# Responses of Scots pine to nickel and copper exposure and herbivory

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Academic dissertation in environmental ecology

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## CONTENTS

## ABSTRACT

The goal of this thesis was to examine the ecophysiological responses of Scots pine (*Pinus sylvestris* L.), with an emphasis on the oxidative enzyme peroxidase and plant phenolics to environmental stresses like elevated levels of nickel (Ni) and copper (Cu), and herbivory. The effects of Ni and Cu were studied in a gradient survey at a sulphur dioxide ($SO_2$) contaminated site in the Kola Peninsula and with experiments, in which seedlings were exposed to Ni mist or to Ni and Cu amended into the soil. In addition, experimental Ni exposure was combined with disturbance of the natural lichen cover on the forest ground layer.

Pine sawfly attack was simulated in the early season defoliation experiment, in which mature Scots pine were defoliated (100 %) during two successive years in a dry, nutrient-poor Scots pine stand. In addition, the effect of previous defoliation on the growth of sawfly (*Diprion pini* L.) larvae was studied.

Apoplastic peroxidase activity was elevated in the needles of pine in an $SO_2$- , Ni- and Cu- polluted environment. Increased foliar peroxidase activity due to Ni contamination was shown in the experiment, in which Ni was added as spray. No such response was found in peroxidase acitivity in the roots exposed to elevated Ni and/or Cu in the soil. Elevated Ni in the soil increased the concentration of foliar condensed tannins, which are able to bind toxic Ni in the cells.

Wet Ni deposition of 2000 mg $m^{-2}$ reduced growth and survival of pine seedlings, whereas deposition levels 200 mg $m^{-2}$ or 20 mg $m^{-2}$ caused no effects in a 2-y lasting experiment. The lichen mat on the forest floor did not act as an effective buffer against the adverse impacts of heavy metals on pine seedlings. However, some evidence was found indicating that soil microbes profited from the lichen mat. Moreover, addition of low levels of Ni in the soil appeared to benefit pine seedlings, which was seen as promoted shoot growth and better condition of the roots.

Artificial defoliation increased peroxidase activity in the Scots pine needles. In addition, defoliation decreased nitrogen, diamine putrescine and glucose concentrations in the needles and increased the concentrations of several phenolic compounds, starch and sucrose. Previous artificial defoliation led to poor growth of sawfly larvae reared on the pines, suggesting a delayed induced resistance in Scots pine. However, there was no consistent relationship between inducibility (proportional increase in a compound following defoliation) and adverse effects on the growth of pine sawfly larvae. The observed inducible responses in needle phenolics due to previous defoliation thus appear to represent non-specific responses against sawflies.

## LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which in the text are referred to by their Roman numerals:

I Roitto M., Ahonen-Jonnarth U., Lamppu J. & Huttunen S. 1999. Apoplastic and total peroxidase acitivities in Scots pine needles at subarctic polluted sites. European Journal of Forest Pathology 29: 399-410.

II Roitto M., Strömmer R., Ahonen-Jonnarth U., Hyvärinen M. & Markkola A.M. 2001. Does the lichen mat alleviate the effects of wet deposited nickel on soil microorganisms and Scots pine (*Pinus sylvestris* L.) seedlings? Plant and Soil 230: 267-277.

III Roitto M., Rautio P., Julkunen-Tiitto, R., Kukkola, E. & Huttunen S. 2005. Changes in the concentrations of phenolics and photosynthates in Scots pine (*Pinus sylvestris* L.) seedlings exposed to nickel and copper. Environmental Pollution 137: 603-609.

IV Ahonen-Jonnarth U., Roitto M., Markkola A.M., Ranta H. & Neuvonen S. 2004. Effects of nickel and copper on growth and mycorrhiza of Scots pine seedlings inoculated with *Gremmeniella abietina*. Forest Pathology 34: 337-348.

V Roitto, M., Markkola, A.M., Julkunen-Tiitto, R., Sarjala, T., Rautio, P., Kuikka, K. & Tuomi, J. 2003. Defoliation-induced responses in peroxidases, phenolics and polyamines in Scots pine (*Pinus sylvestris* L.) needles. Journal of Chemical Ecology 29: 1905-1918.

VI Roitto M., Rautio P., Markkola A.M., Julkunen-Tiitto R., Kuikka K. & Tuomi J.: Induced accumulation of phenolics in Scots pine in response to previous defoliation: does inducibility of individual compounds correlate with adverse effects on sawfly larvae? Submitted manuscript.



## THE AUTHOR'S CONTRIBUTION

Marja Roitto planned and conducted the experiments in II, V and VI together with supervisors A.M. Markkola, P. Rautio and R. Strömmer, and other authors. The study I was supervised by S. Huttunen. In III, the experiment was planned and started by P. Rautio and E. Kukkola. In IV, the experiment was planned and started by S. Neuvonen and H. Ranta. M. Roitto was responsible for and carried out analyses in needle chemistry presented in all articles. In addition, T. Sarjala conducted polyamine analyses in V. Marja Roitto is the corresponding author in I-III, V and VI and has interpreted the results and written the articles together with the other authors.

## ABBREVIATIONS AND CONCEPTS USED IN THE THESIS AND THEIR DEFINITIONS:

| | |
|---|---|
| C | current year |
| C+1 | previous year |
| CNB | carbon nutrient balance |
| $CO_2$ | carbon dioxide |
| Cu | copper |
| DIR | delayed induced resistance |
| DMSO | dimethyl sulphoxide |
| DC | dicoumaryl |
| HPLC | high-pressure-liquid chromatography |
| Fe | iron |
| MC | monocoumaryl |
| Mg | magnesium |
| N | nitrogen |
| Ni | nickel |
| $\cdot O_2^-$ | superoxide anion |
| ·OH | hydroxyl radical |
| POD | peroxidases |
| $qCO_2$ | microbial metabolic quotient |
| ROS | reactive oxygen species |
| $H_2O_2$ | hydrogen peroxide |
| S | sulphur |
| $SO_2$ | sulphur dioxide |
| $SO_4$ | sulphate |
| UV | ultraviolet |

# 1. INTRODUCTION

## 1.1 General framework of the research

Among the severe stresses encountered by Scots pine (*Pinus sylvestris* L.) in boreal forests are those caused by the soil contamination of heavy metals and massive insect herbivore outbreaks. The local accumulation of heavy metals in forests is mainly due to industrial practices, such as non-ferrous metal industry. The emissions from copper and nickel (Cu, Ni) smelter complexes in the Kola Peninsula have caused severe destruction in local forest ecosystems during the last decades (Tikkanen & Niemelä 1995), and the environment around these smelters is among the most studied industrial areas. In 1990, the estimated total emissions from two large nonferrous smelter-complexes were 500 000, 3 300 and 2 180 t of sulphates ($SO_4$), Ni and Cu, respectively (Tuovinen & Ryaboshapko 1995). Around Monchegorsk, the area of forest death is about 40 000–50 000 ha, extending about 10 km to the south and about 15 km to the north of the city (Tikkanen & Niemelä 1995).

Pine sawflies, *Neodiprion sertifer* (Geoff) and *Diprion pini* (L.), are the most important pest species on Scots pine, and occasionally large outbreaks occur. It was estimated that in 1997-1999 *D. pini* caused damage of varying degree on approximately 300 000 ha in Finland. Moderate defoliation by *N. sertifer* and *D. pini* reduced volume growth of Scots pine by 21% and 86% and heavy defoliation by 38% and 94%, respectively, and consequently, the estimates of economic losses indicate a much higher impact of pine sawflies than those revealed by the few earlier studies in Europe (Lyytikäinen-Saarenmaa & Tomppo 2002).

Although the duration of exposure to these severe stresses is often different, heavy metal exposures being long-term and insect outbreaks short-term, both stressors lead to needle damage and loss of foliage, to which trees respond with complex chains of signalling and defensive reactions, including oxidative burst (see Mithöfer *et al.* 2004).

## 1.2 Nickel and copper in plants

Ni is needed for the functioning of urease enzyme in legumes, but its role in the metabolism of other plants is not well known (Marschner 1995). Cu is an essential micronutrient for plants and crucial to the functioning of several proteins, such as superoxide dismutase, ascorbate oxidase, polyphenol oxidase, cytochrome oxidase and plastocyanin (Marschner 1995). On the other hand, excess amounts of heavy metals disturb plant metabolism and retard shoot and root growth (Barceló & Poschenrieder 1990). Plant species, organ, developmental stage and nutrient status affect the critical toxicity level of heavy metals in plants. Foliar Ni concentrations above 10-50 mg $kg^{-1}$ and Cu levels of 20-30 mg $kg^{-1}$ exceed the suggested threshold value (Marschner 1995).

Schützendübel & Polle (2002) listed three different mechanisms for heavy metal toxicity:

- Production of reactive oxygen species (ROS) by autoxidation and Fenton reactions.
- Blocking of essential functional groups in biomolecules. Heavy metals may bind strongly to oxygen (O), nitrogen (N) and sulphur (S) atoms thus inactivating enzymes.
- Displacement of essential metal ions from biomolecules. The activity of certain enzymes is dependent on the presence of metals. E.g. Ni can displace magnesium ions ($Mg^{2+}$) in ribulose-1,5-biphosphate-carboxylase/oxygenase (Rubisco) and inactivate the enzymatic activity.

Heavy metals may cause deficiencies of essential nutrients, interference with photosynthesis, changes in photoassimilate translocation, and alterations in water relations (reviewed by Barceló & Poschenrieder 1990). Toxic levels of Cu damage the photosynthetic

apparatus at the thylakoid level (Clijsters *et al.* 1999) and enhance photoinhibition (e.g. Pätsikkä *et al.* 1998). Heavy metal-induced inhibition of light-dependent carbon dioxide ($CO_2$) fixation is connected with plant-water relations and with stomatal closure (Barcelo & Poschenrieder 1990). In addition, metal-induced nutrient imbalances in the plants may indirectly affect photosynthesis and growth. Cu inhibits iron uptake in plants and subsequently reduces the chlorophyll content of the leaves (Pätsikkä *et al.* 2002).

Mycorrhizal symbiosis may protect plants against the harmful effects of heavy metals (e.g. Jentschke & Godbold 2000). Certain mycorrhizal fungi are able to bind heavy metals in mycelium (Morselt *et al.* 1986, Denny and Wilkins 1987, Wasserman *et al.* 1987, Turnau *et al.* 1993). The metals can be bound to the cell walls, chelated and stored in the vacuoles or bound in complexes with metallothionein-like protein in the cytoplasm (Kahle 1993). Better nutrition and growth of mycorrhizal plants compared with non-mycorrhizal plants may also alleviate toxicity of heavy metals (Cairney & Meharg 1999, Ahonen-Jonnarth & Finlay 2001). Species and populations of ectomycorrhizal fungi may greatly differ in their tolerance to heavy metals (Blaudez *et al.* 2000, Adriaensen *et al.* 2005).

