

Effects of nitrogen fertilization on beech and Norway
spruce and on the preformed defences of their fine roots
against fungal pathogens

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Prof. Dr. Hans-Jakob Wirz

Dekan

This thesis is dedicated to my parents

ABSTRACT

The main objective of this thesis was to determine the effect of nitrogen fertilization on beech and Norway spruce and on the pre-formed defences of their fine roots to fungal pathogens. I investigated the influence of nitrogen fertilization on root parameters of beech and Norway spruce in field experiments. The plots were situated at three sites in Switzerland on acidic and calcareous soils. For nine years, the trees had been treated with dry ammonium nitrate resulting in 0, 10, 20, 40, 80 and 160 kg N ha⁻¹ year⁻¹, respectively.

Fine roots of beech and Norway spruce showed a significant decrease of fungistatic phenolics with increasing nitrogen fertilization. The *in vitro* growth of *Heterobasidion annosum* and *Cylindrocarpon destructans* was inhibited by the presence of most of the fungistatic phenolic compounds in the concentrations found in the roots.

In this thesis I also addressed questions about the effect of increased nitrogen fertilization on fine roots of beech growing on different soil conditions. Relative length of fine roots and the root tip density of beech were reduced by nitrogen fertilization on acidic soil but not on calcareous soil. The nutrient concentrations in beech leaves decreased due to increased nitrogen fertilization.

I suggest that increased nitrogen deposition may increase the susceptibility of trees to pathogens and may affect the water and nutrient uptake of trees.

Keywords:

Cylindrocarpon destructans, *Fagus sylvatica*, fine roots, foliage nutrients, *Heterobasidion annosum*, nitrogen fertilization, nitrogen, phenolic compounds, *Picea abies*, soil acidification.

ABBREVIATIONS

C4L	hydroxycinnamate: coenzyme A ligase
CA4H	cinnamic acid 4-hydroxylase
EMF	ectomycorrhizal fungi
GC	gas chromatography
HPLC	high-pressure liquid chromatography
HPLC-MS	high-pressure liquid chromatography - mass spectrometry
MCF	methanol-chloroform-formic acid
MS	mass spectroscopy
PAL	phenylalanine ammonialyase

LIST OF PUBLICATIONS

This thesis is based on the following articles and previously unpublished results. The articles are referred to in the text by their Roman numbers.

- I Tomova L., Braun S., and Flückiger W. 2005. The effect of nitrogen fertilization on fungistatic phenolic compounds in roots of beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* L. [Karst.]). Forest Pathology. Submitted for publication.

- II Tomova L., Braun S., and Flückiger W. 2005. Effect of nitrogen fertilization on fine root length, root tip density and mineral nutrients in leaves of beech (*Fagus sylvatica* L.) on acidic and calcareous soils. Plant and Soil. Submitted for publication.

Some unpublished results are also briefly presented in this thesis (**Appendix I** and **Appendix II**).

The author's contribution

- I Lila Tomova has performed the field and all of the experimental work, the calculation, and interpretation of the results. She is responsible for writing the manuscript.

- II Lila Tomova has performed the field and the fine root experimental work, calculation, and interpretation of the results. She is responsible for writing the manuscript.

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1 GENERAL INTRODUCTION

1.1 Nitrogen deposition

Nitrogen deposition from the atmosphere has strongly increased in Europe and other industrial regions world-wide since the middle of the 20th century. This is due to increased emissions of ammonia (NH₃) and nitrogen oxides (NO_x). Ammonia is volatilized primarily in regions with intensive agricultural systems. The massive use of nitrogen fertilization and, more importantly, high livestock densities result in enhanced emission of NH₃-N and thus in higher deposition rates of NH₄-N, predominantly as (NH₄)₂SO₄.

Nitrogen oxides originate mainly as a side product when fossil fuel is burnt in automobile engines and in industrial processes. This problem has been considerably reduced but not eliminated through the widespread use of “catalysers”, which convert the nitrogen oxides back into nitrogen and oxygen. Nitrogen oxides are deposited primarily in the form of NO and NO₂.

These anthropogenic processes have increased the input of biologically reactive forms of N into the global N cycle (Vitousek *et al.*, 1997). Compared with natural N fixation, these human-related activities have more than doubled the input of N into the global N cycle (Galloway *et al.*, 1995; Vitousek *et al.*, 1997).

Because of short- and long-range transport of these nitrogenous compounds, atmospheric nitrogen deposition has increased in many ecosystems across the world and has therefore become a major concern for the health of forests. Currently, the areas with high atmospheric nitrogen deposition are nowadays central and western Europe, eastern USA and, since the 1990s, Eastern Asia (Galloway and Cowling, 2002). The deposition of atmospheric N deposition in forest stands in Europe or the USA has reached values between 20-100 kg ha⁻¹ year⁻¹ have been reached, instead of the estimated background inputs of 1-3 kg N ha⁻¹ year⁻¹ in the early 1900s (Asman *et al.*, 1998; Fowler, 2002; Galloway, 1995).

The term “critical load” is widely used in Europe when acceptable levels of pollutants such as sulphur or nitrogen are discussed. The definition of critical load by Nilsson and Grennfelt

(1988) is used here: *A quantitative estimate of the exposure to one or more pollutants below which significant harmful effects on specified elements of the environment do not occur, according to present knowledge.* The critical load of forest for nitrogen deposition has been estimated to be 10-20 kg N ha⁻¹ year⁻¹ (Achermann and Bobbink, 2002). According to Kurz and Rihm (1997), estimated nitrogen deposition exceeds the critical loads in 90% of the Swiss forests.

Atmospheric inputs of excess nitrogen significantly affect fine roots through effects on the plant carbon allocation pattern, biomass, secondary defence chemicals, and the storage of carbohydrates (Vogt *et al.*, 1993). High nitrogen contents in the soil may negatively influence the fine root mycorrhizal symbiosis and thus tree vitality might be affected (Mohr, 1986; Schulze, 1994).

1.2 Fine roots

Fine roots are important structural and functional components of forest ecosystems (Grier *et al.*, 1981; Kolek and Kozinka, 1992; Persson, 1980; Waisel *et al.*, 1991). The definition of fine roots is based on diameter analyses. Usually, roots <2 mm in diameter are termed fine roots (Böhm, 1979). Such analyses show that the major part of the length of tree root systems consist of fine roots and that the quantity and activity of the small-diameter parts of the root systems are of prime significance regarding water and nutrient supply (Lyr and Hoffman, 1967). Lyr and Hoffman (1967) have shown in four tree species that fine roots account for approximately 86 to 99% of the total root length, although their contribution to root weight is only marginal. As another an example for an in-depth study with fine roots, Hendrick and Pregitzer (1993) showed that a large proportion of net primary production in sugar maple is going to roots <0.5 mm in diameter. These roots contribute a large proportion of the root biomass and are the physiologically most active part of the root system, responsible for water and nutrient uptake. Root length is a direct indicator of the potential for the nutrient and water uptake (Atkinson, 2000).

The contributions of fine roots to whole plant carbon budgets (Lambers, 1987) and to ecosystem level carbon and nitrogen cycles have gained wider appreciation (Jackson *et al.*,

1997). Fine root turnover has been estimated to account for as much as 33% of global annual net primary production (Jackson *et al.*, 1997). In trees, belowground net primary production may exceed 50% of total net primary production, with fine roots comprising a substantial part of total belowground net primary production (Nadelhoffer and Raich, 1992). Root turnover is related to precipitation and soil factors (Norby and Jackson, 2000). However, the difficulties associated with studying fine roots have resulted in a paucity of data for plant roots.

1.3 Fine roots and nitrogen deposition

Nitrogen deposition above the critical load may be considered a stress factor for plants. Plants respond to natural or simulated stress factors by changes in their above-ground parts and root systems. Effects of increased nitrogen deposition on leaves were one of the themes of this thesis. Experimental nitrogen addition to saplings of beech and Norway spruce in young stands on acidic and calcareous soils in afforestation plots in Switzerland induced nutrient imbalances and deficiencies. These changes were significant at added nitrogen loads of 10-20 kg N ha⁻¹ year⁻¹ after 4-6 years of nitrogen treatment (modelled atmospheric deposition 12-20 kg N ha⁻¹ year⁻¹). On acidic soil, nitrogen treatment led to acute Mg deficiency whereas on calcareous soil K and P were reduced (Flückiger and Braun, 1999).

A major emphasis of this thesis work was the effect on nitrogen fertilization on beech and Norway spruce fine roots. Quantitative measurements have been carried out already in previous work by other in order to assess the influence of increased nitrogen levels on fine roots (Majdi and Persson, 1995a, b, Olsthoorn *et al.*, 1991; Persson, 1980; Seith *et al.*, 1996). The most frequently used methods to measure the response of root systems include measurements of the fine root biomass, the vertical distribution of fine roots, the number of root tips and mycorrhizas, the number of live and dead root tips, the chemical composition of fine roots, changes in the uptake of nutrients, etc. (Böhm, 1979; Clemensson-Lindell, 1994; Smit *et al.*, 2000). Some of these methods were used in this study.

High nitrogen deposition is expected to produce long-lasting effects on fine-root systems and their function. In some ecosystems, nitrogen fertilization has negative effects on fine-root development, resulting in a 50% reduction of fine roots compared to controls (Persson and Ahlstrom, 1991). Forest trees with a reduced or damaged root system may be sensitive to

environmental stress (Persson, 1988). Nadelhoffer (2000) reported that with increasing nitrogen availability, fine-root biomass decreases and turnover increases. The ratio of NH_4^+ to NO_3^- might also influence the fine root morphology as was shown for beech by Paar (1994). Van Dijk *et al.* (1990) applied 0-48-480 kg N ha⁻¹ year⁻¹ (as $(\text{NH}_4)_2\text{SO}_4$) to young *Pinus sylvestris*, *Pinus nigra* and *Pseudotsuga menziesii* in a pot experiment. After seven months the coarse root biomass had not changed, but the fine root biomass decreased by 36% at the highest nitrogen application. In parallel, a 63% decrease in mycorrhizal infection at the highest nitrogen application was found. Matzner and Murach (1995) showed a negative relation between nitrogen deposition and soil acidification on the one hand and a reduce the fine root biomass and root length of trees. Seith *et al.* (1996) reported for Norway spruce trees a drastic decrease in root length per plant and also per gram root mass with high N availability. Olsthoorn *et al.* (1991) reported that high levels of NH_4^+ deposition (100 kg N ha⁻¹ year⁻¹) may have strong negative effects on root uptake capacity. In one nitrogen saturation study the fine root biomass and the number of root tips of *Pinus sylvestris* have increased after reduction of the present nitrogen deposition to pre-industrial levels, indicating a restricted root growth and nutrient uptake capacity at the ambient nitrogen load of ca. 40 kg N ha⁻¹ year⁻¹ (Boxman 1994, 1995).

Although a majority of studies reported negative effects, there are also reports on van Dijk *et al.*, 1990; Clemensson-Lindell and Persson, 1995, unchanged (Seith *et al.*, 1996), or even increased (Ahlström *et al.*, 1988) root production under high nitrogen deposition. This indicates that root responses to increased nitrogen deposition may depend strongly on the chemical and physical properties of the soil. Majdi and Persson (1995a) found an increased biomass of fine roots in spruce after addition of ammonium sulphate at a dose corresponding to 100 kg N ha⁻¹ year⁻¹ in the first and third year of application.

In forest trees such as beech and Norway spruce, most fine roots are associated with ectomycorrhizal fungi (EMF). Mycorrhizas play an indispensable role in the uptake of nutrients and water by trees. This symbiosis is an important means of nitrogen uptake by many plants, since these fungi secrete extracellular enzymes that break down organic material in the soil (Abuzinadah and Read 1989; Read 1991) and play a critical role in tree nutrition and carbon balance, supplying soil resources to their plant hosts in exchange for sugars (Smith and Read, 1997). Ectomycorrhizal trees dominate nitrogen-limited forest ecosystems (Smith

and Read, 1997). Increased nitrogen inputs might be responsible for the disturbance of mycorrhiza, leading to nutrient imbalances in the plants (Skeffington and Wilson, 1988). Termorshuizen *et al.* (1988) applied 0 to 400 kg N ha⁻¹ year⁻¹ either as ammonium or nitrate to young *Pinus sylvestris*, inoculated with *Paxillus involutus* in a pot experiment. Above application rates of 10 kg N ha⁻¹ year⁻¹, a decrease in the amount of mycorrhizal root tips and the number of sclerotia was found. Hofmann *et al.*, 1990 showed that increased contents of nitrogen in soil induced an apparently decreased frequency of mycorrhizal roots in the humus layer from 80% to 50%. Similarly, fertilization experiments have shown that an application of 3 × 100 kg N ha⁻¹ suppressed the frequency of mycorrhizas from 87% to 77% and the formation of fruiting bodies by more than 50% (Ritter, 1990). The number of fruit bodies and number of species of ectomycorrhizal fungi are lower in areas with high levels of nitrogenous air pollution (Arnolds, 1988). Arnebrandt (1994) found that the growth of external ectomycorrhizal mycelium was negatively affected by nitrogen additions. Reduced growth of mycorrhizal mycelium resulting from high nitrogen deposition is likely to reduce the potential of the fungus to take up minerals and water and transfer them to the tree. Boxman and Roelofs (1988) found that the uptake of cations like Mg²⁺, K⁺, and Ca²⁺ by mycorrhizal fungi growing in symbioses with pine seedlings decreased at high NH₄⁺ levels. Branderud (1995) found already after 1.5 year a decrease in fruit body production of mycorrhizal species at a nitrogen application of 35 kg N ha⁻¹ year⁻¹ (as NH₄NO₃) in a *Picea abies* stand.

1.4 Pollutants and soil acidification

Acidification caused by emissions of nitrogen and sulphur and associated adverse effects on forest ecosystems, surface waters and human health has been an issue on the political agenda for decades. The atmospheric reactions of NO_x and SO₂ lead to nitrous and sulphuric acid formation and to an increased inflow of anions and protons into soils, which can increase ion leaching from soils. Consequently, “acidic deposition” can be defined as the inflow of hydrogen ions from the atmosphere to forests. In addition, the reactions of sulphates and nitrates in soil are considered in studies that analyse the effects of acidic deposition on forests. “Soil acidification” is a general term that is used to cover harmful chemical changes in soils due to acidic deposition, but as a phenomenon soil acidification can also occur as a result of internal proton production in soil. According to Vanmechelen *et al.* (1997), 65% of european

forest topsoils are acidic ($\text{pH} (\text{CaCl}_2) \leq 4.5$) and more than 30% of soils are desaturated (base saturation $\leq 15\%$). De Vries *et al.* (2003) indicate that nitrogen depositions of 13 to 25 kg N $\text{ha}^{-1} \text{year}^{-1}$ lead to elevated NO_3 -leaching, decreasing base cation pools in soil, and increasing soil acidity. Many forest soils have become acidified and have high levels of both Al and NO_3 in the soil solution (Falkengren-Grerup and Eriksson, 1990). Soil acidification has accelerated also in Switzerland (Blasser *et al.*, 1999). The concern on acidification effects has led to the definition of critical loads for acid deposition which take into account the buffering capacity of the soil and the ecological effects (UN/ECE 2004). Acidification has been shown to affect tree roots (Matzner and Murach, 1995; Nadelhoffer, 2000).

1.5 Fine roots and soil acidification

Soils acidify naturally as they weather over thousands of years. The acidity of any soil varies according to the type of rock it comes from, the length of time it has weathered, and the local climate. Hence, some soils are naturally acidic while others are more alkaline. Agricultural practices can greatly increase the rate of acidification. Changes in soils that may occur in acidified soils include: decreased base saturation, increased availability of aluminium and manganese, and competition between base cations and Al for exchange sites in the fine roots (Robarge and Johnson, 1992). Majdi and Persson (1993) reported that the fine roots are considerably influenced by the degree of acidification. The effects of soil acidity depend on the investigated parameter. Total root biomass may be increased in acidic soils (Godbold *et al.*, 2003; Leuschner and Hertel, 2003). Godbold *et al.*, 2003 investigated the fine root distribution and turnover in ca. 40-year-old Norway spruce (*Picea abies* Karst.) stands, growing on four sites that differed in soil acidity. At one of the most acidic sites, fine root density in the humus layer was more than twice as high as at the least acidic site. Most of the roots and the root tips were found in the humus layer and in the first mineral soil horizon (0-10 cm). There was a significantly different decrease in specific root length ($\text{cm g}_{\text{DM}}^{-1}$) and specific root tip density (root tips $\text{g}_{\text{DM}}^{-1}$) in the more acidified stands. However, Leuschner *et al.* (2004) reported that beech fine root tip density (tips per soil volume), were much higher in acidic than in basic soils. Recently, it has been shown that subsoil acidity affects the distribution of fine roots of Norway spruce (Jentschke *et al.*, 2001), confirming ideas expressed in earlier work (Matzner and Murach, 1995).

