KUOPION YLIOPISTON JULKAISUJA C. LUONNONTIETEET JA YMPÄRISTÖTIETEET 251
KUOPIO UNIVERSITY PUBLICATIONS C. NATURAL AND ENVIRONMENTAL SCIENCES 251

ELINA HÄIKIÖ

Clonal Differences of Aspen (*Populus* spp.) in Responses to Elevated Ozone and Soil Nitrogen

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium L21, Snellmania building, University of Kuopio, on Friday 22nd May 2009, at 12 noon

Department of Environmental Science
University of Kuopio

KUOPION YLIOPISTO

KUOPIO 2009

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ISBN 978-951-27-1189-5
ISBN 978-951-27-1284-7 (PDF)
ISSN 1235-0486

Kopijyvä
Kuopio 2009
Finland

Häikiö, Elina. Clonal differences of aspen (*Populus* spp.) in responses to elevated ozone and soil nitrogen. Kuopio University Publications C. Natural and Environmental Sciences 251. 2009. 49 p.
ISBN 978-951-27-1189-5
ISBN 978-951-27-1284-7 (PDF)
ISSN 1235-0486

ABSTRACT

The global background tropospheric ozone concentrations have doubled since the pre-industrial era, and ozone is considered to be one of the most toxic air pollutants to plants. Ozone-induced reductions in the photosynthetic capacity and in the carbon assimilation of plants may lead to increased amounts of carbon dioxide in the atmosphere. *Populus* species have been reported to be among the most sensitive forest trees to elevated ozone, and in the Nordic and Baltic countries, European aspen (*Populus tremula*) and hybrid aspen (crossings of the native *P. tremula* and the American trembling aspen, *Populus tremuloides*) are used especially in the pulp and paper industry for the production of high quality paper, and for biofuel production. The clones in commercial production have been selected primarily based on high yield and desirable wood physicochemical properties, but it is also important to test the stress tolerance of the clones before they are used in large-scale plantations.

We studied the effects of moderately elevated ozone (1.5 x ambient concentration) on the growth, physiology and foliar chemistry of selected aspen and hybrid aspen clones in open-field experiments to find out if there are differences in the ozone sensitivity of the clones, and if the sensitivity is affected by soil nitrogen availability. Both ozone-sensitive and ozone-tolerant clones were found after two years' growth in elevated ozone based on growth responses to ozone. Differences in the foliar physiology and chemistry among the clones and between the ozone-sensitive and tolerant groups were also found. However, the reason for the significant growth reduction of the ozone-sensitive clones could not be determined by the differences in the leaf characteristics studied. Nitrogen amendment counteracted the adverse ozone effects by increasing the growth of all plant parts.

Ozone enters the leaf through stomata and is either detoxified in the apoplast or induces an active production of reactive oxygen species, such as hydrogen peroxide (H_2O_2). We found accumulation of H_2O_2 inside the plasma membrane in the cytoplasm and in the chloroplasts of an ozone-sensitive aspen, whereas in the tolerant clones, H_2O_2 was found only on the outer surface of cell walls, indicating efficient detoxification of ozone in the apoplast. *Populus* species are also rich in phenolic compounds, and we studied the potential role of leaf phenolics as antioxidants in ozone-induced oxidative stress. The ozone-tolerant clones had high concentrations of condensed tannins, which have good radical-scavenging properties, but which are also costly in high concentrations in terms of growth. The ozone-sensitive clones allocated carbon into salicylates, which have no antioxidative capacity but may instead protect the trees from herbivory.

When considering the stress tolerance of a species in a changing climate, the interactive effects of many factors have to be taken into account. High yield does not often correlate with stress tolerance, and we found both good growers and slow growers in both the ozone-sensitive and ozone-tolerant groups. The high intraspecific genetic variation in aspen suggests that natural aspen populations may adapt to changes in the environment through selection of tolerant genotypes but for large-scale clonal plantations the stress tolerance or responsiveness to fertilization should be assessed beforehand and weighed against the desirable wood properties or growth potential of the clones. In low deposition areas, such as Scandinavia, the atmospheric N deposition may not counteract the effects of ozone in native aspen stands, but adequate nitrogen fertilization of hybrid aspen plantations could compensate for the decrease in growth caused by elevated ozone.

Universal Decimal Classification: 504.5, 546.214, 581.1, 581.2, 581.54, 582.681.81, 632.151
CAB Thesaurus: climatic change; ozone; trees; *Populus*; clones; clonal variation; genetic variation; genotypes; hybrids; growth; yields; plant physiology; leaves; nitrogen; soil; hydrogen peroxide; plasma membranes; cytoplasm; chloroplasts; cell walls; detoxification; antioxidants; secondary metabolites; phenolic compounds; tannins; salicylates; stress factors; tolerance

ACKNOWLEDGEMENTS

This study was carried out at the Department of Environmental Science, University of Kuopio. The field work was mainly conducted in Kuopio, at the Ruohoniemi open-air ozone exposure field of the University of Kuopio Research Garden. Samples were also collected at the Aspen FACE site in Rhinelander, Wisconsin (USA). The laboratory work was conducted in Kuopio and at the facilities of University of Joensuu Faculty of Biosciences. I wish to acknowledge the support from these institutions. This work was mainly financed by the Academy of Finland and the University of Kuopio Environmental Risk Assessment Center (ERAC). In addition, I wish to acknowledge the financial support by North Savo Regional Fund of the Finnish Cultural Foundation, Jenny and Antti Wihuri Foundation, the Kuopio Naturalists' Society, Niemi Foundation, the Finnish Konkordia Union, the Finnish Society of Forest Science, and the University of Kuopio.

I wish to express my sincere thanks and gratitude to my two supervisors, Professor Elina Oksanen from the University of Joensuu and Professor Toini Holopainen from the University of Kuopio for their guidance, support, confidence and endless patience throughout this work. They pushed me gently but firmly forward, especially during the (many) writing process(es). Egbert Beuker from the Finnish Forest Research Institute is acknowledged for providing us with the aspen and hybrid aspen clones, as well as the knowledge and background on the cultivation of aspen. A special thanks goes to Professor Riitta Julkunen-Tiitto for introducing me into the world of phenolics and for the warm welcome every time I walked into her lab. I also wish to thank the other co-authors and co-workers Marika Makkonen, Nadezhda Prozherina, Vivek Pandey, Jana Sitte and Teodor Homentcovschi for their help in the lab and in the field, and the reviewers of the thesis, Docent Jaana Bäck and Dr. Anu Sõber, for their constructive comments.

I want to warmly thank all my colleagues and friends at the Department of Environmental Science, especially Tarja Silfver and Vera Freiwald, with whom I spent days (and nights) in Ruohoniemi and in the lab. I also want to thank the rest of the Coffee Room Team (a.k.a the Victorious Lottery Team), Johanna Riikonen, Minna Kivimäenpää, Anne Kasurinen and Päivi Tiiva who understand the importance of coffee breaks and long, in-depth discussions about Life, Universe and Everything Else. A special thanks goes to Timo Oksanen, not only for ozone fumigations and technical expertise, but also for his perceptive opinions and solutions to any practical problem encountered. I also want express my gratitude to the staff of the Research Garden, Marjatta Puurunen, Topi Kuronen and Leena Tilus, for maintaining the plants and helping in the field, and to the lab technicians Virpi Tiihonen and Jaana Rissanen for helping in the lab. Docent Nathan Lillie is acknowledged for revising the language and Vesa Kiviniemi for the advice on statistical analyses.

Finally, thank you Riitta and Kylli, my dearest friends, for keeping in touch and being so close even though being so far away. And above all, thank you Martti for the patience and for all the love and support you have given me during these years, and Leevi, Lassi, Elmo and Jaakko for making life a continuous challenge!

Kuopio, May 2009

Elina Häikiö

LIST OF ABBREVIATIONS

AA	ascorbic acid
ANOVA	analysis of variance
EM	electron microscopy
FACE	free-air carbon dioxide enrichment
GVA	graphical vector analysis
HPLC	high performance liquid chromatography
LM	light microscopy
MS	mass spectrometry
NO_x	nitrogen oxides
Nr	reactive nitrogen (organic nitrogen-containing compounds, inorganic oxidized and reduced forms of N)
PAGE	polyacrylamide gel electrophoresis
PCA	principal component analysis
ROS	reactive oxygen species
VOC	volatile organic compound

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred to in the text by their Roman numerals, and on unpublished results:

I Häikiö, E., Freiwald, V., Silfver, T., Beuker, E., Holopainen, T. & Oksanen E. (2007): Impacts of elevated ozone and nitrogen on growth and photosynthesis of European aspen (*Populus tremula*) and hybrid aspen (*P. tremula x Populus tremuloides*) clones. *Canadian Journal of Forest Research* 37: 2326-2336.

II Häikiö, E., Freiwald, V., Julkunen-Tiitto, R., Beuker, E., Holopainen, T. & Oksanen, E. (2009): Differences in leaf characteristics between ozone-sensitive and ozone-tolerant hybrid aspen (*Populus tremula* x *P. tremuloides*) clones. *Tree Physiology* 29: 53-66.

III Häikiö, E., Makkonen, M., Julkunen-Tiitto, R., Sitte, J., Freiwald, V., Silfver, T., Pandey, V., Beuker, E., Holopainen, T. & Oksanen, E.: Performance and secondary chemistry of two hybrid aspen (*Populus tremula* L. x *P. tremuloides* Michx.) clones in long-term elevated ozone exposure. *Journal of Chemical Ecology* (2009). In press.