### 1.3 Oxidative reactions in plant cells induced by heavy metal exposure and herbivory

Impacts of both heavy metal exposure and certain biotic stresses, such as herbivory, on plant ecophysiology have a common feature in their potential to increase production of ROS in plant cells, although different enzymatic and nonenzymatic reactions may be involved in these reponses (Mithöfer *et al.* 2004). ROS species include hydrogen peroxide ($H_2O_2$), superoxide anion ($\cdot O_2^-$), hydroxyl radicals ($\cdot$OH) and singlet oxygen. $H_2O_2$ is relatively stable, whereas hydroxyl radicals are highly reactive and highly toxic to living cells. $H_2O_2$ and $\cdot O_2^-$ are formed in many biological processes in cells continuously. Their toxicity results mainly from their ability to form more harmful ROS species, such as $\cdot$OH radicals. It should also be noted that the formation of ROS is also part of normal cellular processes, including photosynthesis.

Generally, ROS has been proposed to affect plants in two different ways (reviewed by Apel & Hirt 2004). Firstly, ROS may cause oxidative damage to lipids, DNA and proteins. The toxicity of ROS is often measured by the level of lipid peroxidation, because fatty acids are among the first targets of ROS damage. Secondly, ROS are part of the biological signalling system in plants, which activates and controls various stress responses. The hypothesis presented by Mithöfer *et al.* (2004) suggests that the concept of ROS and unsaturated fatty acid-derived signals (e.g. jasmonic acid) may explain the similarities in responses caused by heavy metals and certain biotic factors in plants. Application of exogenous jasmonic acid induces the activity of oxidative enzymes such as peroxidases (PODs) and polyphenol oxidases (Thaler *et al.* 1996, Redman *et al.* 2001, Walters *et al.* 2002) and increase polyamine concentrations in plants (Walters *et al.* 2002).

Heavy metals can be classified as redox active and inactive metals. Cu is a redox active metal, and $H_2O_2$ can be converted to $\cdot$OH in a metal catalyzed reaction (Scheme; Mithöfer et al. 2004). The oxidized metal is rereduced in a reaction with $\cdot O_2^-$.

Fenton reaction:

$$H_2O_2 + Cu^+ \rightarrow \cdot OH + OH^- + Cu^{2+}$$

$$\cdot O_2^- + Cu^{2+} \rightarrow Cu^+ + O_2$$

Redox-inactive metals, such as Ni do not take part in Fenton-type reactions. Occurrence of ROS and/or symptoms of oxidative injury have also been observed in plants exposed to heavy metals that do not belong to the group of transition metals, such as Ni (Baccouch *et al.* 1998, Kukkola *et al.* 2000, Rao & Sresty 2000, Prasad *et al.* 2005). Schützendübel &

Polle (2002) concluded that redox inactive metals cause a transient loss in antioxidative capacity that may be accompanied by a stimulation of oxidant producing enzymes. This results in $H_2O_2$ accumulation in plant cells and tissues. Prasad *et al.* (2005) suggested that excessive accumulation of ROS in leaves following Ni treatment was the result of inhibition of photosynthetic electron transport activity.

Insects wound tissue mechanically when chewing and induce increases in $H_2O_2$ levels inside the plant tissues. Pathogens trigger similar oxygen burst, but wound-induced genes are different from those induced by pathogens (reviewed by Apel & Hirt 2004). In plant pathogen interactions, various potential enzymatic sources have been described in different plant species, such as cell-wall localized peroxidases and plasmamembrane localized NADPH oxidase. $H_2O_2$ generation occurs both locally and systemically in response to wounding. Bi & Felton (1995) demonstrated significant increases in lipid peroxidation and ·OH formation, elevated activity of oxidative enzymes and depletion of cellular antioxidants in soybean (*Glycine max* L.) in relation to caterpillar feeding. Leitner *et al.* (2005) found that chewing and piercing-sucking herbivores induced accumulation of ROS in legumes. Ruuhola & Yang (2006) observed that feeding by autumnal moth, *Epirrita autumnata* (Borkhausen), larvae induced $H_2O_2$ accumulation in mountain birch, *Betula pubescens* Ehrh. Subsp. *czerepanovii* (Orlova) leaves, while the activity of oxidative enzymes simultaneously increased.

### 1.4 Peroxidases, phenolics and polyamines may counteract the effects of heavy metals and herbivory

Plants have antioxidants that quench ROS before they damage cellular structures. Antioxidant defence systems comprise a variety of antioxidant molecules and enzymes. Enzymatic (superoxide dismutase, catalase, ascorbate POD, unspesific POD, glutathione reductase) and nonenzymatic antioxidants (ascorbate, glutathione, phenolic compounds, polyamines) are involved in these reactions in different cell compartments. In addition to their role as a part of the scavenging system all these compounds also have many other functions in plant cells (Dixon & Paiva 1995, Dowd & Lagrimini 1997, Martin-Tanguay 2001, Keski-Saari *et al.* 2005, Passardi *et al.* 2005).

PODs are a subclass of oxidoreductases that use peroxide such as $H_2O_2$ as an oxygen acceptor. The PODs take part in lignification, suberization and crosslinking of cell-wall polymers (reviewed by Dowd & Lagrimini 1997, Passardi *et al.* 2005). Lignin serves as a defence material, providing both a physical barrier and a chemical deterrent to insect attack. The PODs promote the polymerization of the monolignols coniferyl, p-coumaroyl and sinapyl alcohol into lignin (Greisbach 1981). An overview of the functions of PODs is presented in Figure 1.

The phenolic–peroxidase reaction system may function as a mechanism for $H_2O_2$ scavenging in plants (Takahama & Oniki 1997, Yamasaki *et al.* 1997, Passardi et al, 2005). Apoplastic PODs may also be capable of oxidizing sulphite to sulphate and thus prevent damage to cells against $SO_2$ (Pfanz *et al.* 1990). The response of peroxidases to elevated levels of heavy metals varies depending on plant species and heavy metals utilised (reviewed by Gratão *et al.* 2005).

PODs may also take part in chemical defence mechanisms against herbivores (Felton & Duffey 1991, Dowd &Vega 1996, Dowd and Lagrimini 1997). The oxidative burst in chewed birch leaves followed by increased peroxidase activity was seen as an efficient way to suspend further pathogen invasion in damaged leaves (Ruuhola & Yang 2006).

The phenolics are plant secondary metabolites that are often attached to sugars (as glycosides) or they may occur as aglycones. Many of them are involved in plant growth and reproduction. In addition, phenolics increase plant resistance to different environmetal stress

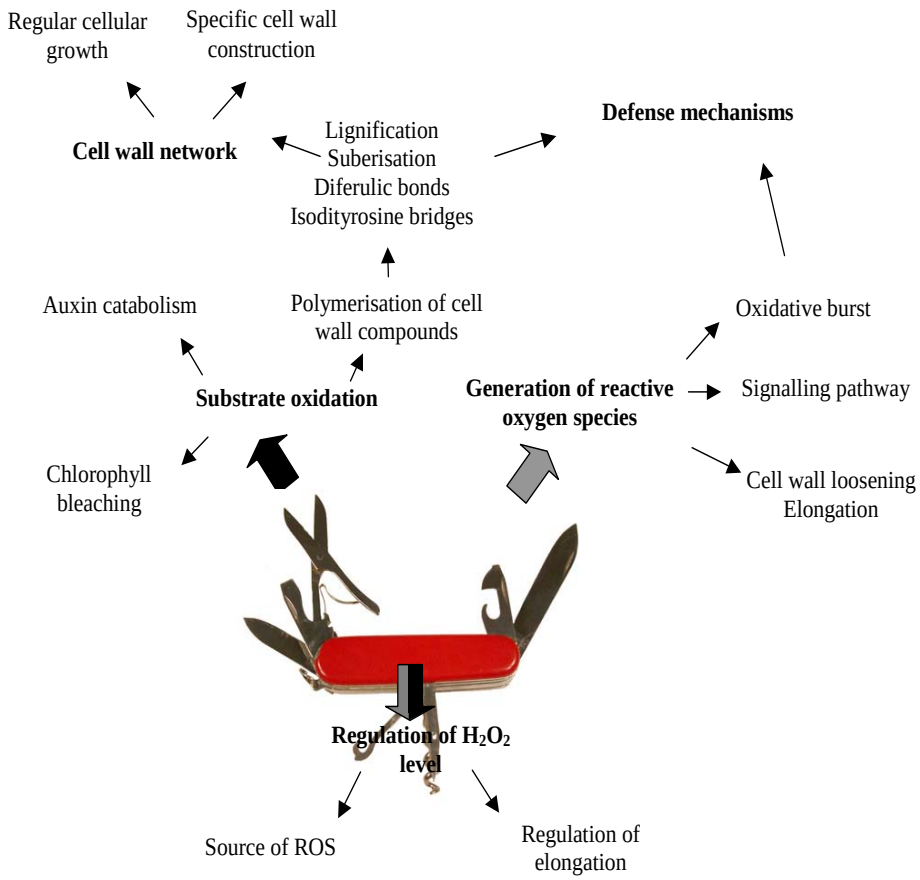


**Figure 1.** Passardi *et al.* (2005) showing the "multifunctional tool" of plants. Peroxidases oxidize various substrates through the peroxidative cycle (black arrow) and generate reactive oxygen species (ROS) through the hydroxylic cycle (grey arrow). The level of hydrogen peroxide can be regulated through both catalytic cycles (figure from Passardi *et al.* 2005, with permission of the authors).

factors, such as pathogens, herbivores, UV-radiation and nutrient stress (e.g. Dixon & Paiva 1995). Abiotic factors, which decrease photosynthesis, are suggested to be the most important elicitors of phenolics (Close & McArthur 2002). Ni and Cu are among the abiotic factors that may induce accumulation of phenolics in plant tissues (Pandolfini *et al.* 1992, Baccouh *et al.* 1998a, Santiago *et al.* 2000, Loponen *et al.* 2001).

The flavonoids are a subgroup of phenolics consisting of two aromatic rings, and they can be classified further into subgroups (e.g. flavones, flavonols, flavanones, dihydroflavonols, flavanols), based on variations in the heterocyclic C ring, and more than 5000 different flavonoids have been found (reviewed by Ross & Kasum 2002). Certain flavonoids may mitigate the effects of oxidative stress by acting as direct radical scavengers, but they are also used as reducing substrates for PODs (Grace & Logan, 2000). Studies have indicated that aglycones (quercetin, myricetin) may have greater antioxidant

capacities than conjugated flavonoids such as quercetin-3-glucoside, quercitrin and rutin (reviewed by Ross & Kasum 2002).

Phenolics may play important roles in plant-herbivore interactions. They may be involved in both constitutive and induced resistance against herbivory in plants. Induction of phenolics could be involved in both structural (lignin formation, thickening of cell walls) and chemical defences against damage (reviewed by Constabel 1999). Phenolics may have direct negative effects on herbivores as feeding deterrents, binding agents and generators of oxygen radicals (Appel 1993). For example, flavonol glycosides extracted from *Pinus* species adversely affected the growth and development of early instar of gypsy moth larvae, and that may be one reason why early instars do not generally feed on pine species (Beninger & Abou-Zaid 1997). On the other hand, if plant damage increases the production of flavonoids that do not deter insects from feeding and are not toxic, the insect might benefit of consuming these flavonoids (Simmonds 2001, Johnson 2005).