The uptake and transport of several essential elements i.e. Ca, Mg, K and P may be adversely affected by Al. In acidic soils Al is soluble and acts as a phytotoxic agent and may be an important growth factor for forest trees (Asp and Berggren, 1990; Godbold *et al.*, 1988; Falkengren-Grerup and Eriksson, 1990; Foy, 1984; Summer *et al.*, 1991). Inhibition of root growth and changes in root morphology are the principal visible symptoms of the Al-toxicity (Godbold, 1994; Horst, 1995). It has been hypothesized that increased Al solubility at low pH could damage the roots of the trees and impair nutrient and water uptake (Ulrich, 1983).

1.6 Secondary plant metabolites

Plants are known to produce a large number of low molecular weight secondary compounds. Due to the remarkable development of analytical methodology; e.g., in gas chromatography (GC) and high-pressure liquid chromatography (HPLC) coupled to mass spectroscopy (MS), it has been possible to characterise even minor components in plants (Rhodes, 1994). Previously, most of these compounds were considered metabolic waste they did not seem to have any clear function in the organisms that produced them. However, more recently, such compounds have been shown to exhibit diverse biological functions (Waterman, 1992; Koes *et al.*, 1994; Strack, 1997).

Many secondary compounds are now known to be of ecological importance, not only due to their role as protectants from effects of UV-light and other abiotic factors (Waterman 1992), but also as a defence against herbivores and pathogens (Hartley and Jones, 1997; Alcobilla *et al.*, 1971; Raffa *et al.*, 1991). In contrast to primary metabolites, secondary compounds vary widely in their distribution among plant species (Rhodes, 1994). Some of these compounds occur only sporadically, whereas others are distributed widely throughout the plant kingdom (Harborne 1980; Rhodes, 1994). In addition, the intraspecific variation in plant secondary metabolite composition is often considerable (Harborne and Turner, 1984). Such variation may also occur at the level of an individual plant; i.e., secondary products are not found uniformly throughout the plant but are often limited to particular organs, and even to particular cells and tissues within that organ (Rhodes, 1994; Wiermann, 1981). Two widely distributed groups of secondary compounds that are present in all plants are phenolics and

terpenoids (Harborne, 1997). In the following, only the phenolic compounds will be addressed.

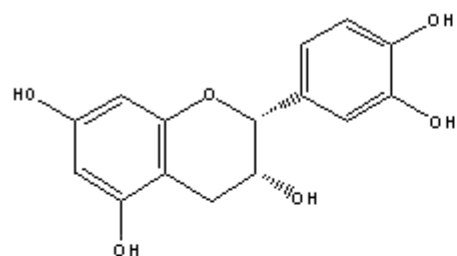
1.6.1 Phenolic compounds

A phenol is a chemical compound with at least one aromatic ring bearing one or more hydroxyl groups. Generally, these compounds occur as different derivatives formed by condensation or addition reactions, thus contributing to the wide variety of chemical compounds found in plants (Harborne, 1980; Strack, 1997).

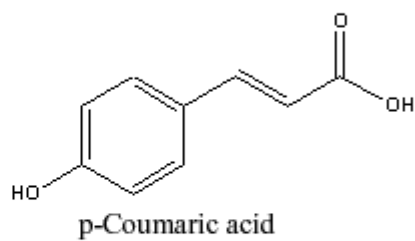
Phenolic compounds have many functions in plants. They are important in the defence mechanisms of plants under different environmental stress conditions. The chemical defence strategy used by trees against atmospheric stress (SO₂, ozone, UV-B) and herbivory includes the synthesis and accumulation of secondary metabolites, such as phenolic compounds (Julkunen-Tiitto *et al.*, 1996; Korolewski *et al.*, 1995; Lavola, 1998). Phenolics can provide resistance for plants against fungal, bacterial, and viral infections (Harborne, 1980).

The compounds of interest in the present thesis were 4-hydroxyacetophenone, piceatannol (3,3', 4,5'-tetrahydroxy-transstilbene), p-hydroxybenzoic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), p-coumaric acid (4-hydroxycinnamic acid), (-)-epicatechin and (+)-catechin in fine roots (Figure 1). These compounds were selected because of they occurred in beech and Norway spruce and of their proposed fungistatic effects.

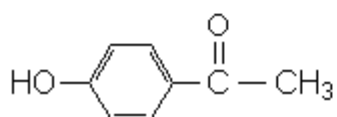
The phenolic compounds catechin and epicatechin found in the roots of beech and Norway spruce in the present thesis had been previously identified by Weiss *et al.* (1996) in mycorrhizal larch roots. Munzenberger *et al.* (1995) found catechin, epicatechin, 4-hydroxybenzoyl-glucose, 4-hydroxybenzoate, and additionally picein in mycorrhizal fine roots of larch.



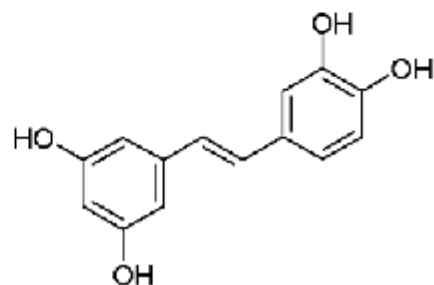
(-)-Epicatechin



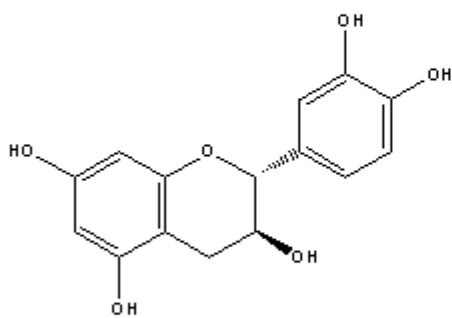
p-Coumaric acid



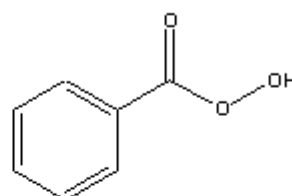
4'-Hydroxyacetophenone



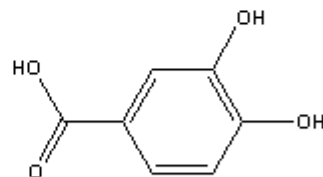
Piceatannol



(±)-Catechin



p-Hydroxybenzoic acid



Protocatechuic acid

Figure 1 Structure of phenolic compounds, used in the present thesis.

1.6.2 Biosynthesis of phenolic compounds in plants

The biosynthetic pathways of phenolic compounds in plants are quite well known (Harborne, 1988; Macheix *et al.*, 1990; Strack, 1997). The pathways are shown in Figure 2. Phenylalanine, produced as a primary metabolite in all plants via the shikimate pathway, is the common precursor for most phenolic compounds (Macheix *et al.*, 1990; Strack *et al.*, 1997). Similarly, hydroxycinnamic acids and particularly their coenzyme A esters, are common structural elements of phenolic compounds, such as cinnamate esters and amides, lignin, flavonoids, and condensed tannins (Macheix *et al.*, 1990). The phenylalanine/hydroxycinnamate pathway is defined as “general phenylpropanoid metabolism”. It includes reactions leading from L-phenylalanine to the hydroxycinnamates and their activated forms (Strack, 1997).

The enzymes catalysing the individual steps in general phenylpropanoid metabolism are phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (CA4H), and hydroxycinnamate: coenzyme A ligase (C4L). These three steps are necessary for the biosynthesis of most phenolic compounds (Macheix *et al.*, 1990; Strack, 1997).

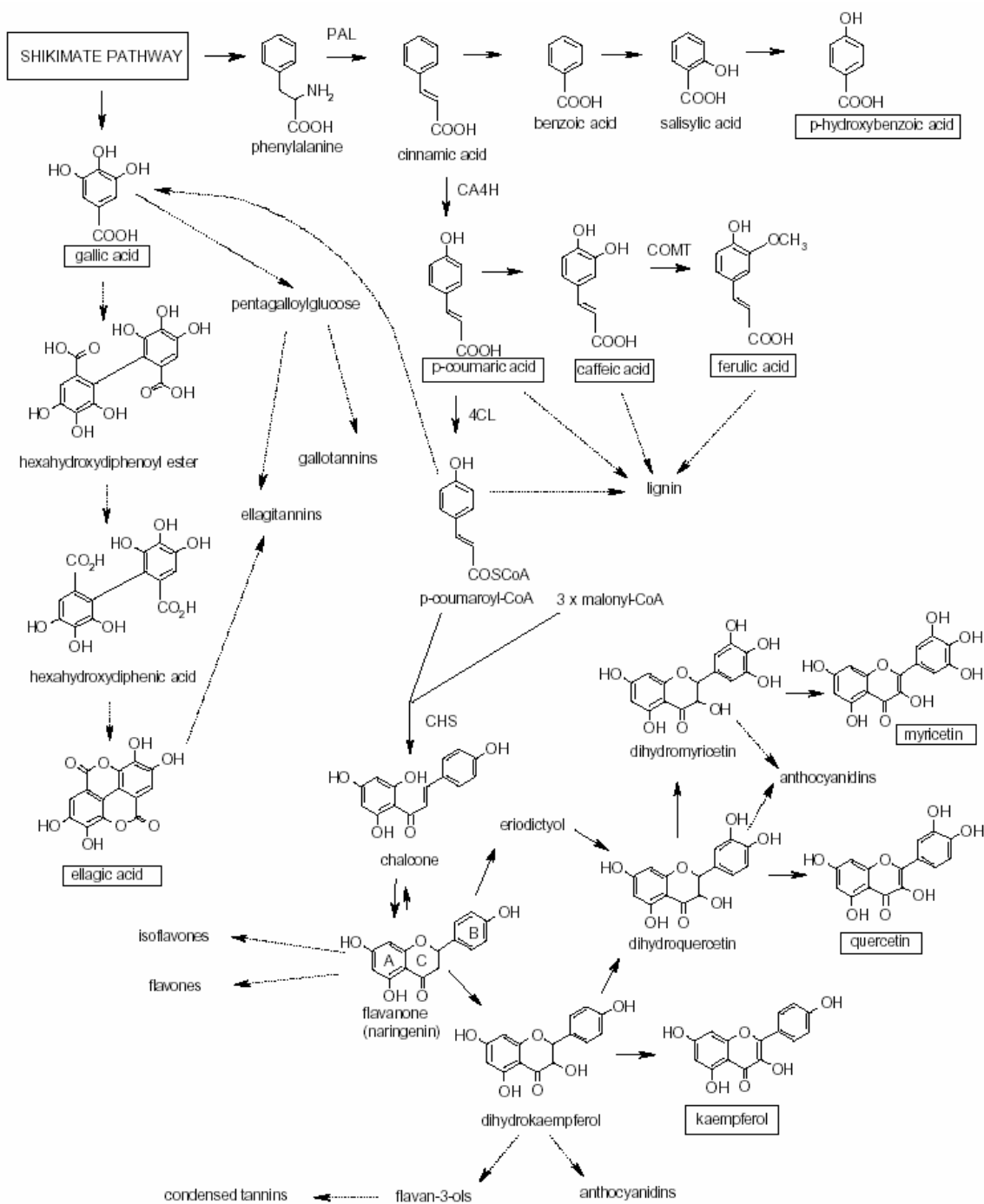


Figure 2 Biosynthesis of hydroxycinnamic acids, hydroxybenzoic acids and flavonoids (Harborne, 1988; Bennet and Wallsgrove, 1994; Strack, 1997). Solid arrows represent well-characterised reactions catalysed by single enzymes. Dashed lines represent transformations that require multiple enzymes that are less characterised, or vary among plant species. Enzymes: CA4H, CHS, 4CL, PAL.

1.6.3 Nitrogen fertilization and phenols

Beech leaves phenolic compounds be decreased by nitrogen fertilization (Balsberg-Påhlsson, 1989). Probably because of a higher demand for carbohydrates during absorption and assimilation of nitrogen, the phenolic concentration of birch leaves decreases (Tuomi *et al.*, 1990).

The composition and concentrations of phenolic compounds were studied from Keski-Saari and Julkunen-Tiitto (2003) in the first true leaves, cotyledons, stems and roots of seedlings of mountain birch. The differences in secondary compounds among these plant parts were both qualitative and quantitative. The seedlings were growth at three levels of nitrogen supply (very low-N 1.5 ppm; low-N 15 ppm and moderate-N 70 ppm) and the effect of nitrogen on concentrations of phenolic compounds was studied. The concentrations in roots were highest at low-N (Keski-Saari and Julkunen-Tiitto, 2003).

In nitrogen fertilization experiments, carbon allocation between different metabolic pathways and into individual phenolic compounds differs considerably from each other (Reichard *et al.*, 1991; Haukioja *et al.*, 1998; Koricheva *et al.*, 1998; Keinänen *et al.*, 1999).

Environmental conditions, such as the fertility of the habitat, also affect the concentrations of secondary compounds (Hartley and Jones, 1997; Keinänen *et al.*, 1998). Nitrogen availability is thought to affect the concentrations of secondary compounds in a predictable manner: The plants growing in nitrogen-poor conditions are thought to have more secondary compounds than plants growing in nitrogen-rich conditions (Bryant *et al.*, 1983; Herms and Mattson, 1992; Jones and Hartley, 1999).

The few studies available comparing phenolic compound production in plants growing on a acidity gradient in natural conditions have found higher phenolic concentrations in plants associated with lower pH (Gylphis and Puttick, 1989; Muller *et al.*, 1987; Nicolai, 1988; Northup *et al.*, 1995b). This correlation is associated with lower N availability found in acidic soils (Northup *et al.*, 1995a) although some studies have found direct effects of pH (Laukkanen *et al.*, 1997).

According to the *carbon/nutrient balance hypothesis* (Bryant *et al.*, 1983), nitrogen availability in the soil is low, the low resource availability limits the growth of plants more than the photosynthesis, and plants allocate their extra carbon that they cannot use for growth to carbon-based secondary compounds. Furthermore, the predictions of the theory seem more reliable in some plants species a other, and the carbon/nutrient balance hypothesis can perhaps only be usefully applied to specific groups of secondary compounds and particular plant species (Hartley *et al.*, 1995).

According to the *growth/differentiation balance hypothesis* (Herms and Mattson, 1992), nitrogen or carbon availability are not the only environmental conditions that influence the secondary concentrations in a predictable way, but the trade-off happens between all growth and differentiation processes.

The *protein competition model* (Jones and Hartley, 1999) predicts that protein synthesis and phenolic synthesis are alternative competitive demands and, for example during the development of leaves the demands for growth and protection alternate.

A possible regulatory step between growth and the synthesis of secondary metabolites are the phenolic amino acid, phenylalanine, and is used for synthesis of both proteins and of secondary compounds produced by the shikimic acid pathway (Margna *et al.*, 1989; Jones and Hartley, 1999). The hypotheses predict increases in the concentration of carbon-rich compounds when nutrients are less available. I therefore examined phenolic concentrations occurring in the roots.

A reduction in the levels of carbon/based secondary compounds in response to fertilisers have been observed experimentally for many plants (Gershenson, 1984; Bryant *et al.*, 1987). Shaw *et al.* (1998) reported unaffected total phenolic and tannin levels in the root collar area of Douglas-fir seedlings by high nitrogen treatments.

Pasqualini *et al.* (2003) showed a significantly negative correlation between total phenolics in needles of Aleppo pine from 6 sites affected by various forms of atmospheric pollutants and nitrogen oxide concentrations. This negative correlation may be explained by the positive impact of these pollutants on the activity of nitrate reductase (Krywult *et al.*, 1996). This

enzyme promotes nitrogen assimilation and several studies have shown the same negative correlation in needles or leaves of various species (Gietrych *et al.*, 1999). In the same study Pasqualini *et al.* (2003) reported that the correlations between air pollutants and simple phenolic compounds showed concordant results. Several enzymes, which take part in the shikimic acid biosynthetic pathways, may be more sensitive than others in relation to different pollutants (Loponen *et al.*, 2001).

Increased infection of Douglas-fir (*Pseudotsuga menziensis* (Mirb.) Franco) by *Armillaria ostoyae* (Romagn.) Herink occurred after fertilization with nitrogen (Entry *et al.*, 1991). Conifer species with increased susceptibility to *Armillaria ostoyae* generally have fewer phenolic compounds and more sugar in root bark (Entry *et al.*, 1992), suggesting that the ratio of available sugars to phenolic compounds may be related to the success of the fungal infection.

