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MINNA KIVIMÄENPÄÄ

The Cell and Tissue Structures of Norway Spruce and Scots Pine Needles as Tools for the Diagnosis of Ozone Impact

Doctoral dissertation

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ABSTRACT

Ozone at its present concentrations is a potential risk for forests in Fennoscandia and there is a need for bioindication methods that specifically reveal the impact of ozone on the trees. Microscopy is one possibility for conifers. The effects of most natural and anthropogenic stress factors on the cell structures are known, especially for Norway spruce (*Picea abies* [L.] Karst.) and Scots pine (*Pinus sylvestris* L.). Ozone-related symptoms are characterized by an electron dense chloroplast stroma, a reduced size of the chloroplasts and a more abundant occurrence of these changes in the outer mesophyll cell layers and in the sky-facing side of the needles than in the inner mesophyll cell layers and in the ground-facing side. The principal aim of this study was to further develop microscopy for the diagnosis of ozone impact. The aim was also to obtain information about the modes of action of ozone stress. Spruce saplings, growing in open-top-chambers (OTCs), and mature field-grown pine and spruce were studied. Effects of ozone, drought and their interaction, aging, senescence and sampling at different times during the day were studied at the cell and tissue levels by transmission electron microscopy (TEM) and light microscopy (LM).

The OTC studies showed that drought did not significantly modify the ozone responses at the cellular level. Ozone exposure increased the numbers of mitochondria and peroxisomes of Norway spruce needles. This suggested active protection, as the most ozone-sensitive organelles, the chloroplasts, were almost completely unaffected in the same cells. The results for the mitochondria suggested that protection was more effective in the current than in the second-year needles. The results for the peroxisomes suggested that oxidative stress was more intensive in the sky-facing side than in the ground-facing side of the spruce needles. The intercellular space was wider in the sky-facing than in the ground-facing side of the spruce needles. The sky-facing side of the spruce needles and the outermost mesophyll cell layer in the pine needles had more plastoglobuli, mitochondria, peroxisomes, less starch and smaller chloroplasts, suggesting that more stressful conditions normally prevail in these parts of the needles. These observations suggest that different light conditions and the diffusion pattern of gases may explain the pattern of ozone-related changes within the mesophyll tissue.

Chloroplasts from the senesced, yellow needles were extremely small and pale, due to the loss of the stroma. Senescence-induced changes occurred rapidly and simultaneously throughout the mesophyll tissue. Mesophyll cells of the oldest needle generation of Scots pine remained intact as long as the needles were green. Chloroplasts of the older needles had more plastoglobuli than the youngest needle generation. This was the only age–related change in the chloroplasts of Scots pine needles. Thus, the ozone-related symptoms cannot be regarded as signs of accelerated senescence or aging in Scots pine needles.

TEM and LM showed typical ozone-induced symptoms in the mesophyll tissue of the needles of mature Scots pines in southern Finland and northwest Russia, and of Norway spruce in southern Sweden. LM results were quantified as the percentage of symptomatic cells (pine) and as an ozone syndrome index (spruce), which also took into account the advance of the symptom in the tissue. Correlations showed that the ozone impact was highest in the needles with the lowest nutrient concentrations. Regression analysis after principal component analysis revealed that both ozone and low nutrient concentrations together reduced the winter hardiness of the needles and bark. The results suggest that LM, with verification by TEM, can be used to monitor the ozone impact on Norway spruce and Scots pine in the field, as long as all features of the ozone-related chloroplast symptoms (reduced size and electron dense stroma, which are changes that are more abundant in the outer cell layers) are present. An attempt to standardize the method is also presented.

Universal Decimal Classification: 504.054, 546.214, 581.2, 582.47, 632.151
CAB Thesaurus: air pollutants; ozone; conifer needles; *Picea abies*; *Pinus sylvestris*; biological indicators; cell ultrastructure; mitochondria; peroxisomes; chloroplasts; mesophyll; drought; aging; senescence; microscopy

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Minna Kivimäenpää

ABBREVIATIONS

a.s.l.	above sea level
AOS	active oxygen species
AOT40	accumulated dose over a threshold of 40 ppb
CF	charcoal-filtered air
C	current year (needle)
C+1, C+2, C+3	second (previous), third, fourth year (needle)
D	drought stress
HF	higher fertility
GLM	general linear model
LF	lower fertility
LM	light microscopy
NF+	non-filtered air + extra ozone
O_3	ozone
OTC	open-top chamber
NO	nitrogen monoxide
NO_2	nitrogen dioxide
PCA	principal component analysis
ppb	parts per billion, nl l^{-1}
Rubisco	ribulose 1,5-bisphosphate carboxylase/oxygenase
TEM	transmission electron microscopy
VOC	volatile organic compound
W	well-watered

LIST OF ORIGINAL PAPERS

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

I Kivimäenpää M, Sutinen S, Karlsson PE, Selldén G. Cell structures of needles of Norway spruce exposed to long-term ozone and drought. Annals of Botany (in press)

II Kivimäenpää M, Sutinen S, Medin EL, Karlsson PE, Selldén G (2001) Diurnal changes in microscopic structures of mesophyll cells of Norway spruce, *Picea abies* (L.) Karst., and the effects of ozone and drought. Annals of Botany 88: 119-130

III Kivimäenpää M, Sutinen S. Microscopic structure of needles of Scots pine, *Pinus sylvestris* (L.), during needle aging and autumnal senescence. Manuscript.

IV Sutinen S, Lumme I, Mäenpää M, Arkhipov V (1998) Light microscopic structure of needles of Scots pine (*Pinus sylvestris* L.) in relation to air pollution and needle element concentrations in S.E. Finland and Karelian Isthmus, N.W. Russia. Trees – structure and function 12: 281-288

V Jönsson AM, Kivimäenpää M, Stjernquist I, Sutinen S (2001) Frost hardiness in bark and needles of Norway spruce in southern Sweden. Trees - structure and function 15: 171-176

VI Kivimäenpää M, Jönsson AM, Stjernquist I, Selldén G, Sutinen S. The use of light and electron microscopy to assess ozone impact on Norway spruce needles. Environmental Pollution (in press)

CONTENTS

1 INTRODUCTION

1.1 Formation and concentrations of ground-level ozone

Tropospheric ozone is formed in a reaction where sunlight (wavelength <420 nm) splits nitrogen dioxide, NO_2, into nitrogen monoxide, NO, and a single oxygen atom, O (Finlayson-Pitts and Pitts 1997). Single oxygen atoms then unite with molecular oxygen, O_2, in the presence of a third molecule (that stabilizes the excited intermediate in the reaction), and ozone, O_3, is formed. In the opposite reaction, ozone reacts with nitrogen monoxide and forms molecular oxygen and nitrogen dioxide. Thus the net reaction is:

$$NO_2 + O_2 \leftrightarrow NO + O_3 \qquad (1)$$

In the presence of volatile organic compounds, VOCs, this balance is disturbed and ozone formation is promoted (PORG 1997). Peroxy radicals, which are formed from VOCs, react with NO, and NO_2 is formed without the consumption of ozone. The tropospheric photochemistry of ozone, as well as the night-time chemistry, is a set of very complex reactions and has been reviewed in PORG (1997).

Both the nitrogen oxides and VOCs needed for the photochemical formation of ozone originate from the burning of fossil fuels but also from natural processes (e.g. NO from soil and lightning, VOCs from vegetation). Episodic influx of ozone from the stratosphere also contributes to tropospheric ozone (Ebel et al. 1991). Before the industrialization, and the increase of anthropogenic VOCs and nitrogen oxides, yearly mean ground-level ozone concentrations were in the range of 5-15 ppb in Europe (Volz and Kley 1988). Since then the yearly mean values have increased two-three-fold (Volz and Gray 1988; Ashmore and Bell 1991; Staehelin et al. 1994).

While the mean annual concentrations have increased, the high peak concentrations have declined lately in Europe (Brönnimann et al. 2000; NEGTAP 2001). The predictions for future ground-level ozone concentrations and distribution depend on changes in the emissions of the ozone precursors (Collins et al. 2000; Jonson et al. 2001). Globally, ground-level ozone concentrations have been predicted to increase by 8% from 1996 to 2010 (Jonson et al. 2001) or by 9% from 1992 to 2015 (Collins et al. 2000). Collins et al. (2000) predicted increased ozone concentrations and doses for most parts of Europe, whereas the more positive scenarios of Jonson et al. (2001) forecast reductions in southern, eastern and central Europe, but either increased or unchanged ozone concentrations in northern and western Europe.

1.2 Phytotoxicity of ozone

Ozone-induced visible damage on vegetation was first noted in the 1940's in California, although ozone as a principal reason was identified later (see Treshow and Bell 2002). Since the 1950s the understanding of photochemical pollution, including ozone formation, as well as the action mechanism of ozone and its effects on vegetation has increased considerably.

The largest part of the total ozone deposition to vegetation is non-stomatal (Fowler et al. 2001), but the phytotoxicity of ozone, based on current knowledge, is mediated by

stomatal uptake (Long and Naidu 2002). Most of the ozone entering the leaf through the stomata reacts with the apoplastic fluids (Moldau and Bichele 2002). In these reactions active oxygen species (AOS), such as hydrogen peroxide, superoxide and hydroxyl radicals are produced (Long and Naidu 2002). In the most extreme acute ozone exposure, ozone itself, or the uncontrolled chain reactions induced by it, can damage the plasmalemma, disturb the ion balance of the cells and lead to necrosis (Rao et al. 2000; Long and Naidu 2002). In less extreme acute ozone exposures (i.e. short-term, high concentrations, 120-500 ppb) AOS produced from the degradation of ozone may act as triggers that activate AOS production by the cell itself, which can lead to programmed cell death and to rapid appearance of chlorotic mottles (Dizengremel 2001; Kangasjärvi et al. 2001). Chronic ozone exposure (i.e. long-term, elevated background concentrations, peak daily concentrations in the range of 40-120 ppb), instead, leads to several biochemical, physiological and structural alterations before any visible injuries and/or enhanced senescence can be seen (Dizengremel 2001, Long and Naidu 2002).

AOS are also involved in signal transduction that induces plant defence reactions directly or through gene expression (Dizengremel 2001; Long and Naidu 2002). The defence reactions may consist of enzymatic (e.g. superoxide dismutase, ascorbate peroxidase, glutathione reductase) or non-enzymatic (e.g. ascorbate, glutathione, α-tocopherol) antioxidative molecules or secondary metabolites (e.g. phenolics, carotenoids, flavonoids) that scavenge AOS, as well as general plant protective compounds (e.g. phytoalexins), and cell wall compounds (e.g. lignin, callose) that could possibly create barriers for ozone diffusion (Long and Naidu 2002). Plants can also emit terpenoids that can increase ozone formation, but also quench ozone (Andreae and Crutzen 1997) before it reacts with cells. Isoprene has been shown to protect leaves from cell structural and photosynthetic damage during high ozone episodes and is suggested to be an effective antioxidant (Loreto et al. 2001).

Thus, ozone injury depends on the intensity of the ozone stress and also on efficiency of the defence mechanisms of the plants. Besides this, the morphology of the leaves (such as stomatal density and intercellular space) plays a key role in ozone sensitivity (Evans et al. 1996).

The negative effects of ambient ozone on forest vitality and growth in North America and Europe are well established (Skärby et al. 1998; Ashmore 2002). The current view of how chronic ozone exposure affects forest trees is that ozone modifies the primary and secondary carbon metabolism for the benefit of detoxification, protection and repair, at the expense of growth (Dizengremel 2001). Ozone also impairs sucrose translocation altering the carbon partitioning in plants above and below ground (Dizengremel 2001; Ashmore 2002).

At the foliage level, the negative effects of chronic ozone exposure are seen in the older leaves or needles, whereas the youngest leaves or current year needles can be unaffected, or even stimulated (e.g. Wallin et al. 1990; Dizengremel 2001). Such stimulative effects at low concentrations are common to most substances that typically inhibit a biological process at higher concentrations (cf. Ries 1976). Ozone-induced stimulations of photosynthesis (Wallin et al. 1990; Beyers

et al. 1992; Barnes et al. 1995) and of the chlorophyll concentration (Senser et al. 1990; Wallin et al. 2002) have been noted in conifer seedlings. The stimulative effect may depend on the nutrient availability. Rantanen et al. (1994) reported enhanced height growth, mycorrhiza development and size of chloroplast starch grains in Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) seedlings when the nutrient availability was adequate, but not in nutrient deficiency.

1.3 Indirect effects of ozone on plants

In addition to that ozone is directly harmful to plants, it can also make plants more susceptible to biotic or other abiotic stresses. These factors may unmask hidden ozone injuries (e.g. biochemical and microscopic changes). Ozone can predispose trees to the drought stress (Maier-Maercker 1998), it can disturb the frost hardening (Lucas et al. 1988; Edwards et al. 1990) and dehardening (Edwards et al. 1990) processes and can increase winter damage (Fincher et al. 1989) of conifers. It can alter the chemical or physical quality of host plants and the production of allelochemicals, and then increase the performance of insect herbivores and pathogens or decrease that of predators and parasitoids (Flückiger et al. 2002, Percy et al. 2002).

1.4 Environmental factors as modifiers of ozone response in plants

Several climatic (humidity, temperature, light, wind speed) and edaphic (soil water content, nutrients, salinity) factors modify the ozone response of plants (Mills 2002). Environmental conditions affect the function of stomata and thus the uptake of ozone, and also modify the responses to ozone by influencing growth as well as the energy and resource availability for repair mechanisms (Mills 2002). The effects of the environmental conditions on ozone responses vary with the plant species (Mills 2002). The following examples are from conifers. Ozone uptake is enhanced by increased light, low leaf-air water vapour pressure difference (VPD) and sufficient water supply (Wieser et al. 2000). During the cold season, but not during the growing season, low temperatures during the previous night hinder stomatal opening (Wieser et al. 2000) and thus affect ozone uptake. High temperatures can amplify the negative effects of ozone as seen in stem growth of loblolly pine (*Pinus taeda*) in a field study of McLaughlin and Downing (1996). Co-occurrence of high ozone and high light induced decreases in photosynthesis and quantum efficiency of photosystem II (Fv/Fm) of Norway spruce needles, indicative of that ozone lowers the level at which photoinhibition occurs or that high light intensities lower the levels at which ozone inhibits photosynthesis (Mikkelsen and Ro-Poulsen 1994).