Another group of plant secondary metabolites involved with radical scavenging is the plant polyamines consisting of the diamine putrescine and the polyamines spermine and spermidine. They have been linked in a variety of plant processes such as cell division, flower induction and senescence (Martin-Tanguay 2001). Putrescine especially is involved in responses to abiotic stress (Bouchereau *et al.* 1999). Polyamines may directly or indirectly function as free radical scavengers (Boucherau *et al.*, 1999). The radical scavenging ability of polyamines is due to their phenolic hydroxygroups. Polyamines may reduce oxidative damage by increasing the acitivity of antioxidant enzymes and thus reducing lipid peroxidation (Tang & Newton 2005). Enzymes involving in polyamine catabolism produce $H_2O_2$.

## 1.5 Carbon-nutrient balance (CNB) hypothesis and delayed induced responses (DIR)

According to CNB hypothesis, concentration of carbon-based metabolites in plants is controlled by availability of C and N (Bryant *et al.* 1983, 1988). This theory predicts that in nutrient limited plants growth is reduced more than photosynthesis, which leads to the use of photosynthates for production of carbon-based metabolites. (Bryant *et al.* 1983, Tuomi *et al.* 1988b). Accumulation of carbon-based secondary metabolites has been observed in N-deficient conditions (Margna 1977, Bryant et al. 1983, Gershenzon 1984, Tuomi et al. 1990). This especially concerns phenylpropanoids associated with the shikimic acid pathway, whereas terpenoids were less affected by N availability (reviewed by Haukioja et al. 1998). Correspondingly, there is a general trend towards increasing concentrations of carbon based secondary metabolites in plants in response to high $CO_2$, especially soluble phenolics and condensed tannins, but not of lignin, structural polysaccharides or terpenes (Peñuelas & Estiarte 1998). CNB hypothesis has also been critisized (Haukioja *et al.* 1998, Honkanen *et al.* 1999, Hamilton *et al.* 2001, Koricheva 2002). Thus, CNB may not apply to a particular taxonomic groups or to a certain carbon-based secondary chemicals (Peñuelas & Estiarte 1998, Haukioja *et al.* 1998, Hamilton *et al.* 2001).

Production and accumulation of inducible defensive chemicals may occur in the same growing season (rapidly induced resistance) or in the next growing seasons (delayed induced resistance) (Haukioja 1980).Tuomi *et al.* (1984) observed that delayed induced accumulation of total phenolics was associated with reduced N content of the foliage in previously defoliated mountain birches. The same response occurred in previously defoliated birch branches (Tuomi *et al.* 1988b). As the inverse relationship between

foliage phenolics and nitrogen was consistent with the carbon/nutrient balance and related theories (Margna 1977, Bryant *et al.* 1983, Tuomi *et al.* 1990, 1991, Herms and Mattson 1992), attempts were made to explain variation in delayed induced responses in terms of these theories (Tuomi *et al.* 1984, Bryant *et al.* 1988, 1991a, 1991b, Tuomi *et al.* 1988a, 1990). Niemelä *et al.* (1984) and Tuomi *et al.* (1988a, 1990) further suggested that delayed induced resistance, when mediated by changes in plant resource balances, should be more pronounced in deciduous than in evergreen trees. Results of a recent meta-analysis have supported this expectation (Nykänen and Koricheva 2004).

## 2. AIMS

In the present study I focused on the physiological responses of Scots pine to Ni and Cu exposure and herbivory. I investigated the physiological responses of mature Scots pine trees to Ni, Cu and defoliation at contaminated sites in a gradient study from the smelters of Monchegorsk (I) and that of seedlings exposed to Ni mist (II) or to Cu and Ni amended into the soil (III, IV). In addition, experimental heavy metal exposure was combined with disturbance of the natural lichen cover of the forest ground layer to determine whether the intact lichen mat could act as a buffer against heavy metal deposition (II). I also studied needle chemistry in previously defoliated Scots pine (V, VI) and tested whether the inducibility of individual phenolic compounds correlates with the effects on sawfly larval growth (VI). My main interest was to determine

(i) whether total or apoplastic PODs are involved in Ni-, Cu- or defoliation- mediated stress (I, II, IV, V)

(ii) whether Ni- and Cu -or defoliation-induced stress gives rise to alterations in phenolics in Scots pine needles (III, V, VI)

## 3. MATERIAL AND METHODS

A summary of the experimental designs and analysed variables of the thesis are presented in Table 1. A gradient survey was carried out to compare the POD activity in Scots pine needles in relation to the distance from the smelters of Monchegorsk, Kola Peninsula, in northwestern Russia (I). Apoplastic and total POD activity and S, Ni and Cu concentrations in the needles of mature trees were measured on plots located 10 -110 km from the pollution source (I).

After this field survey, several experiments were conducted to simulate heavy metal deposition (II, III, IV). A field experiment (II) was conducted in a dry heath forest dominated by Scots pine and a mat-forming lichen *Cladina stellaris*. The pine seedlings were planted in plots covered by a natural lichen layer and in plots from which the lichen layer had been completely removed. The plots were exposed to four levels of Ni deposition: 0 (i.e.distilled water), 20, 200 and 2000 mg $m^{-2}$ during two growing seasons, the most severe treatment thus corresponding to the Ni deposition level near Monchegorsk. This study focused on the effects of a single metal to determine specific response of pine to Ni.

In the second (III) and third (IV) experiments the pine seedlings were exposed to Ni and Cu via soil without direct shoot exposure. The 4-year old seedlings were planted in pots filled with metal treated mineral soil (III). The experimental design consisted of all combinations of Ni and Cu at levels 0, 5, 15 and 25 mg Ni $kg^{-1}$ dw of soil and 0, 25, 40 and 50 mg Cu $kg^{-1}$ dw of soil, respectively.

Scots pine seedlings during their second growing period were exposed to Ni (20.5 mg Ni $l^{-1}$ of soil) or Cu (63.3 mg Cu $l^{-1}$ of soil) (IV). The seedlings were planted in soil originating from two localities with different background levels of Ni and Cu (low "Kaamanen" and high "Svanvik").

The defoliation experiment in the field simulated attack by pine sawflies (V, VI). Mature Scots pine trees, from 8 to 25 years

**Table 1.** General description of the studies

| Study | Experiment design | Measured variables | | |
|---|---|---|---|---|
| | | Biological parameter | Element concentrations in the leaves | Element concentrations in the roots |
| I | A gradient survey in a Ni-, Cu- and S- polluted forest site near Monchegorsk, Kola Peninsula | Foliar apoplastic and total peroxidases | Ni, Cu, S | |
| II | Two-years exposure of wet-deposited Ni on the seedlings, which grew with or without lichen cover in their surroundings | Growth of the seedlings, foliar and root peroxidase activity, soil microbial variables | Ni | Ni |
| III | One-season exposure of pine seedlings to Ni and Cu mixed in the growth medium | Phenolics, soluble carbohydrates | Ni, Cu | Ni, Cu |
| IV | Seedlings were exposed to Ni and Cu via soil without direct shoot exposure | Root peroxidases,. mycorrhizal morphotypes and biomass of fungi | | Ni, Cu |
| V | 100% artificial defoliation of 8-25 -year-old pines in two successive years | Foliar peroxidases, phenolic compounds, polyamines and solube carbohydrates, chlorophyll | N, C | |
| VI | Larvae of *Diprion pini* were reared on the trees (cf. V) one year after two successive years of defoliation | Phenolic compounds, weight of pupae | N, C | |

of age, were artificially defoliated during two successive years at a dry, nutrient poor Scots pine stand in Hailuoto, northern Finland. Thirty trees were selected for the defoliation treatment and the selected trees were randomly divided into two groups (control and defoliation), which showed no difference in age distribution or in height. The trees were defoliated by clipping off all 1-year old and older needles at the time when the current shoot was fully elongated, but when the current year needle growth was just starting. A feeding experiment was performed with larvae of pine sawfly (*Diprion pini* L.) (VI) in the growing season following the two successive defoliations.

Guaiacol POD activity in the needles and fine roots was measured according to Polle *et al.* (1990, 1991) (I, II, IV, V). The soluble polyamine concentration was measured as described by Sarjala & Kaunisto (1993) (V).

The phenolic compounds were measured with high-pressure-liquid chromatography (HPLC) according to Julkunen-Tiitto *et al.* (1996) (III, V, VI). Chlorophyll and carotenoids were extracted from fresh needle samples with dimethyl sulphoxide (DMSO) as described by Barnes *et al.* (1992) (V). Soluble sugar (glucose, fructose and sucrose) and starch were analysed using enzymatic techniques (Beutler *et al.*1978).

The N concentration in the needles was determined with a CHN-analyser (V, VI). The Ni and Cu concentrations in the needles, roots and soil were analysed by ICP-AES, GFAAS (II, IV) or by XRF analyses (I). The exchangeable concentrations of soil cations were measured from ammonium acetate (pH 4.65) extracted samples (II).

The fine roots were visually examined under a dissecting microscope to assess the proportion of clearly senescent short roots, which were then rejected from the morphotype classification. The mycorrhizal short roots were classified into different types based on their morphology (IV). The homogenized humus samples were analysed for the microbial activity, using respirometric analysis of evolved $CO_2$ (Nordgren 1988) (II). The fungal biomass (ergosterol) was analysed in the freeze-dried mycorrhizal roots according to Nylund & Wallander (1992, modified by Ola Kåren) (II).

## 4. RESULTS AND DISCUSSION

### 4.1 Responses of peroxidase activity to heavy metals and defoliation

Apoplastic and total peroxidase activity in Scots pine needles increased towards the smelters of Monchegorsk in the gradient study (I). Scots pine is sensitive to the effects of $SO_2$ and a critical annual mean of as low as 3-4 µg $m^{-3}$ together with higher short-term peaks may cause sufficient oxidative stress to damage the pines in the north (Manninen *et al.* 1998). In the present study, the annual mean $SO_2$ concentration at the most polluted site was 56 µg $m^{-3}$ (Tikkanen & Niemelä 1995). In contrast to our results, Polle *et al.* (1994) found no increased apoplastic or total POD activity in Scots pine needles at polluted sites with even higher aerial $SO_2$ levels in eastern Germany. I also suggest that the high levels of Cu and Ni in the environment are partly responsible for the increased peroxidase activity found in our study. This is also supported by the results of other studies, which showed that Ni induces apoplastic POD activity in plants grown in heavy metal containing nutrient solution (Pandolfini *et al.* 1992, Blinda *et al.* 1997). Basically, the leaf apoplast is the site of the preferential accumulation of divalent heavy metal ions (Blinda *et al.* 1997).

If the effect of metals on the induction of POD activity in leaves and roots were regular and linear, they could be used for evaluating the phytotoxicity of metal-polluted soils, as proposed by Vangronsveld & Clijsters (1994). POD activity vary, however depending on the age of the plant tissue (I, II) and time of the growing season (I, Tarvainen *et al.* 2004 ). POD activity increased in Scots pine seedlings after exposure to 2000 mg Ni $m^{-2}$ for two years (II). However, the increase in foliar POD activity was age-dependent, suggesting a cumulative effect of the Ni: the increase was more evident in previous year (C+1) needles (ANOVA results for Ni $F = 5.17$, $P<0.01$) than in C needles ($F = 2.44$, $P<0.082$). Since the Ni concentration in C needles averaged 109 mg $kg^{-1}$ (cf. at polluted 10 km plot from Monchegorsk 128 mg Ni $kg^{-1}$; I), the POD activity did not appear to be a sensitive indicator for Ni impact (II). Foliar POD activity may be partially induced by the direct toxic effects of deposited Ni on the needles (II). Koricheva *et al.* (1997) reported that enzyme responses in birch leaves were dependent on whether metals were applied directly to shoots or to soil.