1.6.4 Phenolic compounds and plant pathogens

Plants are capable, through their secondary metabolic products to reduce the attack of pathogenic organisms and phenolic compounds may be an important group of secondary metabolites involved in resistance to pathogens (Isaac, 1992; Waterman and Mole, 1994). It has long been known that fungal secondary metabolites are crucial to the pathogenicity of many fungi (Agrios, 1997; Harborne, 1993).

Plant pathologists have traditionally recognized two types of antiinfectional defence chemicals in plants: those that are present constitutively and those where *de novo* synthesis is induced as a result of an infection. These two groups are thus pre- and post-infectional defences, respectively (Isaac, 1992; Waterman and Mole, 1994). Below, I give examples of the substances involved.

The content in the needles of *Picea abies* of 4-hydroxyacetophenone and its glucoside Picein have been proposed as stress indicators (Hoque, 1986; Jensen and Løkke, 1990). Recently the phenols get into the centre of attention as the potential defensive compounds of plants (Levin 1971). The proanthocyanidins have been shown to function as preformed defence metabolites

in their hosts (Schloösser, 1997; Stafford, 1997). For example, Jersch *et al.* (1989) found that as long as proanthocyanidins are present at sufficiently high concentrations in strawberries, *Botrytis cinerea* remains in a quiescent state. When the concentration of proanthocyanidins decrease, *Botrytis cinerea* becomes pathogenic. In the case of pathogenic plant-fungal interaction, the fungus *Heterobasidion annosum* catabolizes catechin and causes a reduction in this compound in infected *Pinus* roots (Bonello *et al.*, 1993).

The fungistatic properties of phenolic compounds have been documented *in vitro*. Terpenes and phenolics showed an inhibitory effect to pathogens at concentrations observed in pines and other conifer species (Alcubilla *et al.*, 1971; Raffa *et al.*, 1991). Blodgett and Stanosz (1997) also examined the effect of phenolic compounds and monoterpenes on mycelial growth of *Sphaeropsis sapinea in vitro*. The bioassays revealed that pinosylvin and several monoterpenes occurring in red pine inhibit mycelial growth of this pathogen. The concentrations of phenolics in the bark of American beech trees (*Fagus grandifolia*) resistant to infection by *Nectria cocconeae* var. *faginata*, remained high even 6 months after the attack (Ostrofsky *et al.*, 1984). The effect of an extract of Norway spruce bark on the growth of the root rot fungus *Heterobasidion annosum* and the blue-stain fungus *Ceratocystis polonica* was investigated *in vitro* by Evensen *et al.* (2000). *Heterobasidion annosum* was not negatively affected, the extract had only fungistatic effects on the blue-stain fungus.

The production and accumulation of phenolic compounds are often associated with injury responses and the wound healing of plants, but these substances also have fungitoxic activity. Previous studies of host-fungus interactions in the xylem of living trees have strongly emphasised the importance of host responses to fungal invasion (Bauch *et al.*, 1980; Pearce, 1996). By contrast, the mechanisms underlying reaction zone penetration and subsequent lesion expansion by wood decay fungi are poorly understood (Schwarze *et al.*, 2000). Recently, however, Schwarze and Baum (2000) described in detail a variety of mechanisms by which reaction zones of beech wood (*Fagus sylvatica* L.) were penetrated *in vivo* or when challenged *in vitro* with decay fungi. When attacked by bark beetles and their associated fungi, conifers form a necrotic reaction zone around the site of infection (Reid *et al.*, 1967; Berryman, 1969). This reaction zone becomes impregnated with secondary metabolites such as phenols and terpenes (Brignolas *et al.*, 1995a; Russel and Berryman, 1976; Shrimpton, 1973; Shrimpton and Whitney, 1968). Métraux (1994) related phenolic accumulation in plants

to defence mechanisms against pathogens. After wounding or infection of Norway spruce (*Picea abies* (L.) Karst.), there are both quantitative and qualitative changes in the phenol content in the reaction zone (Brignolas *et al.*, 1995 a, b, c). These changes, together with the presence of polyphenolic parenchyma cells in the phloem of Norway spruce (Franceschi *et al.*, 1998), suggest an important role of phenols in coniferous resistance reactions. The polyphenolic parenchyma cells are located in continuous sheets in the secondary phloem and contain dense vacuolar deposits of phenols. *In vitro* bioassays have demonstrated a fungistatic or fungitoxic effect of some of the phenols normally found in the sapwood (Shain, 1971) and phloem (Brignolas *et al.*, 1995b) of Norway spruce.

In 1940, K.O. Müller and H. Börger proposed the phytoalexin theory of disease resistance in plants which offered an explanation of certain aspects of disease resistance based on the post-infectious formation of warding-off-compounds (phytoalexins) by the plant. One of the best and extensively-studied defence responses of plants to pathogen infection is the induced accumulation of secondary metabolites such as phytoalexins (Hammerschmidt, 1999). Phytoalexins are a diverse group of low molecular weight anti-microbial compounds that are synthesized and accumulated in appreciable amounts in plants after stimulation by the various types of pathogens or by chemical or mechanical injury and are toxic to pathogens (Mansfield, 1999; Smith, 1996).

1.7 Nitrogen fertilization and pathogens

Nitrogen availability can affect a plant's susceptibility to pathogens (Alcubilla *et al.*, 1971; Alcubilla *et al.*, 1987). Van Dijk *et al.* (1992) found a relation between the availability of nitrogen and the incidence of damage caused by *Sphaeropsis sapinea* (Fr.) Dyko & Sutton. Trees affected more strongly by *Sphaeropsis sapinea* contained more nitrogen.

The present investigation was made in a nitrogen addition experiment designed to evaluate critical loads for nitrogen. Fertilization with 0-160 kg N ha⁻¹ year⁻¹ caused significant changes in nutrient concentrations and ratios in beech in afforestation plots in Switzerland and trees were significantly more infested by *Apiognomonium errabunda*, *Phomopsis* sp., *Phyllaphis fagi* and *Botrytis cinerea* when fertilized with nitrogen (Flückiger and Braun, 1999). However

there are scarce informations about the effect of nitrogen on the interaction between roots and root pathogens.

The effects of nitrogen on *Sphaeropsis* have been studied by De Kam *et al.* 1991. Two-year old plants of *Pinus nigra* were grown for three years in pots and fertilized with 5 treatments of ammonium sulphate (very low to ca. 300 kg N ha⁻¹ year⁻¹), in combination with two levels of potassium sulphate. The 5-year-old plants were then inoculated with *Sphaeropsis*. The bark necroses were much higher in the plants treated with ammonium sulphate, whereas in the plots with high levels of potassium sulphate the necrosis was low. Effects of ammonium sulphate upon fungal damage were already observed at an addition of 75 kg N ha⁻¹ year⁻¹, but were very significant in the plants treated with 150 kg N ha⁻¹ year⁻¹ (De Kam *et al.*, 1991).

The influence of nitrogen on the disease seems to depend on the host-pathogen combination and the form of soil nitrogen (Erwin and Ribeiro, 1996; Utkhede and Smith, 1995). Jung *et al.* (2002) demonstrated a stimulation of *in vivo* production of sporangia by several *Phytophthora* species, with increasing concentrations of nitrate in the soil leachate. This stimulation was probably a major cause of the increasing difference in the fine-root length and the number of fine root tips between uninfected and infected *Quercus robur* seedling with increasing nitrate concentration. In field studies, pH as well as nitrate and Ca concentrations were significantly higher in soils infested with *Phytophthora* than in uninfested soils (Jung *et al.*, 2000).

1.8 Objectives

The main objective of this thesis was to determine the effect of nitrogen on the tree resistance to pathogens. Therefore I investigated the influence of nitrogen fertilization on the tree health, by measuring root parameters in field experiments.

The aims of the individual studies were:

- to study the effect of nitrogen fertilization on fine root fungistatic phenolic compounds on beech and Norway spruce (**Paper I**)
- to test the effect of phenolic compounds on two root pathogenic fungi (**Paper I**)
- to determine the effect of increased nitrogen on fine roots of beech growing on acidic and calcareous soils (**Paper II**)
- to study the influence of nitrogen on beech leaves nutrients upon different soil conditions (**Paper II**)

2 MATERIALS AND METHODS

A brief summary of the methods used is given here. More detailed information can be found in the original publications and in the references cited therein.

2.1 Studied species

This thesis is focused on two tree species, beech (**Paper I** and **Paper II**) and Norway spruce (**Paper I**). Almost in all European countries European beech (*Fagus sylvatica* L.) plays a dominant role concerning the makeup of broadleaved forests. Norway spruce is an important European species with a wide natural range from the Pyrenees, Alps, and Balkans northwards through Germany to Scandinavia, and eastwards to west Russia.

I used two fungal pathogens for the *in vitro* test (**Paper I**). The basidiomycete fungus *Heterobasidion annosum* (Fries: Fries) Brefeld is the causal agent of annosum root rot and is economically one of the most important disease of coniferous forests (Stendlid and Johansson, 1987; Piri, 1996). This thesis deals also with the root pathogen *Cylindrocarpon destructans* (Zins.) Scholten. This fungus is a common cause of root rot in many plants (Seifert and Axelrood, 1998).

2.2 Nitrogen fertilization experiments

Beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* (L.) Karst.) were planted in afforestation plots in 1992 (Flückiger and Braun, 1999). The field plots are situated at three sites in Switzerland at an elevation of 290 m (Möhlin), 1000 m (Zugerberg) and 670 m (Hochwald) (Table 1). The trees (twelve replicates) were treated three times per season - in April, July and October - with dry ammonium nitrate to resulting loads of 0, 10, 20, 40, 80, 160 kg N ha⁻¹ year⁻¹. The modelled atmospheric nitrogen deposition at the plot Möhlin was 12 kg N ha⁻¹ year⁻¹, at the plot Zugerberg 20 kg N ha⁻¹ year⁻¹ and at plot Hochwald (Photo 1) 15.5 kg N ha⁻¹ year⁻¹ (Rihm, 2001).



Photo 1 Nitrogen fertilization experiment on plot Hochwald.

Table 1 Characteristics of the sites used in the nitrogen fertilization experiment in afforestation plots (Flückiger and Braun, 1999).

Plot	Möhlin	Zugerberg	Hochwald
Altitude (m asl)	290	1000	670
Soil type	Haplic Acrisol	Dystric Cambisol	Rendzic Leptosol
Geology	gravel	till	limestone
pH (CaCl ₂) 0-40 cm	3.8	4.0	6.9
Average base saturation (0-40cm) (%)	12	12	100
Min. base saturation (%)	4	2	100

2.3 Chemical analysis

Fine roots (**Paper I**) and beech leaves (**Appendix II**) were suspended in methanol-chloroform-formic acid (MCF), allowed to stand for one hour with continuous stirring and centrifuged. The pellet was re-extracted twice with the same solvent. The combined extracts were taken to dryness and the residual redissolved in MCF and H₂O. The aqueous upper phase was used for HPLC and MS analysis (Strack *et al.*, 1989).

The phenolic compounds were analysed by high-pressure liquid chromatography (HPLC) gradient elution, using UV-detection (**Paper I** and **Appendix II**). The phenolics were identified by comparing their retention time with standards (Figure 3). For further identification of the compounds a high-pressure liquid chromatography - mass spectrometry (HPLC-MS) analysis was used, as described by Radimerski *et al.*, 2000 (**Paper I**).

The leaves (**Paper II**) were analysed for their concentrations of N, P, K, Ca, Mg and Mn after mixed acid digestion (Allen, 1989). N and P were determined colorimetrically (Walinga *et al.*, 1995). Ca, Mg and Mn were analysed using atomic absorption photometry and K using flame photometry.

The same MCF-extracts from phenolics analysis were used to analyse free amino acids. The concentration of total amino acids was estimated by a ninhydrin method (Moore, 1968) with L-leucine as a standard. For detection a Perkin Elmer Lambda 2 UV/VIS spectrometer (Bodenseewerk Perkin-Elmer & Co GmbH, Überlingen, Germany) was used at 570 nm (**Appendix I**). In the present thesis, levels of free amino acids were considered as indicators of roots nitrogen status and responses to nitrogen fertilization.

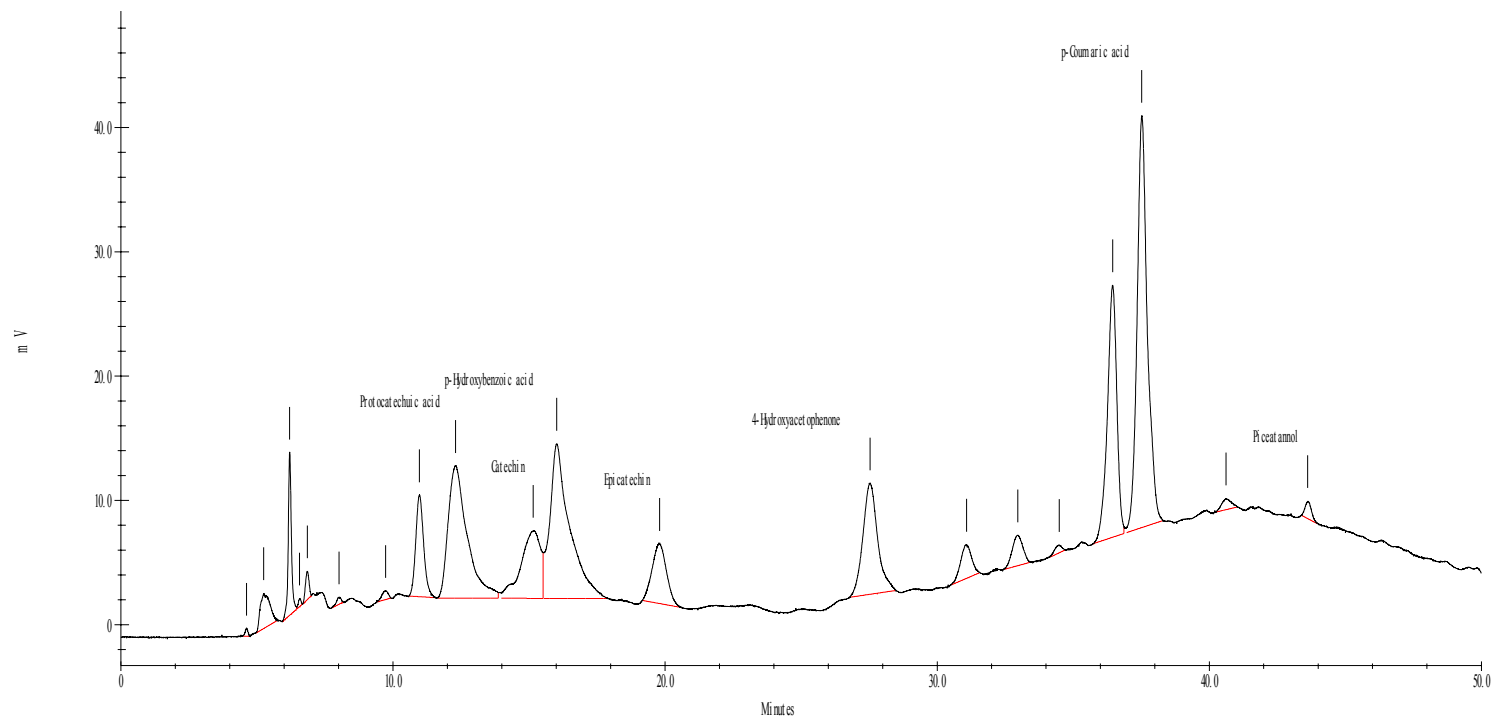


Figure 3 Chromatograms from HPLC analysis of an extract of beech roots.

2.4 Root analysis

Root length and root tips (**Paper II**) were measured using the software WinRHIZO 3.9 (Regent Instruments Inc., Quebec, Canada) (Figure 4).