1.4.1 Drought

Drought can modify the ozone responses of plants by inducing the closure of the stomata and thus reducing ozone uptake (Mills 2002). On the other hand, both ozone (Long and Naidu 2002) and drought (Smirnoff 1993) can induce oxidative stress, and thus the response from interactions might be more severe than that induced by the drought or ozone stress alone. However, both moderate drought and ozone stress can increase the defence reactions in the cells (Smirnoff

1993; Long and Naidu 2002) making the plant more resistant to the other stress. In concert with the several possible action mechanisms for the combined ozone and drought stresses, the observed responses in the conifers are various, depending e.g. on the severity of the drought stress (Karlsson et al. 1995; Inclán et al. 1998), timing of the drought stress (i.e. early or late drought stress) (pointed out by Wallin and Skärby 1992 and Alonso et al. 2001), used clones (Le Thiec et al. 1994; Dixon et al. 1998) and on the age of plant parts (Alonso et al. 2001). The responses may also differ between conifer species. For example, drought appears to protect ponderosa pine (*Pinus ponderosa*) from ozone injury (Beyers et al. 1992; Temple et al. 1992; 1993), while ozone impairs the ability of Aleppo pine (*Pinus halepensis*) to withstand drought (Gerant et al. 1996; Inclán et al. 1998; Alonso et al. 2001). Depending on studied parameters, the responses of Norway spruce are variable: drought has been observed to protect trees from negative effects of ozone (Karlsson et al. 1995), it has been shown to enhance the negative effects of ozone (Le Thiec et al. 1994, Dixon et al. 1998) or drought and ozone have been demonstrated to function independently (Wallin and Skärby 1992; Le Thiec et al. 1994; Dixon et al. 1998; Wallin et al. 2002).

1.4.2 Nutrients

Nutrients as modifying factors in the ozone responses of the trees have been mainly studied with seedlings. Interactions of nitrogen (N) availability and ozone are best known, but vary between plants species. In most studies, birch (*Betula pendula*) responded more negatively to ozone when growing in low N availability than in sufficient N (Pääkkönen and Holopainen 1995; Landolt et al. 1997; Maurer and Matyssek 1997). In conifers, ozone-induced reductions in growth of the above-ground parts of loblolly pine and Scots pine were more pronounced in seedlings grown in high than in low nitrogen supply (Tjoelker and Luxmoore 1991; Utriainen and Holopainen 2001a). On the other hand, cellular alterations were most pronounced in seedlings from soils that were low or deficient in N (Kainulainen et al. 2000; Utriainen and Holopainen 2001a). Sufficient N, however, do not necessarily protect plants from the harmful effects of ozone (Maurer et al. 1997; Utriainen and Holopainen 2001a). Rantanen et al. (1994) reported that the ozone-induced stimulation in height growth, mycorrhiza development and size of chloroplast starch grains was depressed in the Scots pine and Norway spruce growing in soil with low magnesium (Mg) and calcium (Ca) availability. Deficiency of Mg, Ca, potassium (K) and phosphorus (P) have not been shown to drastically affect the ozone responses of conifer seedlings (Edwards et al. 1990; Rantanen et al. 1994; Barnes et al. 1995; Utriainen and Holopainen 2001a,b).

1.5 Critical ozone dose for forests

Critical ozone doses have been established to evaluate the risk of adverse ozone effects on plants. AOT40 (accumulated dose over a threshold of 40 ppb) index with critical value of 10 ppm.h, accumulated over a six-month (April-September) period (Kärenlampi and Skärby 1996; Fuhrer et al. 1997) has been used for forest trees in Europe (Hjellbrekke

and Solberg 2001). AOT40 index is calculated as:

$$AOT40=\Sigma (C_{oz} -40), \text{ for all } C_{oz} > 40 \text{ ppb} \quad (2)$$

where C_{oz} refers to hourly ozone concentration (ppb). Calculations are made for daylight hours only, when clear sky global radiation exceeds 50 W m^{-2}. At the moment, this critical level (threshold concentration, dose and time window over which AOTx is accumulated) is under revision (Bull et al. 2003).

1.6 Ozone as a risk for forests in Fennoscandia

Compared to the central and southern parts of the Europe, the ozone concentrations are lower in northern Europe and there are fewer high peak values (e.g. Hjellbrekke and Solberg 2001). Despite this, the climate conditions, such as long light days during the growing season, favour ozone uptake of the foliage (Emberson et al. 2000) and experimental fumigations in this area have shown that ambient or slightly elevated ozone concentrations have negative effects on young trees. For example, reduced growth and biomass accumulation, decreased photosynthesis, altered winter hardening processes, reduced chlorophyll concentrations, visible injuries on leaves, changed leaf anatomy and microscopic injuries in needles and leaves have been reported (Sutinen et al. 1990; Wallin et al. 1990; Pääkkönen et al. 1995; Anttonen and Kärenlampi 1996; Wallin et al. 2002; Ottosson et al. 2003). In addition, the soils of the coniferous forests in northern Europe are commonly low in nitrogen (Helmisaari 1995; Thelin et al. 1998), a situation that can enhance the negative effects of ozone (Landolt et al. 1997; Utriainen and Holopainen 2001a). On the other hand, nitrogen deposition can increase nitrogen content of the soil, and increase the growth of the trees. This, in turn, can induce nutrient imbalance in the trees via an increase in the demand of other nutrients (Nihlgård 1997). Possible sign of this is the significant decreasing ratios of needle K/N and Cu/N that were reported in southern Sweden during a ten-year-period (Thelin et al. 1998). Both ozone (Lucas et al. 1988; Edwards et al. 1990; Anttonen and Kärenlampi 1996) and imbalance of N, P and K (Klein et al. 1989; Rikala and Repo 1997; Jokela et al. 1998) can increase the sensitivity to frost of seedlings. Thus, based on the studies with young trees, the soil status will be important when predicting the effects of ozone on tree vitality, especially as the climate in Fennoscandia is characterized by cold winters. In addition, drought, an environmental factor, has been reported to have negative effects on forest growth and health in southern Fennoscandia (Bergh et al. 1999; Mäkinen et al. 2001; Alavi 2002). Interactions of ozone and drought in Fennoscandia have been studied to some extent. Study of Pääkkönen et al. (1998) was conducted in Central Finland. They showed that the responses to ozone and drought in birch could be independent, additive or interactive depending on studied parameter, severity of stress, experimental conditions and tree clone. Furthermore, a four-year study in open-top chambers (OTCs) suggests that drought did not largely modify the ozone response of Norway spruce in the southern Sweden (Karlsson et al. 2002; Wallin et al. 2002).

1.7 Evaluation of the ozone impact on mature forest trees in the field

The areas at risk for adverse effects of ozone on forest trees in Europe can be identified by mapping areas where the critical dose of ozone are exceeded (for AOT40, Hjellbrekke and Solberg 2001). Along with risk-based indexes, there is need for biomarkers, i.e. methods that reveal the actual impact of ozone on plants.

For sound ozone diagnosis the used attribute should be specific to ozone, and not mixed with other stress factors. Visible symptoms typical of ozone are now well recognized and documented, especially in broad-leaved plants (e.g. Innes et al. 2001). As a result, the Working Group on Air Quality (Expert Panel on Deposition, ICP Forests) selected the scoring of visible symptoms as the tool for assessing ozone injuries at the European level on the intensive Monitoring Plots, Level II (Submanual for the assessment of ozone injury on European Forest Ecosystems, 2003). More species are continuously added into the Pictorial atlas of sensitive species (2003) together with information of possible leaf symptoms of other origin, which may be mistaken for those of ozone.

Visible injuries on the needles of many conifer species, like Norway spruce and Scots pine, cannot yet be reliably associated with ozone in the field (Pictorial atlas of sensitive species, 2003). Furthermore, visible symptoms of Norway spruce are controversial and rarely reported (Günthart-Goerg 2001). This is due to that visible symptoms occur in the upper sun crown only. Moreover, ozone may decay by reaction with the terpenes, which are emitted by the needles, and the wax layer that covers the needles. In addition, macroscopic injury is difficult to detect, because any cell death is hidden by the compact rhomboid form of the needles (Günthart-Goerg 2001). Therefore, it is important to find additional diagnostic methods. This is especially important in those vegetation zones where conifers are the main woody species.

In addition to visible injuries, ozone induces several biochemical and microscopic changes, which could serve as biomarkers. Langebartels et al. (2001) listed several potential biomarkers at the protein transcript level. Several stress factors has been shown to increase phytol related compounds, but free phytol appeared to be a typical ozone-induced compound and could thus be used as an indicator of ozone stress for Norway spruce (Ekeberg et al. 1995). Relatively few biochemical methods have been tested in the field. Calzada et al. (2001) studied biochemical parameters in mature Monterey pine (*Pinus radiata*) needles in relation to several ozone indices in Spain. They reported that peroxidase activity could be used as a short-term indicator, ascorbate as an intermediate indicator and sulfhydryl as a long-term indicator of ozone exposure in the field. In the leaves of mature beech trees (*Fagus sylvatica*), inositol levels and increased concentrations of lignin-like material can be used to assess ozone impact in the field (Baumgarten et al. 2000). However, it is not known how ozone-specific the above-mentioned biochemical changes are.

Microscopic changes in the inner structure of conifer needles have been extensively studied, and the effects of both natural and anthropogenic stress factors are relatively well known (Holopainen et al. 1992; Sutinen

and Koivisto 1995; Fink 1999). Some of the changes show high specificity to ozone, and are often reported to have diagnostic value (e.g. Sutinen et al. 1990; Holopainen et al. 1996; Kainulainen et al. 2000; Utriainen and Holopainen 2001a). Moreover, microscopic methods have earlier been successfully used to evaluate forest health in the field (e.g. Soikkeli and Tuovinen 1979; Meyberg et al. 1988; Hafner et al. 1989; Wulff et al. 1996a; Kukkola et al. 1997; Alvarez et al. 1998, IV where sulphur dioxide and acidic wet deposition are discussed).

1.8 Microscopy as a method to study stress responses of plants

The structure of mature needles of many conifer species is well described at the light microscopic (LM) and transmission electron microscopic (TEM) levels (e.g. Evans and Miller 1972a,b; Walles et al. 1973; Soikkeli 1980; Sutinen 1987a; Ruetze and Schmitt 1988; Evans and Leonard 1991). In addition, the effects of natural factors, as well as biotic and abiotic stress factors, on the inner needle structure are also known for many conifer species, although best for Scots pine and Norway spruce. A review about these effects is out of the scope of this work, as that is done elsewhere. Holopainen et al. (1992), Sutinen and Koivisto (1995) and Fink 1999 have reviewed the effects of season, aging, drought, excess water, frost, sulphur dioxide, ozone and other oxidants, hydrogen fluoride, chlorine, acid precipitation, nitrogen oxides, ammonia, carbon dioxide, continuous illumination, enhanced UV-B radiation, ionising radiation, deficiency or excess of most macro- and micronutrients, detergents, mixtures of some air pollutants, some biotic factors as well as some of fixation errors on inner needle structure. Other papers have also revealed the effects of trichloroacetic acid (Sutinen et al. 1995), elevated temperature (Sallas et al. 2003), exhaust gas (NO_x and hydrocarbons) (Viskari et al. 2000) and copper and nickel exposure (Kukkola et al. 2000) on needle structure.

Based on above, microscopy is a widely used method to study the stress responses of foliage. The benefits of the method are that early stages of changes can be seen long before any visible injuries appear, site of action of a stress can be localized and hints about the modes of action of a stress obtained (Holopainen et al. 1992). Moreover, the method can be used to diagnose the causal agents of the injuries, as the effects of several stress factors, especially on conifer needle structure, can be specifically distinguished from each other (Holopainen et al. 1992; Sutinen and Koivisto 1995). The approach of the microscopic studies has mainly been descriptive, but in recent years structural changes have been quantified, to allow statistical comparisons between treatments. Histochemical stains to identify the chemical components in the tissue or to identify the cell organelles often accompany conventional light and electron microscopic techniques (e.g. Morré et al. 1990; Fink 1991; Günthardt-Goerg et al. 2000).

1.8.1 Ozone-induced changes in the microscopic structure of conifer needles

Both acute and chronic ozone exposure induce alterations in the cell structures of the plants. Most of the microscopic studies have focused on ozone effects on leaves and needles, because ozone is taken up via the

stomata and thus the earliest effects of ozone are expected to occur in the foliage. Ozone can also affect the cell structures of stems (Günthardt-Goerg et al. 1996) and roots (McQuattie and Schier 1992; Anttonen and Kärenlampi 1996). In the following, microscopic investigations dealing with the effects of ozone on the inner cell structures of the foliage, with emphasis on conifer needles and experimental exposures with ozone concentrations up to around 300 ppb (600 $\mu g\ m^{-3}$), are reviewed.

1.8.1.1 Changes in the mesophyll tissue

Effects of both acute and chronic ozone exposure on needles are first (Sutinen et al. 1990; McQuattie and Schier 1993) or only (Evans and Miller 1972a,b; Evans and Leonard 1991) seen in the mesophyll tissue. At the cellular level the effects are first seen in the chloroplasts (Sutinen 1987b; Sutinen et al. 1990; Holopainen et al. 1996), where the alterations are the same for several coniferous species, like Norway spruce, pitch pine (*Pinus rigida*), Aleppo pine, loblolly pine and Scots pine (Sutinen 1987b; McQuattie and Schier 1993; Anttonen et al. 1995; Anttonen et al. 1996; Holopainen et al. 1996). Chloroplasts are often reported to have an electron dense stroma (e.g. Sutinen 1986a, 1987b; Sutinen et al. 1990; McQuattie and Schier 1993; Anttonen and Kärenlampi 1996; Utriainen et al. 2000) accompanied by increased stroma granulation (Sutinen 1987b; Sutinen et al. 1990; Anttonen and Kärenlampi 1996; Holopainen et al. 1996; Utriainen and Holopainen 2002). Sutinen (1987b) and Sutinen et al. (1990) described the granules as "ribosome-like". In general, the granulation has not been defined in detail. It is possible that the granules reported in different papers are distinct from each other. Composition of used fixatives may also affect the distinctiveness of the granules (Maunsbach and Afzelius 1999). The electron dense chloroplasts are decreased in length and/or width (Sutinen 1986a, 1987b; Sutinen et al. 1990; Anttonen et al. 1996; Anttonen and Kärenlampi 1996; Holopainen et al. 1996). The increased electron density of the chloroplasts seems to precede the decrease in chloroplast size. Namely, there are several reports where increased electron density of stroma is noted without any decrease in chloroplast size (Kainulainen et al. 2000; Utriainen and Holopainen 2000; Utriainen et al. 2000; Utriainen and Holopainen 2001a). Furthermore, Sutinen et al. (1990) reported increased electron density and decreased chloroplast size in six-month-old Norway spruce needles four months after ozone exposure. The chloroplasts continued to decrease in size but the decrease was not significant until the following summer, when the needles were 13 month old. The above-mentioned chloroplast alterations are accompanied by an indistinct chloroplast envelope (Sutinen et al. 1990; Anttonen and Kärenlampi 1996; Anttonen et al. 1996; Holopainen et al. 1996).