Artificial defoliation simulating pine sawfly herbivory increased POD acitivity in Scots pine needles compared with undefoliated trees (V). The POD activity in intact needles of defoliated

trees remained elevated for several months after the stimulus. Thus, the function of these enzymes is apparently not limited to the immediate reactions after physical damage. The rapid reactions are involved in the oxidative burst following leaf damage (see Bestwick *et al.* 1998). Modifications of cell-wall structure against further damage could be one of the defoliation-induced responses (Dowd & Lagrimini, 1997).

Root PODs did not respond to elevated Ni (II, IV) or Cu (IV), and the activity decreased slightly in the roots of defoliated mature Scots pines compared to controls ( t = 2.189, df=27, p=0.037, V, VI, unpubl. results). The results obtained in the heavy metal exposure are consistent with those of Roitto *et al.* (1998) and Kukkola *et al.* (2000) with Ni- and/ or Cu- exposed Scots pine. Koricheva *et al.* (1997) and Mazhoudi *et al.* (1997) also suggested that leaf and root guaiacol PODs may respond differentially to heavy metal loading. Moreover, Schützendübel & Polle (2002) observed that Cd exposure (50 µM) increased POD activity in nonmycorrhizal roots, but not in mycorrhizal roots. The results suggest that heavy metal induced stress reactions were diminished or not perceived in mycorrhizal roots (see also discussion above, and Jentschke & Godbold 2000). Lower root POD activity found in defoliated pines may thus be due to changes in mycorrhizal symbionts affected by defoliation (Kuikka *et al.* 2003) rather than reflecting induced stress reactions of the tree.

### 4.2 Effects of heavy metals on the seedlings

Wet Ni deposition of 2000 mg $m^{-2}$ reduced growth and survival of pine seedlings, whereas deposition levels 200 mg $m^{-2}$ or 20 mg $m^{-2}$ caused no effects in a 2-y lasting experiment (II). Since the metals were sprayed on the seedlings, considerable proportions of the foliar Ni may have consisted of direct surface contamination (element concentrations in Table 2). However, the Ni in our experiment was in soluble form, whereas in the CuNi -smelter area heavy metals were also deposited as particles on the foliage (Rautio *et al.* 1998, Rautio & Huttunen 2003).

Nieminen (2004) observed a lethal threshold value of 80 mg Ni $kg^{-1}$ roots for pine seedlings grown in a mineral soil. In our study (II) the Ni concentration in the pine roots averaged 74 mg $kg^{-1}$ with the highest Ni application level (Table 2). However, seedlings exposed to low levels of Ni in soil (III, IV) had root Ni concentrations ranging 70 – 80 mg $kg^{-1}$ (Table 2) without retarded growth. In contrast, seedlings exposed to Ni had 17% higher shoot growth and fewer clearly senescent short roots compared with controls (IV).

In IV the mean Cu concentration in the roots of seedlings exposed to Cu averaged 256 mg $kg^{-1}$ (Table 2). Cu did not affect shoot growth, but the fungal biomass was about 6% lower in the fine roots of seedlings treated with additional Cu (IV). In III the Cu concentration remained very low in the needles of Scots pine despite high concentrations in the roots (III, Table 2; results of Rautio *et al.* 2004). The growth responses of pine seedlings in III are reported by Rautio *et al.* 2005: root growth showed the strongest response, Ni being more harmful than Cu.

Generally, a positive correlation was found between seedling growth and ergosterol concentration (indicating biomass of fungi) in the fine roots (IV). In a separate study (Markkola et al. 2002) of our other Ni experiment (II), fungal biomass in the roots did not differ among the Ni treatments. However, the seedling growth was retarded more in lichen than in skimmed quadrats with the highest Ni exposure level (II). Accordingly, when fungal biomass was calculated as an amount per seedling, it was significantly reduced by the highest Ni application level in both lichen covered and skimmed plots, the reduction being more distinct in the former (ca. 45%) than in the latter treatment (ca. 15%). Mycorrhizal morphotype composition was only slightly affected in our Ni and/or Cu exposures

**Table 2.** Mean Ni and Cu concentrations in the needles and roots in a gradient from Monchegorksk to Jena (I) and Ni-and Cu- exposed seedlings (II-IV). In the experiment IV Ni and Cu concentrations in the needles were not determined.

| | Study | | Ni needles (mg kg$^{-1}$) | Ni roots (mg kg$^{-1}$) | Cu needles (mg kg$^{-1}$) | Cu roots (mg kg$^{-1}$) |
|---|---|---|---|---|---|---|
| I | A gradient survey on Kola Peninsula | 10 km Monchegorsk | 128 | | 118 | |
| | | 40 km Imandra | 37 | | 67 | |
| | | 80 km Jena | 7 | | 1 | |
| II | Two-years exposure of wet-deposited Ni on the seedlings | Exposure levels mg m$^{-2}$ | | | | |
| | | 0 | 2.2 | 2.5 | | |
| | | 20 | 2.7 | 3.2 | | |
| | | 200 | 19.1 | 10.0 | | |
| | | 2000 | 109.2 | 73.6 | | |
| III | Single-season exposure of pine seedlings to Ni and Cu mixed in the growth medium | Ni exposure levels mg kg$^{-1}$ soil | | | | |
| | | 0 | 1.3 | 10.7 | | |
| | | 5 | 4.6 | 74.1 | | |
| | | 15 | 14.6 | 237.7 | | |
| | | 25 | 27.5 | 392.9 | | |
| | | Cu exposure levels mg kg$^{-1}$ soil | | | | |
| | | 0 | | | 2.1 | 15.8 |
| | | 25 | | | 3.7 | 124.9 |
| | | 40 | | | 4.8 | 240.2 |
| | | 50 | | | 4.7 | 285.7 |
| IV | Seedlings were exposed to Ni and Cu via soil for one season. Kaamanen = low original metal concentration Svanvik = high original metal concentration | Kaamanen | | 3.7 | | 27.6 |
| | | KaamanenNi | | 88.0 | | 20.6 |
| | | Kaamanen + Cu | | 16.1 | | 354.5 |
| | | Kaamanen + NiCu | | 78.1 | | 192.5 |
| | | Svanvik | | 15.9 | | 37.1 |
| | | Svanvik + Ni | | 63.1 | | 30.3 |
| | | Svanvik + Cu | | 27.6 | | 215 |
| | | Svanvik + NiCu | | 87.7 | | 262 |
| | | +Ni = 20,5 mg/l soil +Cu = 63,5 mg /l soil | | | | |

(II, IV). Additional Ni decreased the proportion of the morphotype with the thinnest mantles from 34% to 24% in the roots of seedlings grown in soil with low original heavy metal content (IV). In II, moderate Ni deposition (200 mg m$^{-2}$) increased the proportion of tubercle (*Suillus*, *Rhizopogon* or *Chroogomphus* type) morphotypes in lichen covered quadrats (Markkola et al. 2002). This suggests that fungi forming large amounts of external mycelia and tubercle mycorrhizae could be more beneficial symbionts of Scots pine than fungi forming smooth, thin-mantled ectomycorrhizae with only low amounts of external mycelia.

Different exposure methods, such as spraying, pouring as solutions or mixing in the growth medium, seem to induce different responses in pines (II, III, IV). Ni application on the shoots may be more toxic to seedlings than exposure only via growth media. Differences in soil composition, e.g. amount

of soil organic matter, pH value and concentrations of available nutrients, may influence heavy metal effects on trees. Moreover, the toxicity of Ni is modified in the presence of Cu (Rautio *et al.* 2004, Nieminen 2004, 2005). The results from the present experiments suggest that the toxicity of Ni cannot be evaluated based entirely on Ni concentration in the roots.

### *Effects of lichen mat*

The dense lichen mat covering the soil did not benefit pine seedlings exposed to wet Ni deposition (II). The proportion of Ni captured by lichen was reduced with increasing Ni exposure and approximately 70% of the total Ni applied to the lichen mat had leached to the soil after the most severe Ni treatment (2000 mg $m^{-2}$) in this experiment (Hyvärinen *et al.* 2000). However, the exchangeable Ni concentration in the topsoil (soil organic layer) was lower in the lichen covered ($16.43 \pm 4.97$) mg $kg^{-1}$ ) plots compared with the skimmed plots ($83.02 \pm 49.08$ mg $kg^{-1}$) at the highest Ni application level. The pH value in the soil organic layer did not differ significantly between intact and skimmed plots.

Removal of the lichen layer did not significantly affect the shoot or root growth in the quadrats not exposed to Ni. However, the frequencies of poor and senescent short roots were lower in the skimmed than in the intact plots and removal of the lichen layer increased the diversity of the ectomycorrhizal fungal community in a separate study of the Ni experiment (II) (Markkola *et al.* 2002). The mortality of the pine seedlings tended to be higher in lichen covered plots compared with the skimmed plots ($F = 5.48$, $P<0.025$). Reduced growth and survival of Scots pine in the presence of *Cladina* have been reported (Brown & Mikola 1974, Fisher 1979), and extracts of mat-forming lichens inhibited the growth of ectomycorrhizal fungi in pure culture (Goldner *et al.* 1986, Brown & Mikola 1974). However, the existence of ecologically relevant allelopathy between lichen and forest trees has not been experimentally verified.

Soil conditions, e.g. nutritional status and the activity of soil organisms, are important for the sustainability of trees. Some nutrient leaching was recorded in the soil when the lichen layer was removed (II), similarly to the pine forests extensively grazed by reindeer (Ohtonen & Väre 1998, Stark *et al.* 2000). Some indices of microbial activity were also studied (II). Ni application did not decrease microbial biomass or basal respiration, but when the lichen mat was skimmed the most severe Ni treatment decreased the microbial metabolic quotient ($qCO_2$) and increased the time lag in response to glucose addition (Lag). During this relatively short experiment (2 y), no clear evidence of retarded decomposition of soil organic matter due to Ni was found.

## 4.3 Responses of phenolics to heavy metals and defoliation

Phenolic compounds, such as tannins and flavonoids may detoxify the effects of heavy metals by radical scavenging and metal chelation (Brown *et al.* 1998, Lavid *et al.* 2001). In our study pine seedlings exposed to Ni (15 mg Ni $kg^{-1}$ soil) had higher concentrations of condensed tannins compared with controls, which suggests a role for tannins as Ni chelators in Scots pine needles (Fig. 2a, III). This view was also supported by Kukkola *et al.* (2000), who found dark staining of the central vacuole in the Ni exposed pine seedlings (15 or 25 mg Ni $kg^{-1}$ soil).

Concentrations of MC-isoquercitrin, DC-isoquercitrin and DC-astragalin showed a decreasing trend, when Cu and Ni were added in combination to the soil (Fig. 2b,c,d). Effects of Cu were indirect, since Cu was not effectively transported to the foliage (Table 2). However, Cu treatment reduced above-ground biomass of the seedlings (Rautio et al. 2005). Additive or antagonistic effects of Cu and Ni were found in the growth and element concentrations of the seedlings (Rautio et al. 2004, 2005). Moreover, interaction between Cu and Ni seemed to cause more severe injuries to chloroplasts than Ni alone (Kukkola et al. 2000).