IV Oksanen, E., Häikiö, E., Sober, J. & Karnosky, D.F. (2004): Ozone-induced H_2O_2 accumulation in field grown aspen and birch is linked to foliar ultrastructure and peroxisomal activity. *New Phytologist* 161:791-799.

CONTENTS

1. INTRODUCTION

1.1 The drivers of global climate change

Human activities have resulted in increasing global atmospheric concentrations of the main greenhouse gases carbon dioxide (CO_2), methane (CH_4), ozone (O_3) and nitrous oxide (N_2O), altering the energy balance of the climate system. The concentration of CO_2 has increased from the pre-industrial level of 280 ppm to 379 ppm in 2005, primarily due to fossil fuel use and land use change (IPCC 2007). At the same time, the background concentrations of tropospheric ozone have more than doubled from about 10 ppb to the present concentrations of 25 – 40 ppb (Vingarzan 2004, The Royal Society 2008) and are predicted to increase by another 40 – 70 % by the year 2100 (Grenfell et al. 2003, Zeng et al. 2008). Even if the emissions of O_3 precursor pollutants, nitrogen oxides (NO_x) and volatile organic compounds (VOCs), were successfully cut down in Europe, the concentrations would still be increasing in the rapidly developing regions of South and East Asia, and intercontinental transport links NO_x emissions in one continent with the surface ozone in another (Grenfell et al. 2003, Derwent et al. 2008). Reductions of the emissions of ozone precursors are difficult to implement because of the many emission sources of NO_x (power plants, traffic, biomass burning) and VOCs (industrial emissions, traffic, biogenic emissions), and because of the complicated interactions of these compounds with ozone in the atmosphere (Atkinson 2000, Cape 2008). In addition to providing the third largest increase in direct radiative forcing on climate since the preindustrial times (IPCC 2007), elevated tropospheric ozone adds to the radiative forcing of CO_2, methane and N_2O by reducing the photosynthetic capacity and carbon assimilation of plants thus leading to even higher concentrations of CO_2 in the atmosphere (Sitch et al. 2007). Increased radiative forcing has a warming effect on the climate, and higher temperatures are predicted to increase the biogenic emissions of VOCs. However, high temperatures lead also to higher water vapour concentrations resulting in photolysis of O_3 and formation of hydroxyl radicals which in turn leads to faster turnover of VOCs (Cape 2008). In addition, the inter-annual variability driven by climatic factors makes modelling and predicting future trends of greenhouse gas concentrations and precursor emissions very complex.

1.2 Ozone

Most of the ozone in the atmosphere is found in the stratosphere where it acts as a shield to protect the Earth's surface from harmful ultraviolet radiation. Ozone is transported to the troposphere from the stratosphere but in addition to the stratosphere-troposphere exchange, O_3 is also formed photochemically in the troposphere in the reactions between molecular oxygen (O_2), NO_x and VOCs (Atkinson 2000). O_3 is formed from the photolysis of NO_2 to first give NO and O, and then in the reaction of O with O_2, O_3 is formed. Because O_3 reacts rapidly with NO to generate NO_2, there is a photoequilibrium between NO, NO_2 and O_3 with no net formation or loss of O_3. However, photolysis of O_3 by ultraviolet light in the presence of water, as well as degradation reactions of VOCs, result in the production of hydroxyl radicals and peroxy radicals, which react with NO to form NO_2, thus shifting the equilibrium towards NO_2 and resulting in net production of O_3. Therefore, any factors that affect OH radical concentrations and the conversion of NO to NO_2, affect the amount of O_3 formed (Atkinson 2000, Cape 2008).

1.2.1 Effects of ozone on forest trees

Tropospheric ozone is one of the most phytotoxic air pollutants causing reductions in the photosynthetic capacity, biomass allocation and carbon sequestration of forest trees (Felzer et al. 2007, Wittig et al. 2007, Wittig et al. 2009). When angiosperm trees growing in an average background ozone concentration of 40 ppb were compared with charcoal-filtered controls in a meta-analysis, 14 % and 16 % reductions in the photosynthetic capacity and stomatal conductance, respectively, were found (Wittig et al. 2007) and the total biomass was reduced on average by 7 % (Wittig et al. 2009). Significant reductions in leaf area, transpiration rates and concentrations of chlorophylls and Rubisco were reported in ozone concentrations of 80 to 100 ppb (Wittig et al. 2009).

Ozone may affect stomata directly, by inhibiting stomatal opening or by reducing the stomatal aperture (Torsethaugen et al. 1999, Zheng et al. 2002), but usually the ozone-induced reduction in stomatal conductance is considered to be a secondary response resulting from increased concentrations of internal CO_2 due to a reduced photosynthetic rate (Noormets et al. 2001). Reductions in the photosynthetic rate and stomatal conductance may in turn be secondary responses to reduced phloem loading due to elevated ozone (Grantz 2003), which may lead to reduced carbon allocation to roots, a widely reported ozone effect (see Andersen 2003, and references therein). Ozone may also reduce the whole tree carbon gain by accelerating leaf senescence, i.e. the degradation of chlorophylls, total soluble proteins and especially ribulose-1,5-bisphosphate oxygenase/carboxylase (Rubisco; Reich and Lassoie 1985, Pell et al. 1999). However, species with an indeterminate growth habit (such as *Populus*) may respond to accelerated senescence by compensatory growth of new leaves, and switching from an indeterminate growth pattern to determinate growth may be the reason for increased sensitivity of mature trees compared to young trees (Kolb and Matyssek 2001). Fast-growing trees have also been reported to be more sensitive to ozone than slow growers (Bortier et al. 2000).

Reactive oxygen species (ROS) are generated in plant cells during physiological processes but also in response to many stress factors. To avoid oxidative damage, plants have evolved enzymatic (e.g. superoxide dismutase, peroxidases, catalases and reductases) and non-enzymatic (e.g. ascorbate, glutathione, phenylpropanoids and xanthophylls) ROS scavenging systems. Ozone enters the leaf through stomata and is quickly degraded into ROS in the apoplast. Ascorbate is the first line of defence acting as an antioxidant in the apoplast (Conklin and Barth 2004). When ozone-induced ROS formation exceeds the apoplastic antioxidative capacity, a second burst of ROS production is induced leading to accumulation of mostly hydrogen peroxide (H_2O_2) and appearance of visible symptoms in the vicinity of leaf veins (Schraudner et al. 1998). Ozone-elicited ROS signals trigger downstream processes and induce expression of defence genes which are regulated by the plant hormones abscisic acid, ethylene, jasmonic acid and salicylic acid (Sandermann et al. 1998, Tamaoki et al. 2003, Overmyer et al. 2008).

In Scandinavia the background ozone concentrations are relatively low compared to the concentrations in e.g. Central and Southern Europe (Klumpp et al. 2006*a*, Lindskog et al. 2007). However, the Scandinavian forests may be at an increasing risk of negative effects of even moderately elevated O_3 concentrations due to climatic conditions promoting high rate of O_3 uptake of the leaves (Matyssek et al. 2007), whereas the Mediterranean evergreen broadleaved

trees are more tolerant to O_3 pollution, because of their sclerophyllous leaves, low gas exchange rates and their ability to tolerate oxidative stress by an active antioxidant pool (Bussotti 2008).

1.2.2 Exposure- and flux-based indices for predicting ozone effects on vegetation

The most common exposure-based index for assessing the risk of elevated ozone concentrations to the vegetation in Europe is AOT40, which is the sum of the daylight hourly concentrations accumulated over a threshold of 40 ppb (Kärenlampi and Skärby 1996). The critical level for European forest trees has been reduced from a 5-year average AOT40 value of 10 ppm h (Kärenlampi and Skärby 1996) to 5 ppm h, which is associated with a 5% growth reduction per growing season for the deciduous sensitive tree species beech and birch (LRTAP Convention 2004). Ozone concentrations are usually higher at rural sites than at urban sites, and the critical levels are regularly exceeded all over Europe, with a gradient of increasing ozone levels from northern to Central and Southern Europe (Klumpp et al. 2006*a*). However, the exposure-based critical levels only consider the ozone concentration at the top of the canopy and do not take into account the potential for O_3 uptake, its detoxification or biochemical interaction within the plant. For this reason, flux-based indices including O_3 entry into the leaf through stomata as well as detoxification and repair processes have been developed (Emberson et al. 2000, Tausz et al. 2007). Flux-based indices still have their limitations since they cannot be extrapolated across species and environments outside of specific experimental or field study conditions, and much more data is needed to validate the flux-based concept (Paoletti and Manning 2007). In order to be able to determine the "effective ozone flux" which takes into account both the stomatal flux of O_3 into the leaf as well as the detoxifying barrier (Musselman et al. 2006, Tausz et al. 2007), levels of the reduced pyridine nucleotides (NAD(P)H), Rubisco/PEPcase ratio and water use efficiency have recently been suggested as better indicators of the ability of leaf cells to regenerate antioxidant power, than ascorbate content alone (Dizengremel et al. 2008).

1.3 Nitrogen

Nitrogen gas (N_2) comprises 78 % of the atmosphere, but the triple-bonded molecule is not biologically available to most organisms. Some free-living and symbiotic bacteria and blue-green algae are able to produce reduced forms of nitrogen such as ammonia (NH_3), amines and amino acids, which can be utilized by plants. After the invention of the Haber-Bosch process in 1913 it became possible to convert atmospheric N_2 to NH_3 for the production of fertilizers (Galloway and Cowling 2002). Ammonia is emitted into the atmosphere mainly from intensive livestock agriculture. Ammonia is readily deposited on surfaces close to its sources, but a significant amount dissolves into cloud water forming ammonium ions (NH_4^+) which in turn react with sulphate ions to form ammonium sulphate which has a longer lifetime than ammonia and is transported further away from its sources. Ammonium sulphate deposition may result in increased acidity of soils (Sanderson et al. 2006).