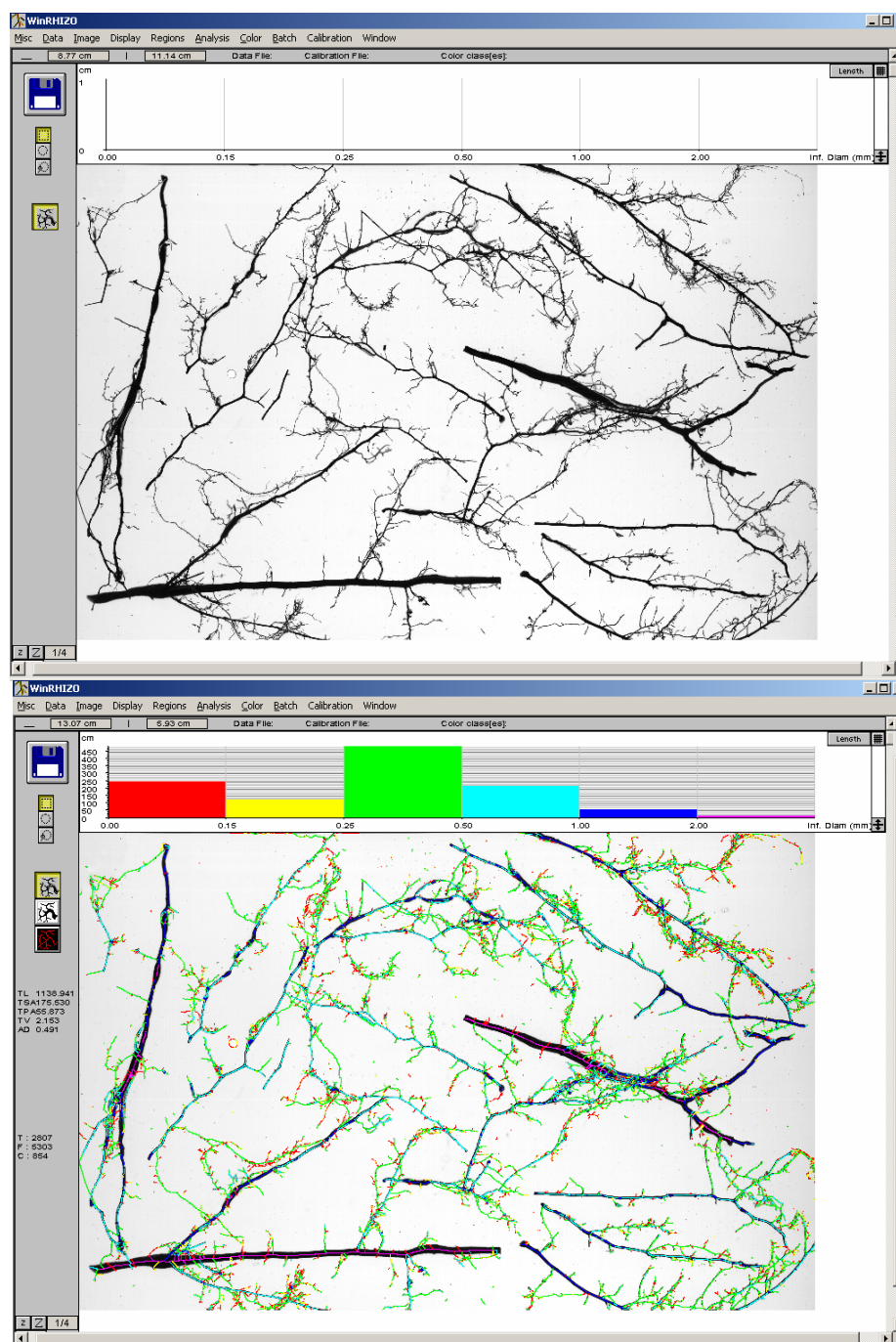


Figure 4 Examples of a beech root from plot Zugerberg before and after analysis with WinRHIZO.

2.5 *In vitro* test

In vitro experiments (**Paper I**) were made with tree different fungal strains each of *Cylindrocarpon destructans* (Zins.) Scholten and *Heterobasidion annosum* (Fries: Fries) Brefeld. The fungal strains were maintained on malt extract agar. The same standards used for chemical analysis were assayed for antifungal activity. The concentrations found in fine roots in mg g⁻¹ fresh weight were used to derive test concentrations in mg ml⁻¹ agar. The phenolic solutions were added to the previously autoclaved malt extract agar and plugs of mycelium were added. Growth of fungi was measured daily as colony diameter.

2.6 Statistical analysis

The effect of nitrogen fertilization on root and leave parameters was evaluated by one-way analysis of variance after root transformation, Fisher's LSD-test was used for testing differences to the control. Overall linear regression was tested after the ANOVA using contrasts (**Paper I**, **Paper II**, **Appendix I** and **Appendix II**). The Spearman Correlation was used to analyse correlations (**Paper II** and **Appendix II**). All calculations were performed with the statistical package SYSTAT® vers.10 (SPSS Inc., Chicago, USA).

3 EXPERIMENTAL RESULTS

PAPER I

**The effect of nitrogen fertilization on fungistatic phenolic
compounds in roots of beech (*Fagus sylvatica* L.) and
Norway spruce (*Picea abies* L. [Karst.]**

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Summary

The effect of nitrogen fertilization on fungistatic phenolic compounds in fine roots of beech and Norway spruce growing in afforestation plots was analysed. The plots were situated at two sites in Switzerland on acidic soil with low base saturation. For nine years, the trees have been treated with dry ammonium nitrate to give 0, 10, 20, 40, 80, 160 kg N ha⁻¹ year⁻¹, respectively.

The phenolic compounds responded differently to fertilization. Fine roots of beech showed a significant decrease of (-)-epicatechin and piceatannol with increasing nitrogen fertilization. The concentration of protocatechuic acid was increased with fertilization. Roots of fertilized Norway spruce showed significantly decreased concentrations of 4-hydroxyacetophenone and piceatannol.

The in vitro growth of *Heterobasidion annosum* and *Cylindrocarpon destructans* was inhibited by the presence of most of the listed fungistatic phenolic compounds in the concentrations found in the roots.

Keywords: Nitrogen, *Fagus sylvatica*, *Picea abies*, phenolic compounds, fine roots, *Heterobasidion annosum*, *Cylindrocarpon destructans*

Introduction

Secondary plant metabolites are known to play an important role in the response of plants towards various biotic and abiotic stress factors. The chemical defence strategy used by trees against atmospheric stress (SO₂, ozone, UV-B) and herbivory includes the synthesis and accumulation of secondary metabolites, such as phenolic compounds (Julkunen-Tiitto *et al.*, 1996; Korolewski *et al.*, 1995, Lavola, 1998).

It has been shown that some phenolic compounds have important biological function such as toxicity against pathogenic fungi. It is well documented that resistance against pathogens is affected by phenolic compounds, which can be decreased by nitrogen fertilization as shown for leaves of beech (Balsberg-Påhlsson, 1989). On the other hand, nitrogen availability can affect a plant's susceptibility to pathogens (Alcubilla, 1971; Alcubilla *et al.*, 1987). Van Dijk *et al.* (1992) found a relation between the availability of nitrogen and the incidence of damage caused by *Sphaeropsis sapinea* (Fr.) Dyko & Sutton. Trees affected by *Sphaeropsis sapinea* contained more nitrogen. The fungistatic properties of phenolic compounds have been documented *in vitro* as well. Terpenes and phenolics showed an inhibitory effect to pathogens at concentrations observed in pines and other conifer species (Alcubilla *et al.*, 1971; Raffa *et al.*, 1991). Blodgett and Stanosz (1997) also examined the effect of phenolic compounds and monoterpenes on mycelial growth of *Sphaeropsis sapinea* *in vitro*. The bioassays revealed that pinosylvin and several monoterpenes occurring in red pine inhibit mycelial growth of this pathogen.

The present investigation was made in a nitrogen addition experiment designed to evaluate critical loads for nitrogen. The nitrogen treatment led to increased parasite attacks. Beech from the two afforestation plots Möhlin and Zugerberg showed a significantly increased dieback caused by *Apiognomonia errabunda*, and Norway spruce on Zugerberg was significantly more infested with *Botrytis cinerea* when fertilized with nitrogen (Flückiger and Braun, 1999). However there are scarce informations about the effect of nitrogen on the interaction between roots and root pathogens.

The basidiomycete fungus *Heterobasidion annosum* (Fries:Fries) Brefeld is the causal agent of annosum root rot and is economically one of the most important diseases of coniferous forests (Stendlid and Johansson, 1987; Piri, 1996). This paper deals also with the root pathogen *Cylindrocarpon destructans* (Zins.) Scholten. This fungus is a common cause of root rot in many plants (Seifert and Axelrod, 1998).

Therefore we investigated the influence of nitrogen fertilization on fine root fungistatic phenolic compounds and their effect on the two root pathogenic fungi mentioned above.

Materials and methods

Fertilization experiment and collection of roots samples

Beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies* (L.) Karst.) were planted in afforestation plots in 1992 (Flückiger and Braun, 1999). The field plots are situated at two sites in Switzerland at an elevation of 290 m (Möhlin) and 1000 m (Zugerberg). The trees (twelve replicates) were treated three times per season - in April, July and October - with dry ammonium nitrate to give loads of 0, 10, 20, 40, 80, 160 kg N ha⁻¹ year⁻¹, respectively. The modelled atmospheric nitrogen deposition at the plot Möhlin was 12 kg N ha⁻¹ year⁻¹ and at the plot Zugerberg 20 kg N ha⁻¹ year⁻¹ (Rihm, 2001).

The soil from the beech plot Möhlin is a haplic acrisol. In the upper 40 cm the average pH (CaCl₂) is 3.8 and the base saturation is 12%. The soil from the plot Zugerberg is a dystric cambisol. The pH is 4.0 and the base saturation is also 12%.

Roots of beech were sampled in the Möhlin plot in September 1999, roots of spruce in the Zugerberg plot in September 2000. The roots were excavated by hand from the uppermost soil layer from an area of 1 m² around the stem. Roots were washed and fine roots with diameter ≤ 2 mm were separated, frozen in liquid nitrogen, lyophilised and stored at -20°C until analysed.

Chemical analysis

Plant extraction

Roots (50 mg) were finely ground with ball grinder in the presence of liquid nitrogen. The resulting powder was suspended in 5 ml methanol-chloroform-formic acid (12:5:3; MCF), allowed to stand for one hour with continuous stirring (Variomag STERICO AG,

Switzerland) and centrifuged (10 min 4300 g). The pellet was re-extracted twice with 2.5 ml each of the same solvent. The combined extracts were taken to dryness (Rotavapor-RE&115Ex, Büchi AG, Switzerland) and the residual redissolved in 2.5 ml MCF and mixed with 2.5 ml MCF and 5 ml H₂O. The aqueous upper phase was used for HPLC analysis (Strack *et al.*, 1989).

HPLC-separation and quantification of phenolic compounds

For the analysis of phenolic compounds a high-performance liquid chromatograph Waters was used, consisting of a 600 E multisolvent delivery system, a sample processor 715 Ultra WISP, a detector 486 with absorbance set at 280 nm and a data module printer/plotter 746 (Millipore-Waters, Harrow, U.K.). Separations were achieved on Nucleosil C18 column (5 µm, 250 mm long, 4 mm i.d., Macherey-Nagel, Düren, Germany). The elution system for phenolics was a linear gradient from solvent A (1.5% H₃PO₄ in H₂O) to 30% solvent B (MeOH/CH₃CN/H₂O, 1/1/1, (v : v)) in A within 60 min. The elution was carried out at 0.5 ml min⁻¹, the injection volume was 20 µl.

Six phenolic compounds were identified in root extracts from beech and seven in extracts from Norway spruce by comparing their retention time with standards and by mass spectroscopy (see section below). In the beech roots, (+)-catechin was not analyzed but it was identified in later samples. The following authentic compounds were used as external standards for quantification and/or identification: 4-hydroxyacetophenone, (+)-catechin (Fluka), piceatannol (3,3, 4,5'-tetrahydroxy-transstilbene), p-hydroxybenzoic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), p-coumaric acid (4-hydroxycinnamic acid) and (-)-epicatechin (Aldrich).

Mass spectral analysis of phenolics

Extracts made for the HPLC-analysis (20 µl extract acidified with 0.2 µl 10% acetic acid, centrifuged at 10'000 rpm for 5 min) were injected onto a C18 reverse-phase column (Vydac C₁₈ 218TP52. 2.1 x 250mm, Grace Vydac, Hesperia, CA) that had been equilibrated in 0.1 % acetic acid. Phenolic compounds were eluted with a linear gradient from 0.1% acetic acid to 50% acetonitrile containing 0.1% acetic acid during 55 min at a flow rate of 150 µl min⁻¹. Standards dissolved in methanol were dried in a speed vac concentrator and dissolved in 30 µl

0.1% acetic acid and centrifuged at 10'000 rpm for 5 min. The elution positions of 4-hydroxyacetophenone, (+)-catechin, piceatannol, p-hydroxybenzoic acid, protocatechuic acid, p-coumaric acid and (-)-epicatechin were established by individually injecting approximately 7 µg of the corresponding compound.

The column effluent was split so that a flow of approximately 2-3 µl min⁻¹ was diverted into the micro ion source constructed as previously described (Radimerski *et al.*, 2000). Measurements were carried out on a TSQ7000 triple quadrupole mass spectrometer (Finnigan, San José, CA) operated in positive ion mode and scanning was over 50-500 daltons in 2 seconds.

***In vitro* test**

In vitro experiments were made with three different fungal strains each of *Cylindrocarpon destructans* (Zins.) Scholten and *Heterobasidion annosum* (Fries:Fries) Brefeld. Isolates were obtained from the Belgian Co-ordinated Collections of Microorganisms: *Heterobasidion annosum* (Fries:Fries) Brefeld was isolated in 1964 from *Picea abies* at Les Failles, Chabre, Belgium (MUCL 6136), in 1990 from decaying stump *Picea abies* at Clairefontaine, Belgium (MUCL 30530) and in 1990 from *Picea* sp. at Louvain-la-Neuve, Bois de Laizelle, Belgium (MUCL 30709). *Cylindrocarpon destructans* (Zins.) Scholten was isolated in 1965 from *Fagus sylvatica* at Heverlee, Arenberg Park, Belgium (MUCL 7864), in 1967 from *Phragmites communis* at Heverlee, Belgium (MUCL 11299) and in 1972 from *Taxus baccata* at Heverlee, Belgium (MUCL 18715).

The fungal strains were maintained on 2% (w : v) malt extract agar (30 g Malt Extract Agar Oxoid CM59; 4.5 g Bacteriological Agar Oxoid L11; 1 l water) in the dark at 20°C.

The same standards as used for chemical analysis (dissolved in 25% aqueous methanol (v : v)) were assayed for antifungal activity. The concentrations found in fine roots in mg g⁻¹ fresh weight were used to derive test concentrations in mg ml⁻¹ agar. Control was 25% v : v methanol in distilled water. The phenolic solutions (0.2 ml per 200 ml agar) were added to the previously autoclaved 2% (w : v) malt extract agar (buffered at pH 5.5 with KH₂PO₄) (Table 1). Plugs (5 mm in diameter) of actively growing mycelium were taken from the margins of fungal colonies, transferred to the centre of plates with medium and incubated at 20°C in the dark. Growth of fungi was measured daily as colony diameter. The *in vitro* test was replicated two times.

Table 1 Concentrations of phenolic compounds used for *in vitro* test.

Phenolic compounds	Concentrations (mg ml ⁻¹ in 2% malt extract agar) used for <i>in vitro</i> test with <i>Heterobasidion annosum</i>			
	I	II	III	IV
protocatechuic acid	0.0038	0.0075	0.0150	0.0300
(+)-catechin	0.0163	0.0325	0.0650	0.1300
(-)-epicatechin	0.0009	0.0018	0.0035	0.0070
4-hydroxyacetophenone	0.0001	0.0003	0.0005	0.0010
p-hydroxybenzoic acid	0.1588	0.3175	0.6350	1.2700
p-coumaric acid	0.0038	0.0075	0.0150	0.0300
piceatannol	0.0138	0.0275	0.0550	0.1100

Phenolic compounds	Concentrations (mg ml ⁻¹ in 2% malt extract agar) used for <i>in vitro</i> test with <i>Cylindrocarpon destructans</i>			
	I	II	III	IV
protocatechuic acid	0.0075	0.0150	0.0300	0.0600
(+)-catechin	0.0088	0.0175	0.0350	0.0700
(-)-epicatechin	0.0063	0.0125	0.0250	0.0500
4-hydroxyacetophenone	0.0005	0.0010	0.0020	0.0040
p-hydroxybenzoic acid	0.0875	0.1750	0.3500	0.7000
p-coumaric acid	0.0025	0.0050	0.0100	0.0200
piceatannol	0.0004	0.0008	0.0015	0.0030

Statistical analysis

The effect of nitrogen fertilization on phenolic compounds was evaluated by one-way analysis of variance after root transformation, Fisher's LSD-test was used for analysing the significance of differences to the control. Overall linear regression was tested after the ANOVA using contrasts. The effect of phenolic compounds on fungal inhibition of growth was evaluated by ANOVA analysis treating the concentration as categorical variable. Linear regression was assessed with concentration as numerical variable. All calculations were performed with the statistical package SYSTAT® vers.10 (SPSS Inc., Chicago, USA).