Ozone-induced changes in the thylakoids are variable: McQuattie and Schier (1993) and Anttonen et al. (1996) reported increased thylakoid swelling, while Holopainen et al. (1996) showed that the thylakoids retained their structure also in the later stage of injury when the chloroplasts had otherwise lost their integrity. Decrease in the amount thylakoids has also been noted (Sutinen et al. 1990). Ozone does not induce any typical

changes in the plastoglobuli number: increases (Sutinen 1987b; Holopainen et al. 1996), decreases (Rantanen et al. 1994; Wulff et al. 1996b) or no effects (Anttonen and Kärenlampi 1996; Utriainen et al. 2000) have been reported. Increase in the diameter of the plastoglobuli has been reported by Anttonen et al. (1996) and increased translucence of plastoglobuli in combination with ozone and low nitrogen status by Utriainen and Holopainen (2001a). Most studies with high and low ozone concentrations show that ozone decreases the size of the starch grains (Sutinen et al. 1990; Anttonen et al. 1995; Anttonen et al. 1996; Anttonen and Kärenlampi 1996; Holopainen et al. 1996; Utriainen and Holopainen 2000). However, Wellburn and Wellburn (1994) reported increased starch in the mesophyll and endodermis of Aleppo pine needles in the summer but not in the autumn. Rantanen et al. (1994) showed that needles from ozone-fumigated Norway spruce and Scots pine seedlings had larger starch grains compared to ambient air in autumn, and Anttonen and Kärenlampi (1996) noted that the ozone-fumigated Scots pine needles had starch left during the winter, even if the starch grains had been smaller in the ozone-fumigated seedlings compared to ambient air control earlier in the season.

In the mesophyll cells of Norway spruce needles, peroxisomes (microbodies) are the next organelles, after the chloroplasts, to degenerate by ozone exposure (Sutinen 1987b; Sutinen et al. 1990). Along with the ozone-induced chloroplast alterations (electron dense and granulated stroma, decreased size) peroxisomes from Norway spruce and Scots pine needles were hypertrophied (Holopainen et al. 1996). Enlargement, as well as proliferation, of the peroxisomes in ozone-exposed Norway spruce needles was noted by Morré et al. (1990). When ozone exposure continues, also mitochondria start to disintegrate (Sutinen 1987b; Sutinen et al. 1990). At this stage of ozone injury also the cytoplasm loose integrity (Sutinen et al. 1990). Besides disintegration, chronic ozone exposure has other effects on the cytoplasm, such as an increased amount of ribosomes (Utriainen and Holopainen 2000; Utriainen et al. 2000), increased (Anttonen and Kärenlampi 1996; Wulff et al. 1996b) or decreased (Utriainen and Holopainen 2002) amount of lipid bodies, formation of darkly stained bodies (Anttonen and Kärenlampi 1996), and in the autumn and winter, increased vacuoles (Anttonen and Kärenlampi 1996; Wulff et al. 1996b) and thinning of the cytoplasm (Anttonen and Kärenlampi 1996). Other reported findings in the mesophyll tissue are formation of protrusions in the chloroplasts (Holopainen et al. 1996), darkly stained bodies in the chloroplasts (Anttonen and Kärenlampi 1996) or between the plasma membrane and the cell wall (McQuattie and Schier 1993), decrease in the vacuolar tannin (Utriainen and Holopainen 2002) and poor resolution of the plasma membrane (Anttonen and Kärenlampi 1996; Anttonen et al. 1996).

The changes described above are mainly from studies with chronic ozone exposure. Typical effects of acute ozone concentrations are a general disintegration of the cellular structures (e.g. swelling and rupture of membranes, condensation of organelles), and finally collapse of the cells with disappearance of the intracellular contents, and death of the cells (Evans and Miller 1972a, Evans and Fitzgerald 1993, Anttonen et al. 1996;

Holopainen et al. 1996). Intercellular debris may also accumulate (McQuattie and Schier 1993). Anttonen et al. (1996) reported that in loblolly pine the early symptoms of acute ozone exposure were seen in the chloroplasts thylakoids (swelling and rupture), while the stroma was unaffected. They noted that a round shape of the chloroplasts accompanied the thylakoid alterations. These chloroplast changes were followed by cell collapse. Ultrastructural studies accompanied with histochemical staining have shown that ozone in high concentration alters the compartmentalization of the minerals within the cells (Fink 1991). Outgrowths, which contain callose, cellulose and Ca-oxalate, are formed from the inner mesophyll cell walls (Fink 1991). Ca-oxalate crystals can also be seen in the vacuole (Fink 1991; Holopainen et al. 1996) from which they can protrude into the cytoplasm (Holopainen et al. 1996). In dead mesophyll cells, the crystals can also be seen between the tonoplast and the cell wall (Fink 1991). Ca-oxalate crystals have not been reported in experiments with low ozone concentrations.

The structural alterations induced by chronic and acute ozone exposures are not always easy to differentiate. For example, acute ozone concentrations induced a similar increase in electron density of stroma and decrease in chloroplast size as chronic ozone exposure in the needles of Norway spruce and Scots pine (Sutinen 1987b; Holopainen et al. 1996), although not in the loblolly pine (Anttonen et al. 1996). Moreover, if the chronic exposure continues for several seasons, quite similar disintegration of the cells takes place as in acute exposure (Sutinen et al. 1990).

1.8.1.2 Changes in relation to ozone exposure, diffusion dynamics, needle age and species sensitivity

Effects of both chronic and acute ozone concentrations are first seen, and are most severe, in the cells of the outermost cell layer or near to stomata or substomatal cavities of the mesophyll tissue (Evans and Miller 1972a,b; Sutinen 1987b; Sutinen et al. 1990; McQuattie and Schier 1993; Anttonen et al. 1995; Anttonen and Kärenlampi 1996). With increasing exposure time or concentrations, deeper laying tissues are also affected (Evans and Miller 1972b, Sutinen 1987b; Sutinen et al. 1990; McQuattie and Schier 1993). Finally, the whole mesophyll tissue can be affected (Evans and Miller 1972a). In Norway spruce, mesophyll cells under the endodermis in the ground-facing side of the needle are last and least affected (Sutinen et al. 1990), and in several pine species the cells between the endodermis and the resin ducts are the least damaged (Evans and Miller 1972a,b). In general, injuries within a tissue follow the diffusion dynamics of ozone (Fink 1988). Sutinen et al. (1990) collected needles from upper side of the spruce branches and could then study the differences between the sky-facing and the ground-facing sides of the needles. They noted that mesophyll cells in the sky-facing side were earlier and more severely affected than the cells in the ground-facing side of the needle.

In general, the higher the ozone concentration or dose is, the more severe the cellular injury and the faster the advance of the cellular injury within the tissue (Sutinen 1987b; Sutinen et al. 1990; Evans and Fitzgerald 1993; Holopainen et al. 1996).

However, Anttonen et al. (1996) showed that loblolly pine needles from treatments with elevated ozone concentrations (1.5x and 2x ambient concentration) had less structural alterations compared to the treatment with ambient or below-ambient concentrations. This was suggested to be due to stomatal closure after the initial acute injury, symptoms of which were pronounced in the highest ozone levels.

Studies about the effects of ozone on cell structures are mainly concentrated on the youngest needle generation or newest flush. In short-term exposures, ozone effects in some cases can be more intensive in the youngest needle generation (Holopainen et al. 1996). In longer lasting experiments older needle flushes or generations are more affected due to longer exposure or greater susceptibility of the older needles (Anttonen et al. 1996; Wulff et al. 1996b). In contrast to that, the darkening of the chloroplast stroma of Scots pine needles was more evident in the current than in the previous-year-needles (Utriainen et al. 2000). When Norway spruces were fumigated with ozone for three seasons, cellular alterations steadily increased as needles aged (i.e. ozone dose per needle increased) (Sutinen et al. 1990). This means that the older needles are expected to show higher amount and more severe cellular injuries than younger ones. It must be noted, however, that mild effects such as increase in the electron density of the stroma, can be reversed when the ozone concentrations are lowered, at least if needles are young and ozone exposure is low (Anttonen and Kärenlampi 1996).

Microscopic studies have revealed differences in the species sensitivity to ozone. While ozone exposures lasting for several years may induce only mild changes in the chloroplasts of the youngest needle generation of Norway spruce and Scots pine (Utriainen et al. 2000; Utriainen and Holopainen 2001a,b) slightly elevated ozone concentrations and doses can induce widespread cell death in slash pine (*Pinus elliotti)* (Evans and Fitzgerald 1993) and giant sequoia (*Sequoiadendron giganteum*) (Evans and Leonard 1991). Evans and Miller (1972b) noted differences in ozone sensitivity of several pine species. Ozone damage was seen in the substomatal area or in cells adjacent to stomata in the insensitive species, while the whole mesophyll tissue was damaged in the sensitive species.

1.8.1.3 Changes in other needle tissues

With increasing ozone concentrations or exposure time, endodermis (bundle sheath) and the tissues of the vascular cylinder can be affected after the mesophyll (Sutinen et al. 1990; McQuattie and Schier 1993). Endodermal cells can have similar changes as mesophyll cells, for example small chloroplasts with electron dense and granular stroma (Sutinen 1987b; Sutinen et al. 1990). Ozone-induced starch accumulation has been seen in the endodermis of Aleppo pine (Wellburn and Wellburn 1994). Large accumulations of densely stained compounds were noted in the endodermis and the transfusion tissue of pitch pine needles (McQuattie and Schier 1993). The albuminous cells of Norway spruce needles were affected at the same time as the endodermis and showed general breakdown of the cell organelles and disintegration of the cytoplasm (Sutinen 1987b; Sutinen et al. 1990). High ozone concentrations can induce

phloem collapse (McQuattie and Schier 1993; Wellburn and Wellburn 1994). McQuattie and Schier (1993) noted accumulation of dense material in the sclerenchyma cells and epithelial cells around the resin ducts of the pitch pine needles. Utriainen and Holopainen (2002) reported increased number of the resin ducts when high ozone concentration was combined with phosphorus deficiency. The area of the resin ducts did not decrease in the low ozone exposure (Utriainen et al. 2000). Accumulation of calcium oxalate inside the epidermal cells, subsidiary cells, guard cells of stomata and lumina of hypodermis were noted in Norway spruce (Fink 1991). Sutinen et al. (1990) reported that guard cells had similar changes in the chloroplasts as those found in the mesophyll. Cytophotometrical analysis of Norway spruce needles revealed that ozone delignified the cell walls of the guard and subsidiary cells (Maier-Maercker 1989). Accumulation of dense material was noted in the hypodermis (McQuattie and Schier 1993). Common to most of these changes, found in needle tissues other than the mesophyll tissue, is that they are induced by high ozone concentrations or doses.

1.8.2 Interactions of ozone and other abiotic factors on needles

There is limited information about the interactions of ozone and other abiotic factors on cell structure of foliage. To give some examples from the needle structural studies, Kainulainen et al. (2000) and Utriainen and Holopainen (2001a) noted that the ozone-induced darkening of the chloroplast stroma of the Scots pine and Norway spruce needles was more enhanced when the soil was deficient or low in N compared to sufficient N. Fincher et al. (1989) reported that ozone increased the disruption of the mesophyll of the red spruce (*Picea rubens*) needles after frost. Anttonen and Kärenlampi (1996) showed that ozone altered the typical winter hardening processes of Scots pine, such as the gathering of the chloroplasts in the mesophyll cells, and increased symptoms of frost, like increased cytoplasmic thinning, cytoplasmic dark bodies and small vacuoles (seen between chloroplasts). Sutinen (1987b) showed that the ozone-induced alterations in the ultrastructure of Norway spruce needles were enhanced when ozone stress was combined with SO_2 exposure. McQuattie and Schier (1993) noted the same on pitch pine needles in combined exposure of ozone and increased aluminium in soil.

1.8.3 Conifers vs. angiosperms

Ozone-induced chloroplast alterations in conifer needles and leaves of angiosperms have similar characteristics. Increased electron density of the stroma and decreased size of the chloroplasts has been noted in *Betula pendula, Fagus sylvatica*, *Prunus serotina*, *Carpinus betulus*, *Fraxinus excelsior*, *Sorbus aucuparia* and *Populus tremuloides* (Pääkkönen et al. 1995, 1996, 1998; Günthardt-Goerg et al. 2000, Oksanen et al. 2001). Decreased chloroplast size was also noted in spring wheat (*Triticum aestivum*) (Ojanperä et al. 1992) and radish (*Raphanus sativus*) (Miyake et al. 1989). In contrast to conifers, increase in the plastoglobuli number and size in the angiosperms appears to be general ozone-induced change in the chloroplasts (Miyake et al., 1989; Ojanperä et al. 1992; Pääkkönen et al. 1995, 1996, 1998;

Mikkelsen and Heide-Jørgensen 1996; Günthardt-Goerg et al. 2000, Oksanen et al. 2001). Ozone changes the anatomy of leaves of several broad-leaved tree species (Pääkkönen et al. 1995; Günthardt-Goerg et al. 2000) and affects the thickness of the cell walls (Günthardt-Goerg et al. 2000; Oksanen et al. 2001), although the direction of these changes depends on the ozone sensitivity of the species or genotype. Pectinaceous cell wall projections are also typical ozone-induced changes in the tree leaves (Günthardt-Goerg et al. 1997, 2000). In conifers, slightly elevated ozone concentrations have not been reported to cause anatomical modifications (Utriainen et al. 2000; Utriainen and Holopainen 2001a,b, 2002) or to cause changes in the cell wall thickness (Anttonen et al. 1996). Many of the ozone-induced changes in angiosperms resemble those of senescence and aging, and ozone has been suggested to accelerate senescence in many angiosperms (e.g. Ojanperä et al. 1992; Pääkkönen et al. 1995, Mikkelsen and Heide-Jørgensen 1996; Günthardt-Goerg et al. 2000).

1.8.4 Observations of structural ozone responses in the field

The impact of ozone on forest trees has been evaluated by transferring young trees into field sites with different ozone levels (Pääkkönen et al. 1997, Soda et al. 2000, Gravano et al. 2003) or by studying mature trees at sites with different ozone levels (Alvarez et al. 1998). Pääkkönen et al. (1997) noted that green *Betula pendula* leaves growing at the site with highest ozone concentrations and doses (24-h mean concentrations 32 and 30 ppb, AOT40 for 24 h day^{-1} 6.0 and 3.4 ppm.h, calculated from bud break to leaf senescence, for two successive years), had narrower chloroplasts or a higher proportion of chloroplasts with spherical or abnormal shape, dense stroma and swollen or curled thylakoids, compared to the less ozone-exposed sites. Furthermore, the number and size of plastoglobuli was positively correlated to the ozone exposure at the three sites in Finland. Soda et al. (2000) compared green and chlorotic areas of Aleppo pine needles in one site in Italy (maximum 24-h mean concentration around 60 ppb; AOT40 for May-September 20 ppm.h), while indoor chambers with charcoal filtered air served as controls. Symptomatic areas showed collapsed mesophyll cells, increased lipid and starch accumulation in the mesophyll and in the bundle sheath, collapsed phloem, shorter and rounder chloroplasts, granulated stroma, larger and abundant plastoglobuli and accumulation of calcium oxalate-like crystals in the epidermal tissue. These symptoms were not noted in the charcoal-filtered chamber control, and they were less evident or absent in the green areas of the needles. Gravano et al. (2003) reported very intensive darkening of the chloroplast stroma of symptomatic leaves of *Ailanthus altissima* in a central Italian forest site with high ozone exposure (maximum 24-h mean concentration 60 ppb, AOT40 "more than three times higher" than the current critical level 10 ppm.h). The visible symptoms of ozone injury developed later in the less exposed site (maximum 24-h mean concentration 40 ppb, AOT40 "just above the current critical level" 10 ppm.h), and the asymptomatic leaves there had lightly stained stroma, although thylakoid swelling was noted. Alvarez et al. (1998) used light microscopy to analyse structure of *Abies religiosa* needles collected from three

ozone-polluted forest sites (monthly maximum average concentration 150 ppb) and from one low-ozone-exposed site (ozone data not available) in Mexico. Necrosis was noted in the upper layer of the palisade tissue in the polluted sites and it increased with needle age. Vacuolar degradation and a cell type characterized by scarce cytoplasm, fully developed vacuole and unperceivable chloroplasts were described as alterations from general structure of *Abies religiosa*. These symptoms increased with needle age and were abundant in the polluted sites, but scarce in the control site. Although not discussed by the authors, the figures reveal that chloroplasts were dark-stained in the polluted sites and light-stained in the control site.