Oxidized forms of nitrogen (NO_x) are produced in high temperature natural processes such as lightning but also as a consequence of fossil fuel combustion in industry and traffic. Since the beginning of the 20th century, fossil fuels have replaced the use of biofuels in energy production, and by 1990 the biologically reactive nitrogen (Nr) created by anthropogenic

activities had increased 9-fold from the year 1890 (Galloway and Cowling 2002). Emissions of NO are readily oxidized to NO_2 by ozone and deposited onto vegetation and soil.

When the creation of reactive nitrogen exceeds the conversion of Nr back to N_2 by denitrification, nitrogen will accumulate in environmental systems. Elevated N deposition may impair the cycling of inorganic N in the forest ecosystem leading to nitrogen saturation and elevated nitrogen leaching below the root zone (Gundersen et al. 2006).

1.3.1 Effects of nitrogen addition on forests

Plant growth in temperate and boreal forests is often limited by nitrogen availability, and low levels of N deposition may lead to stimulated tree growth and increased carbon sequestration, as reported after 30 years of experimental nitrogen application on an unpolluted boreal forest (Högberg et al. 2006). Plants mainly take up NH_4^+ and NO_3^- from the soil, but they are also capable of incorporating NO_x through stomata and assimilating this N into the leaf metabolism, especially under N-limited conditions (Wellburn 1998, Vallano and Sparks 2008). Nitrogen addition, whether taken up through leaves or from soil, favours the growth of canopy biomass at the expense of roots (Siegwolf et al. 2001, Cooke et al. 2005), leading to a higher demand for water and risk of water shortage. Near industrial sources atmospheric Nr is usually dominated by nitrogen oxides, which enter the leaves through stomata. Fumigation of tomato or tobacco plants with realistic concentrations of NO_2 (20 ppb or 40 ppb) was not detrimental to growth, and fertilizing effects occurred at relatively low concentrations (Vallano and Sparks 2008). In regions further away from N sources, the atmosphere is often dominated by nitric acid (HNO_3) which is water soluble and is deposited on surfaces rather than taken up through stomata. HNO_3 and its associated anion NO_3^- can also be assimilated in active leaf metabolism, but HNO_3 vapour may also damage the epicuticular waxes thereby leaving epidermal cells more vulnerable to e.g. ozone toxicity (Padgett et al. 2009*a*, 2009*b*).

Current levels of N deposition of 10 to 30 kg N ha^{-1} yr^{-1} in Northern and Central Europe, respectively, are unlikely to impact soils and affect tree growth negatively (Högberg et al. 2006). Reduced growth after 15 years of addition of 150 kg N ha^{-1} yr^{-1} was reported in a pine stand but not in a mixed hardwood stand, with a large net retention of added N in the soil (Magill et al. 2004). In contrast, many tropical forests with highly-weathered soils are limited by phosphorus and calcium, and are naturally N saturated and poorly buffered (Matson et al. 2002). In long term, high N deposition has been suggested to lead to decreased tree growth due to acidification, leaching of nutrient base cations together with nitrate and mobilization of toxic aluminum ions (Emmett 1999, Gundersen et al. 2006). In areas with low background N deposition, high soil nitrogen availability may lead to loss of species naturally adapted to low N levels, and to a reduction in species richness (Nordin et al. 2005, Emmett 2007).

1.3.2 Critical loads of nitrogen deposition

The critical load concept has been established to determine N deposition levels which ecosystems can tolerate without significant harmful effects (LRTAP Convention 2004). Critical N loads are exceeded and will be exceeded also in the future despite the emission reduction agreements in Europe (Spranger et al. 2008). For mixed boreal forests the critical load range has been suggested to be between 10 and 20 kg N ha^{-1} yr^{-1} and even lower (<10 kg N ha^{-1} yr^{-1}) for the low deposition areas (Kuylenstierna et al. 1998, LRTAP Convention 2004). To protect the

sensitive understorey vegetation in low deposition areas such as the forests of Scandinavia the empirically determined critical load of N has been suggested to be lowered to 6 kg N ha^{-1} yr^{-1} (Nordin et al. 2005). A threshold for nitrate leaching at 10 kg ha^{-1} yr^{-1} of N in atmospheric N deposition was suggested by (Gundersen et al. 2006).

1.4 Do increased nitrogen loads or carbon dioxide concentrations counteract the impacts of elevated ozone on forest trees?

Nitrogen addition usually leads to enhanced growth of the aboveground biomass at the expense of belowground allocation (Landolt et al. 1997, Grulke et al. 1998, Coleman et al. 2004, Cooke et al. 2005). High nitrogen availability may also lead to carbon allocation to growth instead of defensive compounds, thereby leading to reduced stress tolerance (Bryant et al. 1987, Leser and Treutter 2005).

The results of studies with combined effects of elevated ozone and nitrogen are controversial: nitrogen amendment has been reported to either mitigate the ozone-induced adverse effects by slowing down the ozone-induced accelerated senescence (Pääkkönen and Holopainen 1995), to have no effect on ozone responses (Volin and Reich 1996) or to result in greater ozone-induced biomass reductions than nitrogen deficiency (Pell et al. 1995). In Scots pine, ozone-induced structural damage on the needles was most evident in low N treatment, whereas shoot growth and root biomass were reduced due to ozone in high N treatment but not in low N treatment (Utriainen and Holopainen 2001). When a gradient of ozone exposure and nitrogen deposition was studied in a ponderosa pine forest, the above-ground growth was greatest at the most polluted site (Grulke and Balduman 1999). When growth increase due to nitrogen deposition was compared to reduction of carbon storage due to ozone, the benefits of nitrogen deposition were concluded to outweigh the negative effects of ozone on carbon sequestration in temperate forests (Felzer et al. 2007).

Elevated CO_2 is generally thought to act as a growth enhancer, and in short term it may mitigate the adverse ozone effects on trees by inducing stomatal closure (Paoletti and Grulke 2005). However, it is not known if the CO_2-induced reduction in stomatal conductance may be sustained, or if stomatal acclimation is decreased over time (Paoletti and Grulke 2005). The combined effects of elevated ozone and CO_2 were studied in a young, aggrading forest, where elevated O_3 at relatively low concentrations significantly reduced the growth enhancement by elevated CO_2 (Karnosky et al. 2003). However, the responses depend on the forest type: the combined fumigation with elevated O_3 and CO_2 led to reductions in biomass in a pure aspen stand, but not in a mixed aspen-maple stand (King et al. 2005). Allocation of carbon to fine roots instead of stem biomass in elevated CO_2 may reduce the tentative long-term enhancement of carbon sequestration in biomass (Norby et al. 2004). No stimulation in stem growth or litter production after four years of 530 ppm CO_2 exposure in a mature deciduous forest was found, with a wide variation between species in the responses to elevated CO_2 (Körner et al. 2005).

1.5 European aspen and hybrid aspen in Finland

European aspen (*Populus tremula* L.*)* is a native tree in Finland and widely distributed in Europe and Asia, whereas trembling aspen (*Populus tremuloides* Michx.) is the most widely distributed tree in North America. In Finland, aspen mostly occurs in mixed stands with pine,

spruce and birch almost throughout the country and makes up about 1.5 % (30 million m^{-3}) of the standing volume of the Finnish forests. Pure aspen stands cover only about 0.3 % of the forest area in Finland (Haapanen and Mikola 2008). Aspen is a species of great ecological value for biodiversity especially in old mixed forests being a host to many specialist and even threatened species (Siitonen 1999). However, since aspen is the intermediate host for the pine rust fungus *Melampsora pinitorqua*, it has been systematically eliminated from managed forests in Finland (Kouki et al. 2004). Until the mid-1990s aspen was of economical value mostly for the match industry but since then, the pulp and paper industry have shown new interest in the use of aspen fibres as raw material for high quality paper. Aspen has short, bright fibres with small diameters and thin walls, which is ideal for producing homogenous, opaque printing paper (Ranua 2001). Aspen wood is also used e.g. in sauna benches and panels, in furniture, as well as in various special products such as hockey sticks (Verkasalo 2002). *Populus* species can also be grown for energy purposes in short rotation forestry in the boreal regions with rotation times of 5 - 10 years, reducing the use of fossil fuels and adding only marginally to the increasing quantities of greenhouse gases in the atmosphere (Weih 2004).

Hybrid aspen (also known as *Populus* x wettsteinii Hämet-Ahti) is a crossing between European aspen and trembling aspen, and it has shown superior growth compared to native aspen (Yu et al. 2001a). The papermaking properties of hybrid aspen are also better than those of native aspen due to a higher fibre count and a lower fibre shape factor (Tigerstedt 2002). The breeding of hybrid aspen in Finland started in the 1950s for the needs of the match industry. Crossings were made between selected European aspen trees in Finland and trembling aspen from Ontario and British Columbia, Canada. Several experimental forests were established in southern Finland (Viherä-Aarnio 1999). The field performances of the hybrid aspen stands were first studied in the 1970s, and the clones that are in commercial production today have been selected from these experimental forests at the end of 1990s.