Results

Phenolic compounds

Beech

In fine roots of beech the phenolic compounds 4-hydroxyacetophenone, piceatannol, p-hydroxybenzoic acid, protocatechuic acid, p-coumaric acid and (-)-epicatechin were identified.

The concentrations of (-)-epicatechin and of piceatannol in fine roots of fertilized beech from the plot Möhlin were significantly decreased at a nitrogen treatment of ≥ 10 kg N ha⁻¹ year⁻¹ compared to the control (Fig. 1, Table 2).

The concentration of protocatechuic acid was increased with fertilization. A significant difference to the control was, however, only found at a treatment of 160 kg N ha⁻¹ year⁻¹. P-hydroxybenzoic acid increased up to 40 kg N ha⁻¹ year⁻¹ fertilization and decreased afterwards. Nitrogen had no significant effect on 4-hydroxyacetophenone and p-coumaric acid.

1.1.1 Norway spruce

In fine roots of Norway spruce the phenolic compounds 4-hydroxyacetophenone, piceatannol, p-hydroxybenzoic acid, protocatechuic acid, p-coumaric acid, (+)-catechin and (-)-epicatechin were identified.

The concentrations of piceatannol and 4-hydroxyacetophenone showed a reduction in fine roots of plants treated with ≥ 10 kg N ha⁻¹ year⁻¹ compared to the control (Fig. 2, Table 2). Concentrations of (+)-catechin and of protocatechuic acid were reduced as well in fertilized trees but only at intermediate treatments (10, 20 and 40 kg N ha⁻¹ year⁻¹). Fertilization had no overall significant effect on the concentrations of p-coumaric acid, p-hydroxybenzoic acid and (-)-epicatechin.

Table 2 Results of ANOVA with nitrogen as factor for beech and Norway spruce root phenolic compounds.

Data were root transformed for analysis. ns - Not significant

Species	Phenolic compounds	Significant differences compared to the control at nitrogen treatments of (in kg N ha ⁻¹ year ⁻¹)	Overall regression	
			P _{linear}	P _{quadratic}
Beech	(-)-epicatechin	10, 20, 40, 80, 160	< 0.001	< 0.001
	piceatannol	10, 20, 40, 80, 160	< 0.001	ns
	p-hydroxybenzoic acid	40	ns	< 0.05
	4-hydroxyacetophenone	10, 20	ns	ns
	p-coumaric acid		ns	ns
	protocatechuic acid	160	<0.01	ns
Norway spruce	(-)-epicatechin		ns	ns
	piceatannol	10, 20, 40, 80, 160	< 0.001	< 0.05
	p-hydroxybenzoic acid	10, 160	ns	ns
	4-hydroxyacetophenone	10, 20, 40, 80, 160	< 0.01	ns
	p-coumaric acid		ns	ns
	protocatechuic acid	10, 20, 40	ns	< 0.01
	(+)-catechin	10, 20, 40	ns	< 0.01

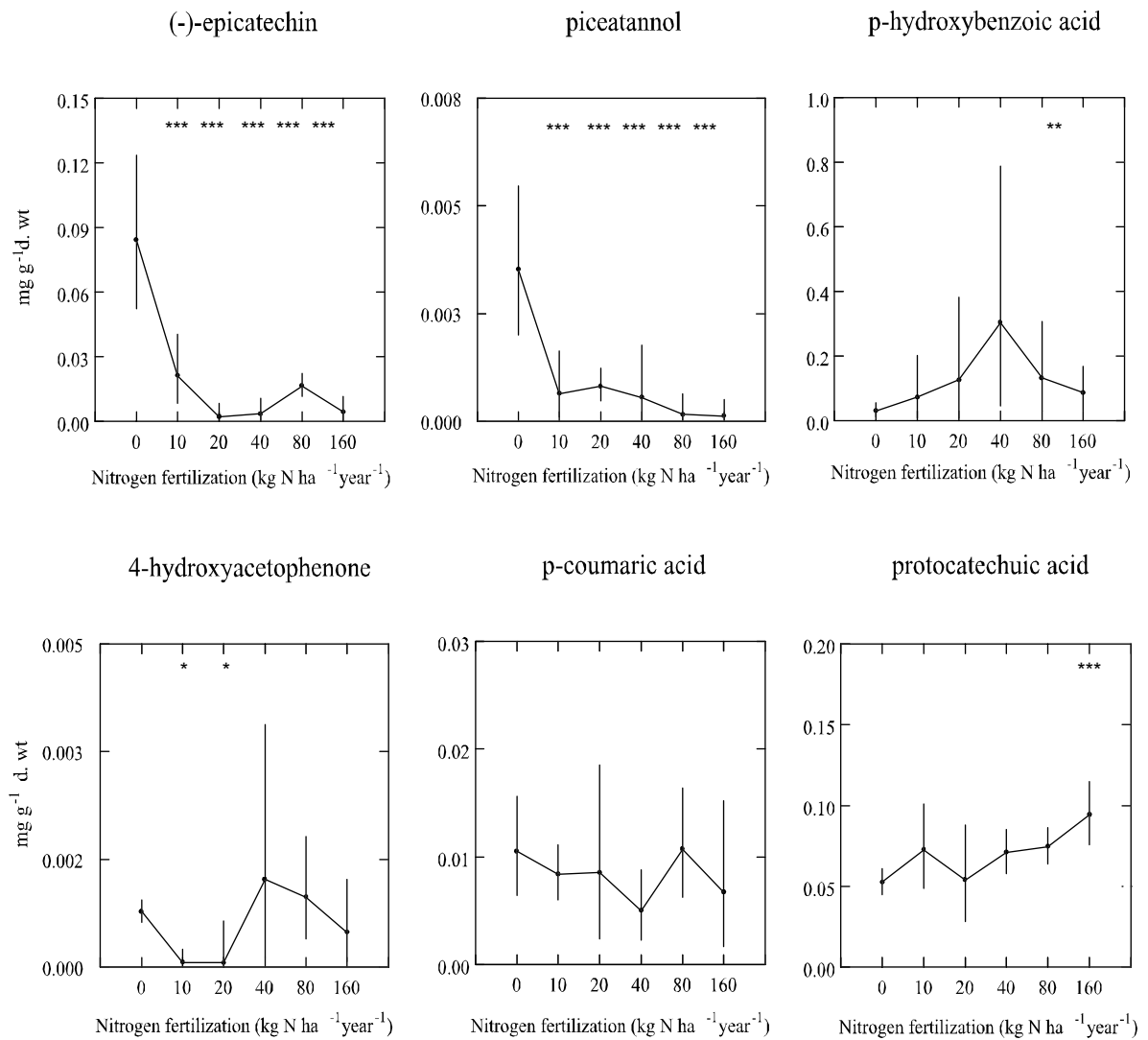


Fig. 1 Changes in the concentration of phenolic compounds in the fine roots of beech (mg g⁻¹ d. wt) in relation to nitrogen fertilization. Bars indicate 95% confidence interval. Difference to control significant at: P < 0.05 (*), P < 0.01 (**), P < 0.001 (***).

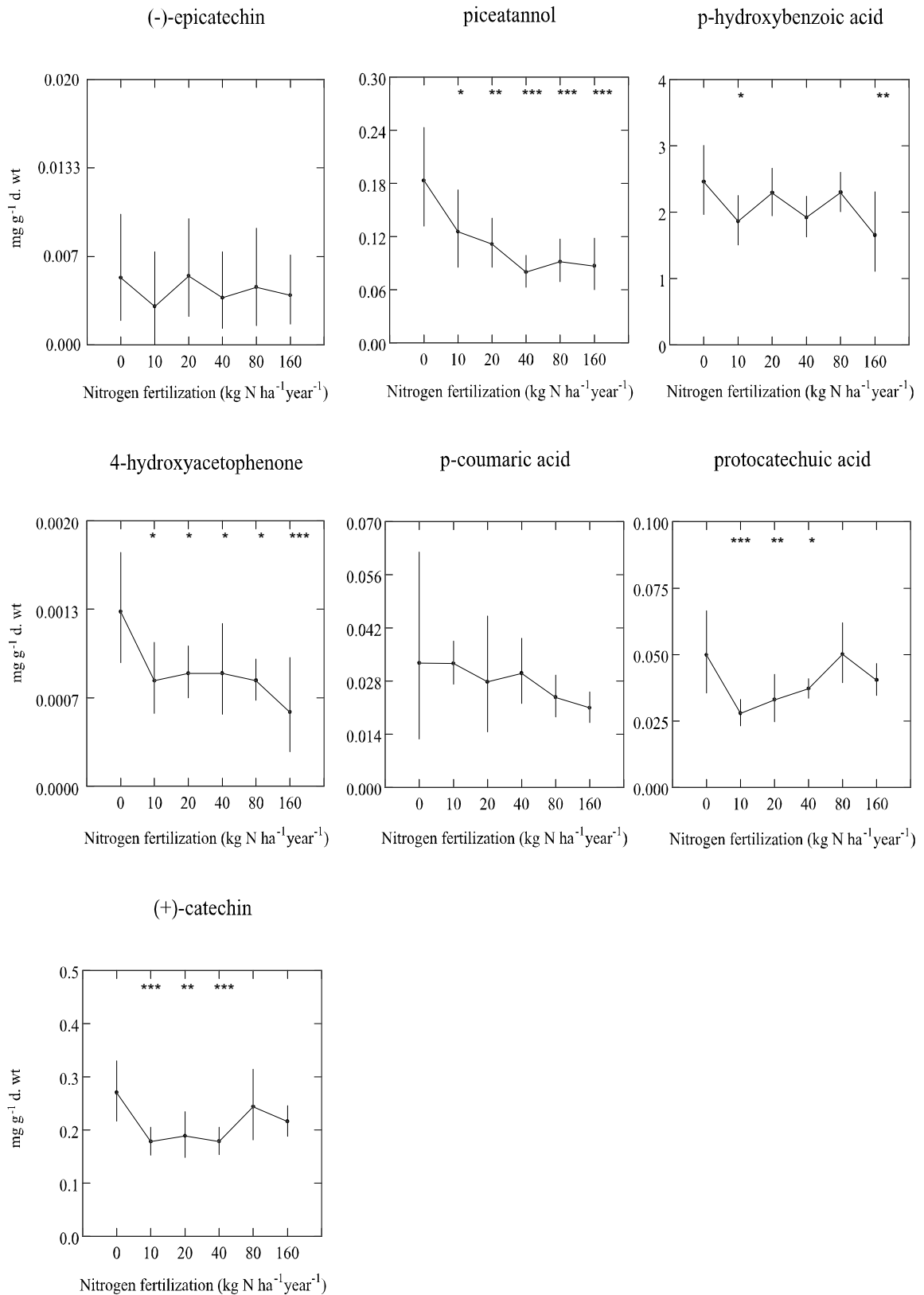


Fig. 2 Changes in the concentration of phenolic compounds in the fine roots of Norway spruce (mg g⁻¹ d. wt) in relation to nitrogen fertilization. Bars indicate 95% confidence interval. Difference to control significant at: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)

Identification of phenolics by mass spectral analysis

Plant extracts contained molecular ion peaks which corresponded to 4-hydroxyacetophenone (m/z 138.0, M - H -), p-hydroxybenzoic acid (m/z 140.0, M - H -), protocatechuic acid (m/z 155.9, M - H -), p-coumaric acid (m/z 165.9, M - H -), piceatannol (m/z 245.9, M - H -), (+)-catechin (m/z 291.8, M - H-), (-)-epicatechin (m/z 291.8, M - H-). (+)-Catechin and (-)-epicatechin displayed the most abundant peaks (Fig. 3).

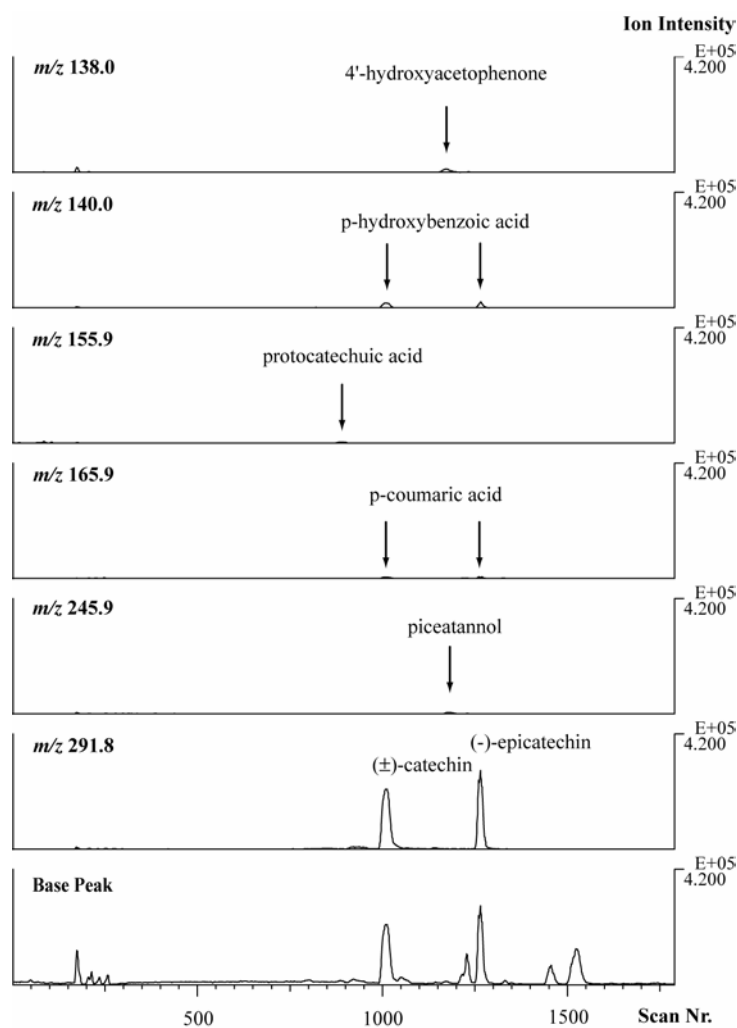


Fig. 3: Chromatograms from MS analysis of an extract of beech roots. Base peak: chromatogram with all molecular weights.

In vitro test of phenolic compounds

Fungal growth of three isolates of *Heterobasidion annosum* (Fries:Fries) Brefeld in the media containing phenolic compounds is shown in Fig. 4. The mycelial growth of all tree isolates was inhibited by p-hydroxybenzoic acid, p-coumaric acid and (-)-epicatechin (Table 4). P-coumaric acid and (-)-epicatechin significantly inhibited the mycelial growth of *Heterobasidion annosum* by 8-100% and 23-100%, respectively. The average mycelial growth for controls was 0.866 cm day⁻¹. The *Heterobasidion annosum* isolate MUCL 30709 was negatively affected by the presence of all seven phenolics. There were significant differences in the inhibitory effect of p-hydroxybenzoic acid, p-coumaric acid, 4-hydroxyacetophenone, piceatannol and protocatechuic acid on fungal radial growth between the isolates and for p-hydroxybenzoic acid, p-coumaric acid, (-)-epicatechin and 4-hydroxyacetophenone between the concentrations (Table 3).