Typical ozone effects on chloroplasts of conifer needles (decreased size, electron dense, granular stroma) have been noted in mature Norway spruce and Scots pine needles collected from southern Sweden (Sutinen 1989) and in Norway spruce needles from a damaged stand in southern Finland (Sutinen 1990) and several areas in Germany (Sutinen 1986b). In German areas, these chloroplast changes were described under the necrotic spots in the sky-facing side of the Norway spruce needles. The cellular injury and the chloroplast changes were milder further away from the visibly damaged mesophyll zone and intact chloroplast could be detected in the needle side facing the ground. A similar gradual decrease in chloroplast size and amount of thylakoids and increase in number of plastoglobuli from the sky-facing side to the ground-facing side of the needles was noted in another field study in Germany (Sutinen 1987c). Increased electron density of the chloroplast stroma was occasionally seen in the Scots pine needles in the summer in the ambient air control (maximum 7-h mean 53 ppb and 47 ppb in June and July, respectively) in central Finland (Anttonen and Kärenlampi 1996). Kukkola et al. (1997) studied structure of Scots pine needles in subarctic industrial sites in northern Finland and, based on chloroplast changes, reported ozone effects on the trees among the influence of other stress factors.

Based on above, microscopy has a great potential to be used in diagnosis of ozone effects in conifers in the field. The chosen microscopic parameters should be specific to ozone and be expressed quantitatively to allow statistical comparisons between the forest sites or other parameters. No other stress factor than ozone is known to induce, at the same time, the following chloroplasts alterations: electron dense stroma and decreased size, being more intensive in the outer mesophyll cell layers in the needles (cf. Holopainen et al. 1992; Sutinen and Koivisto 1995; Fink 1999). Therefore, these chloroplast changes, when seen at the same time, could be used for ozone diagnosis in the field. The broad knowledge about how natural and anthropogenic stress factors affect needle structure is needed when interpreting the microscopic findings, as well as their connection to the physiological function of the plants.

1.9 Aims of the study

The overall aim of this study was to further develop microscopy for the use in diagnosis of the ozone impact on Scots pine and Norway spruce in the field. This aim was approached by open-top-chamber studies with Norway spruce saplings (I, II) and field studies with mature Norway spruce and Scots pine trees (III-VI). The aim was also to obtain information about the modes of action of ozone stress, and give background knowledge for other microscopic field studies.

From the point of these aims, the included papers aim to answer to the following questions:

What are the effects of ozone on needle structure (I, II)?

Does drought modify the ozone responses of needles at the cell and tissue structural levels (I, II)?

Are ozone effects more pronounced in the sky-facing side of the needle than in the ground-facing side (I)?

Are there differences at the cellular level within the mesophyll tissue (I, III)?

What kind of structural changes can be seen during needle aging (I, III) and senescence (III) and are these changes similar to ozone?

Can microscopic structures show diurnal changes (II)?

Can ozone-specific chloroplast changes be seen in mature spruce and pine in the field (IV, VI)?

Can previously reported relations between ozone, nutrients and frost hardiness be found in the field (Results, IV,V,VI)?

2 MATERIAL AND METHODS

The material and methods for the studies are described in detail in the original papers and manuscripts (I-VI). Only brief descriptions are presented here. More detailed descriptions are given for those analyses not included in the papers.

2.1 Open-top chamber studies

Long-term (four growing seasons, 1992-1995) open-top chamber experiment with low ozone concentration (charcoal filtered air, CF) and 1.5 x ambient ozone concentration (non-filtered air with extra ozone, NF+) was carried out with Norway spruce saplings at Östad, southern Sweden (I-II). Half of the saplings in each chamber were kept well watered (W) while the other half was exposed to several periods of reduced water supply (D) during late summers of the last three years. The studies of the microscopic structures of needles were from the last year, 1995. In study I, the samples were collected five times during the drought period. In the study II, the diurnal changes during one day were studied. For more details, see Table 1.

2.2 Field studies

Needle aging and autumnal senescence of young and mature Scots pine were studied in Suonenjoki, central Finland (III). The light microscopic structure of the needles of mature Scots pine were studied in relation to air pollution and nutrient concentrations at a survey line from the Karelian Isthmus in north-western Russia to south-eastern Finland (IV). Frost hardiness and nutrient concentrations in the bark and needles (V) and the microscopic symptoms of ozone stress in the needles (VI) of mature Norway spruce were studied at two stands with different soil fertility at Asa, in southern Sweden. For more details, see Table 1.

2.3 Sampling and preparation for microscopy

Procedures of sampling for microscopic analyses are summarized in Table 1. Care was taken to collect samples in a way, which provides as much as possible, similar microclimatic conditions for every needle in each study (Table 1).

The needles were immediately placed into cold (+4°C) fixative: 1.5% glutaraldehyde, 1.5% paraformaldehyde, 0.15 M sucrose, 2 mM $CaCl_2$ in cacodylate buffer, pH 7.0. The composition of the used fixative was the same in every study; only the molarity (0.05-0.1 M) was changed according to the season (Soikkeli 1980). The 0.5-1.0 mm thick sections were cut from the same needle position within a study. Samples were post-fixed in 1% OsO_4, dehydrated in a graded ethanol series followed by propylene oxide and embedded in Ladd's epon. For LM 2 µm thick cross-sections were stained first with toluidine blue and then with p-phenylene diamine. For TEM thin sections (40-70 nm) were mounted on copper grids, and stained with uranyl acetate and lead citrate.

Table 1. Summary of the study types and description of the materials and the sampling for microscopic analyses.

	I	II	III	IV	V, VI
Study type	OTC; Sweden	OTC; Sweden	Field; central Finland	Field; S.E. Finland, N.W. Russia	Field, southern Sweden
Main effects studied / Purpose of study	Ozone, drought, needle age, needle side (sky- vs. ground-facing), sampling time	Ozone, drought, diurnal changes	Needle age, tree age, senescence, sampling time, location of cells (close epidermis vs. endodermis)	Cellular changes in air pollution gradient, effect of nutrients	Relation of winter hardiness, nutrients and symptoms of ozone stress
Tree species	Norway spruce saplings	Norway spruce saplings	Scots pine	Scots pine	Norway spruce
Tree age	6	6	approx. 20 and 60	50-100	30-40
Needle generations	C, C+1	C	C, C+1, C+2, C+3	C, C+1	C, C+1, C+2
Sampling days	28.7.,[a] 16.8., 18.8.,[a] 18.9., 4.10.[a] 1995	18.9. 1995 four times during a day	21.8.-6.9. 1996 every day	15.11.-6.12.1992 15.11.-12.12.1994	16.11.1999
Time of day	5-11 am	7 am–6 pm	10 am–1 pm	10 am-15 pm	10 am–15 pm
Crown position	Third whorl from the top	Third whorl from the top	Young: upper third of the crown Mature: lower part of the crown	Upper third of the crown facing south	Seventh whorl from the top of the crown facing south-west
Position of needles	Upper side, middle of shoot of several branches	Upper side, middle of shoot of several branches	Upper side of same branch every occasion	Upper side of branch, same trees both occasions	Upper side, middle of several shoots of one branch

Needle generations current C, second year C+1, third year C+2, fourth year C+3. [a] Drought-stressed trees were watered.

2.4 Analysis of microscopic parameters

Using a light microscope with a magnification of 400-500x, mesophyll tissues were photographed on positive film (I, II, IV) or digitally (III). Pictures were taken both from the abaxial and adaxial sides of the mesophyll tissue. An approximate mesophyll area in one photograph can be seen in Fig. 1. Thin sections for TEM (Fig. 1.) were cut either only from the abaxial side of all needles (III, V, VI) or from the abaxial side of one and from the adaxial side of another needle (I, II). In each section, photographed mesophyll cells were located next to the hypodermis or the endodermis (I, II, III) or in the middle of the mesophyll tissue (V, VI). Microscopic parameters studied in the experiments I-III are summarizes in Table 2.

In studies IV-VI microscopy was used for field applications. Electron density of the chloroplast stroma and decreased chloroplast size (IV, VI) and higher occurrence of these chloroplast symptoms in the outer cell layers and sky-facing side of the needle (VI) were used to assess the effect of ozone. A change in the chloroplast orientation and shape was used to assess the hardening status of the needles (V). The chosen parameters for these three studies were based on literature on seasonal and air pollution effects on microscopic structure of conifer needles (e.g. Holopainen et al. 1992; Sutinen and Koivisto 1995; Fink 1999).

Electron microscopic paper photographs, slides on a slide projector monitor or digital images on a computer monitor were analysed with point counting. The measuring tools of Adobe Photoshop were also used. Some measurements were done directly under the light microscope with help of an ocular scale.

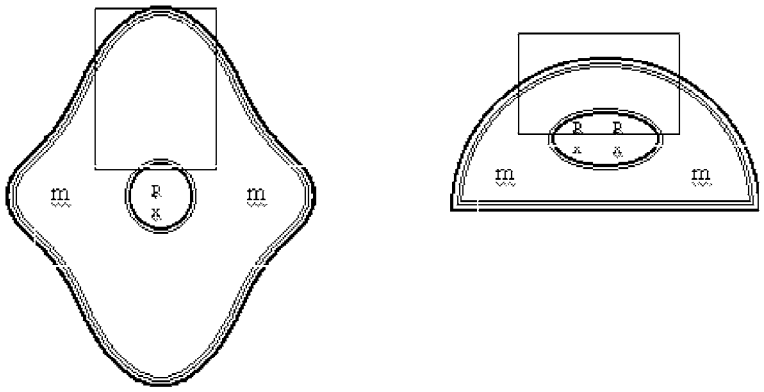

Figure 1. Schematic illustrations of the cross-sections of the spruce (left) and pine (right) needles. The outermost line represents the cuticle and the narrow regions under it epidermis and hypodermis. p and x are for phloem and xylem in the conducting tissue. Narrow region around vascular cylinder represents the endodermis. Mesophyll tissue (m) is located between the hypodermis and endodermis. Squares in the abaxial sides show an example of the mesophyll region photographed using LM. Approximately the same areas were cut for thin sections.

Table 2. Light microscopic (LM) and electron microscopic (TEM) features studied in the experiments I-III.

	Ozone and drought (I)	Diurnal changes (II)	Senescence and aging (III)
Mesophyll			
Chloroplast size	TEM	TEM	LM, TEM
Chloroplast number or area per cytoplasm		TEM	
Amount of starch	TEM	LM, TEM	TEM
Granulation of stroma	TEM		
Electron density of stroma	TEM		TEM
Amount of plastoglobuli	TEM		TEM
Staining of plastoglobuli	TEM		
Swelling of thylakoids	TEM	TEM	
Staining of thylakoids	TEM		
Amount of thylakoids	TEM		TEM
Shape of thylakoids	TEM		
Chloroplast shape	TEM		TEM
Mitochondrion size, number or area per cytoplasm	TEM	TEM	TEM
Mitochondrion shape		TEM	
Mitochondrion condition		TEM	
Peroxisome size, number or area per cytoplasm	TEM	TEM	TEM
Cytoplasmic vacuoles	LM	LM	
Vacuole size	LM	LM	LM
Tonoplast proliferation	TEM		
Appearance of tannin			LM
Intercellular space	LM		LM
Other tissues			
Amount of endodermal starch	LM		
Area of vascular cylinder	LM		
Condition of tissues in vascular cylinder	LM		LM
[a]Thickness of hypodermis and epidermis	LM		
[a]Number of hypodermal cells	LM		

[a]The thickness of epidermis and the outermost cell layer of hypodermis were measured from the upper corner of the needle (Fig. 1). The number of hypodermal cells under the outermost hypodermal layer was counted from the same place.

2.5 Water status, nutrients, frost sensitivity

The water potential of the needles was measured using a pressure-bomb, and water content by measuring the fresh weight and dry weight after drying to constant weight (II). Needle N concentrations were analysed by the Kjehdahl-method and those of the other elements from the bark and needles by digesting by HNO_3, heating to 125 °C and analysing with Inductively Coupled Plasma spectrometry, ICP (V). Frost sensitivity of the bark at the two test temperatures (V) was analysed according to Thomas and Hartman (1992). Samples for needle water content (II) and nutrients (V) were collected from the same branches as for microscopy and those for bark analysis from the same trees as needles were collected (V). Samples for water potential were from different trees from the same treatment (II).

2.6 Statistics

In principle, mean values from a certain needle generation and sampling time were calculated per tree. Exceptions were the cases where the location of the cell in the needles was of interest (I, III). Variance analysis (General linear model, GLM) was used in the statistical analysis where effects of group factors, such as ozone, drought and sampling time, and their interactions, were studied on continuous parameters. In case the group factors depended on each other, for example needles were collected from same tree at several sampling occasions (I, II) or cells for studies of cell location in the tissue were from the same needle (III), GLM for repeated measures was used. Post-hoc tests (I) and orthogonal contrasts (II, III) were used in more detailed analysis, e.g. to study the differences between several needle generations (III) or sampling occasions (II, III). Several data transformations were done for the homogeny of variances (Zar 1984). For non-continuous parameters, Mann-Whitney test and Kruskal-Wallis test were used instead of variance analysis (I, III, V, VI) and Wilcoxon test (III) and Friedman test (II) instead of GLM for repeated measures. In case the interaction effects of group variables on non-continuous parameters were desired to know, non-continuous parameters were classified into two groups, and studied by binary logistic regression analysis with hierarchical models (I). Main effects on the parameters of the ordinal scale were studied with Somers' d (III). Dependence between variables was studied by Spearman or Pearson correlation tests (I-VI). Stepwise regression analysis, forward selection, was used to model the continuous variable by other continuous variables (V).

The data from studies V and VI were combined and reanalyzed to study the simultaneous effect of ozone and nutrients on frost sensitivity of bark and winter hardiness of needles in multivariate analyses. For index of hardiness status of needles (mean index value from C, C+1 and C+2 needles, V), ozone syndrome index for tree (i.e. sum of the index values of C, C+1 and C+2 needles, VI) and nutrient concentrations (V) from the C needles were used in analysis. C-needles were used, because deficiency limits of nutrients are determined for them (Linder 1995). For frost sensitivity of bark (i.e. index of injury at -10 °C and -20 °C, V), ozone syndrome index for tree and nutrient concentrations of bark (V) were used in analysis.