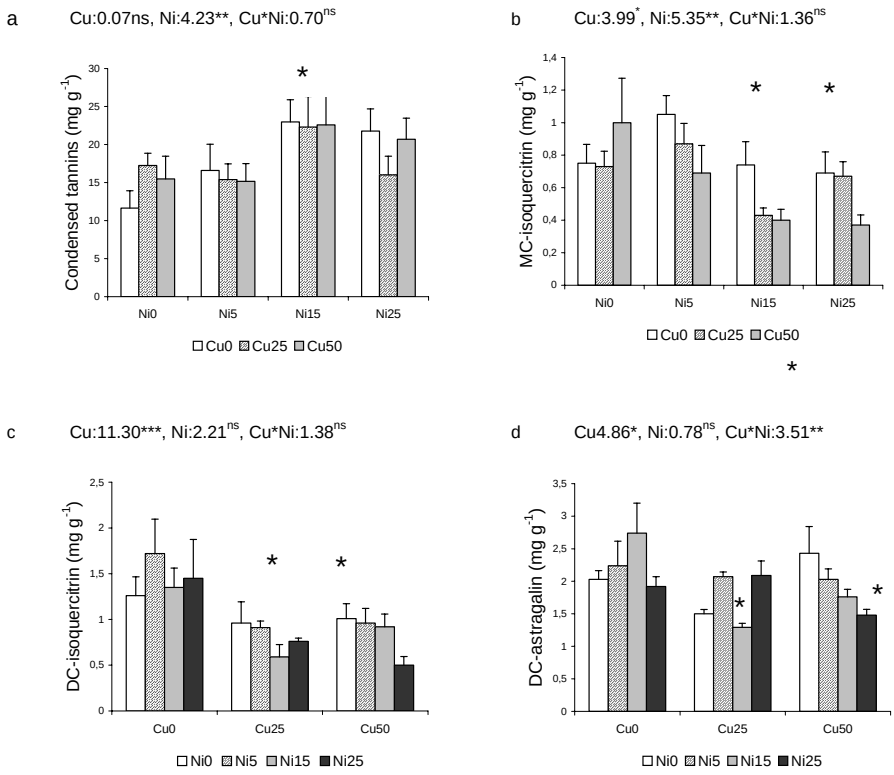


**Figure 2**. Concentrations of condensed tannins, MC-isoquercitrin, DC-isoquercitrin and DC-astragalin in the NiCu exposed Scots pine seedlings. F-values (with significance symbols:* =P<0.05, **=P<0.01, ***=P<0.001) are given separately for Cu, Ni and CuNi interaction. Each bar represent mean (±SE) of 4-5 seedlings. DF-value for Cu is 2 and Ni is 3. Ni exposure levels were 0, 5, 15 and 25 mg $kg^{-1}$ and Cu 0, 25 and 50 mg $kg^{-1}$, respectively. Differences within Ni and Cu treatments are indicated in the figure (Ni0 versus Ni5, Ni15 and Ni25 and Cu0 versus Cu25 and Cu50). Differences between the Cu0Ni0 and metal treatments are indicated, when significant interaction between Cu and Ni (d).

Previously it was shown that total phenolics increased after simulated whole-tree defoliation in Scots pine (Honkanen et al. 1999). Our results show that responses of individual phenolic compounds to defoliation vary (Table III, V, VI). When phenolics were studied 3 months after the last defoliation, quercitrin (=quercetin-3-rhamnoside) was the compound with the highest increase (V; Table 3). During the following summer, more than one year after the latest defoliation, the concentrations of several compounds were higher in the defoliated trees than in the controls (VI; Table 3). The concentrations of most flavanols and flavonols were higher in C+1 needles than in C needles. On the contrary, DC-astragalin was found in higher concentrations in C needles.

Only damaged plant tissues show disorder in cellular structures, which are associated with changes in metabolism (reviewed by Léon et al. 2001). Therefore, in addition to intact needles we also analysed needles that were chewed by the sawfly larvae (VI). Damaged

**Table 3**. Changes (%) of concentration of phenolic compounds and nitrogen affected by simulated defoliation (V, VI). Trees (n = 15) were totally defoliated in two concomitant years. Needles were sampled in September after the second defoliation (V) and a year later in August (VI). The same needle age-class was monitored (flushed after the second defoliation). The difference between control and defoliated trees is indicated for each year separately ($P < 0.05$*, $P < 0.01$**, $P < 0.001$***). Gallocatechins, condensed tannins and a neolignan were studied only in the latest analyses (VI). MC=monocoumaryl, DC=dicoumaryl.

| Compound | Control vs defoliated (V) | Control vs defoliated (VI) |
|---|---|---|
| *Flavonols* | | |
| Quercetin-3-rhamnoside | +136%*** | +94% *** |
| Quercetin-3-galactoside | ±0% | +89%*** |
| Myricetin-3-galactoside | ±0% | +70%** |
| MC-isoquercitrin I | -3% | +15% |
| MC-isoquercitrin II | +17% | +71%** |
| DC-astragalin | -22%** | -41%* |
| *Flavanols* | | |
| (+)-catechin | +25%* | +27% |
| Catechin derivative I | +37%* | +37%* |
| Gallocatechin | nd | +26%* |
| Condensed tannins | nd | +36%* |
| *Neolignans* | | |
| 3-Hydroxymethyl-5-(γ-hydroxy-*n*-propyl)-2(3´-methoxy-4´-O- β-glucopyranolsylphenyl)-2,3-dihydrobenzofuran | nd | +48%** |
| Nitrogen | -19% | -7% |

needles had higher (+)-catechin and DC-astragalin concentrations compared with intact needles. (+)-Catechin is known as an effective antioxidant (Pedrielli et al. 2001), and the increase could thus be involved in increased oxidative stress in wounded tissue.

In our defoliation study (V, VI), the C/N ratio in the needles was distinctly increased due to defoliation. Simulated defoliation of Scots pine led to decreased N and increased content of several phenolic compounds (Table 3), and consequently, the nutritional quality of the needles for insect herbivores was assumed to decrease. Hence, we further examined the growth of larvae of a sawfly *Diprion pini* on the previously defoliated and control Scots pine trees (VI).

### *Growth of sawfly on the previously defoliated pines*

The pupal weights of larvae grown on the defoliated trees were lower than those on control trees. A similar trend was observed with hatched sawflies. We thus suggest that severe needle loss together with a nutrient-poor growth site altered pine needle chemistry leading to adversely affected performance of *D. pini*. The reduced pupal mass and weight of hatched *D. pini* feeding on repeatedly

defoliated Scots pine suggests delayed induced resistance. By contrast, Niemelä *et al.* (1984, 1991) observed no delayed induced resistance in the mature needles of Scots pine against diprionid sawflies. Niemelä *et al.* (1991) concluded that their results either indicated a lack of delayed inducible resistance or that diprionid sawflies, including *D. pini*, as specialized folivores of Scots pine may be highly tolerant to inducible chemical changes in foliage. Niemelä *et al.* (1984) and Tuomi *et al.* (1988a, 1990) actually suggested that delayed induced resistance, when mediated by changes in plant resource balances, should be more pronounced in deciduous than in evergreen trees, and a recent meta-analysis supported this expectation (Nykänen and Koricheva 2004, but see, Bryant *et al.* 1991b).

In general, the estimated effect of identified phenolics on pupal weight was negative, but in some cases even slightly positive effects were found. Correlations of larval growth with concentration of the indvidual phenolic compounds varied from positive to negative, depending on the phenolic compound and whether control or defoliated trees and C or C+1 year needles are considered. Furthermore, we found no consistent relationship between inducibility (proportional increase in a compound following defoliation) and adverse effects on the performance of pine sawfly larvae. Consequently, the observed inducible responses in needle phenolics may represent nonspecific responses that are expressed in defensively effective as well as ineffective secondary compounds.

## 4.4 General trends in the stress responses of Scots pine

In the present study Scots pine showed certain parallel changes in response to both heavy metal exposures and defoliation. Thus, both elevated levels of Ni in the growth medium (III) and severe defoliation (V) resulted in higher foliar sucrose and starch concentrations in Scots pine needles in the end of the growing season (Table 4). These results are consistent with those of earlier studies, i.e. heavy metals such as Ni, Co, Zn and Cd resulted in photoassimilate accumulation in leaves of *Phaseolus vulgaris* L. (Rauser 1978, Samarkoon and Rauser 1979) and *Zea mays* (L'Huiller *et al.* 1996, Baccouch *et al.* 1998b). Balsberg-Påhlsson (1989) observed increased sucrose and starch concentrations in Scots pine needles at Cu, Zn and $SO_2$ polluted forest site, and suggested that hydrolysis of starch or the transport of sucrose were inhibited by heavy metals. In addition, reduced root sink activity due the Ni toxicity to roots (L'Huiller *et al.* 1996) or nutrient imbalances (Ericsson, 1979; Baccouch *et al.*, 1998) could be reasons for decreased photoassimilate transport from the foliage. In contrast to our results, Honkanen *et al.* (1999) found that total defoliation of Scots pines did not affect the allocation of resources to different carbohydrates despite reduced growth after the second growing season.

Both certain heavy metal treatments and defoliation increased peroxidase activity in Scots pine needles. Further, concentrations of important carbon-based secondary compounds, such as condensed tannins were increased in Scots pine needles both due to certain Ni treatments and defoliation (V, VI, Table 3). Several other phenolics studied (quercetin derivatives, catechins, kaempferol-3-glucoside) showed variable responses to either heavy metal exposure or defoliation, whereas concentration of DC-astragalin decreased both due Cu+Ni treatment and simulated defoliation. It is known that UV-inducible compounds (DC-astragalin and DC-isoquercitrin) locate mainly in the epidermal layer of the needles (Schnitzler *et al.* 1996). However, both defoliation and severe heavy metal treatments led to reduced needle (needle pair) mass (V, III, see also Rautio *et al.* 2005). Hence, the format for the output of the results may be important (per unit of weight or per unit of leaf area).

**Table 4.** Observed trends in peroxidases (POD), phenolics, polyamines, soluble carbohydrates and nitrogen in Scots pine after heavy metal exposure or defoliation. ↑=increase, ↓ = decrease, 0= no change. Nd = not determined

| | | Ni+Cu | Defoliation |
|---|---|---|---|
| Peroxidases | Needles | ↑ | ↑ |
| | Roots | **0** | ↓ |
| Phenolics | Condensed tannins | ↑ | ↑ |
| | Flavonoids | **↓,0** | **↑, 0, ↓** |
| Carbohydrates | Glucose | **0** | ↓ |
| | Sucrose | ↑ | ↑ |
| | Starch | ↑ | ↑ |
| Polyamines | Putrescine | **nd** | ↓ |

Stress reactions induced by heavy metals and herbivory could be affected by nutrient availability. Simulated insect herbivory led to decreased foliar N concentration in Scots pine (V, VI, Table 3). After the second defoliation total N was 0,90% in the control trees and 0.73% in the defoliated trees (V). In the following year the N concentration was 0.96% in the C needles in the control trees and 0.85% in the defoliated trees (VI). In the C+1 needles the N concentration was 0.83% (controls) and 0.77% (defoliated). Although not documented in the present study, the mature Scots pines in Kola Penisula may also have suffered from nutrient deficiency as suggested by drastically lowered N availability in soil (Lukina *et al.* 1999, Rautio & Huttunen 2003, see also Zvereva & Kozlov 2001). Further, Ni exposure is reported to decrease foliar P concentration in Scots pine seedlings in experimental conditions (Ahonen-Jonnarth & Finlay 2001).