Table 3 ANOVA results for the *in vitro* test. ns - Not significant

<i>In vitro test with Heterobasidion annosum</i>			
Phenolic compounds	Significant differences between fungal strains	Significant differences between concentrations	Significant linear gradient
p-hydroxybenzoic acid	P < 0.05	P < 0.01	ns
(+)-catechin	ns	ns	ns
p-coumaric acid	P < 0.05	P < 0.001	P < 0.05
(-)-epicatechin	ns	P < 0.01	P < 0.01
4-hydroxyacetophenone	P < 0.05	P < 0.05	ns
piceatannol	P < 0.001	ns	ns
protocatechuic acid	P < 0.01	ns	ns
<i>In vitro test with Cyindrocarpon destructans</i>			
Phenolic compounds	Significant differences between fungal strains	Significant differences between concentrations	Significant linear gradient
p-hydroxybenzoic acid	ns	ns	ns
(+)-catechin	P < 0.01	ns	ns
p-coumaric acid	P < 0.001	ns	ns
(-)-epicatechin	P < 0.001	ns	ns
4-hydroxyacetophenone	P < 0.01	ns	P < 0.05
piceatannol	P < 0.01	ns	ns
protocatechuic acid	P < 0.001	ns	ns

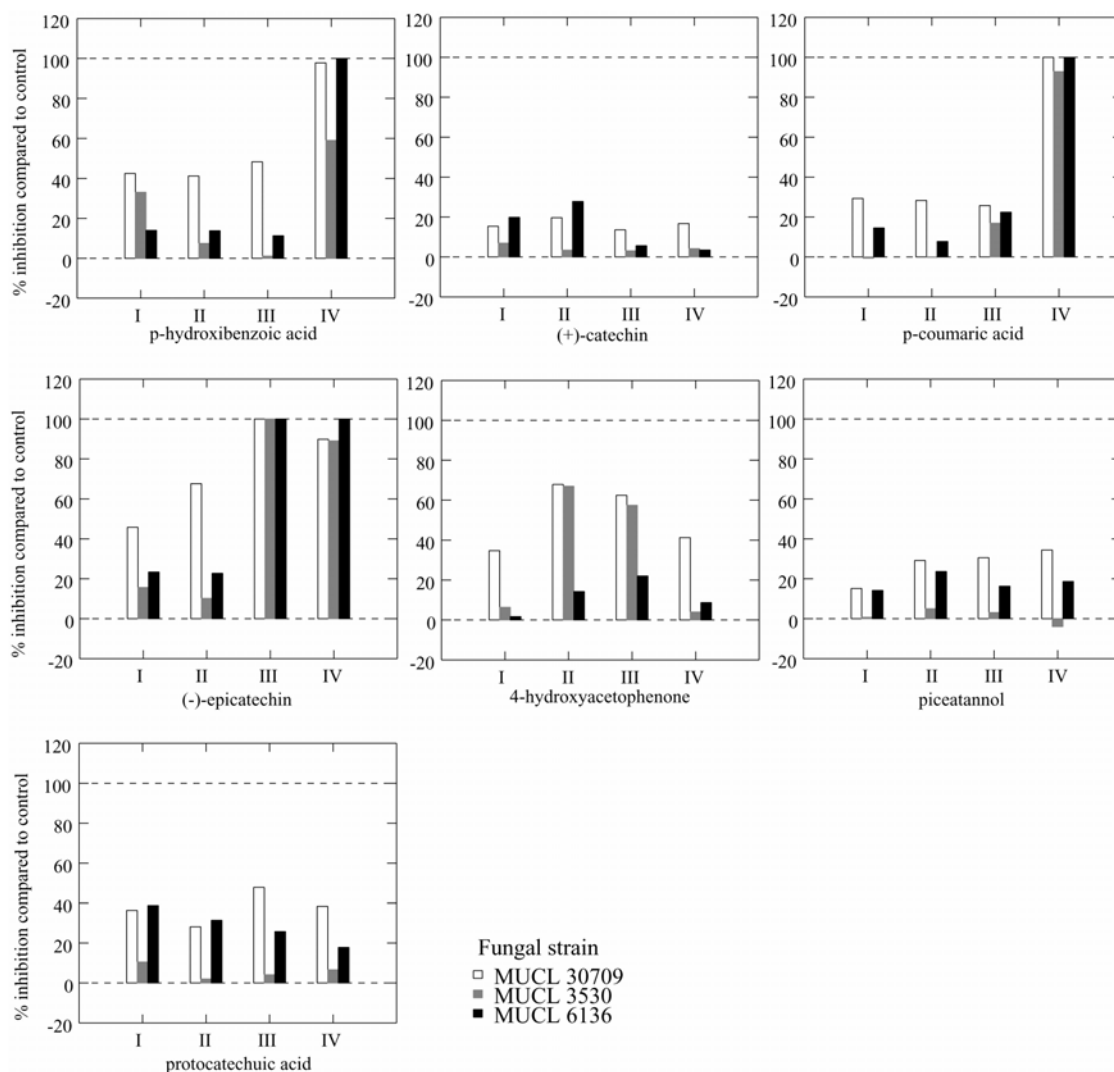


Fig. 4 Inhibition of mycelial growth of three isolates of *Heterobasidion annosum* (Fries:Fries) Brefeld *in vitro* by different concentrations of phenolic compounds. For concentrations see Table 1.

The effects of phenolic compounds on the growth of the three isolates of *Cylindrocarpon destructans* (Zins.) Scholten is shown in Fig. 5. There were no significant differences between the concentrations (Table 3). 4-hydroxyacetophenone significantly inhibited the growth of *Cylindrocarpon destructans*. The average mycelial growth of the controls was $0.384 \text{ cm day}^{-1}$. The inhibitory effect of (+)-catechin, p-coumaric acid, 4-hydroxyacetophenone, piceatannol and protocatechuic acid on fungal radial growth was

significantly different between the isolates (Table 3). The isolate MUCL 18715 was not inhibited by phenolic compounds (Table 4).

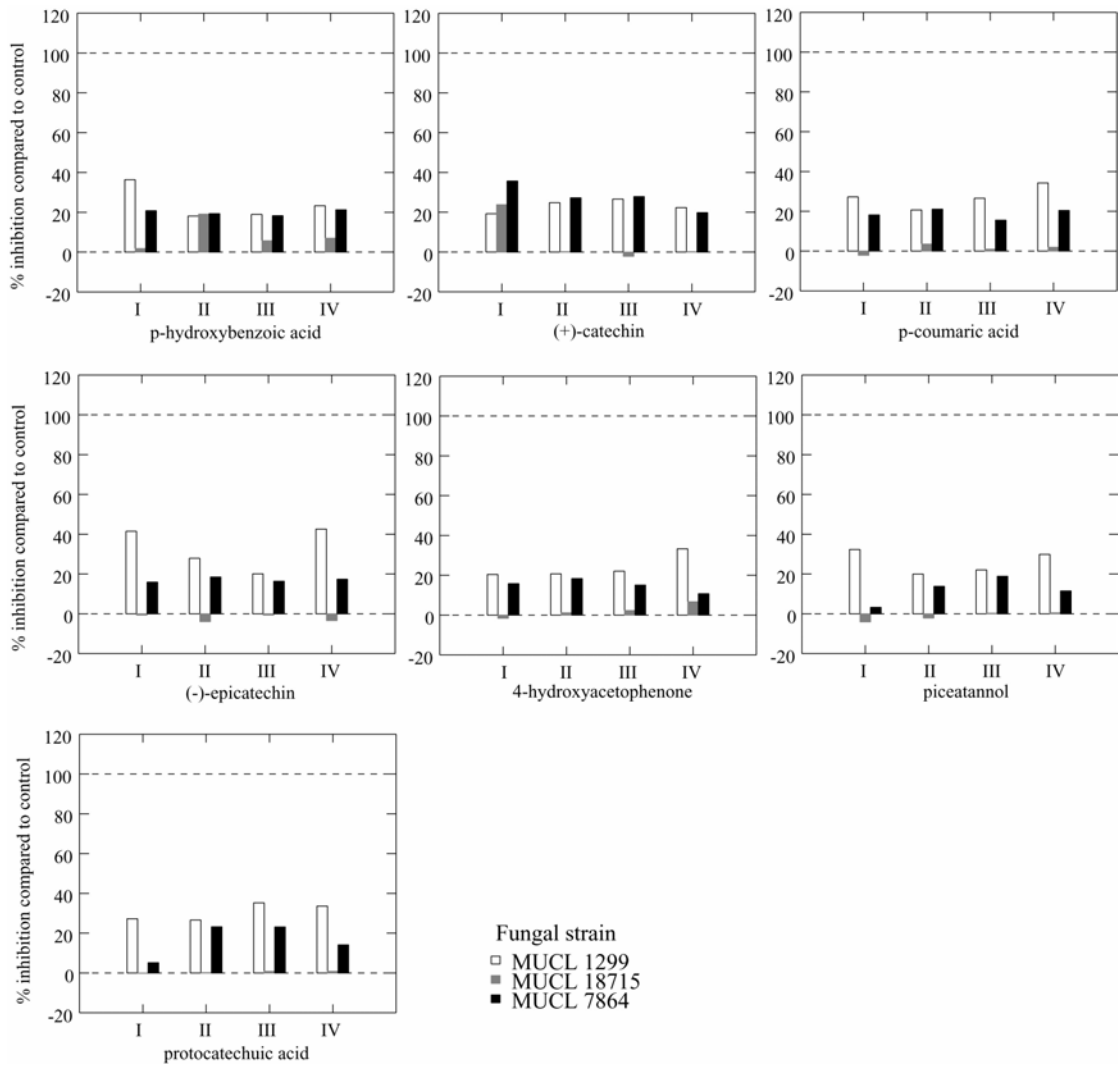


Fig. 5 Inhibition of mycelial growth of three isolates of *Cylindrocarpon destructans* (Zins.) Scholten. *in vitro* by different concentrations of phenolic compounds. For concentrations see Table 1.

Table 4 Number of strains with significant inhibition by phenolic compounds.

Phenolic compounds	Number of strains	
	<i>H. annosum</i>	<i>C. destructans</i>
p-hydroxybenzoic acid	3	2
(+)-catechin	2	2
p-coumaric acid	3	2
(-)-epicatechin	3	2
4-hydroxyacetophenone	2	2
piceatannol	2	2
protocatechuic acid	2	2

Discussion

In foliage and root bark of tree species a large number of phenolics have been found (Alcubilla *et al.*, 1971; Alcubilla *et al.*, 1987; Osswald, 1987; Richter and Wild, 1992; Strack, 1989). However, information on phenolic compounds in fine root are scarce. Our study documents the presence of 4-hydroxyacetophenone, piceatannol (3,3', 4,5'-tetrahydroxy-transstilbene), p-hydroxybenzoic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), p-coumaric acid (4-hydroxycinnamic acid), (-)-epicatechin in fine roots of both beech and Norway spruce and (+)-catechin in Norway spruce. Earlier studies have shown that the concentration of total phenolics decrease after nitrogen fertilization and decreased the defence chemistry (Bryant *et al.* 1987; Rousi *et al.*, 1993; Tuomi *et al.*, 1984;), although individual phenolics may show the opposite effect (Muzika and Pregitzer, 1992). However terpenes, which are also carbon-based compounds, did not respond to nitrogen fertilization, suggesting that production of carbon-based resistance compounds is not invariably linked to nitrogen availability (Muzika, 1993).

Our results show a reduction in the concentration of the fungistatic compounds (-)-epicatechin and piceatannol in beech and of piceatannol, 4-hydroxyacetophenone, (+)-catechin, p-hydroxybenzoic acid and protocatechuic acid in fine roots of spruce by nitrogen fertilization already at 10 kg N ha⁻¹ year⁻¹. The mechanisms causing the reduction in the carbon-based secondary compounds are unknown, but could be based on various processes. Phenolic, phenylpropanoids and derived compounds (hydroxycinnamic acids, flavonoids,

condensed tannin and lignin) are produced from phenylalanine, and therefore their synthesis competes directly with protein synthesis and thus with plant growth. Fertilization may cause a reduction in carbon-based secondary metabolites (Haukioja *et al.*, 1998).

One current hypothesis attempts to explain how and why nitrogen availability affects plant defence. The carbon/nutrient balance hypothesis predicts that fertilization with growth-limiting nutrients will lead to decreased concentrations of carbon-based secondary metabolites (Bryant *et al.*, 1987), but does not explain how this carbon is distributed among different pathways and compounds. The hypothesis has been validated for woody plant/herbivore interaction but not for plant/fungal pathogen interaction and the protection mechanisms against pathogenic fungi in roots might be different from those in needles and leaves.

Muzika and Pregister (1992) suggested that limited availability of nitrogen affects phenolics that are produced in the same shikimic acid pathway as aromatic amino acids. High availability of nitrogen accelerates protein synthesis and less phenylalanine is directed to phenolic production.

According to Graham (1983), the balance between primary and secondary metabolic pathways is probably shifted onto the shikimate pathway under nitrogen limiting condition, providing for phenolic compounds.

Elevated deposition of nitrogen and reduced chemical defence might explain the increased susceptibility of trees to pathogens (Bryant *et al.*, 1987; Rousi *et al.*, 1993; Tuomi *et al.*, 1984). Increased infection of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) by *Armillaria ostoyae* (Romagn.) Herik occurred after fertilization with nitrogen (Entry *et al.*, 1991). The root bark of the fertilized trees had lower concentrations of fungistatic phenolic compounds and higher nitrogen than control trees. Additionally, conifer trees with increased susceptibility to *Armillaria ostoyae* generally had fewer phenolic compounds and more sugar in roots, (Entry *et al.*, 1992) suggesting that the ratio of available sugars to phenolic compounds may be related to the success of the fungal infection.

Phenolic compounds are well known as antifungal compounds in some plants (Alcubilla *et al.*, 1971; Alcubilla, 1987; Alcubilla *et al.*, 1987; Yamamoto *et al.*, 2000). Our assay of fungal growth demonstrated some differences in phenolic sensitivity between the examined fungal species and isolates. The *Heterobasidion annosum* isolate MUCL 30709 were negatively affected by the presence of all seven phenolics substances. Two strains of *Cylindrocarpon destructans* were inhibited by the presence of all seven phenolics, whereas the growth of *Cylindrocarpon destructans* isolate MUCL 18715 was not inhibited.

The identified phenolic compounds have been previously identified as fungistatic aromatic substances implicated in allelopathy (Bennett and Wallsgrave, 1994; Yamamoto *et al.*, 2000; Macheix *et al.*, 1990; Stendlid & Johansson, 1987; Dübeler *et al.*, 1997; Osswald, 1987).

The cell wall phenolics (ferulic acid, caffeic acid and p-coumaric acid) at 0.025% inhibited growth of *Heterobasidion annosum in vitro* (Asiegbu, 2000). This is in accordance with the present results where p-coumaric acid inhibited fungal growth all three isolates of *Heterobasidion annosum* and two of *Cylindrocarpon destructans*. Piceatannol showed a fungistatic effect against *Heterobasidion annosum* in the inner bark of stems and roots of *Picea abies* L. (Karst.) (Alcubilla, 1987; Alcubilla *et al.*, 1987). Taxifolin, D-catechin, quercetin, piceatannol glucoside and isorapontigenin glucoside have been shown to inhibit *Heterobasidion annosum* as well (Alcubilla *et al.*, 1971). The effect of an extract of Norway spruce bark on the growth of the root rot fungus *Heterobasidion annosum* and the blue-stain fungus *Ceratocystis polonica* was investigated *in vitro* by Evensen *et al.* (2000). *Heterobasidion annosum* was not negatively affected, the extract had only fungistatic effects on the blue-stain fungus. In contrast to our results, catechin showed no inhibitory effect on *Heterobasidion annosum in vitro* (Bonello *et al.*, 1993) although these authors used similar concentrations than in the present study.

In conclusion, our results show that concentrations of most phenolics compounds in roots were reduced by nitrogen fertilization of 10 kg N ha⁻¹ year⁻¹ at an ambient nitrogen deposition of 12 kg N ha⁻¹ year⁻¹. Hence, critical loads for nitrogen must be set at below 20 kg N ha⁻¹ year⁻¹ to prevent the decrease of fungistatic phenolic compounds in tree roots. It is suggested that increased nitrogen deposition makes fine roots of trees more susceptible to pathogens.

Acknowledgements

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PAPER II

**Effect of nitrogen fertilization on fine root length, root tip density
and mineral nutrients in leaves of beech
(*Fagus sylvatica* L.) on acidic and calcareous soils**

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Abstract

The effect of nitrogen fertilization on fine root length, root tips and foliar nutrient concentrations of beech growing on acidic and calcareous soils was analysed in experimental field plots situated at two sites in Switzerland. Since 1992 the trees have been treated with dry ammonium nitrate to give 0, 10, 20, 40, 80, 160 kg N ha⁻¹ year⁻¹, respectively.

On acidic, not on calcareous soil, a decrease in the length proportion of finest roots (<0.25 mm diameter) and root tip density were found with increasing nitrogen treatment.

The foliar concentrations phosphorus, calcium and magnesium on acidic soil and phosphorus, potassium and calcium on calcareous soil were decreased with increasing nitrogen fertilization. There was a correlation between the length proportion of finest roots and foliar calcium and magnesium concentrations.

Key words: acidic and calcareous soils, fine root length, foliage nutrients, nitrogen fertilization, root tips

Introduction

Fine roots are important structural and functional components of forested ecosystems (Grier *et al.*, 1981; Persson, 1980). Diameter analyses show that the major part of the length of tree root systems consist of fine roots (Lyr and Hoffman, 1967). Roots <2 mm in diameter are termed fine roots (Böhm, 1979). Hendrick and Pregitzer (1993) showed that a large proportion of net primary production in sugar maple is going to roots <0.5 mm in diameter, and that they contributed a large proportion on the root biomass and this roots are the physiologically most active part of the root system, responsible for water and nutrient uptake. Root length is a direct indicator of the potential for the nutrient and water uptake (Atkinson, 2000).

Increased nitrogen deposition has become a major concern for the health of European forests. Nitrogen deposition has increased within the last decades, exceeding the critical nitrogen loads for forests of 10-20 kg N ha⁻¹ year⁻¹ (Achermann and Bobbink, 2002). In Switzerland in large parts of the country Kurz and Rihm (1997) estimate that critical loads for nitrogen are exceeded in 90% of the Swiss forests.