If explanatory factors, such as nutrients, are correlated, there is a risk that important factors are dropped away from the linear regression analysis. Therefore, principal component analysis (PCA) was used to transform a number of (possibly) correlated variables into a smaller number of uncorrelated variables called principal components. Promax rotation with Kaiser Normalization was done for both needle data and for bark data. Principal components with Eigenvalues higher than 1 were extracted from needle data and four principal components, all having Eigenvalues higher than 1, from bark data. The scores of the principal components were then used in the linear regression analysis (forward selection) to study the changes in frost sensitivity of bark and winter hardiness of needles in relation to nutrients and ozone impact.

3 RESULTS

The most important results are summarized here. The effects of ozone, drought, aging of needles and the location of the cells in the mesophyll tissue on certain microscopic structures of the needles from studies I, II and III are summarized in Table 3.

3.1 Effects of ozone in experimental exposure

In the OTC experiment (I), the effects of ozone were noted in the cells of mesophyll tissue and in the endodermis, but not in the vascular cylinder, epidermis or hypodermis. As an average for the whole sampling period, the cross-section areas of chloroplasts from both C- and C+1 needles were slightly larger in NF+ compared to CF (Table 3). No differences in the electron density of the chloroplast stroma were seen. Fifteen weeks after the start of the ozone exposure, larger starch grains were noted in the chloroplast of the endodermis in needles from the NF+ saplings, but at the end of the season, 25 weeks after the start of the exposure, starch grains were smaller in NF+ compared to CF (Table 3). The same trend could be seen in the size of starch grains in the mesophyll cells. Non-osmiophilic plastoglobuli increased in the C needles, while they decrease in the C+1 needles.

Ozone-induced changes in the peroxisomes and mitochondria persisted during the whole study period. Peroxisomes were more numerous in the sky-facing side compared to ground-facing side of the needles from NF+ (Table 3), this difference between needle sides was not seen in the needles from CF. Moreover, the peroxisomes in the sky-facing side of the C needles from NF+ were smaller than those in the ground-facing side (Table 3). Mitochondria were smaller in NF+ compared to CF (Table 3). Furthermore, their number was higher in NF+ compared to CF, although this was only seen in the C needles (Table 3). Ozone also affected the shape of the mitochondria: in NF+, the proportion of tube-shaped mitochondria increased and that of circular mitochondria decreased from early morning to the later sampling occasions during the day (II). In CF, instead, the proportion of tube-shaped mitochondria decreased and that of circular mitochondria increased from early morning (II). In the cytoplasm, the effects of ozone were seen as an increase in lipid accumulations in the C needles and as a decrease in the C+1 needles (I). There was an overall increase in the volume of the central vacuole towards the end of the season which, however, did not take place in the C+1 needles from NF+. In addition, in summer, at the first sampling time, cytoplasmic vacuoles encircling nucleus were more numerous in NF+ compared to CF, and the NF+ saplings did not show similar seasonal increasing pattern in the number of vacuoles as the CF saplings.

Table 3. Increases (↑), decreases (↓) or non-significant effects (-) of selected ultrastructural characteristics of chloroplasts, peroxisomes and mitochondria affected by ozone, drought, increasing needle age and location of cells in the mesophyll. For location of cells, sky-facing side of the spruce needle is compared to the ground-facing side and outer cell layers (close to the epidermis) of pine needles are compared to the inner cell layers (close to the endodermis). Spruce was investigated in the studies I and II and pine in the study III. Note that the samples in I were collected in the morning and that only C-needles were collected in II. The extent of the changes in the chloroplast area or length is shown, emboldened values indicate chloroplasts without starch.

	Ozone	Drought	Aging	Location of cells	
				Upper side of needle	Outer cell layer
Chloroplasts					
Size (area or length)	↑ (I) **0.1 µm²** - (II)	↑ (I) **0.1 µm²** - (II)	↓ (I) **1.2 µm²** - (III)	↓ (I) 0.3 µm[a] **0.8 µm²**	↓ (III) 1.5 µm², **0.8 µm²**, 0.5 µm
Plastoglobuli number	- (I)	- (I)	↑ (I, III)	nd	↑ (III)
Amount of starch	↑↓ (I)[b] - (II)	↓ (I, II)	↓ (I) - (III)	↓ (I)	↓ (III)
Peroxisomes					
Size	↓ (I)[c] - (II)	↓ (I) ↓↑[d] (II)	↓ (I) - (III)	- (I)	- (III)
Number or density	↑ (I)[e] - (II)	↑ (I)[f] - (II)	- (I) - (III)	↑ (I)	↑ (III)
Mitochondria					
Size	↓ (I) - (II)	- (I, II)	- (I) ↓ (III)[g]	- (I)	↓ (III)
Number or density	↑ (I)[h] ↑ (II)	↑↓ (I)[i] - (II)	- (I) - (III)	↑ (I)	↑ (III)

nd, not determined
[a]measured on 28.7 and 16.8
[b]transient increase, then decrease, measured from mesophyll and endodermis
[c]only in the sky-facing side of C-needles
[d]decreased in the morning, increased later during the day
[e]only in the sky-facing side
[f]noted when D saplings were watered
[g]between C, C+1 and C+2
[h]only in C-needles
[i]dependent on needle age and severity of stress.

3.2 Effects of drought in experimental exposure

Effects of drought in spruce needles were seen in the vascular cylinder, endodermis and mesophyll (I). The vascular cylinder was larger, the proportion of sclerenchyma cells with thin cell walls higher and the number of sclerenchyma cells lower in D compared to W. During severe drought stress the D saplings had more often precipitated or disintegrated cytoplasm compared to the W saplings (II). Also, the adjacent thylakoids were more often separated or whole grana stacks separated from the stroma in D compared to W (I not shown, II). Other effects of drought on the chloroplasts were seen as a small increase in the cross-section area of chloroplasts (I, Table 3) and increase in the non-osmiophilic plastoglobuli at the first sampling time, decrease in the amount of starch (I) and lowered starch accumulation during the day (II) (Table 3). However, at the end of the experiment, in the beginning of October, the D saplings had more starch both in the mesophyll and in the endodermis compared to the other treatments (I). The area of the central vacuole was larger in D compared to W (I, II). Drought also increased the proliferation of the tonoplast. An increasing effect of drought on the number of peroxisomes was seen before and between the drought periods when the D saplings were watered (I, Table 3). Peroxisomes were smaller in D compared to W when the samples were collected during the morning hours (I, II, Table 3). Later during the day, however, peroxisomes from the D saplings increased in size over those from the W saplings. Effects of drought on the number of mitochondria were variable and depended on the severity of the drought stress and needle age (I, Table 3). Effects of drought on the mitochondria shape were also seen: mitochondria from D were more often circular than tubular compared to W (II).

3.3 Combined effects of ozone and drought

Interactions of ozone and drought were few (I, II). However, an increase in the amount of starch was not seen in chloroplasts from NF+/D as in CF/D (I). Chloroplasts from NF+/D did not increase in size as in CF/D and NF+/W, but were even smaller than those from CF/W (I). Drought enhanced the increasing effect of ozone on the nucleus-encircling vacuoles (I).

3.4 Aging

In both spruce (I) and pine (III) the structural changes occurring with increasing needle age were increased cytoplasmic lipid bodies and chloroplast plastoglobuli (Table 3) and larger central vacuole. In spruce, C+1 needles had smaller starch grains, peroxisomes, the shape of the chloroplasts was more rounded and an amount of cells with non-osmiophilic plastoglobuli was higher compared to the C needles (Table 3). In pine, the mitochondria decreased in size from C to C+2 needles and enlarged from C+2 to C+3 needles (Table 3). The tannin of the C needles was seen as fine homogenous granules in the whole area of the vacuole while tannin from the older needles was commonly seen as an electron dense, wavy or foamy-looking large mass, often occupying the whole area of the vacuole. Hypertrophy and accumulation of tannin in the phloem parenchyma cells in

one or two rows facing to the neighbouring phloem tissue was typical to C and C+1 needles. As the needles got older, the phloem parenchyma cells were more often strongly hypertrophied, appearing empty or filled with tannins. The occurrence of cavities between the phloem and sclerenchyma cells was noted in the C+3 needles, but not in the younger ones. The thickness of the phloem increased with needle age.

The field studies showed that the hardening status of the needles (V), based on chloroplast shape and location, did not significantly differ between needle generations in any of the field sites ($p>0.1$ for Friedman test). The ozone-specific chloroplast changes were most intensive in the oldest (C+2) needle generation (VI).

3.5 Senescence

Senescence was studied during a phase of autumnal yellowing of C+3 needles of Scots pine (III). Cells from the yellow needles had larger central vacuoles, higher proportion of cells with disintegrated cytoplasm and ruptured cell walls compared to the oldest green needles. However, the cytoplasm was healthy-looking in the majority of the cells of the yellow needles. The cytoplasm had large lipid deposits. The tannin in the central vacuole was in the form of granules, which, however, were smaller in size than in the green C+3 needles. The chloroplasts were so small and light in colour that they were difficult to discern at the LM level. This change was seen uniformly in the whole mesophyll tissue. TEM showed that the light appearance of the chloroplasts was due to loss of stroma, and that they were at maximum one third of the size of the chloroplasts form green needles. The envelopes of the extremely small chloroplasts were distinguishable. Diffuse patches, possibly leftovers from the stroma and plastoglobuli, remnants of the thylakoids and sometimes starch grains were seen in the empty-looking chloroplasts. Mitochondria even in the most disintegrated cytoplasm were intact. The strong hypertrophy of the phloem parenchyma cells, appearing empty or filled with tannins, as well as the occurrence of cavities between the phloem and sclerenchyma cells, were more common in the yellow than in the green C+3 needles.

An early phase of the above described changes was found only once in one green C+3 needle in one day. Otherwise, the structure of green C+3 needles remained intact until the yellowing occurred. The only significant difference in the green C+3 needles that could be related to the senescence process was the larger cross-section area of the mitochondria.

3.6 Winter hardening

The samples for the studies I, II and III were collected between the end of July and the beginning of October, i.e. at a time when the winter hardening of Scots pine and Norway spruce in Fennoscandia takes place. Therefore, the time-related changes in the structures of the trees may be related to the season. Such changes in time in Norway spruce (I) were enlargement of the central vacuole, increased proportion of the small vacuoles around the nucleus, decline in the starch, increase of the mitochondria number at the last sampling time and a rounding of the chloroplasts. The occurrence of shorter chloroplasts in Scots pine was also possibly

due to a change in shape (III). Previously defined changes related to the winter hardening (Soikkeli 1980, Holopainen et al. 1992, Öquist and Martin 1986) were seen in several studies as irregular or rounded chloroplasts (I, III, V), grouping of the chloroplasts close to each other (V), small or absent starch grains (I, II, IV, V), increased chloroplast protrusions (III) and division (III) and occasional decrease in grana thylakoids (III).

3.7 Location of cells in the tissue

Cells next to the epidermis, compared to those close to the endodermis, in pine needles (III) and in the sky-facing side, compared to the ground-facing side, of spruce needles (I) had many similarities: chloroplasts and starch grains were smaller and the number of peroxisomes and mitochondria were higher (Table 3). Furthermore, in spruce, the intercellular space was wider in the sky-facing side of the needle, and in pine, the number of plastoglobuli was higher and the cross-section area of the mitochondria was smaller in the cells next to the epidermis (Table 3).

3.8 Diurnal changes in cell structures

Several changes in the shape, volume and location of cell organelles, as well as in the storage products were noted during the day (II). The proportion of cells with starch increased and that with cytoplasmic lipid accumulations decreased from the morning to the evening, the chloroplasts were more tightly packed in the mesophyll cells in the morning than later in the day, the mitochondria increased in size from early morning to midday, after which they progressively decreased in size, and the peroxisomes increased in size from early morning to midday. Some of the diurnal changes were modified by ozone and drought (II, 3.1. and 3.2.). Preservation of the cell structures also changed during the day: a higher proportion of injured mitochondria, condensed cytoplasm, disintegration of the cytoplasm and chloroplasts, plasmolysis, separation of adjacent thylakoids and separation of the grana stack from the stroma were frequent early in the morning, but the occurrence of these features decreased or disappeared during the day (II). Similar drastic disintegration has not been reported elsewhere with same fixative (Soikkeli 1980; Sutinen 1987a, 1995, III, IV, V, VI), apart from the plasmolysis of the young needles (Sutinen et al 1995, III). Although the diurnal changes were investigated on only one day, the results from the same study from other sampling occasions in the morning (I, data not presented) supported the results from morning hours (II). These were seen e.g. in the size of peroxisomes and in the preservation of the cell structures.

3.9 Diagnosing impact of ozone in the field

3.9.1 Microscopic symptoms of ozone stress

Ozone-related symptoms were seen in 30-50% (stand averages) of the mesophyll cells of Scots pine needles in SE Finland and NW Russia in 1992, while in 1994 the proportions were lower, 6-45% (IV). The differences between stands were not

significant in 1992. Instead, differences were noted in 1994.

Three different approaches were used to study ozone effects on Norway spruce in study VI. TEM studies, which were restricted to a few cells in the needle cross-section, showed that there were more trees at the low fertility (LF) site compared to the high fertility (HF) site with increased electron density of the chloroplasts. The difference between the sites was not significant. The electron dense chloroplasts had a high amount of ribosome-like granules. The diameter of the granules was 17-20 nm. Electron dense chloroplasts with decreased lengths, studied from whole needle cross-sections by LM, were most evident in the sky-facing side of the needles and in the outer cell layers (VI). Symptoms were more advanced in the oldest needle generation compared to the younger ones, and at the LF site compared to the HF site. The results were expressed as an ozone syndrome index at needle generation, tree and stand level. The index was based on chloroplast changes at the cell and tissue level. The ozone syndrome index had the highest values in the oldest needle generation and at the low fertility site.

3.9.2 Relation of microscopic symptoms of ozone stress, nutrients and winter hardiness

Pearson and Spearman correlations showed that the ozone-induced microscopic symptoms in the field were more pronounced in the sites with low N, S and P concentrations in the needles of Scots pine (IV) and with low P, N, Mg, S concentrations and P/N and Mg/N in the needles of Norway spruce (VI).

Table 4. Correlation matrix of Principal Component Analysis with Promax rotation, Kaiser Normalization, for needle nutrients and ozone syndrome index for tree (O_3 index). High loadings are emboldened.

	Component			
	N1	N2	N3	N4
K	**0.900**	-0.086	0.128	0.114
Zn	**0.865**	-0.196	0.245	-0.166
S	**0.692**	0.392	-0.189	-0.012
N	**0.604**	**0.544**	-0.192	0.114
P	-0.011	**0.929**	-0.152	0.214
O_3 index	0.204	**-0.822**	-0.195	0.279
Mg	0.156	**0.615**	0.164	-0.381
Cu	0.294	**0.509**	0.175	0.166
Al	-0.239	0.214	**-0.798**	-0.211
Ca	0.110	0.069	**0.706**	-0.222
B	0.244	-0.344	**-0.692**	-0.369
Mn	0.028	0.012	0.169	**0.924**

The results from V and VI were combined for PCA. The correlation matrix (pattern matrix) of PCA for the needle nutrients and the ozone syndrome index is presented in Table 4 and that for the bark nutrients and the ozone syndrome index in Table 5. The values represent the correlation between the explanatory factor and the rotated component. Large loadings for particular factors within a component indicate that they have a simultaneous effect. For example, in Table 4, component 2 (N2) gets highest loadings from Cu, Mg, P, N and ozone syndrome index. In that case, the influence of the nutrients is of opposite direction than that of ozone, as seen from the opposite signs.