The recuded foliar N concentration in defoliated Scots pines (V, VI) could partially result from loss of nutrient storage in the removed needles. Moreover, the Scots pine stand was originally very nutrient poor (Kuikka *et al.* 2003). In addition to the direct loss of foliar N, defoliation can influence negatively nutrient acquisition e.g., due to increased fine-root mortality, or reduced sink-strength of the foliage. However, reduced N as a consequence to defoliation is not a general phenomenon in conifers. In fact, increased N concentration have been observed in several defoliation studies (e.g. Niemelä *et al.* 1991, Hódar *et al.* 2004, Roitto *et al.* unpublished results).

Our results are in accordance with the reported effects of N on phenolics. Concentrations of total phenolics in the needles increased in response to low N availability in the growth medium of *Pinus sylvestris* (Kainulainen et al. 1996), *Pinus palustris* (Pritchard et al. 1997), *Pinus taeda* (Gebauer et al. 1998) and *Pinus elliottii* (Saxon et al. 2004). Apparently individual phenolic compounds in Scots pine respond differentially to low N. In Scots pine different levels of N in the soil also affeced responses of phenolics to UV-radiation (Lavola *et al.* 2003). In *Betula* spp, N deficiency resulted in the induction of largely the same phenolic compounds as in the present experiment, i.e. myricetin and quercetin glycosides, flavonoid coumaryl derivatives and soluble condensed tannins (Keski-Saari & Julkunen-Tiitto 2003, Keski-Saari *et al.* 2005). N limited plants may be more

susceptible to photodamage, which could partly explain increased levels of certain phenolics (see Close & McArthur 2002)

It should be noted that decreased concentration of the polyamine putrescine (ca. 40% lower in the defoliated trees compared with controls) may be due to lowered foliar N, since N availability is suggested to affect the amount of putrescine in plant tissues (Altman & Levin, 1993). Accordingly, N fertilization increased foliar-free putrescine in *Pinus sylvestris* (Taulavuori *et al.* 1998) and red pine (*Pinus resinosa)* needles (Minocha *et al.* 2000). In addition to increased synthesis, changes in free putrescine concentration may also result from conjugation and/or catabolism. Thus, the reduced putrescine levels in defoliated trees in our study could also have been due to degradation by oxidases.

## 5. CONCLUSIONS

Peroxidases and phenolics are involved in inducible chemical changes or mechanisms and recovery reactions that occur in the needles of Scots pine due to exposure to heavy metals or defoliation. Heavy metal- and defoliation-induced changes in the phenolics and peroxidases may further affect many functions in plant cells, because they are often involved in responses to wounding, pathogen infection, chilling, ozone or nutrient deficiency. In this study, heavy metal treatments and defoliation in several cases increased peroxidase activity and concentration of condensed tannins in Scots pine needles. In contrast to increases in several of the phenolics examined, decreased or not changed levels of many phenolics were observed due to Cu+Ni treatment and defoliation. Elevated levels of Ni and Cu in the soil variously affected needle phenolics and carbohydrate concentration. Both Ni treatment and defoliation resulted in higher foliar sucrose and starch concentrations in Scots pine needles suggesting changes in carbohydrate transport.

The reduced pupal mass and weight of hatched *D. pini* feeding on the repeatedly defoliated Scots pines suggests delayed induced resistance. To my knowledge, this is among the first findings of delayed induced resistance as regards to the conifers. Concerning foliar nitrogen and phenolics, the chemical changes in the foliage are largely consistent with the patterns suggested by the carbon/nutrient balance theory of delayed inducible responses. However, the observed inducible responses in needle phenolics did not constitute specific defensive responses against sawflies.

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## 7. REFERENCES

Adriaensen, K., Vrålstad, T., Noben, J-P., Vangronsveld, J. & Colpaert, J.V. 2005: Copper-adapted *Suillus luteus*, a symbiotic solution for pines colonizing Cu mine spoils. – *Appl. Environ. Microbiol*. 71: 7279-7284.

Ahonen-Jonnarth, U. & Finlay, R. 2001: Effects of elevated nickel and cadmium concentrations on growth and nutrient uptake of mycorrhizal and non-mycorrhizal *Pinus sylvestris* seedlings. – *Plant Soil* 236: 129-138.

Altman, A. & Levin, N. 1993: Interactions of polyamines and nitrogen nutrition in plants. – *Physiol. Plant.* 89: 653–658.

Apel, K. & Hirt, H. 2004: Reactive oxygen species: metabolism, oxidative stress, and signal transduction. – *Annu. Rev. Plant Biol.* 55: 373–399.

Appel, H. M. 1993: Phenolics in ecological interactions: The importance of oxidation. – *J. Chem. Ecol.* 19: 1521-1552.

Baccouch, S., Chaoui, A. & El Ferjani, E. 1998a: Nickel-induced oxidative damage and antioxidant responses in *Zea mays* shoots. – *Plant Physiol. Bioch.* 36: 689–694.

Baccouch, S., Chaoui, A. & El Ferjani, E. 1998b: Nickel toxicity: effects on growth and metabolism of maize. – *J. Plant Nutr.* 21: 577-588.

Balsberg-Påhlson, A-M 1989: Effects of heavy-metal and $SO_2$ pollution on the concentrations of carbohydrates and nitrogen in tree leaves. – *Can. J. Bot*. 67: 2106–2113.

Barceló, J. & Poschenrieder, Ch. 1990: Plant water relations as affected by heavy metal stress: a review. – *J. Plant Nutr*. 13: 1–37.

Barnes, J. D., Balaguer, L., Manrique, E., Elvira, S. & Davison, A. W. 1992: A reappraisal of the use of DMSO for the extraction and determination of chlorophylls a and b in lichens and higher plants. – *Environ. Exp. Bot.* 32: 85–100.

Beninger C. W. & Aboud-Zaid, M. M. 1997: Flavonol glycosides from four pine specis that inhibit early instar gypsy moth

(*Lepidoptera: Lymantriidae*) development. – *Biochem Syst. Ecol*. 25: 505-512.

Bestwick, C. S., Brown, I. R., & Mansfield, J.W. 1998: Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. – *Plant Physiol*. 118: 1067–1078.

Beutler, H.-O., Michal, G., & Beistingl, G. 1978: Enzymatische Analyse von komplexen Kohlenhydratgemischen. – *Deutsch. Lebensm.-Rundsch*. 74: 431–434.

Bi, J. L. & Felton, G. W. 1995: Foliar oxidative stress and insect herbivory: Primary compounds, secondary metabolites and reactive oxygen species as components of induced resistance. – *J. Chem. Ecol*. 21: 511–1530.

Blaudez, D., Jacob, C., Turnau, K., Colpaert, J. V., Ahonen-Jonnarth, U., Finlay, R., Botton, B. & Chalot. M. 2000: Differential responses of ectomycorrhizal fungi to heavy metals in vitro. – *Mycol. Res*. 104: 1366-1371.

Blinda, A., Koch, B., Ramanjulu, S. & Dietz, K. J. 1997: De novo synthesis and accumulation of apoplastic proteins in leaves of heavy metal-exposed barley seedlings. – *Plant Cell Environ.* 20: 969-981.

Boucherau, A., Aziz, A., Larher, F., & Martin-Tanguy, J. 1999: Polyamines and environmental challenges: Recent development. – *Plant Sci.* 140: 103–125.

Brown J. E., Khodr H., Hider, R. C. & Rice-Evans, C. A. 1998: Structural dependence of flavonoid interactions with $Cu^{2+}$ ions: implications for their antioxidant properties. – *Biochem. J.* 330: 1173-1178.

Brown, R. T. & Mikola, P. 1974: The influence of fruticose soil lichens upon the mycorrhizae and seedling growth of forest trees. – *Acta Forest. Fenn.* 141: 1–22.

Bryant, J. P., Chapin, F. S. & Klein, D. R. 1983: Carbon/ nutrient balance of boreal plants in relation to vertebrate herbivory. – *Oikos* 40: 357-368.

Bryant, J. P., Tuomi, J. & Niemelä, P. 1988: Environmental constraints of constitutive and long-term inducible defenses in woody plants. – In: Spencer, K (ed.) *Chemical mediation of coevolution*: 367-389. Academic Press, New York.

Bryant, J. P. Danell, K., Provenza, F. B., Reichardt, P. B. & Clausen, T. P. 1991a: Effects of mammal browsing upon the chemistry of deciduous woody plants. – In: Tallamy, D. W. & Raup, M. J. (eds.) *Phytochemicali induction by herbivores*: 135-154. John Wiley and Sons, New York.

Bryant, J. P., Heitkonig, I., Kuropat, P. J. &.Owen-Smith, N. 1991b: Effects of severe defoliation upon the long-term resistance to insect attack and chemistry of leaves of six southern African savanna woody species. – *Am. Nat.* 137: 50-63.

Cairney J.W. G. & Meharg A. A. 1999: Influences of anthropogenic pollution on mycorrhizal fungal communities. – *Environ. Pollut.* 106: 169-182.

Clijsters, H., Cuypers, A. & Vangronsveld, J. 1999: Physiological responses to heavy metals in higher plants; defence against oxidative stress. – *Z. Naturforsch*. 54c: 730–734.

Close, D. C. & McArthur, C. 2002: Rethinking the role of many plant phenolics –protection from photodamage not herbivores? – *Oikos* 99: 166–172.

Constabel, P. C. 1999: A survey of herbivory-inducible defensive proteins and phytochemicals. – In: Agrawal, A., Tuzun, S. & Bent, E. (eds.) *Induced Plant Defenses Against Pathogens and Herbivores. Biochemistry, Ecology and Agriculture:* 137–166. APS Press, St. Paul, Minnesota.

Denny, H. J. & Wilkins, D. A. 1987: Zinc tolerance in Betula spp. I. Effect of external concentration of zinc on growth and uptake. – *New Phytol*. 106: 517–524.

Dixon, R. A. & Paiva, N. L. 1995: Stress-induced phenylpropanoid metabolism. – *Plant Cell* 7: 1085-1097.

Dowd, P. F. & Lagrimini, L. M. 1997: The role of peroxidases in host insect defences. In: Carozzi, N & Koziel, M. (eds.) *Transgenic Plants for Control of Insect Pests*: 195–223. Francis and Taylor, London.

Dowd, P. F., & Vega, F. E. 1996: Enzymatic oxidation products of allelochemicals as a basis for resistance against insects: Effects on the corn leafhopper *Dalbulus maidis*. – *Nat. Toxins* 4: 85–91.

Ericsson, A. 1979: Effects of fertilization and irrigation on the seasonal changes of carbohydrate reserves in different age-classes of needles on 20-year-old Scots pine trees (Pinus sylvestris). – *Physiol. Plant*. 45: 270-280.

Felton, G. W. & Duffey, S. F. 1991: Protective action of midcut catalase in *Lepidopteran*

larvae against oxidative plant defences. – *J. Chem. Ecol*. 17: 1715–1732.