High nitrogen deposition is expected to produce long-lasting effects on fine-root systems and their function. Nitrogen fertilization has negative effects on fine-root development, resulting in a reduction in the amount of fine roots to about 50% (Persson and Ahlstrom 1991). Nadelhoffer (2000) reported that with increasing N availability, fine-root biomass decreases and turnover increases. Matzner and Murach (1995) showed a negative relation between nitrogen deposition and soil acidification on the one hand and a reduction the fine root biomass and root length of trees.

Nitrogen deposition of more than 13 to 25 kg N ha⁻¹ year⁻¹ lead to elevated NO₃ - leaching, decreasing base cation pools in soil, and increasing soil acidity (de Vries *et al.*, 2003). Many forest soils have become acidified and have high levels of both Al and NO₃ in the soil solution (Falkengren-Grerup and Eriksson, 1990). Changes in soils that may occur during soil acidification include reduced soil pH, decreased base saturation, increased availability of aluminium and manganese and competition between base cations and Al for exchange sites in the fine roots (Robarge and Johnson, 1992). The effects of soil acidity depend on the investigated parameter. Total root biomass may be increased in acidic soils (Godbold *et al.*, 2003, Leuschner and Hertel, 2003). Specific root length and tip density are, however, decreased (Godbold *et al.*, 2003)

The present investigation deals with the effect of nitrogen on two afforestation plots differing in soil acidity: one is base poor, the other one is situated on a calcareous rendzina. Fine root length, fine root tips and well as foliar nutrient concentrations were investigated.

Materials and methods

Site description

The plots are situated at two sites in Switzerland on acidic and calcareous soils (Table 1). Trees were planted in 1992 and treated three times per season, in April, July and October with dry ammonium nitrate to give loads of 0, 10, 20, 40, 80, 160 kg N ha⁻¹ year⁻¹, respectively. The plants were arranged in a fully randomised block design with twelve replicates per treatment. The modelled atmospheric nitrogen deposition at the plot Zugerberg was 20.0 kg N ha⁻¹ year⁻¹ and at the plot Hochwald 15.5 kg N ha⁻¹ year⁻¹ (Rihm, 2001).

Table 1 Characteristics of the sites used in the nitrogen fertilization experiment in afforestation plots (Flückiger and Braun, 1999).

Plot	Zugerberg	Hochwald
Altitude (m asl)	1000	670
Soil type	Dystric Cambisol	Rendzic Leptosol
Geology	till	limestone
pH (CaCl ₂) 0-40 cm	4.0	6.9
Base saturation (%)		
- 0-2 cm	44.9	100
- 2-10 cm	26.9	100
- 10-60 cm	2.5	100

Sample collection

Foliage for nutrient analysis was sampled in August 2002 from all trees in Zugerberg and Hochwald plots.

The roots were excavated by hand from the soil layer (0-30 cm) from an area of 1 m² around the stem in March 2003. Roots were washed and dead roots from living roots were

separated using macroscopic criteria (Vogt and Persson, 1991). Roots were scanned and analysed for different diameter classes using the software WinRHIZO 3.9 (Regent Instruments Inc., Quebec, Canada). Root length proportion of the finest root class (≤ 0.25 mm) in total root length (further called “length proportion of fine roots” and root tips (number of tips cm^{-1}) were measured.

Nutrient analysis in leaves

The leaves were dried at 70° , ground and analyzed for their concentrations of N, P, K, Ca, Mg and Mn after mixed acid digestion with $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ (Allen, 1989). N and P were determined colorimetrically (Walinga *et al.*, 1995). Ca, Mg and Mn were analyzed using atomic absorption photometry (air/ C_2H_2 with lanthanum addition) and K using flame photometry.

Statistical analysis

The effect of nitrogen fertilization on fine root length, fine root tip density and mineral nutrients in leaves was evaluated by one-way analysis of variance after root transformation, with nitrogen fertilization as class variable, Fisher’s LSD-test was used for testing differences to the control. Overall linear regression was tested after the ANOVA using contrasts. The Spearman Correlation was used to analyse whether the root parameters correlated with the nutrient concentrations in leaves. All calculations were performed with the statistical package SYSTAT® vers.10 (SPSS Inc., Chicago, USA).

Results

Mineral nutrients in leaves

The nutrient status of beech in the plot Zugerberg was changed significantly by the N fertilization (Table 2). N concentrations in leaves remained unchanged. P concentrations decreased significantly by 11% with nitrogen fertilization at $40 \text{ kg N ha}^{-1} \text{ year}^{-1}$. At the same treatment, also Mg decreased by 26% and Ca concentrations by 22%. K and Mn were not

significantly affected by nitrogen fertilization. Foliage N/P and N/Mg ratios increased significantly at 40 kg N ha⁻¹ year⁻¹. There was no significant change in N/K ratio.

In the plot Hochwald both P and K in beech leaves decreased significantly with nitrogen fertilization by 6% and 10%, respectively at 40 kg N ha⁻¹ year⁻¹ (Table 2). Ca decreased by 17% at 160 kg N ha⁻¹ year⁻¹. No change was observed with N, Mg and Mn. N/P and N/K ratios increased significantly at 160 kg N ha⁻¹ year⁻¹. There was no significant change in N/Mg ratio.

Table 2 Nutrient concentrations in beech leaves in mg g⁻¹ d. wt. (mean ± standard error of mean). Significant differences to control or significance of linear trend: * p < 0.05 , ** p < 0.01, ***p < 0.001.

Plot	N fertilization (kg N ha ⁻¹ year ⁻¹)	Nutrient concentrations (in dry weight)								
		N mg g ⁻¹	P mg g ⁻¹	K mg g ⁻¹	Ca mg g ⁻¹	Mg mg g ⁻¹	Mn mg kg ⁻¹	N/P w/w	N/K w/w	N/Mg w/w
„Zugerberg“										
	0	24.43±1.47	1.56±0.12	4.42±0.63	9.23±1.64	1.61±0.51	3023±1406	15.72±1.44	5.62±0.79	16.58±5.12
	10	24.10±2.21	1.63±0.15	4.76±0.49	8.34±1.72	1.45±0.47	3451±1136	14.87±1.87	5.09±0.57	18.72±7.53
	20	24.38±2.81	1.53±0.15	4.93±0.71	8.47±1.66	1.61±0.25	2946±973	16.04±2.38	5.03±0.86	15.34±2.12
	40	24.06±2.55	1.39±0.09 *	4.79±0.91	7.21±1.58 *	1.20±0.39 *	3107±961	17.34±2.32 *	5.19±1.10	21.95±7.21 *
	80	23.80±1.72	1.35±0.11 ***	4.58±0.82	6.94±0.44 **	1.10±0.44 **	2687±757	17.69±0.91 *	5.31±0.70	26.94±17.21 **
	160	23.64±2.69	1.40±0.17 ***	4.42±1.30	4.93±1.34 ***	0.73±0.42 ***	2931±684	17.06±2.43	5.77±1.68	44.18±27.87 ***
Linear trend		ns	***	ns	***	***	ns	**	ns	***
“Hochwald”										
	0	19.89±2.14	0.99±0.12	3.01±0.71	14.68±2.97	1.80±0.55	67.76±18.59	20.22±2.56	6.82±1.16	12.15±4.42
	10	19.86±1.46	0.95±0.20	3.28±0.61	14.28±2.42	1.69±0.43	59.33±19.05	21.92±5.36	6.27±1.31	12.52±3.52
	20	18.94±2.16	0.90±0.15	2.79±0.46	14.51±2.52	1.66±0.42	60.99±12.29	21.82±5.74	6.92±1.14	12.03±3.03
	40	19.60±1.03	0.93±0.08 *	2.72±0.40 *	13.66±1.88	1.87±0.41	65.31±10.86	21.28±2.15	7.32±1.04	10.95±2.62
	80	18.83±1.41	0.82±0.18 ***	2.48±0.31 ***	13.72±3.45	1.67±0.38	61.43±22.53	23.81±4.69	7.68±1.00	11.82±2.82
	160	19.81±1.60	0.75±0.17 ***	2.28±0.44 ***	12.24±2.66 *	1.49±0.36	77.59±46.69	27.43±6.21 ***	8.99±1.94 ***	14.17±4.43
Linear trend		ns	***	***	*	ns	ns	**	***	ns

Fine root length and root tips

In the experimental plot Zugerberg on acidic soil, nitrogen fertilization decreased the length proportion of fine roots significantly by 30% at the highest treatment (from 24.5 ± 4.8 to $17.0 \pm 3.1\%$ of total root length), with $40 \text{ kg N ha}^{-1} \text{ year}^{-1}$ as lowest treatment giving a significant difference to the control (Figure 1). The root tip density decreased from 2.5 cm^{-1} in non fertilized trees to 2.1 in the highest treatment, with the first significant difference found already at $20 \text{ kg N ha}^{-1} \text{ year}^{-1}$.

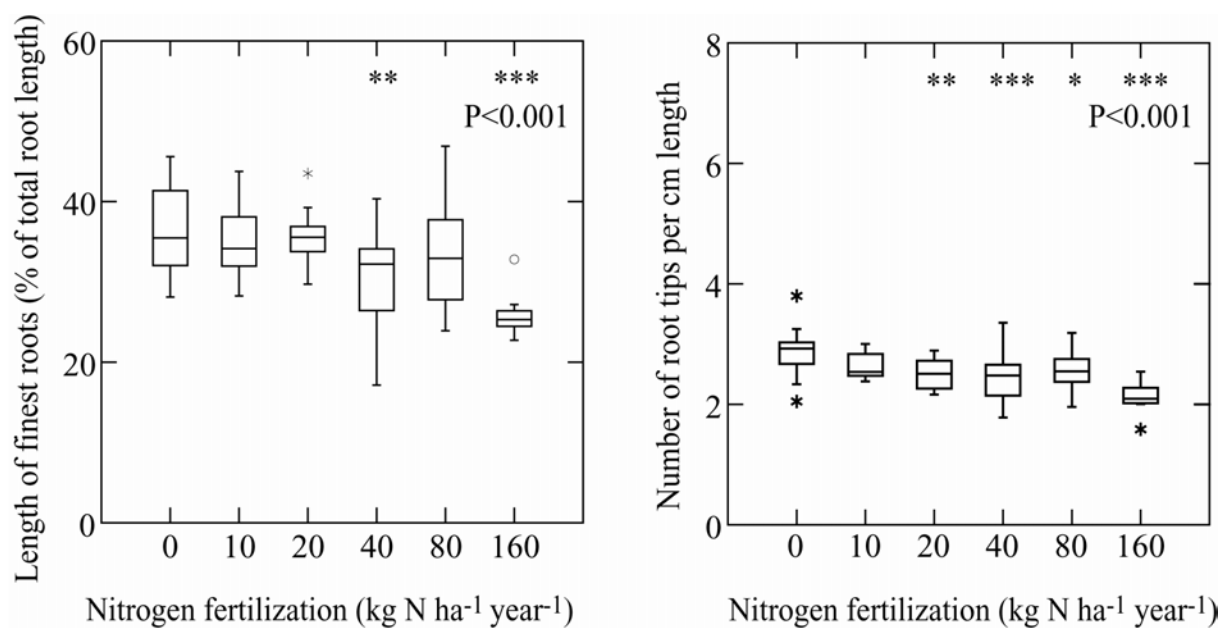


Figure 1 Length proportion of finest roots (<math><0.25 \text{ mm}</math> diameter) and root tip density from plot Zugerberg. Difference to control significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Nitrogen had no significant effect on fine root length and tip density in the experimental plot Hochwald on calcareous soil (Figure 2), with generally higher levels of these two parameters.

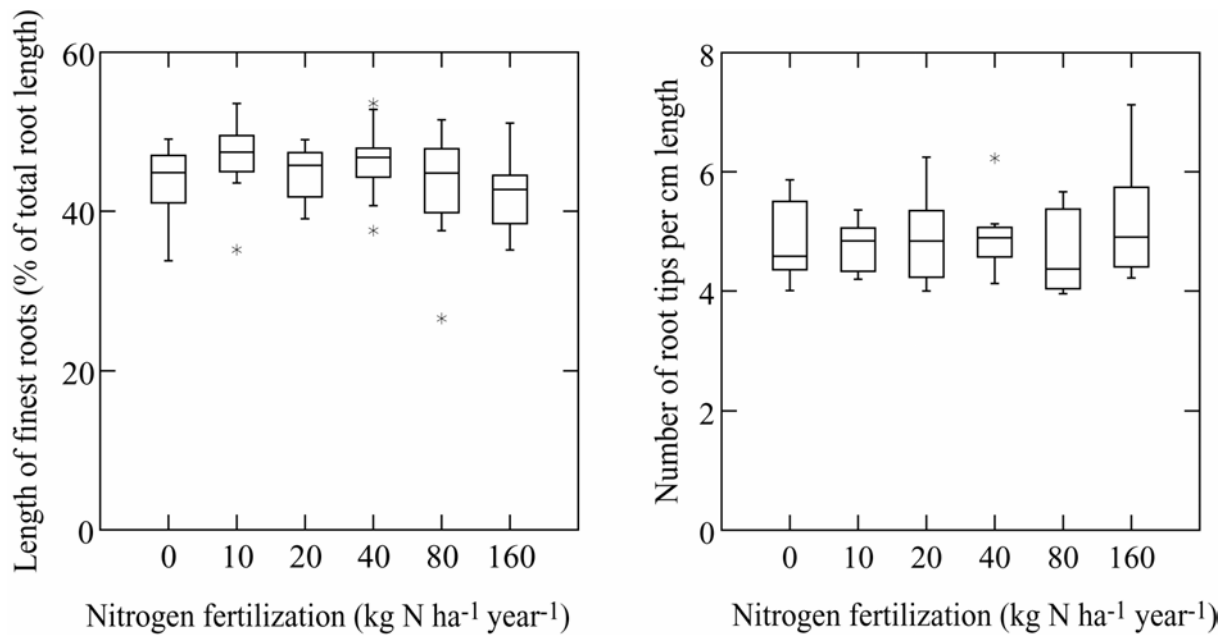


Figure 2 Length proportion of finest roots (<0.25 mm diameter) and root tip density from plot Hochwald.

Correlation of length proportion of fine roots and leaves nutrients

The length proportion of fine roots (≤ 0.25 mm diameter) from beech in the plot Zugerberg significantly correlated with the leaf concentrations of Ca and Mg (Figure 3). We found no correlation on plot Hochwald, with substantially higher overall leaf concentrations of Mg and Ca and higher length proportion on fine roots.

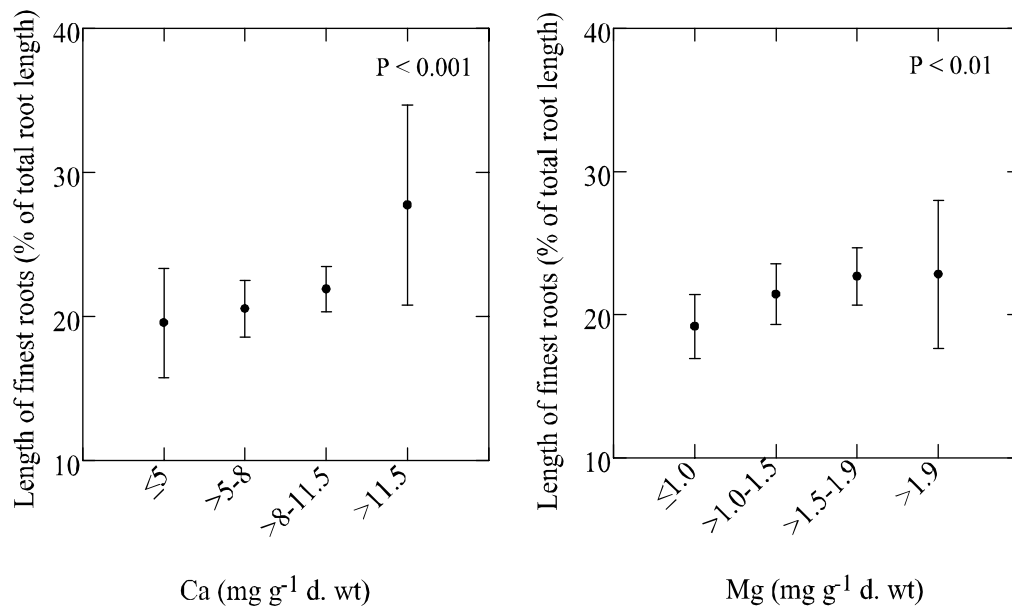


Figure 3 Correlations between the concentrations of Ca ($R_{\text{Spearman}}=0.361$) and Mg ($R_{\text{Spearman}}=0.333$) in beech leaves and relative length of finest roots from plot Zugerberg.