Hybrid aspen is aimed to be cultivated in highly productive clonal plantations with rotation times of 25 – 30 years. Clonal material of selected genotypes can be produced by micropropagation or root cuttings (Stenvall et al. 2005). Significant clonal differences have been found in growth-associated characters and wood physicochemical properties, as well as in the sprouting and rooting ability of aspen and hybrid aspen (Yu et al. 2001*a*, 2001*b*, Stenvall et al. 2005). Wood physicochemical properties are genetically quite stable, but improvement based on fibre count was reported to be negatively correlated with growth (Yu et al. 2001*b*). Selection of superior clones is difficult also because environmental conditions affect the performance of the clones. There is great variation in the growth of the clones depending on e.g. soil properties (DesRochers et al. 2003, Yu and Pulkkinen 2003, Tullus et al. 2007). The superior growth of hybrid aspen has been attributed to a longer growth period (Yu et al. 2001*a*), and stress factors affecting the length of the growing season (delayed bud opening, accelerated autumn senescence) may decrease the hybrid vigour of high-yielding clones. Therefore, in forest tree breeding, it is important to select clones for a combination of genotypic stability and productive quality to obtain clones that are both superior in performance and stable over a range of environments (Yu and Pulkkinen 2003).

1.6 Research objectives

The main aim of the experiments was to study the interactive effects of elevated ozone and different soil nitrogen regimes on native European aspen and hybrid aspen. In the first part of the field experiment in Kuopio with potted saplings, we wanted to assess the ozone sensitivity of selected superior native aspen and hybrid aspen clones, and to find out which characteristics determine the ozone sensitivity or tolerance of the clones. After studying the growth responses of the different clones to elevated ozone (I), the clones were assigned to ozone-sensitive and ozone-tolerant groups, and the differences in the foliar characteristics of the two groups were studied in order to find biochemical markers for ozone sensitivity or tolerance. We were particularly interested in senescence-associated parameters, such as concentrations of pigments and proteins, and on the phenolic compounds of the leaves tentatively affecting the antioxidative capacity of the leaves under ozone stress (II). In the second part of the experiment in Kuopio with two soil-grown hybrid aspen clones, we wanted to study the longer-term effects of elevated ozone on the competitive ability of a moderately ozone-sensitive clone and a moderately ozone-tolerant clone (III). We also identified individual leaf phenolic compounds of hybrid aspen (III). In a separate experiment performed at the Aspen FACE (Free-Air Carbon Enrichment) site in Wisconsin, USA, we studied ozone-induced oxidative stress on the cellular level by examining foliar ultrastructure and accumulation of H_2O_2 inside the leaf cells, and the effect of CO_2 enrichment on the cellular ozone responses of birch and three clones of trembling aspen (IV).

1.6.1 Working hypotheses

We expected to find ozone-induced reductions in growth, because *Populus* has been reported to be among the most sensitive tree species to ozone. We wanted to see if there is variation in ozone sensitivity between hybrid aspen and native European aspen or among different clones within species. Hybrid aspen was expected to be superior in growth compared to native aspen, and because slow-growing trees have been found to be less sensitive to ozone than fast-growing trees, we expected to find a positive correlation between good growth and ozone sensitivity in our trees. Since increased nutrient availability has been found to result in photosynthate allocation to growth instead of carbon-based allelochemicals, we predicted that nitrogen amendment would lead to lower concentrations of phenolics and reduced ozone tolerance in our clones. We also hypothesized that the higher resource availability of the soil-grown trees would result in lower foliar phenolic concentrations as compared to the potted trees. Ozone exposure was expected to result in higher concentrations of foliar phenolic compounds, especially flavonoids. We studied the effects of chronic ozone stress on two soil-grown hybrid aspen clones differing in ozone sensitivity, and expected to find reduced competitive ability of the more ozone-sensitive clone after three years of ozone exposure. We also expected to find increased H_2O_2 accumulation, proliferation of peroxisomes and increased transcript levels of catalase in the ozone-exposed leaf cells, whereas CO_2 enrichment was expected to alleviate the signs of oxidative stress in the ozone-exposed leaves.

2. MATERIALS AND METHODS

2.1 Outline of the field experiments

A field experiment was established at the Ruohoniemi open-air ozone exposure field of the Kuopio University Research Garden (62°37'N, 26°11'E) in the spring in 2002 to study the impacts of elevated ozone and soil nutrient availability on several native European aspen and hybrid aspen clones (I – III). The ozone exposure field consists of four ambient ozone plots and four elevated ozone plots (Fig. 1). In the first part of the experiment, eight hybrid aspen clones and two native aspen clones were planted in pots and exposed to two ozone levels (ambient and 1.4 x ambient ozone concentrations; ca. 25 and 35 ppb, respectively), and within plots, to two soil nitrogen levels (39/78 kg N ha^{-1} yr^{-1} in the first year, and 60/140 kg N ha^{-1} yr^{-1} in the second year for low-N/high N treatments, respectively) for two growing seasons (2002 – 2003; I - II). The pots were watered daily and they were rotated within the plots twice during each growing season (I – II). Parallel with the pot experiment, two hybrid aspen clones with tentatively different ozone sensitivities were planted on soil in the middle of the same plots and exposed to ambient ozone or elevated ozone for three growing seasons (2002 – 2004; III). The soil-grown trees were not fertilized or watered during the experiment. The biggest broad-leaved weeds were removed once in a growing season (III).

Fig. 1 Ruohoniemi open-air ozone exposure field of the Kuopio University Research Garden on the left (http://maps.live.fi/) and AspenFACE site in Wisconsin, USA on the right (photo by Rick Anderson).

The last part of the study was performed on samples collected in 2001 at the Aspen FACE experimental site (Wisconsin, USA; 45°6'N, 89°5'W), where the effects of elevated ozone (ambient and 1.5 x ambient ozone concentrations; ca. 38 and 57 ppb, respectively) and elevated carbon dioxide (ambient and 200 ppm above ambient; ca. 360 and 560 ppm, respectively) on the ultrastructure of the leaves and on the ROS production and peroxisomal activity of three

trembling aspen clones and paper birch seedlings were studied (IV). The experimental site consists of 12 treatment rings, with three replicate rings per treatment (i.e. ambient CO_2 + O_3, elevated CO_2, elevated O_3 and elevated CO_2 + O_3).

2.2 Plant material

Hybrid aspen and native aspen clones used in experiments I – III were selected from clones used in the Finnish hybrid aspen breeding programme. The hybrid aspen clones were originally produced in the 1950s and 1960s by crossing mostly *Populus tremula* from Finland and *Populus tremuloides* from Canada (Table 1). The crossings were planted in experimental forests in southern Finland, and their performance was first studied in the 1970s. Superior hybrid aspen and native aspen trees from these experimental forests have been selected for clonal propagation and commercial production. The clones for studies I – III were produced by micropropagation in the laboratory of the Foundation for Forest Tree Breeding (Haapastensyrjä, Finland) and they were planted at the University of Kuopio ozone exposure field as one-year-old saplings in May - June, 2002. The potted saplings were studied during the second growing season and harvested in September 2003 (I, II). Two of the hybrid aspen clones were planted in soil in the middle of the plots, and were studied during the third growing season and harvested in September 2004 (III).

In the Aspen FACE experiment (IV), three trembling aspen (*Populus tremuloides)* clones (Clones 216, 259 and 271) and seedlings of paper birch (*Betula papyrifera*) were studied. Clone 259 had been previously determined to be ozone-sensitive, whereas Clones 216 and 271 were more ozone-tolerant (Karnosky et al. 1999). The trees had been planted in 1997, and samples for experiment IV were taken at the end of the fourth growing season in 2001 (IV).

2.3 Analyses of growth, physiology and foliar chemistry

Table 2 gives a summary of the topics studied and the methods used in experiments I – IV.

2.3.1 Gas exchange and fluorescence

Leaf-level net photosynthesis was measured several times during the growing season with a CI-510 Portable Photosynthesis System (CID, Vancouver, WA) in 2003 and 2004 (I – III). All measurements were made on one sun leaf per tree in the middle of the canopy in saturating light (>1000 μmol $m^{-2}s^{-1}$), and ambient CO_2 concentration and temperature. The same leaves were used for measuring the maximum photochemical efficiency of photosystem II (F_v/F_m) in 2003 and 2004 with a portable pulse-modulated FMS2 fluorometer (Hansatech Instruments Ltd., Norfolk, England) after a 30 minute dark-adaptation (I – III). Stomatal conductance was measured on the soil-grown trees in 2004 with LI-1600 Steady State Porometer (LI-COR Inc., NE, USA) on the abaxial side of the same leaves that were used for photosynthesis measurements (III).

Table 1. Origins of the hybrid aspen and native aspen clones used in experiments I – III.