Fisher, R. F. 1979: Possible allelopathic effects of reindeer-moss (*Cladonia*) on jack pine and white spruce. – *Forest Sci*. 25: 256–260.

Gebauer, R. L. E., Strain, B. R. & Reynolds, J. F. 1998: The effect of elevated $CO_2$ and N availability on tissue concentrations and whole plant pools of carbon-based secondary compounds in loblolly pine (*Pinus taeda*). – *Oecologia* 113: 29–36.

Gershenzon, J. 1984: Changes in the levels of plant secondary metabolites under water and nutrient stress. – *Recent Advances in Phytochemistry* 18: 273-320.

Goldner, W. R., Hoffman, F. M. & Medve, R. J. 1986: Allelopathic effects of *Cladonia cristatella* on ectomycorrhizal fungi common to bituminous strip-mine soils. – *Can. J. Bot* 64: 1586–1590.

Grace, S. C. & Logan, B. A. 2000: Energy dissipation and radical scavenging by the the plant phenylpropanoid pathway. – *Phil. Trans. R. Soc. Lond.* B 355: 1499–1510.

Gratão, P. L., Polle, A., Lea, P. J. & Azevedo, A. 2005: Making the life of heavy metal-stressed plants a little easier. – *Funct. Plant Biol.* 32: 481-494.

Greisbach, H. A. 1981: *Lignins*. In: Conn, E. E. (ed.) *The biochemistry of plants*: 177-220. New York. Academic.

Hamilton, J., Zangerl, A. R., DeLucia, E. H. & Berenbaum, M. 2001: The carbon-nutirent balance hypothesis; ist raise and fall. – *Ecol. Lett*. 4: 86-95.

Haukioja, E. 1980: On the role of plant defences in the fluctuation of herbivore populations. – *Oikos* 35: 202-213.

Haukioja, E., Ossipov, V., Koricheva, J., Honkanen, T., Larsson, S. & Lempa, K. 1998: Biosynthetic origin of carbon-based secondary compunds: cause of variable responses of woody plants to fertilization. – *Chemoecology* 8: 133-139.

Herms, D. A. & Mattson, W. J. 1992: The dilemma of plants: To grow or defend? – *Quarterly Rev. Biol.* 67: 283-335.

Hódar, J. A., Zamora, R., Castro, J. & Baraza, E. 2004: Feast and famine: previous defoliation limiting survival of pine processionary caterpillar *Thaumetopoea pityocampa* in Scots pine *Pinus sylvestris*. – *Acta Oecol*. 26: 203-210.

Honkanen, T., Haukioja, E. & Kitunen V. 1999: Responses of *Pinus sylvestris* branches to simulated herbivory are modified by tree sink/source dynamics and by external resources. – *Funct. Ecol.* 13. 126-140.

Hyvärinen, M., Roitto, M., Ohtonen, R. & Markkola, A. 2000: Impact of wet deposited nickel on cation content of a mat-forming lichen *Cladina stellaris*. – *Environ. Exp. Bot.* 43: 211–218.

Jentschke, G. & Godbold, D. L. 2000: Metal toxicity and ectomycorrhizas. – *Physiol. Plant.* 109: 107–116.

Johnson, K. S. 2005: Plant phenolics behave as radical scavengers in the context of insect (*Manduca sexta*) hemolymph and midcut fluid. – *J. Agric. Food. Chem*. 53: 10120-10126.

Julkunen-Tiitto R., Rousi, M., Bryant, J., Sorsa, S., Keinänen, M. & Sikanen, H. 1996: Chemical diversity of several *Betulaceae* species: comparison of phenolics and terpenoids in northern birch stems. – *Trees* 11: 16–22.

Kahle, H. 1993: Response of roots of trees to heavy metals. – *Env. Exp. Bot*. 33: 99–119.

Kainulainen, P., Holopainen, J., & Palomäki, V. 1996: Effects of nitrogen fertilization on secondary chemistry and ectomycorrhizal state of Scots pine seedlings and on growth of grey pine aphid. – *J. Chem. Ecol*. 22: 617–636.

Keski-Saari, S. & Julkunen-Tiitto, R. 2003: Resource allocation in different parts of juvenile mountain birch plants: effect of nitrogen supply on seedling phenolics and growth. – *Physiol. Plant.* 118: 114-126.

Keski-Saari, S., Pusenius, J. & Julkunen-Tiitto, R. 2005: Phenolic compound in seedlings of *Betula pubescens* and *B. pendula* are affected by enhanced UVB radiation and different nitrogen regimens during early ontogeny. – *Global Change Biol.* 11: 1180-1194.

Koricheva, J. 2002: The carbon-nutrient balance hypothesis is dead; long live the carbon-nutrient balance hypothesis. – *Oikos* 98: 537-539.

Koricheva, J., Roy, S., Vranjic, J. A., Haukioja, E., Hughes, P. R. & Hänninen, O. 1997: Antioxidant responses to simulated acid rain and heavy metal deposition in birch seedlings. – *Environ. Pollut.* 95: 249–258.

Kuikka, K., Härmä, E., Markkola, A. M., Rautio, P., Roitto, M., Tuomi, J., Ahonen-Jonnarth. U. & Finlay, R. 2003: Severe defoliation of Scots pine reduces reproductive investment

by ectomycorrhizal symbionts. – *Ecology* 84: 2051-2061.

Kukkola, E., Rautio, P. & Huttunen S. 2000: Stress indications in copper- and nickel-exposed Scots pine seedlings. – *Environ. Exp. Bot*. 43: 197-210.

L'Huiller, L., d'Auzac, J., Durand, M. & Michaud-Ferriére N. 1996: Nickel effects on two maize (*Zea mays*) cultivars: growth, structure, Ni concentration, and localization. – *Can. J. Bot*. 74: 1547–1554.

Lavid, N., Schwartz, A., Yarden, O. & Tel-Or, Ee. 2001: The involvement of polyphenols and peroxidase activities in heavy metal accumulation by epidermal glands of the waterlily (Nymphaeaceae). – *Planta* 212: 323-331.

Lavola, A., Aphalo, P., Lahti, M. &. Julkunen-Tiitto, R. 2003: Nutrient availability and the effect of increasing UV-B radiation on secondary compounds in Scots pine. – *Environ. Exp. Bot.* 49: 49-60.

Leitner, M., Boland, W. & Mithöfer, A. 2005: Direct and indirect defenced induced by piercing-sucking and chewing herbivores in *Medicago truncatula*. – *New Phytol*. 167: 597-606.

León J., Rojo, E. & Sánchez-Serrano, J. J. 2001: Wound signalling in plants. – *J. Exp. Bot*. 52: 1-9.

Loponen, J., Lempa, K., Ossipov, V., Kozlov, M. V., Girs, A., Hangasmaa, K., Haukioja, E. & Pihlaja, K. 2001: Patterns in content of phenolic compounds in leaves of mountain birches along a strong pollution gradient. – *Chemosphere* 45: 291-301.

Lukina, N. V. & Nikonov, V. V. 1999: Pollution-induced changes in soils subjected to intense air pollution. In: Nikonov, V. V. & Koptsik, G. N. (eds.) *Acidic deposition and forest soils*: 79-126. Kola Science Centre, Apatity, Russia. (in Russian).

Lyytikäinen-Saarenmaa, P. & Tomppo, E. 2002: Impact of sawfly defoliation on growth of Scots pine *Pinus sylvestris* (Pinaceae) and associated economic losses. – *B. Entomol. Res.* 92: 137-140.

Manninen, S., Huttunen, S. & Perämäki, P. 1998: Effect of ambient $SO_2$ levels on S fractions in *Pinus sylvestris* foliage growing in subarctic. – *Scand. J. Foest Res.* 13: 306-316.

Margna, U. 1977: Control at the level of substrate supply – an alternative in the regulation of phenylpropanoid accumulation in plant cells. – *Phytochemistry* 16: 419-426.

Markkola, A. M., Ahonen-Jonnarth, U., Roitto, M., Strömmer, R. & Hyvärinen, M. 2002: Shift in ectomycorrhizal community composition in Scots pine (*Pinus sylvestris* L.) seedling roots as a response to nickel deposition and removal of lichen cover. – *Environ. Pollut*. 120: 797–803.

Marschner, H. 1995: *Mineral Nutrition of Higher Plants*. Academic Press, New York.

Martin-Tanguay, J. 2001: Metabolism and function of polyamines in plants: recent development (new approaches). – *Plant Growth Regul*. 34: 135-148.

Mazhoudi, S., Chaoui, A., Ghorbal, M. H. & El Ferjani, E. 1997: Response of antioxidant enzymes to excess copper in tomato (*Lycopersicon esculentum*, Mill.). – *Plant Sci*. 127: 129–137.

Minocha, R., Long, S., Magill, A. H, Aber, J. & McDowell, W. H. 2000: Foliar free polyamine and inorganic ion content in relation to soil and soil solution chemistry in two fertilized forest stands at the Harvard Forest, Massachusetts. – *Plant Soil* 222: 119-137.

Mithöfer, A., Schulze, B. & Boland, W. 2004 : Biotic and heavy metal stress response in plants: evidence for common signal. – *FEBS Letters* 566: 1-5.

Morselt, A. F. W., Smits, W. T. M. & Limonard, T. 1986: Histochemical demonstration of heavy metal tolerance in ectomycorrhizal fungi. – *Plant Soil* 96. 417–420.

Niemelä, P., Tuomi J. & Lojander, T. 1991: Defoliation of the Scots pine and performance of Diprionid sawflies. – *J. Animal Ecol*. 60: 683-692.

Niemelä, P., Tuomi, J., Mannila, R. & Ojala, P. 1984: The effect of previous damage on the quality of Scots pine foliage as food for Diprionid sawflies. – *Z. Angew. Ent.* 98: 33-43.

Nieminen, T. M. 2004: Effects of copper and nickel on survival and growth of Scots pine. – *J. Environ. Monit*. 6: 888-896.

Nieminen, T. M. 2005: Response of Scots pine (*Pinus sylvestris* L.) to a long-term Cu and Ni exposure. – *Metsäntutkimuslaitoksen tiedonantoja (The Finnish Forest Research Institute, Research Papers)* 942. 63 p.

Nordgren, A. 1988: Apparatus for the continuous, long-term monitoring of soil respiration rate in large number of samples. – *Soil Biol. Biochem*. 20: 955–957.

Nylund, J.-E. & Wallander, H. 1992: Ergosterol analysis as a means of quantifying mycorrhizal biomass. – In: Norris, J. R., Read, D. J., Varma, A. K. (eds.) *Methods in Microbiology vol. 24*: 77–88. Academic Press, London.

Nykänen, H. & Koricheva, J. 2004: Damage-induced changes in woody plants and their effects on insect herbivore performance: a meta-analysis. – *Oikos* 104: 247-268.

Ohtonen, R. & Väre, H. 1998: Vegetation composition determines microbial activities in a boreal forest soil. – *Microb. Ecol.* 36: 328–335.

Pandolfini, T., Gabbrielli, R. & Comparani, C. 1992: Nickel toxicity and peroxidase activity in seedlings of *Triticum aestivum* L. – *Plant Cell Environ.* 15: 719–725.

Passardi, E., Cosio, C., Penel, C. & Dunand, C. 2005: Peroxidases have more functions than a Swiss army knife. – *Plant Cell Rep.* 24: 255-265.