Discussion

The reduction in P concentration in response to nitrogen was found on both the acidic and the base rich site and has also been observed in numerous European studies (Flückiger and Braun, 1999; Gundersen, 1998; Mohren *et al.*, 1986; Roelofs *et al.*, 1993). The effect of nitrogen on the other macronutrients depends more on soil conditions. Mg deficiency in forest trees is often observed on acidic soils (Hüttl, 1991), whereas K deficiency is more likely on calcareous soils (Fiedler *et al.*, 1973). Nitrogen may then amplify these deficiencies. In the case of Mg, decreases by nitrogen were described by Gundersen (1998), Hüttl (1990) and Kölling *et al.* (1997). The decrease of Ca by N fertilization in the Zugerberg plot can also be explained by the low reserves in the soil. In the plot Hochwald with very high Ca reserves in the soil, however, only changes in uptake may explain the Ca decrease at $160 \text{ kg N ha}^{-1} \text{ year}^{-1}$.

The results from this study showed that the length proportion of finest roots and the root tip density of beech were reduced by nitrogen fertilization on acidic soils but not on

calcareous soils. The nitrogen treatment caused a substantial soil acidification on Zugerberg within 10 years (IAP 2004) and low base saturation was found to be correlated in the field with a decreased length proportion of finest roots and root tip density (Braun *et al.*, 2005). It is therefore suggested that the observed results are rather an acidity than a nitrogen effect although Seith *et al.* (1996) demonstrated that the specific root length from Norway spruce decreased with an increase of inorganic nitrogen supply. A similar acidity effect on roots was observed by Godbold *et al.* (2003), with a reduction of the specific length and specific root tips of fine roots in Norway spruce.

The decreased root length may have consequences for water and nutrient uptake. During the dry summer 2003, significantly increased drought damages were observed in N fertilized plots (IAP 2004; Thomas *et al.*, 2005).

The correlation between the relative length of fine roots and the Ca and Mg concentration in the leaves may be either a result of a decreased uptake in consequence of the changed root parameters or an expression of a parallel acidity effect on both root length and leaf concentrations. The present data do not allow to decide between the two scenarios although the significance of root tips for the uptake of Ca and Mg has been pointed out by Mengel and Kirkby (1982). P nutrition decreased independently of the changes in root length observed on Zugerberg. In the case of this element, the importance of mycorrhiza for uptake is well known; and mycorrhiza may react quite sensitively to nitrogen (Nilsson and Wallander, 2003).

In conclusion, we found that the relative length of finest roots and the root tip density of beech were reduced by nitrogen fertilization on acidic soil but not on calcareous soil.

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4 GENERAL DISCUSSION

In foliage and root bark of tree species a large number of phenolics have been found (Alcubilla *et al.*, 1971; Alcubilla *et al.*, 1987; Osswald, 1987; Richter and Wild, 1992; Strack, 1989). However, information on phenolic compounds in fine roots is scarce. This thesis documents the presence of 4-hydroxyacetophenone, piceatannol, p-hydroxybenzoic acid, protocatechuic acid, p-coumaric acid, and (-)-epicatechin in fine roots of both beech and Norway spruce and (+)-catechin in Norway spruce.

Earlier studies have shown that the concentrations of total phenolics decreased after nitrogen fertilization and decreased the defence chemistry (Bryant *et al.* 1987; Rousi *et al.*, 1993; Tuomi *et al.*, 1984), although individual phenolics may show the opposite effect (Muzika and Pregitzer, 1992). However terpenes, which are also carbon-based compounds, did not respond to nitrogen fertilization, suggesting that production of carbon-based resistance compounds is not invariably linked to nitrogen availability (Muzika, 1993).

The results from **Paper I** show a reduction in the concentration of the fungistatic compounds (-)-epicatechin and piceatannol in fine roots of beech and of piceatannol, 4-hydroxyacetophenone, (+)-catechin, p-hydroxybenzoic acid, and protocatechuic acid in fine roots of spruce by a nitrogen fertilization of 10 kg N ha⁻¹ year⁻¹. The mechanisms causing the reduction in the carbon-based secondary compounds are unknown, but could be based on various processes. Phenolic compounds are produced from phenylalanine, and therefore their synthesis competes directly with protein synthesis and thus with plant growth. Fertilization may cause a reduction in carbon-based secondary metabolites (Haukioja *et al.*, 1998).

The carbon/nutrient balance hypothesis predicts that fertilization with growth-limiting nutrients will lead to decreased concentrations of carbon-based secondary metabolites (Bryant *et al.*, 1987), but does not explain how this carbon is distributed among different pathways and compounds. The hypothesis has been validated for woody plant/herbivore interaction but not for plant/fungal pathogen interaction and the protection mechanisms against pathogenic fungi in roots might be different from those in needles and leaves.

Muzika and Pregister (1992) suggested that limited availability of nitrogen affects phenolics that are produced in the same shikimic acid pathway as aromatic amino acids. High availability of nitrogen accelerates protein synthesis and less phenylalanine is directed to phenolic production. According to Graham (1983), the balance between primary and secondary metabolic pathways is probably shifted onto the shikimate pathway under nitrogen limiting condition, providing for phenolic compounds. More experimental data concerning this issue are needed in order to understand how nitrogen fertilization may affect phenolic compound concentrations in tree fine roots.

Elevated deposition of nitrogen and reduced chemical defence might explain the increased susceptibility of trees to pathogens (Bryant *et al.*, 1987; Rousi *et al.*, 1993; Tuomi *et al.*, 1984). The identified phenolic compounds have been previously identified as fungistatic aromatic substances implicated in allelopathy (Bennett and Wallsgrave, 1994; Dübeler *et al.*, 1997; Macheix *et al.*, 1990; Osswald, 1987 ; Stendlid and Johansson, 1987; Yamamoto *et al.*, 2000). I showed that the *in vitro* growth of *Heterobasidion annosum* and *Cylindrocarpon destructans* was inhibited by the presence of most of the listed fungistatic phenolic compounds in the concentrations found in the roots. The cell wall phenolics inhibited growth of *Heterobasidion annosum in vitro* (Asiegbu, 2000). This is in accordance with the present results where p-coumaric acid inhibited growth of all three isolates of *Heterobasidion annosum* and two of *Cylindrocarpon destructans*. Piceatannol showed a fungistatic effect against *Heterobasidion annosum* in the inner bark of stems and roots of *Picea abies* L. (Karst.) (Alcubilla, 1987; Alcubilla *et al.*, 1987). In contrast to my results, catechin showed no inhibitory effect on *Heterobasidion annosum in vitro* (Bonello *et al.*, 1993) although these authors used concentrations similar to the ones in the present study.

In **Paper II**, I have demonstrated the effect of nitrogen fertilization on beech leaf nutrients. The reduction of P concentration in response to nitrogen was found on both the acidic and the base rich site and has also been observed in numerous European studies (Flückiger and Braun, 1999; Gundersen, 1998; Mohren *et al.*, 1986; Roelofs *et al.*, 1993). The effect of nitrogen on the other macronutrients depends more on soil conditions. Mg deficiency in forest trees is often observed on acidic soils (Hüttl, 1991), whereas K deficiency is more likely on calcareous soils (Fiedler *et al.*, 1973). Nitrogen may then amplify these deficiencies. In the case of Mg, decreases by nitrogen were described by Gundersen (1998), Hüttl (1990), and

Kölling *et al.* (1997). The decrease of Ca by N fertilization in the Zugerberg plot can also be explained by the low reserves in the soil. In the plot Hochwald with very high Ca reserves in the soil, however, only changes in uptake may explain the Ca decrease at $160 \text{ kg N ha}^{-1} \text{ year}^{-1}$.

The results from this thesis showed that the length proportion of finest roots and the root tip density of beech were reduced by nitrogen fertilization on acidic soils but not on calcareous soils. Braun *et al.* (2005) found decreases of the relative length of fine roots ($<0.25 \text{ mm}$ in diameter) in soils with low base saturation and modelled nitrogen deposition between 13 and $33 \text{ kg N ha}^{-1} \text{ year}^{-1}$. The observed results are rather an acidity than a nitrogen effect although Seith *et al.* (1996) demonstrated that the specific root length of Norway spruce decreased with an increase of inorganic nitrogen supply. A similar acidity effect on roots was observed by Godbold *et al.* (2003), with a reduction of the specific length and specific root tips of fine roots in Norway spruce.

The correlation between the relative length of fine roots and the Ca and Mg concentration in the leaves may be either a result of a decreased uptake in consequence of the changed root parameters or an expression of a parallel acidity effect on both root length and leaf concentrations. The present data do not allow deciding between the two scenarios although the significance of root tips for the uptake of Ca and Mg has been pointed out by Mengel and Kirkby (1982). P nutrition decreased independently of the changes in root length observed on Zugerberg. In the case of this element, the importance of mycorrhiza for uptake is well known, and mycorrhiza may react quite sensitively to nitrogen (Nilsson and Wallander, 2003).

5 CONCLUSION

The main objective of this thesis was to determine the effect of nitrogen on beech and Norway spruce and on the pre-formed defences of their fine roots against fungal pathogens. The specific aims were to examine the effect of nitrogen fertilization on fine root fungistatic phenolics (**Paper I**), to test the effect of phenolics on pathogenic fungi (**Paper I**), and to study the effect of increased nitrogen on beech fine roots length, tips, and leaves nutrients growing on different soil conditions (**Paper II**).

The results of this series of studies can be summarised as follow:

1. Concentrations of most phenolic compounds in beech and Norway spruce roots were reduced by nitrogen fertilization.
2. The *in vitro* growth of *Heterobasidion annosum* and *Cylindrocarpon destructans* was inhibited by the presence of most of the fungistatic phenolic compounds in the concentrations found in the roots.
3. The level of phenolics in beech leaves was negatively correlated with the nitrogen concentration in leaves.
4. Increase in beech leaves phenolic compounds correlate with increased leaf necrosis caused by *Phomopsis* sp.
5. Nitrogen fertilization at 160 kg N ha⁻¹ year⁻¹ increased the concentrations of free amino acids in beech and Norway spruce fine roots on acidic soils.
6. The relative length of fine roots and the root tip density of beech were reduced by nitrogen fertilization on acidic soil but not on calcareous soil.
7. The nutrient concentration in beech leaves decreased due to increased nitrogen fertilization.

In summary, the study indicates that nitrogen deposition has a potential to increase the susceptibility of tree roots to pathogens.

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APPENDIX I

Total amino acid concentrations in fine roots of beech and Norway spruce.

Table 1 Concentration of total amino acid (mg leucin equivalent mg g⁻¹ d. wt.) in the fine roots of beech (plots Möhlin) and Norway spruce (plot Zugerberg) from control and fertilized plots. Mean±SE (n=12). Levels of significant: ** p<0.01.

Tree species	Beech	Norway spruce
0 kg N ha ⁻¹ year ⁻¹	23.66±11.20	30.28±7.30
10 kg N ha ⁻¹ year ⁻¹	24.31±7.78	31.84±13.83
20 kg N ha ⁻¹ year ⁻¹	20.76±9.18	29.59±12.01
40 kg N ha ⁻¹ year ⁻¹	24.75±10.0	26.02±6.27
80 kg N ha ⁻¹ year ⁻¹	33.39±8.94	33.71±16.64
160 kg N ha ⁻¹ year ⁻¹	47.49±17.34 **	45.56±12.0 **

Table 2 Concentration of total amino acid (mg leucin equivalent mg g⁻¹ d. wt.) in the fine roots of beech from plots Zugerberg and Hochwald. Mean±SE (n=12). Levels of significant: * p<0.05, *** p<0.001.

Plots	Zugerberg	Hochwald
0 kg N ha ⁻¹ year ⁻¹	53.90±16.35	34.31±8.81
10 kg N ha ⁻¹ year ⁻¹	56.10±27.90	32.37±8.84
20 kg N ha ⁻¹ year ⁻¹	63.73±19.43	31.61±6.84
40 kg N ha ⁻¹ year ⁻¹	63.95±13.51	31.90±5.86
80 kg N ha ⁻¹ year ⁻¹	72.02±17.29 *	42.71±17.45
160 kg N ha ⁻¹ year ⁻¹	98.26±27.61 ***	33.13±10.09

APPENDIX II

Correlations between leaf phenolic compounds and nitrogen contents and leaf necrosis (*Phomopsis* sp.) in beech leaves from plot Hochwald

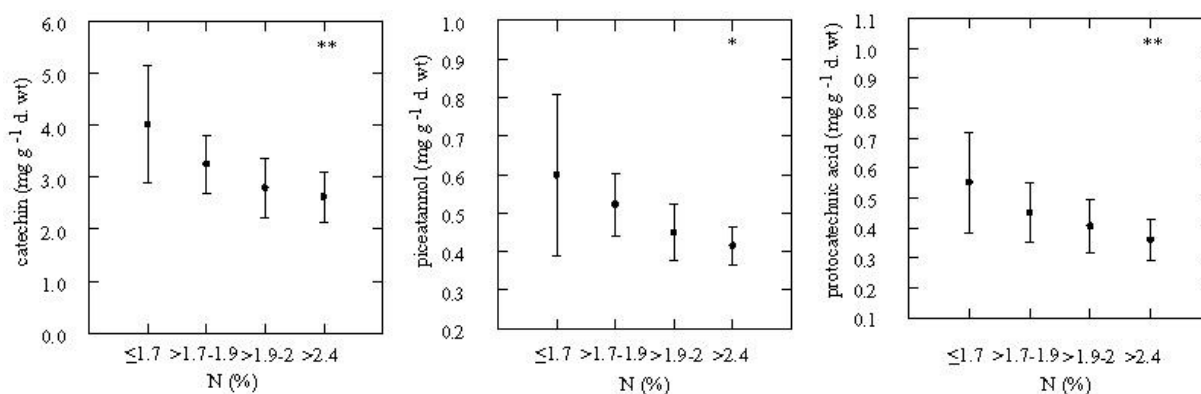


Figure 1 Correlation between catechin ($R_{\text{Spearman}}=-0.329$), piceatannol ($R_{\text{Spearman}}=-0.320$), protocatechuic acid ($R_{\text{Spearman}}=-0.356$) and nitrogen concentration in beech leaves from plot Hochwald (2002). Bars indicate 95% confidence interval. * $p < 0.05$ and ** $p < 0.01$.

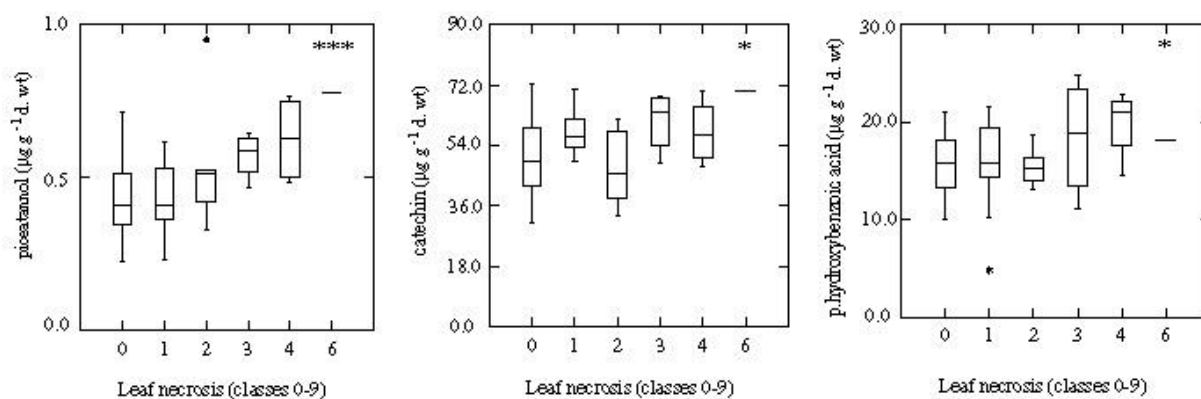


Figure 2 Correlation between piceatannol ($R_{\text{Spearman}}=0.489$), catechin ($R_{\text{Spearman}}=0.302$) and p-hydroxybenzoic acid ($R_{\text{Spearman}}=0.298$) and leaves necrosis (*Phomopsis* sp.) in beech leaves from plot Hochwald (2002). * $p < 0.05$ and **** $p > 0.001$.

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- 1978 - 1981** Elementary school in Algeria (Bulgarian Embassy).
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During my Ph. D. studies at the University of Basel I have attended lectures of the following professors and docents: T. Boller, W. Flückiger, Ch. Körner and V. Wiemken.