Clone	Mother	Lat.	Long.		Father	Lat.	Long.		Year of selection	Lat.	Long.
1	E1732	60°26'	24°57'	*P. tremula* (FI)	U2554	45°17'	78°58'	*P. tremuloides* (CA)	1992	60°37'	24°27'
14	E969	61°45'	29°18'	*P. tremula* (FI)	U2576	?	?	*P. tremuloides* (B. C., CA)	1998	60°37'	24°27'
55	E295	60°22'	24°55'	*P. tremula* (FI)	U2502	?	?	*P. tremuloides* (Ontario, CA)	1998	60°45'	26°59'
110	E1571	61°33'	26°55'	*P. tremula* (FI)	U2566	?	?	*P. tremuloides* (Ontario, CA)	1998	60°37'	26°11'
193	E1095	61°50'	29°20'	*P. tremula* (FI)	kok73(sv101)	?	?	*P. tremuloides* (CA)	1998	60°30'	24°42'
200	U2530	54°06'	122°03'	*P. tremuloides* (CA)	kok73(sv101)	?	?	*P. tremula* (FI)	1998	60°30'	24°42'
218	E1446	60°13'	24°55'	*P. tremula* (FI)	U2565	43°21'	80°26'	*P. tremuloides* (Ontario, CA)	1998	60°20'	24°26'
280	U2006	57°45'	12°00'	*P. tremuloides* (SWE)	E294	60°21'	24°57'	*P. tremula* (FI)	1998	62°15'	25°54'
31	E1433	61°04'	28°12'	*P. tremula* (FI)	E293	60°21'	24°57'	*P. tremula* (FI)	1998	61°19'	23°49'
147	E1214	62°15'	25°30	*P. tremula* (FI)	E828	60°21'	24°57'	*P. tremula* (FI)	1998	61°19'	23°49'

2.3.2 Leaf chemistry

Samples for the concentrations of total nitrogen, chlorophylls, carotenoids, total soluble proteins, Rubisco, soluble sugars and starch were collected three times during the growing season (mid-July, mid-August and mid-September, 2003; I, II). Total organic nitrogen was analyzed from dried and ground leaf samples by the standard Kjeldahl method (Allen 1989; I – III). For the determination of chlorophylls, carotenoids, total soluble proteins and Rubisco, frozen leaf samples were homogenized in extraction buffer (II), and aliquots of the crude extract were used for the different analyses. Concentrations of chlorophylls and carotenoids were analyzed in 80 % acetone by the methods of Arnon (1949) and Lichtenthaler and Wellburn (1983). Rubisco concentrations were determined by native PAGE (polyacrylamide gel electrophoresis; Rintamäki et al. 1988) and total soluble protein concentrations were determined by the method of Bradford (1976; II). Concentrations of soluble sugars and starch were analyzed spectrophotometrically by the anthrone method: soluble sugars were extracted in 80 % ethanol, starch was hydrolyzed with amyloglucosidase, and the amount of the resulting glucose was determined by boiling the samples with anthrone in sulphuric acid and measuring the absorbance at 630 nm (Hansen and Møller 1975; II).

Samples for leaf phenolics were collected at the end of the growing seasons 2003 and 2004 (II, III). Phenolics were extracted from dried leaf samples by 100 % methanol, dried, and dissolved in 50 % methanol for the HPLC analysis (II, III). The tentative identification was based on retention times and spectral characteristics. From the two soil-grown hybrid aspen clones, the individual flavonol glycosides were identified by HPLC/MS (III). Concentrations of condensed tannins were determined by the acid butanol test (Porter et al. 1986; II, III).

2.3.3 Biomass

The trees were measured for height and base diameter at the end of each growing season in 2002 – 2004. At the end of the pot experiment in 2003, 480 trees (3 trees per plot per clone per treatment) were harvested, the plant parts were separated and dried, and the dry masses of roots, stems with branches, and leaves were determined. Only the coarse roots were collected (I, II). The soil-grown trees were harvested at the end of the growing season in 2004 and the dry masses were determined. In addition to the coarse root system, samples for fine root biomass were collected by coring four soil samples from each plot (III). The mycorrhizal biomass of fine roots was determined by ergosterol analysis (III).

2.3.4 Leaf ultrastructure and cellular signs of oxidative stress

In the Aspen FACE experiment (IV), samples from young and old leaves of three aspen clones and paper birch were collected and prepared for light microscopy (LM) and electron microscopy (EM) as described in (IV). Before fixing, the samples were incubated with $CeCl_3$ to localize the accumulation of H_2O_2 within the leaf cells. LM sections were studied for total leaf thickness and thickness of palisade and spongy parenchyma layers. EM thin sections were analyzed for the size of chloroplasts and starch grains, mesophyll cell wall thickness, number of peroxisomes and localization of H_2O_2 accumulation. Young leaves were also analyzed for the expression of catalase by RNA gel blot analysis (IV).

Samples for light microscopy were also collected twice in 2003 in the Ruohoniemi pot experiment, as described in Freiwald (2008). Shortly, one leaf sample per clone per treatment from two replicate ozone plots and control plots were collected (32 samples in total) on 16 June and 19 August 2003, prepared for light microscopy and studied for leaf thickness, thickness of palisade and spongy mesophyll tissues and thickness of upper and lower epidermis.

2.4 Statistical analyses

The main effects and interactions of different factors were analyzed by linear mixed model ANOVA using SPSS for Windows version 14.0 (SPSS, Chicago, IL) with ozone, nitrogen, species/clone/sensitivity group, and date (in case of repeated measurements) as fixed factors, and plot as a random factor (I – III). In case of statistically significant interactions, pairwise *post hoc* comparisons of the simple main effects with Bonferroni corrections were used to elucidate the roles of different factors in higher order interactions (data usually not shown; I – III). The plot was used as a statistical unit (n=4). In the Aspen FACE experiment (IV), ANOVA using general linear models procedure (SPSS 10.0) was used to test for the main effects and interactions of ozone, CO_2, clone and leaf age on leaf structural characteristics. For H_2O_2 accumulation and *Cat* transcript levels, nonparametric Kruskal-Wallis H test was used (IV).

The hybrid aspen clones were divided into ozone-sensitive and ozone-tolerant groups based on their growth responses to ozone by using the two-step clustering analysis of SPSS 14.0 (I). The clustering was verified by testing the biomass variables showing ozone x clone interaction by the mixed model ANOVA with the sensitivity group as one of the fixed factors (II).

To study the phenolic profiles of different clones and the correlations of phenolics with growth or *Venturia tremulae* infection, principal component analysis (PCA) was used (II, III; Fig. 7). Graphical vector analysis (GVA) was used to determine if the increased/reduced accumulation of a phenolic compound was the result of increased/decreased synthesis, or concentration/dilution of the compound due to changes in leaf biomass (Haase and Rose 1995, Koricheva 1999; III).

Table 2. Summary of the topics studied and the methods used in experiments I - IV

Publication	Year [1]	Treatments	Plants used	Parameters measured	Aims of the study
I	(2002-) 2003	Ruohoniemi, University of Kuopio, Finland 1) two levels of O_3 (ambient O_3: 25 ppb, elevated O_3: 35 ppb) 2) two levels of N (low N: 39/60 kg ha^{-1} yr^{-1}, high N: 79/140 kg ha^{-1} yr^{-1})	*Populus tremula* (2 clones), *P. tremula* x *P. tremuloides* (8 clones); potted saplings	**Biomass accumulation:** height, base diameter, dry mass **Photosynthesis:** leaf-level net photosynthesis, chlorophyll fluorescence (F_v/F_m)	* Effects of ozone and soil nitrogen on the growth of native aspen and hybrid aspen * Differences in ozone responses between species and among clones
II	(2002-) 2003	Ruohoniemi, University of Kuopio, Finland 1) two levels of O_3 (ambient O_3: 25 ppb, elevated O_3: 35 ppb) 2) two levels of N (low N: 39/60 kg ha^{-1} yr^{-1}, high N: 79/140 kg ha^{-1} yr^{-1})	*P. tremula* x *P. tremuloides* (4 ozone-sensitive and 4 ozone-tolerant clones); potted saplings	**Biomass accumulation:** height, base diameter, dry mass **Photosynthesis:** leaf-level net photosynthesis, chlorophyll fluorescence (F_v/F_m) **Biochemistry:** concentrations of foliar nitrogen, chlorophylls, proteins, Rubisco, soluble sugars, starch and phenolics	* Differences in leaf characteristics between ozone-tolerant and ozone-sensitive hybrid aspen clones * The role of phenolics as antioxidants in ozone-induced oxidative stress
III	(2002-) 2004	Ruohoniemi, University of Kuopio, Finland two levels of O_3 (ambient O_3: 25 ppb, elevated O_3: 35 ppb)	*P. tremula* x *P. tremuloides* (2 clones); soil-grown saplings	**Biomass accumulation:** height, base diameter, dry mass **Photosynthesis:** leaf-level net photosynthesis, chlorophyll fluorescence (F_v/F_m), stomatal conductance **Biochemistry:** concentrations of foliar phenolics	* Effects of elevated ozone on the competitive ability of two clones differing in ozone sensitivity (comparison of soil-grown trees to potted trees) * Identification of individual phenolic compounds of hybrid aspen
IV	(1998-) 2001	Aspen FACE, Rhinelander, USA 1) two levels of O_3 (ambient O_3: 38 ppb, elevated O_3: 57 ppb) 2) two levels of CO_2 (ambient CO_2: 360 ppm, elevated CO_2: 560 ppm)	*Populus tremuloides* (3 clones), *Betula papyrifera*; soil-grown saplings	**Oxidative stress:** H_2O_2 accumulation, number of peroxisomes, expression of catalase **Leaf ultrastructure:** leaf thickness, thickness of palisade and spongy parenchyma, thickness of cell walls of mesophyll cells, area of chloroplasts and starch grains	* Signs of ozone-induced oxidative stress and detoxification in trembling aspen and paper birch * Effects of elevated ozone and CO_2 on the ultrastructure of the leaves

[1] Year of planting in parenthesis, followed by the year when the samplings/measurements were performed.

3. RESULTS

3.1 Hybrid aspen was superior in growth but had higher mortality than native aspen

In our experiment with potted saplings, hybrid aspen showed superior growth compared to native aspen (I). The native aspen clones suffered from severe *Venturia tremulae* (aspen shoot blight) infection (Freiwald 2008), which restricted especially the growth of Clone 147 in both years. On the other hand, hybrid aspen was more susceptible to terminal shoot dieback than native aspen (II). In our experiment, the trees of some of the hybrid aspen clones had not set bud by the time of the biomass harvest in early September 2002 and 2003, and some trees had even restarted terminal growth after bud set, leading to delayed frost hardening and shoot tip dieback in the following winter (I). In the soil-grown Clone 55, ozone exposure during the first growing season seemed to improve the winter hardening, since there was a significant difference in the shoot tip dieback between control and ozone trees after the first winter. Clone 110 had on the average 7,6 dead buds in the terminal shoot after the first winter, whereas Clone 55 had 4,9 and 7,5 dead buds in elevated ozone and ambient ozone treatments, respectively (P=0.056).