Table 5. Correlation matrix of Principal Component Analysis with Promax rotation, Kaiser Normalization, for bark nutrients and ozone syndrome index for tree (O_3 index). High loadings are emboldened.

	Component			
	B1	B2	B3	B4
Ca	**0.851**	0.028	-0.131	-0.089
S	**0.816**	0.127	0.212	-0.122
Mn	**0.698**	-0.256	0.140	0.232
Cu	**0.589**	0.343	-0.151	-0.452
O_3 index	0.067	**-0.942**	0.159	-0.183
P	-0.174	**0.904**	0.396	0.102
Mg	0.192	**0.782**	0.097	0.189
K	0.084	0.180	**0.862**	0.055
Fe	-0.484	-0.059	**0.794**	-0.310
Zn	0.266	0.180	**0.727**	-0.131
B	0.401	-0.309	**0.563**	0.240
Al	-0.057	0.338	-0.140	**0.941**

Linear regression analyses (Table 6) and correlations (V) showed that the hardiness status of both needles and bark were related to the combination of nutrients as well as their simultaneous effect with ozone (indicated by ozone syndrome index). In the needles, the high ozone syndrome index (indicative of high ozone impact at the cellular level) was accompanied by low N, P, Mg and Cu, and in the bark with low P and Mg (Table 4, 5). Even if the ozone syndrome index was included in the analysis and a different statistical method was used compared to the study V, the same nutrients were seen in the models, although not always in the same composition. Al, however, was not included in the models based on PCA.

Table 6. The hardiness index of the needles (Needle hard.) measured from the three youngest needle generations in relation to the principal components (N), calculated from mineral nutrient concentrations of the current year needles and ozone syndrome index of the tree (measured from three youngest needle generations), and the sensitivity to frost in bark, measured as an index of injury at two temperatures, -10°C (I_t10) and -20°C (I_t20) in relation to principal components (B), calculated from the mineral nutrient concentrations of the bark and ozone syndrome index of the tree. The components are ranked in importance according to the standardized regression coefficients produced by stepwise linear regression analysis, forward selection (n=16).

Index	Model	r^2	F	p
Needle hard.	N2, N4	0.675	13.50	0.001
I_t10	B2	0.358	7.80	0.014
I_t20	B2, B3	0.614	10.35	0.002

Principal components (N, B) are presented in the Tables 4 and 5.

4 DISCUSSION

4.1 Ozone-induced changes in needle structure in relation to function

4.1.1 Chloroplasts

Decreased photosynthesis is a general result from chronic ozone exposure in trees (Dizengremel 2001). Ozone can affect the biochemical reactions of both the stroma (Fontaine et al. 1999; Pelloux et al. 2001) and the thylakoids (Lütz et al. 1992; Kellomäki and Wang 1997) of conifer needles. Alterations in both thylakoids and stroma can also be seen using electron microscopy (Sutinen 1987b; Anttonen et al. 1996). While thylakoid alterations may be observed in acute ozone exposures (Anttonen et al. 1996), increased electron density and increase in ribosome-like granules in the stroma, together with a simultaneous decrease in chloroplast size, are characteristic responses to chronic ozone exposure (Sutinen et al. 1990; Anttonen and Kärenlampi 1996; Utriainen and Holopainen 2001a). In this thesis, these alterations in the stroma were seen in Scots pine and Norway spruce needles in the field (IV, VI).

The exact mechanism behind these changes is not known. However, the changes cannot be due to a direct effect of ozone or its first reaction products, as these are formed in the apoplast (Long and Naidu 2002). The changes in the stroma are shown to be developed into severe cellular damage and eventually into chlorotic mottle when the exposure continues (Sutinen et al. 1990). However, the darkening of the stroma can be a reversible phenomenon in young conifers at low ozone exposure, but recovery does not take place when a certain ozone concentrations or dose is exceeded. This statement is supported by the findings of Anttonen and Kärenlampi (1996) which reported that the darkening of the stroma in ozone-exposed Scots pine seedlings disappeared from summer to winter. Furthermore, Sutinen (1986a) and Sutinen et al. (1990) exposed Scots pine and Norway spruce saplings with slightly higher ozone concentrations than Anttonen and Kärenlampi (1996), and did not note any recovery in needles collected up to 24 weeks after the end of the ozone fumigations (Sutinen 1986a) or at any time during the three-year-lasting study (Sutinen et al. 1990).

Several explanations including changes in the activity, quantity and quality of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Anttonen and Kärenlampi 1996; Holopainen et al. 1996; Utriainen and Holopainen 2001a), as well as involvement of other stromal enzymes (Anttonen and Kärenlampi 1996) have been proposed for the microscopic ozone-induced alterations in the stroma. An ozone-induced perturbation in the envelope membranes (seen as indistinctness by TEM) was suggested to be due to changes in the membrane integrity (Anttonen and Kärenlampi 1996; Holopainen et al. 1996). Altered membrane integrity was then proposed to affect the osmotic status of the chloroplasts resulting in shrinkage and darkening of the stroma (Anttonen and Kärenlampi 1996). Condensation of organelles, indeed, can take place as a result of osmotic changes even during normal metabolic activities of plant cells (Gamalei et al. 2000). The decreased dimension of the chloroplasts cannot, however, be the sole

reason for the stroma darkening, as it has been reported several times without changes in the chloroplast cross-section area (Kainulainen et al. 2000; Utriainen et al. 2000; Utriainen and Holopainen 2001a). In the present work (II), significant changes in the chloroplast area were not found although daily changes in the osmotic properties of the needles, especially in those from the drought treatments, most likely occurred.

The ozone-induced granules in the stroma have been designated as "ribosome-like" (Sutinen 1987b) because the granules have similar size and appearance to the chloroplast ribosomes (cf. Jacobson et al. 1963; Thomson 1974). Increased amount of such granules were noted in Norway spruce needles in the field (VI). Their diameter was 17-20 nm, and their appearance and distribution was similar as found in ozone-exposed Norway spruce and Scots pine saplings (Sutinen 1987b; Sutinen et al. 1990; Holopainen et al. 1996). Thus, based on size, the ozone-induced ribosome-like granules may be ribosomes. Increased amount of granules in ozone-exposure could be indicative of activated repair procedures in the chloroplasts as suggested earlier by Holopainen et al. (1996).

Ozone has been reported to decrease (e.g. Sutinen et al. 1990), increase (e.g. McQuattie and Schier 1993) or have no effects (e.g. Utriainen and Holopainen 2001a) on the size of the starch grains in conifer needles. Here (I), a transient increase in the size of the starch grains was noted in the ozone-exposed spruce saplings. McQuattie and Schier (1993) and Wellburn and Wellburn (1994) noted that the accumulation of starch in the needles of conifer seedlings exposed to relatively high ozone concentrations was associated with the collapse of the phloem, probably causing problems with the phloem loading processes (Wellburn and Wellburn 1994). In the present study (I), all the tissues of the vascular cylinder were unaffected by the moderately elevated ozone. Therefore, the transient ozone-induced increase in the size of the starch grains (I) may be similar to the ozone-induced stimulation of photosynthesis and chlorophyll concentrations (e.g. Wallin et al. 1990; Senser et al. 1990; Beyers et al. 1992). Possibly, the slightly larger chloroplasts in the needles from the well-watered, ozone-exposed spruce saplings could also be due to such stimulation (I). Usually, the stimulative effect of ozone has been noted in the C needles, but here the effects on size of the starch grains and chloroplasts (I) as well as on chlorophyll concentrations (Wallin et al. 2002) were noted also in the C+1 needles, while the chlorophyll concentrations in the C+2 and C+3 needles were significantly decreased by ozone. The nutrient concentrations of the needles indicated luxurious nutrient availability for the spruces (I, Wallin et al. 2002), which might have prolonged the stimulative effect of ozone (cf. Rantanen et al. 1994).

4.1.2 Other cell organelles

Most ultrastructural studies have been concentrated on chloroplasts due to their sensitivity to ozone and to the ozone-induced decrease in photosynthesis. Peroxisomes and mitochondria retain their integrity longer than chloroplasts in ozone-exposure (Sutinen 1987b, Sutinen et al. 1990). The results of the OTC studies (I, II) show that changes in the number, size and shape of peroxisomes and mitochondria occur without any typical chloroplast alterations in the same cells.

Ozone has been shown to proliferate peroxisomes, in Norway spruce needles (Morré et al. 1990). Here, ozone increased the number of peroxisomes in the sky-facing side of the needles over that of the ground-facing side (I). Proliferation of peroxisomes is stated to be indicative of a common mechanism for dealing with oxidative stress (Lopez-Huertas et al. 2000). Peroxisomes contain several non-enzymatic and enzymatic antioxidants (Corpas et al. 2001). Morré et al. (1990) reported that proliferation of peroxisomes in ozone-exposed Norway spruce needles was accompanied by an increase in particulate catalase. Furthermore, peroxisomes are also a source for signal molecules, AOS and nitric oxide, which are involved in many physiological processes, including stress responses (Corpas et al. 2001). Increased number of the peroxisomes in the sky-facing side over that of the ground-facing side of the needles of ozone-exposed saplings was accompanied by a decrease in size, although the decrease in the peroxisome size was only seen in the current year needles (I).

Mitochondria divide and fuse continually and can thus rapidly respond to the demand of the environment (van der Bliek 2000). Furthermore, mitochondrial polymorphism, motility and, putatively, shape are connected to the energy status of the organelles, as discussed by Logan and Leaver (2000). Thus, the observed decrease in the mitochondrion size and increase in number (I) as well as the diurnal change from circular to tubular (II) in ozone-exposed spruce needles may indicate that ozone has affected energy status of the organelles. Ozone has been shown to decrease the activity of Rubisco, to increase that of phosphoenolpyruvate carboxylase (PEPc) and the pathways of carbohydrate breakdown (Dizengremel 2001). These reactions lead to increased mitochondrial NAD malic enzyme and NADH concentrations that can be used for maintenance respiration (Dizengremel 2001). Increased respiration is a common response in the current year needles of conifers exposed to ozone (Barnes et al. 1990; Wallin et al. 1990, Kellomäki and Wang 1998), and was shown to be due to maintenance respiration by Kellomäki and Wang (1998). Wallin et al. (1990) reported that ozone increased respiration in the current, but not in the older needles of Norway spruce. Correspondingly, at the structural level here (I), the increased number of mitochondria was seen only in the current year needles. This increase may be a reflection of an ozone-induced increase in maintenance respiration in the youngest needles. In addition, the decreased size of mitochondria in the ozone-exposed spruces (I) may be connected to the changed respiration, since ADP availability regulates the respiratory activity of the mitochondria (Buchanan et al. 2000) and high ADP concentrations shrink the mitochondria (Bereiter-Hahn and Vöth 1983).

Vacuoles are multifunctional compartments involved e.g. in cellular responses to environmental stress (Marty 1999). Two types of vacuoles, central vacuole and cytoplasmic vacuoles, as seen here (I, II), are common in plant cells, and they may have distinct functions (e.g. Fleurat-Lessard et al. 1997; Swanson et al. 1998). In the present study (I), ozone increased the area of central vacuoles of the previous year needles compared to control treatment and the current year needles at the end of the growing season. The cytoplasmic vacuoles, which were about the size of the chloroplasts and encircled the

nucleus, were more frequent in ozone treated needles compared to the controls in the summer, but did not increase towards the end of the season as in the controls. Vacuoles are involved in the change of the osmotic properties of the cells (Marty 1999), which take place e.g. during winter hardening processes (Öquist et al. 2001). Moreover, the number of small vacuoles has been reported to increase during the induction of winter tolerance in *Bromus inermis* (Tanino et al. 1991). Changes in solute transport and osmoregulation mechanisms were suggested to be the reason for ozone-induced alterations in the timing of hardening in loblolly pine (Edwards et al. 1990). Therefore, it can be suggested that the vacuolar changes in our study (I), at least partly, reflect the osmotic properties of the cells and may indicate that ozone has interfered with the winter hardening processes.

4.1.3 Location of cells within the mesophyll tissue

Sutinen et al. (1990) described that ozone-induced chloroplast alterations, and the subsequent more severe damage, were always first seen in the outermost mesophyll cell layer facing the sky. With increasing ozone dose, the symptoms advanced gradually from the outer to inner cell layers, which has also been noted in other studies and conifer species (Evans and Miller 1972b; Sutinen 1987b; McQuattie and Schier 1993). The symptoms were last or not at all noted in the mesophyll cells under the endodermis in the ground-facing side of the needle (Sutinen et al. 1990). The same pattern of changes in the chloroplast length and electron density of the stroma was seen in the field (VI). In addition, other organelles showed differences between outer and inner cell layers (III) and between sky-facing and ground-facing side of the needle (I).

One reason for the observed differences between the outer and inner cell layers may be the diffusion dynamics of the gases (Fink 1988). However, the proximity of the cell to the stomata does not always explain the cellular injuries. For example, the cellular damage can be seen in the palisade tissues in upper side of the tree leaves, even if the stomata are in the lower side of the leaf (Fink 1988). As stomata are located on all four sides of the spruce needles, their distribution cannot directly explain why the sky-facing side of spruce needles is affected before the ground-facing side. This may be explained by the wider intercellular space of the mesophyll tissue in the upper, sky-facing side of the needle, compared to the lower, ground-facing side (I). This, in turn, might indicate a more effective diffusion of gases in the sky-facing side, and be one reason for more pronounced ozone impact in the sky-facing side. The role of the intercellular space in ozone responses has also been discussed e.g. by Evans et al. (1996).

Another obvious reason for more intensive responses in the outer cell layers compared to inner cell layers, and in the sky-facing compared to ground-facing side, is the light environment in the needle tissue. Inside the needles of *Abies lasiocarpa* and *Picea engelmannii*, visible light attenuates steeply from the outer mesophyll cell layers to the vascular cylinder from the direction of light source (DeLucia et al. 1992). Light as such can induce oxidative stress at the cellular level in plant foliage (Karpinski et al. 2001). Negative effects of ozone on photosynthesis

in Norway spruce have been shown to be more pronounced in the high light (Wallin et al. 1992; Mikkelsen and Ro-Poulsen 1994). On the other hand, high light is also known to increase stomatal conductance, and thus the ozone uptake, in Norway spruce, at crown level (Wieser et al. 2000).