Pedrielli P., Holkeri, L. M. & Skibsted L. H. 2001: Antioxidant activity of (+)-catechin. Rate constant for hydrogen-atom transfer to peroxyl radicals. – *Eur Food Res Tech*. 213: 405-408.

Penuelas, J. & Estiarte, M. 1998: Can elevated $CO_2$ affect secondary metabolism and ecosystem function? – *Trees* 13: 20-24.

Pfanz, H., Dietz, K-J., Weinerth, I. & Oppmann, B. 1990: Detoxification of sulfur dioxide by apoplastic peroxidase. – In: Rennenberg, H., Brunold, Ch., De Kok, L. & Stulen, I. (eds.) *Sulfur nutrition and sulfur assimilation*: 229-235. SPB Academic Publishing BV, The Hague.

Polle, A., Chakrabarti, K., Schürmann, W. & Rennenberg, H. 1990: Composition and properties of hydrogen peroxide decomposing systems in extracellular and total extracts from needles of Norway spruce. – *Plant Physiol.* 94: 312–319.

Polle, A. & Rennenberg, H. 1991: Superoxide-dismutase activity in needles of Scots pine and Norway spruce under field and chamber conditions – lack of ozone effects. – *New Phytol*. 117: 335-343.

Polle, A., Eiblmeier, M. & Rennenberg, H. 1994: Sulphate and antioxidants in needles of Scots pine (*Pinus sylvestris* L.) from three $SO_2$-polluted field sites in eastern Germany. – *New Phytol*. 127: 571-577.

Prasad, S. M., Dwivedi, R. & Zeeshan, M. 2005: Growth, photosynthetic electron transport, antioxidant responses of young soybean seedlings to simultanoeus exposure of nickel and UV-B stress. – *Photosynthetica* 43: 177-185.

Pritchard, S., Peterson, C., Runion, G. B., Prior, S. & Rogers, H. 1997: Atmospheric $CO_2$ concentration, N availability, and water status affect patterns of ergastic substance deposition in longleaf pine (*Pinus palustris* Mill.) foliage. – *Trees* 11: 494–503.

Pätsikkä, E., Aro, E.-M. & Tyystjärvi, E. 1998: Increase in the quantum yield of photoinhibition contributes to copper toxicity in vivo. – *Plant Physiol*. 117: 619-627.

Pätsikkä, E., Kairavuo, M., Sersen, F., Aro, E.-M. & Tyystjärvi, E. 2002: Excess copper predisposes photosystem II to photoinhibition in vivo by outcompeting iron and causing decrease in leaf chlorophyll. – *Plant Physiol.* 129: 1359-1367.

Rao, K. V. M. & Sresty, T. V. S. 2000: Antioxidative parameters in the seedlings of pigeonpea (*Cajanus cajan* (L.) Millspaugh) in response to Zn and Ni stresses. – *Plant Sci*. 157: 113-128.

Rauser, W. E., 1978: Early effects of phytotoxic burdens of cadmium, cobalt, nickel, and zinc in white beans. – *Can. J. Bot.* 56: 1744-1749.

Rautio, P., Huttunen, S., Kukkola, E., Peura, R. & Lamppu, J. 1998: Deposited particles, element concentrations and needle injuries on Scots pine along an industrial pollution transect in northern Europe. – *Environ. Pollut.* 103: 81-89.

Rautio, P. & Huttunen, S. 2003: Total vs. internal element concentrations in Scots pine needles along a sulphur and metal pollution gradient. – *Environ. Pollut.* 122: 273-289.

Rautio, P., Kukkola, E. & Huttunen, S. 2004: Alterations in the nutrient status of Scots pine seedlings in copper- and nickel-treated soil. – *J. Appl. Bot. Food Qual.* 78: 48-57.

Rautio, P., Kukkola, E. & Huttunen, S. 2005: Growth alterations in Scots pine seedlings grown in metal-polluted forest soil: Implications for restorative forest management. – *J. Appl. Bot. Food Qual*. 79: 52-58.

Redman, A. M., Cipollini, D. F. & Schultz, J. C. 2001: Fitness costs of jasmonic acid-induced defense in tomato, *Lycopersicon esculentum.* – *J. Oecol*. 126 : 380-385.

Roitto, M., Ahonen-Jonnarth, U., Ranta, H. & Saikkonen, K 1998: Peroxidase activity in defoliated Scots pines exposed to heavy

metals and acid rain. – In: De Kok, L. J. & Stulen, I. (eds.) *Responses of plant metabolisim to air pollution*: 445-447. Blackhuys Publishers, Leiden.

Ross, J. A. & Kasum , C. M. 2002: Dietary flavonoids: Bioavailability, metabolic effects, and safety. – *Annu. Rev. Nutr*. 22: 19-34.

Ruuhola, T. & Yang, S. 2006: Wound-induced oxidative responses in mountain birch leaves. – *Ann. Bot. – London* 97: 29-37.

Samarkoon, A. B. & Rauser, W. E. 1979: Carbohydrate levels and photoassimilate export from leaves of *Phaseolus vulgaris* exposed to excess cobalt, nickel and zinc. – *Plant Physiol*. 63: 1165-1169.

Santiago, L. J. M., Louro, R. P. & De Oliveras, D. E. 2000: Compartmentation of phenolic compounds and phenylalanine ammonialyase in leaves of *Phyllanthus tenellus* Roxb. and their induction by copper sulphate. – *Ann. Bot. – London* 86: 1023-1032.

Sarjala, T. & Kaunisto, S. 1993: Needle polyamine concentrations and potassium nutrition in Scots pine. – *Tree Physiol*. 13: 87–96.

Saxon, M. E., Davis, M. A., Pritchard, S. E., Runion, G. B., Prior, S. A., Stelzer, H. E., Rogers, H. H. & Dute, R. R. 2004: Influence of elevated $CO_2$, nitrogen, and Pinus elliottii genotypes on performance of the redheaded pine sawfly, *Neodiprion lecontei*. – *Can. J. For. Res*. 34: 1007-1017.

Schnitzler, J-P., Jungblut, T. P., Heller, W., Kofferlein, M., Hutzler, P., Heinzmann, U., Schmelzer, E., Ernst, D., Langebartels, C. & Sandermann Jr., H. 1996: Tissue localization of u.v.-B-screening pigments and of chalcone synthase mRNA in needles of Scots pine seedlings. – *New Phytol*. 132: 247-258.

Schützendübel, A. & Polle, A. 2002: Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protections by mycorrhization. – *J. Exp. Bot*. 53: 1351-1365.

Simmonds, M. S. J. 2001: Importance of flavonoids in insect-plant interactions: feeding and oviposition. – *Phytochemistry* 56: 245-252.

Stark, S., Wardle, D. A., Ohtonen, R., Helle, T. & Yates, G. W. 2000: The effect of reindeer grazing on decomposition, mineralization and soil biota in a dry oligotrophic Scots pine forest. – *Oikos* 90: 301–310.

Takahama, U. & Oniki, T. 1997: A peroxidase/ phenolic/ascorbate system can scavenge hydrogen peroxide in plant cells. – *Physiol. Plant*. 101: 845–852.

Tang, W. & Newton, R. J. 2005: Polyamines reduce salt-induced oxidative damage by increasing the activities of antioxidant enzymes and decreasing lipid peroxidation in Virginia pine. – *Plant Growth Regul.* 46: 31-43.

Tarvainen, O., Markkola, A. M., Ahonen-Jonnarth, U., Jumpponen, A. & Strömmer, R. 2004: Changes in ectomycorrhizal colonization and root peroxidase activity in Pinus sylvestris nursery seedlings planted in forest humus. – *Scand. J. For.Res*. 19: 400-408.

Taulavuori, E., Taulavuori, K., Sarjala, T., & Laine, K. 1998: Polyamines and glutathione metabolism in N fertilized Scots pines during cold hardening. – *J. Plant Physiol*. 156: 179–184.

Thaler, J., Stout, M. J., Karban, R. & Duffey, S. 1996 : Exogenous jasmonate simulates insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. – *J. Chem. Ecol.* 22: 1767–1781.

Tikkanen, E. & Niemelä, I. 1995: *Kola Peninsula Pollutants and Forest Ecosystems in Lappland*. Final Report of The Lapland Forest Damage Project. Gummerus, Jyväskylä. 81 p.

Tuomi, J., Niemelä, P., Rousi, M., Sirén, S. & Vuorisalo, T. 1988a: Induced accumulation of foliage phenols in mountain birch: branch response to defoliation? – *Am. Nat.* 132: 602-608.

Tuomi, J., Niemelä, P., Chapin, F. S., Bryant, J. P. & Sirén S. 1988b: Defensive responses of trees in relation to their carbon/nutrient balance. – In: Mattson, W. J., Levieux, J. & Bernard-Dagan, C. (eds.) *Mechanisms of woody plant defenses against insects: Search for pattern*: 57-72. Springer-Verlag, New York.

Tuomi, J., Niemelä, P., Haukioja, E., Sirén, S. & Neuvonen, S. 1984: Nutrient stress: an explanation for plant anti-herbivore responses to defoliation. – *Oecologia* 61: 208-210.

Tuomi, J., Niemelä, P. & Sirén, S. 1990: The Panglossian paradigm and delayed inducible accumulation of folir phenolics in mountain birch. – *Oikos* 59: 399-410.

Tuomi, J., Niemelä, P. & Fagerström. T. 1991: Carbon allocation, phenotypic plasticity and inducible defences. – In: Tallamy, D. W. &

Raupp, M. J. (eds.) *Phytochemical induction by herbivores*: 85-104. Wiley, New York.

Tuovinen, J-P. & Ryaboshapko, A. 1995: *Saastepäästöt*. – In: Tikkanen, E. (ed.) *Kuolan saastepäästöt Lapin metsien rasitteena. Itä-Lapin metsävaurioprojektin loppuraportti:* 46-48. Gummerus, Jyväskylä.

Turnau, K., Kottke, I. & Oberwinkler, F. 1993: *Paxillus involutus–Pinus sylvestris* mycorrhizae from heavily polluted forest. I. Element localization using electron energy loss spectroscopy and imaging. – *Bot. Acta* 106: 213–219.

Walters, D., Cowley, T., & Mitchell, A. 2002: Methyl jasmonate alters polyamine metabolism and induces systemic protection against powdery mildew infection in barley seedlings. – *J. Exp. Bot*. 53: 747–756.

Vangronsveld, J. & Clijsters, H. 1994: Toxic effects of metals. In: Farago, M. E. (ed.) *Plants and the Chemical Elements. Biochemistry, Uptake, Tolerance and toxicity*: 149–177. VCH Verlagsgesellschaft, Weinham.

Wasserman, J. L., Mineo, L. Majumdar S. K. & van Tyne C. 1987: Detection of heavy metals in oak mycorrhizae of northeastern Pennsylvania forests, using x-ray microanalysis. – *Can. J. Bot.* 65: 2622–2627.

Yamasaki, H., Sakihama, Y. & Ikehara, N. 1997: Flavonoid–peroxidase reaction as a detoxification mechanism of plant cells against $H_2O_2$. – *Plant Physiol*. 115: 1405–1412.

Zvereva, E. L. & Kozlov, M. V. 2001: Effects of pollution-induced habitat disturbance on the response of willows to simulate herbivory. – *J. Ecol.* 89: 21-30.