Hybrid aspen showed also higher mortality than native aspen: all native aspen saplings survived the second growing season whereas there was 5 % mortality in hybrid aspen (main effect of species: P=0.001). Somewhat surprisingly, there was no correlation between the survival of the saplings and the extent of winter damage of the main shoots. Clones 218 and 280 with severe winter damage also had the highest growth rates among the hybrid aspen clones during the second growing season, thus compensating for the lost biomass due to frost damage (data not shown).

3.2 Nitrogen amendment mitigated the adverse effects of ozone on growth

We found few ozone effects on the trees when the clones were pooled (I - III). In the pot experiment, photosynthetic rates were significantly reduced in all clones early in the second growing season (I). Ozone also reduced the coarse root dry mass in native aspen (I). We did not find accelerated senescence, but the concentrations of chlorophylls were significantly lower early in the growing season in ozone-exposed hybrid aspen (II). Elevated ozone also lowered the concentrations of Rubisco (II). In the two soil-grown hybrid aspen clones, no statistically significant effects of ozone on gas exchange, fluorescence or biomass accumulation were found (III).

Nitrogen amendment mitigated the minor adverse ozone effects on growth in both species by significantly increasing growth and thus affecting biomass accumulation, even if there were small ozone-induced reductions in growth also in high N plants (I). High soil nitrogen enhanced photosynthesis and increased the amounts of chlorophylls and proteins (II).

Hybrid aspen was found to have lower survival than native aspen in the pot experiment after the second growing season (Fig. 2), but high-N plants had lower mortality (3 %) than low-N plants (6 %) in hybrid aspen (P=0.017; Fig. 2). In contrast, high nitrogen treatment resulted in a 75 % increase in the length of the dead shoot-tip of pooled hybrid aspen clones during the first winter after planting (P=0.009; Fig. 3): the lengths of the dead shoot tips were on the

average 11 cm and 19 cm representing 7 % and 11 % of the final height after the first growing season in the low-N and high-N treatments, respectively (I; Fig. 3). There were significant differences in the winter damage among the clones (I; Fig. 3). In hybrid aspen Clone 1, there was a statistically significant four-fold increase in the length of the dead top due to elevated ozone (P=0.002; Fig. 3).

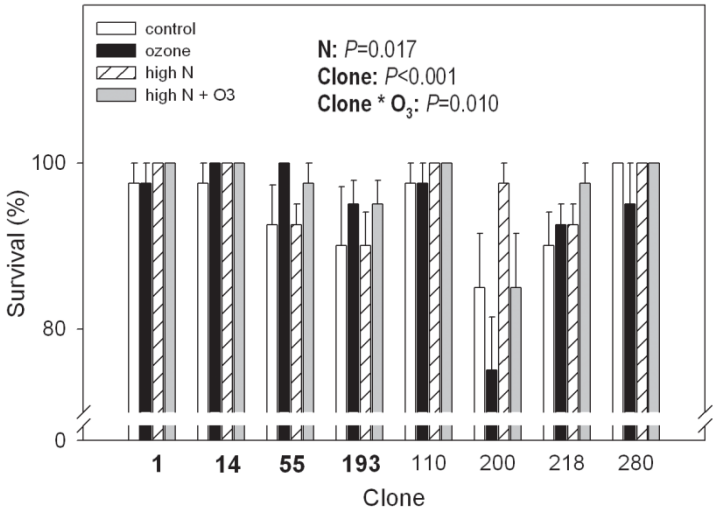

Fig. 2 Survival of hybrid aspen clones after the second growing season. *P* values are from mixed model ANOVA, with ozone, nitrogen and clone as fixed factors, and plot as a random factor. The ozone-tolerant clones are shown in bold.

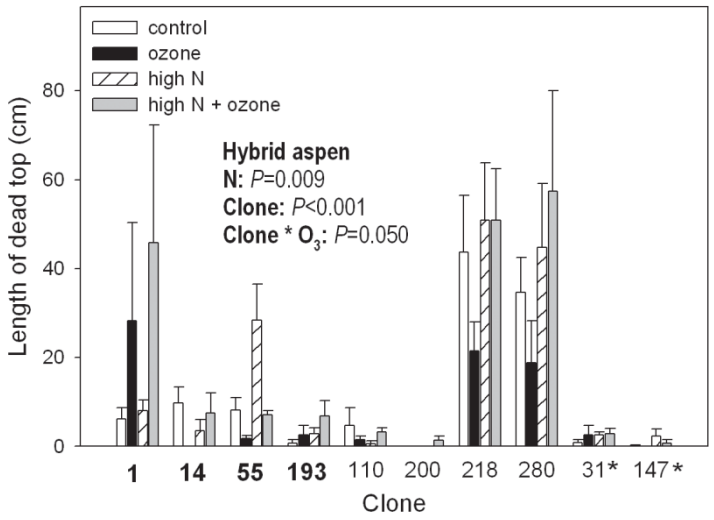

Fig. 3 Effects of ozone and nitrogen on shoot-tip dieback of hybrid aspen and native aspen clones in the winter 2002 – 2003. *P* values are from mixed model ANOVA, with ozone, nitrogen and clone as fixed factors, and plot as a random factor. The ozone-tolerant clones are shown in bold and the native clones are marked with an asterisk.

3.3 Ozone sensitivity of the clones based on reductions of biomass

We found statistically significant differences among hybrid aspen clones in the responses of dry mass to ozone in the pot experiment (I). The clones showing reduced growth in elevated ozone (hybrid aspen Clones 110, 200, 218 and 280, and native aspen Clones 31 and 147) were clustered into the ozone-sensitive group and the clones showing no effect or enhanced growth in elevated ozone (hybrid aspen Clones 1, 14, 55 and 193) were addressed to the ozone-tolerant group (I; Fig, 4). The dry mass of coarse roots was significantly reduced in ozone-exposed native aspen, as well as in the ozone-sensitive hybrid aspen Clones 110, 218 and 280, whereas the dry mass of stems was significantly reduced in the ozone-sensitive hybrid aspen Clone 280, but significantly increased in the ozone-tolerant Clone 193 in elevated ozone (data not shown; O_3 x clone interaction in I). Ozone tolerance or sensitivity could not be determined by photosynthesis measurements because all clones responded to elevated ozone with reduced photosynthesis (I, II). However, there was a clone x O_3 interaction in F_v/F_m in hybrid aspen, and when all three measuring dates were pooled, Clones 218 (hybrid aspen) and 31 (native aspen) showed a reduction of F_v/F_m in elevated ozone (Fig. 5). Both clones were clustered into the ozone sensitive group based on biomass data (I; Fig. 5).

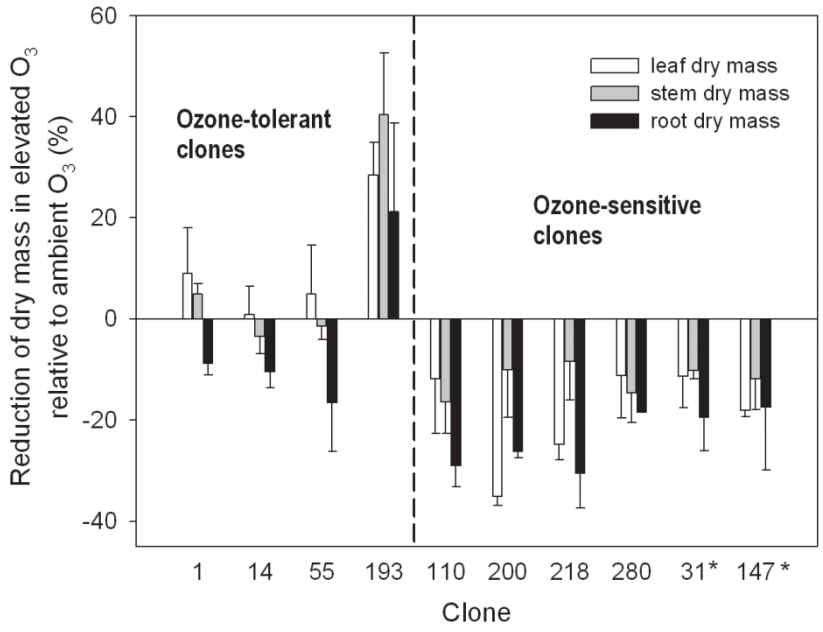

Fig. 4 Clustering of clones based on relative changes in growth of potted hybrid aspen and aspen saplings in elevated ozone in 2003. The means of all ozone-exposed trees from each clone were compared with the means of all control trees of the same clone (n=2 from the low-N and high-N treatments). The native aspen clones are marked with an asterisk.