As both light and ozone can induce oxidative stress at the cellular level, it could be assumed that the early occurrence of chloroplast alterations in the sky-facing side of the needle and in the outer mesophyll cell layers is due to the combined stress of ozone and light. High light alone has not been shown to induce ultrastructural changes similar to ozone (Fink 1999). The assumption of combined oxidative stress effects of ozone and light is supported by the increased number of peroxisomes in the sky-facing side of the needle of Norway spruce (I, see above) and by the fact that ozone-induced visible symptoms is seen in the sky-facing side of the foliage (VanderHeyden et al. 2001). The higher number of peroxisomes, mitochondria, chloroplast plastoglobuli, and the decreased size of chloroplasts and starch grains in the outer mesophyll cell layers and sky-facing side of the needles all reflect that these mesophyll areas are in general more exposed to stress compared to the inner cell layers and ground-facing side (I, III).

4.1.4 Ozone, senescence and aging

Ozone-induced changes in the chloroplasts of conifers needles, decrease in the size and darkening of the chloroplast, are quite similar to that noted in ozone-exposed leaves of angiosperms. In angiosperms, these symptoms are also induced by senescence and thus have been regarded to be indicative of ozone-induced accelerated senescence (Ojanperä et al. 1992; Pääkkönen et al. 1995, Günthardt-Goerg et al. 2000). Other typical changes of ozone-exposure and senescence in angiosperms are increased number and size of the plastoglobuli and decreased amount of the grana thylakoids (Ojanperä et al. 1992; Pääkkönen et al. 1995; Mikkelsen and Heide-Jørgensen 1996). None of these changes were found in the chloroplasts of the oldest green needle generation of Scots pine during a week's sampling period before the autumnal yellowing or during the phase of yellowing (III). Instead, the chloroplasts, as well as the whole mesophyll tissue, of the oldest needles remained intact as long as the needles were green. The chloroplasts in the whole mesophyll tissue from the yellow needles were extremely small and pale, because of the loss of the stroma. The cytoplasm was healthy-looking in most of the mesophyll cells. Last phase in obviously dead cells were characterized by disintegrated cytoplasm where extremely small empty-looking chloroplasts with some remnants of thylakoids and possibly of plastoglobuli could be seen. In contrast, the late stage of ozone damage is characterized by disintegrated cytoplasm and small chloroplasts with electron dense stroma rich in ribosome-like granules (Sutinen et al. 1990, Holopainen et al. 1996). Thus, the changes seen in the chloroplasts from the senescing needles were different from those found in the early and late stages of ozone exposure. In addition, contrary to the slow gradual advance of the ozone injury (Sutinen 1987b; Sutinen et al. 1990), the senescence-induced changes occurred rapidly and simultaneously in all parts of the mesophyll tissue (III). Chloroplasts of the three oldest needle generations had more

plastoglobuli than the youngest one (III). This was the only needle age –related change in the chloroplasts of Scots pine (III). Thus, the ozone-related symptoms can neither be regarded as signs of accelerated senescence nor as signs of accelerated aging in Scots pine.

The needles of Norway spruce do not show visible senescence similar to Scots pine. In concert with that, even the oldest studied green spruce needles, up to nine years (Sutinen 1987a, Ruetze and Schmitt 1988; Wulff et al. 1996a), do not show drastic cellular changes in the mesophyll or in other tissue types and do not share similarities with ozone-induced symptoms. Thus, similarly as in pine needles, the well-characterized symptoms of ozone stress in the chloroplasts cannot be regarded as signs of the accelerated senescence or aging in spruce needles.

4.2 Interaction of ozone and abiotic factors

4.2.1 Drought

Combined effects of drought and ozone on trees depend e.g. on studied parameters, experimental conditions, plant species and genotype (cf. chapters 1.4.1. and 1.6.). In the present study (I, II) the severity and timing of the ozone and drought exposures were realistic for conditions of southern Sweden (Karlsson et al. 2000, 2002; Wallin et al. 2002). The ozone-protective role of drought was observed in Norway spruce in the previous years of the OTC study (I, II): after two seasons, ozone had decreased the stem biomass of saplings, but to a lesser extent in the drought-treated saplings than in the well-watered ones (Karlsson et al. 1995). After four seasons, the protective role of drought could not be seen any more (Karlsson et al. 2002; Wallin et al. 2002). Rather, the stresses seemed to function independently, although the effect on the stem length was synergistic (Karlsson et al. 2002; Wallin et al. 2002). The findings at the cellular level (I, II) support these results (Karlsson et al. 2002; Wallin et al. 2002): no ozone-protective role of drought could be noted after prolonged exposure and few interactions were found. Drought alone increased the amount of starch at the end of the experiment but it was not seen when ozone and drought were combined (I). This interaction at the end of the season resembles that found for gas exchange in Aleppo pine (Inclán et al. 1998) and may indicate that ozone impaired or delayed the recovery from drought. The NF+/D treatment increased the cytoplasmic vacuoles that were encircling nucleus in the C needles and decreased them in the C+1 needles, which may indicate alterations in osmotic properties of the cells (I). Based on this study, ozone and drought act mostly independently at the cell structural level in Norway spruce needles. The effects of drought alone on the structure of the needles in relation to function are discussed in the papers I and II.

4.2.2 Ozone, nutrients and winter hardening

Nitrogen availability can modify the ozone response in conifers, and plants in general, but the plant responses in combined exposures are variable (e.g. Tjoelker and Luxmoore 1991; Pääkkönen and Holopainen 1995; Landolt et al. 1997; Utriainen and Holopainen 2001a, Mills 2002). Imbalance of other nutrients (Mg in Edwards et al. 1990; Mg and Ca in Rantanen et al. 1994; K in Barnes et al. 1995; P in Utriainen and Holo-

painen 2001b) have not been shown to drastically affect the ozone responses of conifer seedlings despite that field observations have suggested the opposite. For example, Mg deficiency in combination with ozone and light was the most likely cause for the biochemical changes related to the oxidative stress and the yellowing of *Picea abies* foliage in mountain areas in Germany (Schmieden and Wild 1994; Siefermann-Harms 1996). The discrepancy between field investigations and controlled exposure studies may be due to differences in environmental conditions, and responses or response rates to low nutrient status of the soil between seedlings and mature trees. The negative correlations between the nutrient concentrations and proportion of the mesophyll cells with ozone-symptoms (IV) or the ozone syndrome index (VI) also suggests that effects of ozone at the forest level can be more enhanced at sites with low nutrient availability than at sites with sufficient nutrient availability. These cellular responses are supported by the findings from Kainulainen et al. (2000) and Utriainen and Holopainen (2001a) who reported that the electron density of the stroma was more enhanced in ozone-exposed Scots pine and Norway spruce needles with low and deficient N concentrations compared to those with a good nutrient status. Furthermore, Utriainen and Holopainen (2002) reported increased electron density of the chloroplast stroma with the same species in the combined treatment with ozone and phosphorus deficiency. Ozone itself, on the other hand, can affect the nutrient concentrations of the conifer needles (e.g. Lucas et al. 1993) e.g. by inducing reallocation to current year needles and nutrient leaching. However, this effect is expected to be minor at low ozone concentrations (Rantanen et al. 1994), such as in the field studies here (IV, VI).

The nitrogen concentration of the needles in the OTC studies here (I, II) was luxurious compared to that of the field-grown conifers in southern Sweden (Thelin et al. 1998; V) and over the optimal value for the Norway spruce (Linder 1995). Good nitrogen availability may be the reason why typical ozone-induced chloroplast symptoms were not seen in the OTC study (I, II), although the symptoms have been described in needles of the same age (C and C+1) from young conifers exposed to ozone in closed (Holopainen et al. 1996) or open-top chambers (Sutinen et al. 1990) or in open-field conditions (Anttonen and Kärenlampi 1996) with similar or lower ozone concentrations and doses than here (I, II). Good N status may have induced the effective defence in the needles (seen as increased numbers of peroxisomes and mitochondria), and/or prolonged the stimulative phase of the stress (cf. Rantanen et al. 1994).

Both ozone (Lucas et al. 1988; Fincher et al. 1989; Edwards et al. 1990; Anttonen and Kärenlampi, 1996) and imbalance of nutrients (Klein et al. 1989; Rikala and Repo 1997; Jokela et al. 1998) have been shown to negatively affect winter hardiness or frost sensitivity of conifer seedlings. These studies are supported by the findings from field-grown mature spruces here (V, 3.9.2). Relatively low nutrient concentrations were reflected in a less advanced hardiness status of the needles and higher frost sensitivity of the bark (V). Regression analysis after PCA (3.9.2) revealed that both ozone and low nutrient concentration together reduced the winter hardiness of the needles and bark. The

latter result should, however, be ascertained at several field sites with different ozone regimes and soil fertilities.

4.3 Microscopic structure of needles in ozone diagnostics

Ecological indicators are used at various levels of ecological hierarchy, from genes to species and ultimately to e.g. the regional scale (Dale and Beyeler 2001). Changes in the cell structures of conifer needles belong to the lower level of this hierarchy. Ecological indicators can be used 1) to assess the condition of the environment, 2) to provide an early warning signal of changes in the environment, or 3) to diagnose the cause of an environmental problem (Dale and Beyeler 2001). Microscopic changes in the cell structures of conifer needles can be analogously used for all these three roles of ecological indicators. 1) Certain microscopic structures, like increase of chloroplast plastoglobuli, large cytoplasmic lipid aggregations and swelling of thylakoids are induced by several stresses and can be regarded as general stress symptoms indicating stress and/or poor needle condition (Holopainen et al. 1992; Sutinen and Koivisto 1995, I, II), 2) Effects of many air pollutants and natural stress factors (e.g. nutrient imbalance) can be seen in the cell structures far before any visible symptoms in needles can be seen (e.g. Sutinen et al. 1990; Palomäki 1995), 3) Several stress factors induce typical symptoms in the cell structures and these have been used to diagnose effects of certain air pollutants, nutrient deficiencies etc. (Palomäki and Raitio 1995; IV, VI). It is noteworthy, that all this information can be obtained from the same samples prepared for microscopy. For example, effects of several air pollutants were identified from the same samples in the study IV, here. Furthermore, other factors, such as seasonal changes can be followed from the same samples (I, V).

The findings from the present studies bring out new background information for diagnosis of the ozone impact on conifers in the field. Important findings regarding ozone diagnostics was that the chloroplast area of spruce needles did not show significant diurnal variation (II), that new information about the variation in chloroplast size within a needle was obtained (I, III) and that drought overall did not affect the ozone responses (I, II). Results showing effects of drought (I, II) and senescence (III) on cell structure, as well as those revealing the diurnal (II) and seasonal (I, III, V) variations, provide important background information for any field study. The studies also confirm many earlier findings about the structural changes in the needles during winter hardening period (III, V) and during aging (III). The effects above, as well as the unspecific changes observed in ozone exposure, non-osmiophilic staining of plastoglobuli and accumulation of cytoplasmic lipid aggregates (I) (Holopainen et al. 1992; Sutinen and Koivisto 1995), are discussed in relation to function of the needles in the original papers (I-VI).

4.3.1 Methodological implications

The present study supports the earlier field observations (Sutinen 1986b, Sutinen 1987c; Sutinen 1989; Sutinen 1990) that specific symptoms of ozone stress, decreased size of chloroplasts, accompanied with an electron dense stroma and ribosome-like granules, being more abundant in the outer than inner

mesophyll cell layers and in the sky-facing side than ground-facing side of the needle, are present in the mature conifers in the field in southern Fennoscandia (IV, VI). Thus the method has great potential to be used in ozone monitoring in the field. Before that the sampling, analysis of needles, criterion of the ozone impact etc. should be standardized. High variation or confusing results between the different studies will be obtained, if the samples are collected and analysed in different ways. These aspects were highlighted in some papers of the present work (II, VI). The next chapters discuss the important aspects of sampling, sample preparation and analysis. An attempt to standardize the methodology for ozone diagnostics for the conifers is also presented. The discussion is based on the earlier published literature and the new observations from this study (I-VI).

4.3.1.1 Season

Samples for ozone diagnostic should be collected during the dormant season. The first reason is that starch grains, which affect the chloroplast size (I, III, Holopainen et al. 1996; Pritchard et al. 1997), are absent (Soikkeli 1980; Linder 1995). Second, the sample preparation of conifer needles is more successful at that time compared to the growing season, possibly due to the low metabolic activity during the dormant season. This was mentioned by Kärenlampi (1986) and was seen between the studies from this work, although not reported in the papers (I-VI). Good preservation of the samples is essential for evaluation of ozone effects. For example, if the cytoplasm is very dense due to suboptimal fixation processes (II), chloroplasts may be difficult to discern by light microscope. In addition, due to differences in osmotic properties between the tissue and fixative, there is a risk that also the stroma is stained electron dense (Sutinen and Koivisto 1995). Furthermore, diurnal changes in cell structures should be smaller during the dormant season compared to active season (II). A third reason to collect the samples during the dormant period is the importance of simultaneous nutrient concentration analysis, because nutrients may modify ozone response at the cellular level (Landolt et al. 1997; Utriainen and Holopainen 2001b; I, IV, VI). According to the Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests (Stefan et al. 2000) samples for nutrient analysis of the needles must be taken during the dormant season. If needles have to be collected in the active season, and if the fixative is the same as generally used for "day-time sampling", needles should not be collected during the morning hours due to the increased risk of fixation artefacts (II).

4.3.1.2 Sampling

The light conditions affect the stomatal conductance, ozone uptake (Wieser et al. 2000) and the ozone responses of the trees (Wallin et al. 1992; Mills 2002). Furthermore, the ozone concentration can differ between the upper and lower canopy (Samuelson and Kelly 2001). In addition, typical ozone-induced changes in chloroplasts are more pronounced in the upper than lower canopy in the field (Sutinen 1990). Therefore, the needles in the present studies (IV, V, VI) were collected from the same height from the

upper canopy of the conifers, where light conditions are similar, and the effect of ozone effects probably highest. This approach can be recommended for common use. Similar light conditions are also important for sampling for nutrient analyses (e.g. Walker 1991). The Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests (Stefan et al. 2000), where sampling for nutrients is standardized, can be used as a guideline for the sampling for microscopy. There, for example, is pointed out that the collected branches should be from the same side of the tree, i.e. from the same point of the compass. Even if the basic structure of the needles does not differ between young and mature trees (Wulff et al. 1996a), ultrastructural differences, pointing out to the different metabolic activity, can be observed (III). It may, therefore, be important to collect the samples from the trees of approximate same age. To ensure similar light conditions for all collected needles they should be taken from the upper side of shoot. This is especially important for tree species, such as Norway spruce, where a clear difference between the sky- and ground-facing sides of the needles in the response to ozone can then be seen (I).

Visible symptoms of ozone injury are more intensive in the older than in the younger needles and leaves, which is a fact that has been taken into consideration for ozone diagnosis in the field (Submanual for the assessment of ozone injury on European Forest Ecosystems 2003). Due to the accumulative effects of ozone, older needles are also expected to have more severe cellular injuries than younger needles (Sutinen et al. 1990), which were also seen here (VI). Therefore, both current and older needle generations should be collected, if available. The needles should be detached carefully from the base of the needle to avoid mechanical damage to the tissues. The collected needles should be directly placed into fixative to ensure preservation of the cell structures.

The sufficient number of trees per study site and needles per needle generation per tree is not known, and should be studied in the future for standardisation. So far, the number of samples per study site has been based on the economical and time resources.

