al., 1989; Durr et al., 1998). Yeast cells lacking the PMR1 transporter are extremely sensitive to manganese and accumulate high concentrations of the metal, presumably in the cytosol (Lapinskas et al., 1995).

Another way to prevent metal toxicity is compartmentalization. Several transporters can potentially mediate transport of metals and compartmentation. These include the heavy

metal ATPases (HMAs), the Nramps, the cation diffusion facilitator (CDF) family, the ZIP family, and the cation antiporters (Hall and Williams, 2003). For example, among multiple Ca^{2+} pumps and Ca^{2+}/H^{+} antiporters in *Arabidopsis*, ECA1, which pumps Ca^{2+} and Mn^{2+} into the endoplasmic reticulum, is required to support plant growth under conditions of Ca^{2+} deficiency or Mn^{2+} toxicity (Zhongyi et al., 2002) (see Introduction Fig. 1).

The main storage compartment for toxic compounds in plants is the vacuole (Vögeli-Lange and Wagner, 1990). Mutants of vacuolar ATPases exhibited manganese sensitivity (Ramsay and Gadd, 1997). In plants and fungi, vacuolar transporters help to remove potentially toxic cations from the cytosol. Metal/H^{+} antiporters are involved in metal sequestration into the vacuole. The *Arabidopsis thaliana* cation exchangers, CAX2 is the only characterized CAX-transporter capable of vacuolar Mn^{2+} transport when expressed in yeast (Shigaki et al., 2003) (see Introduction Fig. 1). The subdomain analysis identified 3 amino acid regions responsible for Mn^{2+} specificity in CAX2 (Shigaki et al., 2003). Tobacco plants overexpressing *CAX2* accumulated more Ca^{2+}, Cd^{2+}, and Mn^{2+} than wild type plants and were more tolerant to elevated Mn^{2+} levels. Expression of CAX2 in tobacco increased Cd^{2+} and Mn^{2+} transport in isolated root tonoplast vesicles (Hirschi et al., 2000).

The ABC-transporter superfamily is one of the largest transporter families, and members can be found in bacteria, fungi, plants and animals. The first reports on plant ABC transporters showed that they are implicated in detoxification processes (Martinoia et al., 2002). The recent completion of the genomic sequencing of *Arabidopsis thaliana* showed that Arabidopsis contains more than 100 ABC-type proteins; 53 genes code for so-called full-size transporters. Work on the cyanobacterium *Synechocystis* sp. PCC 6803 suggests possible roles for ABC transporters in Mn^{2+} transport (Bartsevich and Pakrasi, 1995). Photosynthesis activity and growth rates were restored in photosynthesis-deficient mutants by the addition of Mn (Bartsevich and Pakrasi, 1995, 1996). Yamaguchi and co-workers (2002) using a DNA microarray, screened knockout libraries of His kinases and response regulators of *Synechocystis* sp. PCC 6803 to identify possible participants in the mechanisms for maintaining cytoplasmic Mn^{2+} ion homeostasis. They identified a His-kinase, ManS, which might sense the extracellular concentration of Mn^{2+} ions, and a response regulator, ManR, which might regulate the expression of the *mntCAB* operon for the ABC-type transporter of Mn^{2+} ions (Yamaguchi et al., 2002). It was suggested that

ManS produces a signal that activates ManR, which represses the expression of the *mntCAB* operon during normal Mn concentration in cell (Yamaguchi et al., 2002) (see Introduction Fig.1).

The intracellular trafficking of manganese in yeast was also highly dependent on SMF2, another member of the Nramp family (West et al., 1992). Yeast cells devoid of the SMF2 transporter exhibited deficiencies in invertase glycosylation and manganese SOD2 activity indicating cell-wide disturbance caused by inappropriate manganese trafficking (Luk and Cullota, 2001). One protein, a member of the mitochondrial carrier family (MCF) appeared to be specifically involved in the delivery of Mn to Mn-SOD2 and, thus was termed MTM1 (manganese trafficking factor for mitochondrial SOD2) (Luk et al., 2003b). MTM1 does not globally supply the mitochondria with manganese and does not behave like a classical membrane transporter for manganese, but rather delivers manganese specifically to SOD2. It was concluded that MTM1 is a Mn-chaperone (Luk et al., 2003a) (see Introduction Fig. 1).

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PUBLICATIONS

Tanja Dučić

(1) **Dučić T.**, Leinemann L., Finkeldey R. Polle A. (2006). Uptake and translocation of manganese in seedlings of two varieties of Douglas fir (*Pseudotsuga menziesii* var. *viridis* and *glauca*). *New Phytologist* **170**: 11-20.

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Curriculum vitae

Tanja Dučić

Persönliche Daten

Geburtsdatum:	23.08.1973
Geburtsort:	Užice (Jugoslawien, Serbien)
Nationalität:	Serbisch
Eltern:	Branka und Milić Dučić

Akademischer Werdegang:

1980-1988	Grundschule "Braća Ječmenica"
1988-1992	Gymnasium "M. Milovanović Lune"
1992-1998	Studium der Biochemie Diplom an der Universität Belgrad, Chemische Fakultät.
1998-2002	Masterstudium an der Universität Belgrad, Zentrum für Multidisziplinäre Studien. Abschluss: Master of Science (M. Sc.)
2000	Diplom am Alternativen Akademischen Ausbildungs- netzwerk, Belgrad, „Environment - Challenge for Science, Technology and Society."
2003-2006	Der Promotion an der Biologischen Fakultät, an der Georg-August-Universität, Göttingen

Berufserfahrung

1998 – 2002	Forschungsassistent am Zentrum für Multidisziplinäre Studien, Universität Belgrad, Serbien
2002-2006	Forschungsassistent an der Georg-August-Universität, Göttingen, Deutschland

Auszeichnungen

2001/2002	Postgraduelles Stipendium des World University Service - Austrian Committee, Belgrade Office
Derzeitige Position:	Mitarbeiterin am DFG-Projekt "Charakterisierung der Mangantoxizität in den Varietäten der Douglasie *Pseudotsuga menziesii"*an der Georg-August-Universität, Göttingen