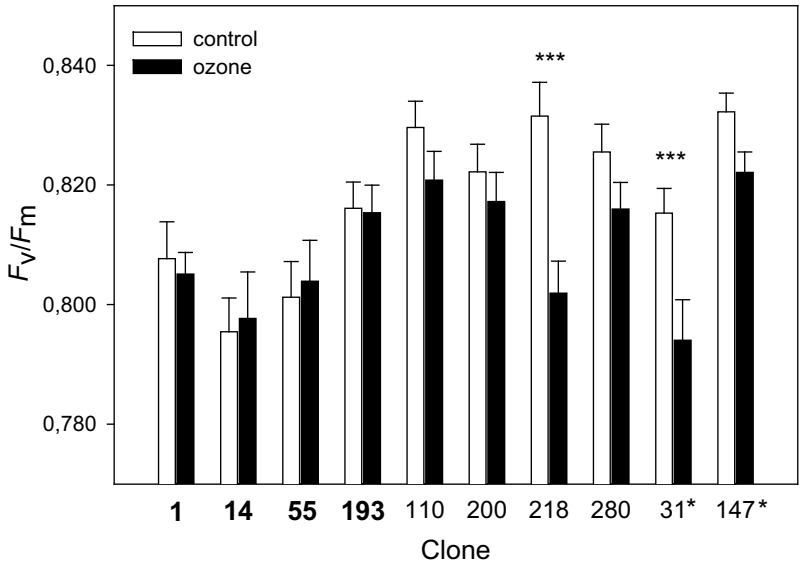

Fig. 5 The effect of elevated ozone (with the N levels pooled) on F_v/F_m of potted hybrid aspen and aspen clones in 2003. Statistically significant differences between the treatments were found in Clones 218 and 31 (*** *P* value <0.050; mixed model ANOVA, *post hoc* pairwise comparisons of the simple main effects). The ozone-tolerant clones are shown in bold and the native clones are marked with an asterisk.

There was statistically significant variation among hybrid aspen clones in survival after the second growing season ($P<0.001$) with Clones 200, 193 and 218 having the highest mortality rates (Fig. 2). Interestingly, there was a significant O_3 x clone interaction which was shown as increased survival of Clone 55 of the ozone-tolerant group ($P=0.059$) and a statistically significant decrease in survival of Clone 200 of the ozone-sensitive group ($P=0.001$) in elevated ozone (Fig. 2).

3.3.1 Nitrogen amendment did not affect the ozone sensitivity of the clones

Nitrogen amendment delayed autumn senescence by enhancing the photosynthetic capacity and slowing down the breakdown of chlorophylls and Rubisco in both sensitivity groups (II). However, there was variation among the clones in the pot experiment in their responses to nitrogen enhancement based on growth data (I): hybrid aspen Clones 14, 110, 193 and 280 showed statistically significant nitrogen-induced enhancement of growth (Fig. 6) whereas the other clones did not.

We found practically no N x O_3 interactions in biomass variables or foliar characteristics indicating that high nitrogen availability did not affect the ozone sensitivity of the clones (I).

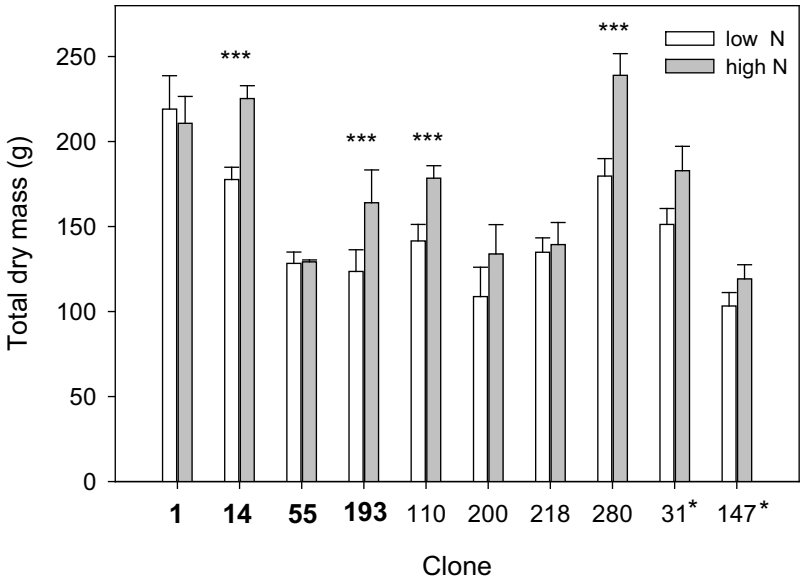

Fig. 6 The effect of nitrogen fertilization (with the O_3 levels pooled) on the total dry mass of potted hybrid aspen and aspen clones in 2003. Statistically significant differences between the nitrogen treatments were found in hybrid aspen Clones 14, 110, 193 and 280 (*** *P* value <0.050; mixed model ANOVA, *post hoc* pairwise comparisons of simple main effects). The ozone-tolerant clones are shown in bold and the native clones are marked with an asterisk.

3.3.2 Differences in leaf characteristics between ozone-sensitive and ozone-tolerant groups

The ozone-sensitive group had higher concentrations of Rubisco, higher photosynthetic capacity and higher concentrations of foliar phenolics than the ozone-tolerant group in the pot experiment (II). Ozone-tolerant clones had high amounts of condensed tannins, whereas the sensitive clones allocated carbon in salicylates (II). However, we did not find differences in the ozone responses between the two groups (no interaction of O_3 x group) and thus were not able to determine the reason for the reduced growth of the ozone-sensitive group (II). The only O_3 x group interaction was found in F_v/F_m, which was significantly reduced only in the ozone-sensitive group (II).

When the leaf structure was studied in the Aspen FACE experiment (IV), the aspen clones and birch responded variably to elevated ozone and CO_2. In general, elevated CO_2 had no main effects on the ultrastructural parameters measured, whereas leaf thickness (both palisade and spongy mesophyll tissues) was increased in elevated O_3 in aspen (IV). The ozone-sensitive aspen Clone 259 had thicker leaves, thinner mesophyll cell walls and a lower palisade to spongy mesophyll layer ratio than the tolerant Clones 216 and 271 (IV). Also in the Ruohoniemi

experiment, the ozone-sensitive group had thicker leaves (P=0.002) and lower palisade to spongy ratio (P=0.075) than the ozone-tolerant group. Elevated ozone did not affect leaf thickness, but the lower epidermis was thicker (P=0.001) in ozone-exposed hybrid aspen leaves. The leaves became thicker toward the end of the growing season (main effect of date: P=0.006) and this was due to a significant increase in the thickness of the palisade tissue (data not shown).

3.4 Ozone-induced oxidative stress and the role of leaf phenolics

3.4.1 ROS accumulation and detoxification in the leaves

In the Aspen FACE experiment, ozone-induced H_2O_2 accumulation was found in both birch and aspen (IV). Birch and the ozone-sensitive aspen Clone 259 showed more H_2O_2 accumulation than the tolerant clones 216 and 271, and the H_2O_2 accumulation was more evident in older leaves than in younger leaves (IV). In the aspen Clones 216 and 271, the H_2O_2 accumulation was restricted to the outer cell walls, whereas in birch and aspen Clone 259 H_2O_2 accumulated also in the cytoplasm and chloroplasts (IV). The number of peroxisomes and the transcript levels of catalase were the highest in elevated ozone in the ozone-tolerant Clones 216 and 271 (IV).

3.4.2 Ozone induced the synthesis of condensed tannins, catechins and chlorogenic acids

Elevated ozone significantly increased the concentrations of (+)-catechin and chlorogenic acid, and to some extent also the concentrations of condensed tannins in hybrid aspen in the pot experiment (II). No effects of nitrogen amendment were found in the concentrations of phenolics in the pot experiment, but the concentrations of foliar phenolics were markedly lower in the soil-grown plants than in the potted plants of the same clones (III).

3.4.3 Different clones had different foliar phenolic profiles

The amounts of most phenolic compounds in *Populus* seem to be genetically determined and different clones had very different foliar phenolic profiles. In the pot experiment, the concentrations of total phenolics were 14 % higher in hybrid aspen than in native aspen (Table 3). Hybrid aspen contained more condensed tannins and flavonol glycosides but no differences between the species in the concentrations of total salicylates were found (Table 3). Only neochlorogenic acid and one quercetin derivative were more abundant in native aspen than in hybrid aspen (Table 3).

In hybrid aspen, the ozone-sensitive clones had higher concentrations of salicylates, whereas the ozone-tolerant group contained more condensed tannins and catechins (II). In total, the ozone-sensitive group contained 20 % more phenolics than the tolerant group.

3.4.4 High amounts of condensed tannins were negatively correlated with growth

High concentrations of condensed tannins were associated with both ozone tolerance and poor growth (II, III). Except for a kaempferol derivative which was found in high concentrations in the ozone-tolerant Clones 1 and 14, the bulk of flavonol glycosides were associated with the ozone-sensitive group (II). High concentrations of salicylates were found in

the ozone-sensitive clones with high growth rates (Clones 280 and 218; II). When the two native aspen clones were compared, Clone 147 which was more susceptible to *Venturia* infection contained more salicylates, whereas the more disease-resistant Clone 31 contained more condensed tannins (data not shown) indicating no role of salicylates in mitigating the oxidative stress, whether caused by ozone or pathogen attack. When the foliar phenolics and *Venturia* infection were studied by PCA, condensed tannins and chlorogenic acid correlated negatively with *Venturia* infection associated with Clone 147, which was characterized with high concentrations of neochlorogenic acid and a quercetin derivative (Fig. 7).