4.3.1.3 Sample preparation

The composition of used fixative and sample preparation may need to be standardized for the diagnosis of ozone impact. The influences of different fixative components and sample preparation on qualitative characteristics, including artifacts, are known to some extent (Holopainen et al. 1992; Sutinen and Koivisto 1995; Maunsbach and Afzelius 1999). Instead, the effects on organelle dimensions, which usually shrink during chemical fixation (Hayat 1999), are not known, and that aspect should be studied in detail. In general, the comparison of organelle dimensions between different studies is difficult as organelle dimensions are measured by different techniques (e.g. LM vs. TEM, for explanation see VI) and from different number of organelles (e.g. cross-section area from the largest chloroplast in the cell vs. mean cross-section area of several chloroplasts in the cell). However, the studies with different methodologies for sampling and sample preparation show quite similar dimensions for the chloroplasts and mitochondria in the needles from same

conifer species, collected during the same time of season (data from this work and Sutinen et al. 1998 compared to e.g. Utriainen and Holopainen 2001a; b; 2002). This suggests that the changes in organelle dimensions due to sample preparation are small. Moreover, the chloroplasts, the organelles used in ozone diagnosis, do not necessarily shrink during chemical fixation, similar to other organelles (Hayat 1989). Thus, it is unlikely that sample preparation could affect chloroplast dimensions to the same extent as ozone.

How soon after sampling the further fixation must be done, depends on plant material. Based on experience, Norway spruce and Scots pine needles can safely be stored in a cold fixative up to five days (Sutinen pers.com.), but the samples must not freeze. Possible differences in the structure or stress sensitivity of the cells between the base and tip of the mature Norway spruce and Scots pine needles are not known. Alvarez et al. (1998) showed that cellular damage progressed from base to the tip in the needles of *Abies religiosa*. Therefore, it may be important that the samples for microscopy are cut from the same position of the needles. Furthermore, before polymerizing the embedded samples they must be orientated so that cross-section of a needle can be cut for microscopy.

The staining for LM depends on the desired information (see e.g. Günthardt-Goerg et al. 2000). In all studies of this thesis, double-staining with toluidine blue, which stains the general cell structures, and with p-phenylene diamine, which stains structures containing lipids, were used. By this method, e.g. chloroplast can be identified and their dimensions measured, if the thickness of the section is thin enough (1-2 μm). Also, differences in the electron-density of the stroma as well as lipid aggregates in the cytoplasm can be seen (II, IV, VI).

TEM sections should be cut from the same position of the same needle samples used for LM. Abaxial and adaxial sides in cross sections of the Scots pine needles are easy to determine from the shape of the needles. In Norway spruce, the needle sides can be identified from the embedded sample under the stereomicroscope, based on the location of the sclerenchyma cells next to phloem, always on abaxial side of the needle (VI). If necessary, the location of the sclerenchyma cells can be ensured from the cross-sections used for LM. If the spruce needles are collected from the sky-facing sides of the lateral branches, the abaxial side is the sky-facing side of the needle. If the side of a needle and location of a cell in the cross section are not known, confusing results or high variation will be obtained.

4.3.1.4 Analysis of samples

For reliable ozone diagnosis certain requirements for microscopic changes must be fulfilled. Small chloroplast size as such is not a sufficient criterion, as certain nutrient deficiencies, such as that of nitrogen (Palomäki and Holopainen 1995) can have similar effect. Electron dense stroma alone is neither a sufficient criterion, as it can be induced by drought (Palomäki et al. 1994). Therefore, both symptoms must be seen at the same time. To ensure that both symptoms, when seen together, are due to ozone, one should see that the symptoms are more abundant in the outer mesophyll cell layers than in the inner ones, which is typical to air pollutants

(Fink 1988) and that the symptoms are more abundant in the sky-facing than ground-facing sides (Sutinen et al. 1990) for those conifers with easily determined needle sides in relation to the sky and ground. I recommend that all these changes must be seen for sound ozone diagnostics in conifers.

All above-mentioned characteristics of ozone specific symptoms can be studied by LM with high magnifications (IV, VI). TEM also shows these differences (Sutinen 1987b; Sutinen et al. 1990; VI), except that whole needle cross-section cannot be studied from TEM section. When reported, TEM cross-sections consist maximally half of the needle cross-section (Sutinen 1987b). Sample preparation, as well as the use of the LM is generally cheaper than that of the TEM. LM is also a more easily available microscope than TEM. Furthermore, the modern digital techniques allow considerable magnifications of digital images increasing the study applications of LM (cf. Figs 1-3 and 5 in III). For all these reasons, LM techniques are encouraged to be used in ozone diagnosis. However, TEM is recommended to ascertain the LM results concerning electron density of the stroma at least from some of the samples.

The most problematic issue in ozone diagnostics in the field is to determine the normal length of the chloroplasts (i.e. how small the chloroplasts should be). Chloroplast lengths or areas can be compared to the values reported in the literature for the same species. In the study IV (not mentioned in the paper), chloroplasts from Scots pine needles were regarded as small, when they were approximately or less than 4 μm long, i.e. shorter than in the ozone-exposed Scots pine seedlings (Anttonen and Kärenlampi 1996). The longest chloroplasts were 7 μm long (IV), i.e. longer than in the ambient air control (Anttonen and Kärenlampi 1996). However, the chloroplast dimensions from the control or ozone fumigations with seedlings are not directly comparable to those from the mature conifers in the field, due to e.g. tree age (III), different nutrient availability (Palomäki and Holopainen 1995) and used microscopic technique (VI).

The problem of chloroplast size was solved in the present study (VI) by comparing a considered chloroplast length to the healthiest part of the needles at the same height, from the same tree, i.e. chloroplasts from mesophyll cells under the endodermis in the ground-facing side of the current-year needle, where ozone effects occur last (Sutinen et al. 1990). In such an approach the difference in chloroplast size must be larger than the within needle variation. Sutinen et al. (1990) reported 0.4 μm difference in length between the upper and lower sides in the mesophyll cells of the spruce needles collected from the control fumigations. In the control fumigations of the present study (I, Table 3), the difference was 0.3 μm in length and 0.8 μm^2 in area (starch excluded). Differences of 0.5 μm and 0.8 μm^2 (starch excluded), was seen between outer and inner mesophyll cell layers from pine needles from low-polluted site (III, Table 3). The significant differences in chloroplast length between ozone-treated and controls have been approximately 1 μm (Sutinen et al. 1990; Anttonen and Kärenlampi 1996). Thus, I suggest that in the approach where chloroplast length is compared to those located in the healthiest part of the same tree, i.e. youngest needles at the same height, the difference in the chloroplast length must be 1 μm or larger.

If samples are collected during an active phase of winter hardening, as in (VI), the shape of the chloroplast must be considered in the analysis. For example, if length is measured both from lens-shaped (not winter hardened) and rounded (typical to winter hardened spruce needles, V), confusing results or high variation may be obtained.

The person(s) who will be responsible for analysis of the samples must be thoroughly trained, as for any other method. The modern digital technique connected to the light microscopy makes the training easy and allows the training of several persons simultaneously, if necessary.

4.3.1.5 Quantification of data

To allow statistical comparisons between forest areas or in relation to other quantitative parameters, microscopic findings should be quantified. Measurement of chloroplast length or area from TEM pictures is a commonly used quantification method in the experimental studies (e.g. Anttonen and Kärenlampi 1996; Utriainen and Holopainen 2000). This approach was also used here (VI). In addition, light microscopic data was expressed as proportion of mesophyll cells with ozone-related symptoms (i.e. dark, under 4 μm long chloroplasts) for pine (IV) and ozone syndrome index for spruce (VI). The benefit of the approach in the study IV is that the percentage is easier to understand than the index in the study VI. The benefit of VI, instead, is that the analysis was much faster to do and that the chloroplast changes at the tissue level are included in the index, which means that all criterions for ozone diagnosis are included in the index. In addition, as the tree itself acted as its own control, the effects of possible nutrient deficiency on chloroplast size are minimized, and the variation between the trees is reduced. Ozone syndrome index showed significant differences between forest sites (VI), which were not detected by TEM. This suggests that the ozone syndrome index, and LM, is more sensitive method compared to the commonly used TEM measurements in ozone diagnostics in the field (VI).

4.3.2 Magnitude of symptoms of ozone stress in the field

Even if the mechanism behind the microscopic ozone syndrome is not fully understood, it has been shown to develop into severe cellular damage and eventually into visible injury (chlorotic mottle) (Sutinen et al. 1990). These chloroplast alterations were accompanied by decrease in the photosynthesis (Wallin et al. 1990). Karlsson et al. (2003) reported that ozone concentrations and prevailing daylight AOT40 had significant negative effects on the increment of the stem basal area of mature Norway spruce in the field. The study was done during the years 1993-1999 at the same forest area at Asa, where the ozone syndrome in the needles was seen (VI) in 1999. Thus the studies (IV, VI) indicate that the ambient ozone concentrations can have negative effects on mature conifers in southern Fennoscandia. The study VI also showed that the syndrome can be seen with ozone dose lower than the present critical dose of AOT40 (10 000 ppb.h). Also other methods and tree species e.g. *Betula pendula* (Pääkkönen et al. 1997) and *Pinus radiata* (Calzada et al. 2001) have exhibited effects of ozone on the foliage far below the current critical dose for AOT40. No ozone-induced symptoms were seen in the structure

of pine needles at Suonenjoki, Central Finland (III). This is most likely due to that samples were collected at a low height and from lower parts of the canopy (cf. Sutinen 1990; Wieser et al. 2000; Samuelson and Kelly 2001) from the area with relatively low ozone concentrations. Some unidentified modifying factors, such as nutrients, may have also affected.

The presented studies (IV, VI) are the first where microscopic symptoms of ozone stress have been quantified in the field from mature conifers. Even though the microscopic findings here (IV, VI) are qualitatively similar compared to the ozone-exposed seedlings (Sutinen 1987b, Sutinen et al. 1990; Anttonen and Kärenlampi 1996, Holopainen et al. 1996) the analyses have not been done in exactly the same way. Thus, the severity of the ozone stress in the mature conifers in the field (IV, VI) compared to the seedling from the exposure studies cannot yet be evaluated. The analysis done by Sutinen et al. (1990) is closest to the one used here for Norway spruce (VI). By comparing the concentrations, and severity of the chloroplast changes at tissue level, it appears that the symptoms of ozone stress are more pronounced in the mature conifers than in young conifers. In addition, it seems that the symptoms of ozone stress in mature Scots pine (IV) in the field are more severe than in the seedlings from the ozone-exposure (Anttonen and Kärenlampi 1996), where ozone concentrations were considerably higher than in the field (IV). It is not known, if these differences in the severity of the microscopic alterations between the seedlings and mature conifers, are due to the dissimilarities in the stress tolerance of differently aged trees, or for example, due to different nutrient availability, climatic conditions or length of the growing period.

4.3.3 Future research prospects

Ozone-induced visible injuries on forest species in Europe are studied under the ICP-forest program. The microscopy is recommended to be used for species and areas where ozone-induced visible injuries can rarely be seen, or when the visible symptoms need to be validated (Submanual for the assessment of ozone injury on European Forest Ecosystems, 2003). The presented quantitative microscopic methods could be used to diagnose the impact of ozone on mature conifers in the framework of the ICP-forests.

Knowledge about the dose-response relationship of ozone and the structural changes in needles of young conifers is scarce and nothing is known about the ozone dose-response relationship in mature conifers. These aspects should be investigated. The possible recovery of ozone-induced chloroplast symptoms should be studied more, i.e. if it occurs below a certain dose or accumulated flux, and if the recovery depends on environmental conditions or on the species. In general, the mechanism behind the chloroplast changes is also one of the challenges for future research. Moreover, the combined effects of ozone and nutrients and their relation to climatic factors in mature conifers need more attention.

5 CONCLUSIONS

This work shows that forests in southern Fennoscandia are affected by ozone, and microscopy can be used for ozone diagnostics in the field. Typical ozone-induced syndrome (decreased size and simultaneous appearance of an electron dense stroma, showing gradual advance from the outer to inner cell layers, and being more abundant in the sky-facing than in the ground-facing side of the needles) described earlier for ozone-exposed seedlings were present in the mature, field-grown, Scots pine and Norway spruce. The syndrome was expressed quantitatively, which allowed the comparisons to other parameters and between forest sites.

For sound ozone diagnostics in conifers, all features of the ozone-related chloroplast symptoms must be seen at the same time, i.e. chloroplasts have electron dense stroma, they are small, and these changes are more abundant in the outer than inner cell layers. Needles for ozone diagnostics are recommended to be collected during the dormant season, from similar light conditions from top of the crowns and both from the youngest and older needle generations. Furthermore, simultaneous sampling for nutrient analysis is advisable.

The study showed that it is important to know the location of the examined cells in the tissue. Several differences were noted in numbers, sizes and metabolic products of organelles between the sky-facing and ground-facing sides of the needles and between outer and inner cell layers of the mesophyll tissue. In addition, the effect of sampling time during a day turned out to be important. Shape, volume, location, and the amount of storage products of the cell organelles of Norway spruce needles changed during a day, and the diurnal changes were modified by ozone and drought. Moreover, preservation of the cellular structures was not optimal during early morning. However, the cross-section area of chloroplast, the organelle used in ozone diagnostics, did not show diurnal changes.

The results from the study give new information about the mode of action of ozone stress at the cell structural level. Ozone-related changes in the cell and tissue structure are not signs of accelerated senescence or aging in Scots pine. The abundance of chloroplast symptoms in outer vs. inner cell layers and sky-facing vs. ground-facing side of the needles is suggested to be related to the larger intercellular space in the sky-facing side and thus, more effective diffusion of ozone there, and to the more stressful and energy-demanding cellular environment in the outer and sky-facing sides due to the higher exposure to light. The latter conclusion was drawn from the differences noted in the dimensions of organelles and their metabolic products within the cells. Moreover, the view that sky-facing side of the needle is more exposed to ozone-induced oxidative stress is supported by the finding that the number of peroxisomes was larger in the sky-facing than ground-facing side of the ozone-exposed spruce needles.

The results from OTC experiment refer to that moderately elevated ozone concentrations activate the defence reactions of the Norway spruce needles, and that the defence is more effective in the youngest needle generation. This was shown by the increase in the numbers of mitochondria in the current year needles and peroxisomes in response to the ozone-induced oxidative stress,

while the chloroplasts, the most ozone-sensitive organelles, were almost completely unaffected.

The study gives new information and supports the earlier reported findings about the interaction of ozone and other abiotic factors. In the OTC experiment, drought did not modify the ozone responses; instead the ozone and drought stresses acted independently at the cellular level in Norway spruce. This finding is important also for ozone diagnostics. The earlier reported relation between low nitrogen status and increased intensity of ozone-induced chloroplast symptoms in the needles of seedlings was also seen in the mature conifers in the field. Ground-level ozone coincidently with low nutrient availability negatively affected winter hardiness of mature conifers. The OTC studies with seedlings suggest that the negative effect of ozone on winter hardening processes is partly mediated by changes in osmotic properties, which was seen as alterations in the seasonal changes in the size of the central vacuoles and the number of cytoplasmic vacuoles.

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