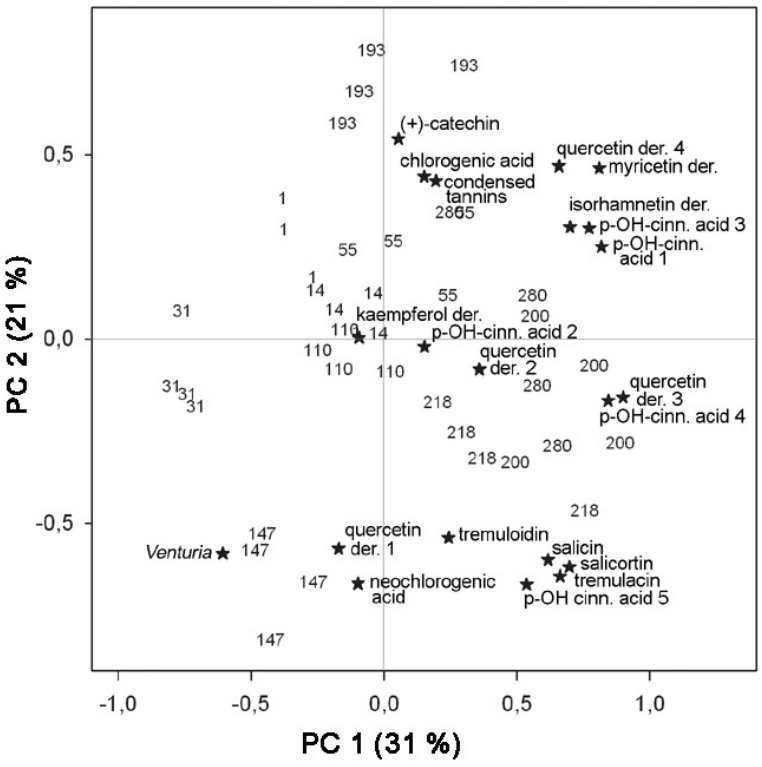

Fig. 7 PCA biplot showing the loading plot of foliar phenolic compounds and *Venturia* infection (star symbols) superimposed on the score plot of hybrid aspen and native aspen clones. The variances explained by principal components 1 and 2 are shown in parenthesis.

3.5 Chronic ozone exposure did not affect the competitive ability of two hybrid aspen clones

Hybrid aspen Clones 55 and 110 had been tentatively determined to be ozone-tolerant and ozone-sensitive, respectively, in short-term experiments (Oksanen et al. 2001; I). However, we found no effects of elevated ozone on either clone during the third growing season under ozone exposure indicating that both clones were relatively insensitive to ozone (III). Additionally, when the data from the pot experiment was reanalyzed, no differences between the clones in their ozone responses were found (III; Fig. 9). Instead, differences between the two clones were reported in almost all variables measured, and Clone 110 was outcompeting Clone 55 when growing in soil (III). There was variation among the potted hybrid aspen clones in their responses to nitrogen enhancement based on growth data: Clones 1, 55 and 218 showed less nitrogen-induced enhancement of growth compared to the other clones (data not shown). This suggests that other resources than nitrogen restricted the growth of Clone 55. An excess of Rubisco can accumulate in response to a high N supply (Millard et al. 2007), and in the potted trees, high-N treatment increased the amount of Rubisco on average by 62 % in Clone 55, whereas the high-N trees of Clone 110 had 24 % more Rubisco than the low-N trees (data not shown). The photosynthetic rate was lower in Clone 55 than in Clone 110, indicating that an inactive pool of Rubisco may have acted as a nitrogen storage protein in Clone 55. Elevated ozone did not affect the competitive ability of the clones, but Clone 110 was better able to exploit the ample soil resources when growing in soil, outperforming Clone 55 in competition for light and nutrients (III). In addition, the bud opening in the spring of Clone 55 was slower than that of Clone 110, reducing, for its part, the growth of Clone 55 (Freiwald et al. 2008).

Elevated ozone seemed to improve the winter hardening of the soil-grown Clone 55 (Chapter 3.1). We did not study the timing of bud set in our experiments, but elevated ozone delayed bud opening in the spring in both of the soil-grown hybrid aspen clones (Freiwald et al. 2008). Even though we found no evidence of ozone-induced accelerated senescence in the potted trees, elevated ozone may have resulted in earlier bud set in the autumn, leading to earlier growth cessation, and thus less biomass allocation, but better winter hardening in Clone 55.

Table 3. Mean concentrations (mg g^{-1} dry mass) and standard errors (SE) of secondary compounds of pooled hybrid aspen and aspen clones (*n*=4) in ambient and elevated ozone concentrations (with nitrogen effects pooled) at the end of the second growing season 2003. *P* values are from mixed model ANOVA, with ozone, nitrogen and species as fixed factors, and field as a random factor (non-significant main effects and interactions of nitrogen are not shown)

	Hybrid aspen				Native aspen				Main effects and interactions		
	Control		O_3		Control		O_3		O_3	species	O_3 * species
	mg g^{-1}	SE	mg g^{-1}	SE	mg g^{-1}	SE	mg g^{-1}	SE	*P* values		
Condensed tannins	107,55	5,67	122,39	5,41	90,73	10,68	106,99	9,58	**0,035**	**0,020**	0,981
(+)-catechin	16,03	1,06	19,43	1,52	17,19	2,32	16,67	2,85	0,273	0,505	0,109
Total tannins and catechins	*123,58*	*5,88*	*141,82*	*5,71*	*107,92*	*12,93*	*123,66*	*12,13*	***0,024***	***0,025***	*0,773*
Neochlorogenic acid	2,36	0,21	2,32	0,20	2,98	0,79	3,63	1,15	0,193	**<0,001**	0,152
Chlorogenic acid	4,97	0,59	6,33	0,79	5,29	0,84	5,72	0,81	0,123	0,748	0,431
p-OH-cinn. acid der. 1	0,48	0,04	0,50	0,03	0,16	0,04	0,21	0,06	0,294	**<0,001**	0,619
p-OH-cinn. acid der. 2	0,58	0,08	0,61	0,06	0,57	0,04	0,72	0,06	0,343	0,606	0,557
p-OH-cinn. acid der. 3	0,07	0,00	0,07	0,01	0,03	0,00	0,04	0,01	0,587	**<0,001**	0,840
p-OH-cinn. acid der. 4	0,18	0,02	0,17	0,02	0,09	0,02	0,10	0,02	0,936	**<0,001**	0,658
p-OH-cinn. acid der. 5	0,09	0,02	0,07	0,01	0,09	0,03	0,08	0,03	0,113	0,767	0,831
Total phenolic acids	*8,73*	*0,66*	*10,09*	*0,80*	*9,22*	*0,17*	*10,50*	*0,58*	***0,049***	*0,530*	*0,976*
Myricetin der.	2,39	0,12	2,49	0,12	0,42	0,05	0,44	0,05	0,777	**<0,001**	0,718
Quercetin der. 1	0,59	0,07	0,61	0,07	1,48	0,12	1,57	0,07	0,394	**<0,001**	0,566
Quercetin der. 2	4,63	0,19	4,93	0,22	4,86	0,30	5,33	0,58	0,187	0,218	0,759
Quercetin der. 3	0,25	0,02	0,22	0,01	0,12	0,02	0,15	0,03	0,952	**<0,001**	0,087
Quercetin der. 4	0,09	0,01	0,10	0,02	0,01	0,00	0,02	0,00	0,580	**<0,001**	0,863
Kaempferol der.	3,98	0,36	3,89	0,27	3,36	0,19	3,35	0,15	0,810	**0,035**	0,844
Isorhamnetin der.	0,42	0,04	0,47	0,05	0,08	0,05	0,09	0,06	0,358	**<0,001**	0,591
Total flavonol glycosides	*12,35*	*0,50*	*12,71*	*0,37*	*10,33*	*0,38*	*10,95*	*0,79*	*0,487*	***0,001***	*0,822*
Salicin	2,46	0,24	2,17	0,18	2,08	0,36	1,86	0,24	0,151	**0,050**	0,814
Salicortin	22,29	4,25	17,62	3,09	15,59	4,31	13,76	2,15	0,327	0,123	0,647
Tremuloidin	1,07	0,14	1,00	0,13	1,28	0,17	0,99	0,15	0,335	0,584	0,504
Tremulacin	29,65	5,74	24,08	4,49	23,30	7,73	20,28	4,50	0,376	0,268	0,786
Total salicylates	*55,48*	*10,22*	*44,87*	*7,74*	*42,25*	*12,28*	*36,89*	*6,65*	*0,343*	*0,189*	*0,748*
Total phenolics	*200,14*	*12,15*	*209,48*	*9,13*	169,73	2,45	182,01	7,63	*0,283*	***0,009***	*0,879*

4. DISCUSSION

4.1 Visible leaf injuries as determinants of ozone sensitivity

Visible leaf injuries are often used as evidence of adverse ozone effects, and ozone-sensitive species can be used for biomonitoring of background ozone concentrations and other conditions causing plant stress (Smith et al. 2003, Klumpp et al. 2006*b*). However, visible leaf injuries are not often correlated with reductions in biomass (e.g. Yamaji et al. 2003, Timonen et al. 2004, Novak et al. 2008). We did not assess the visible leaf injuries systematically in our studies. Instead, we determined the ozone-sensitivity of the clones based on reductions in growth (I – III) which is reasonable when dealing with a species grown for wood production. We did observe ozone-induced leaf injuries which usually appeared in early September in older leaves with no visible senescence (Fig. 8). Leaf symptoms of *Populus* species appear first as light-green spots between the veins on the upper side of the leaf, and later develop into stippling or necrotic spots (http://www.gva.es/ceam/ICP-forests/htmspecies/populus.htm; Fig. 8). Symptoms increase with ozone dose, i.e. leaf age, which is in agreement with the greater accumulation of H_2O_2 in the older leaves reported in our study (IV). However, symptom development and cell death late in the growing season at the onset of senescence probably had no effect on photosynthesis and biomass yield in our studies.

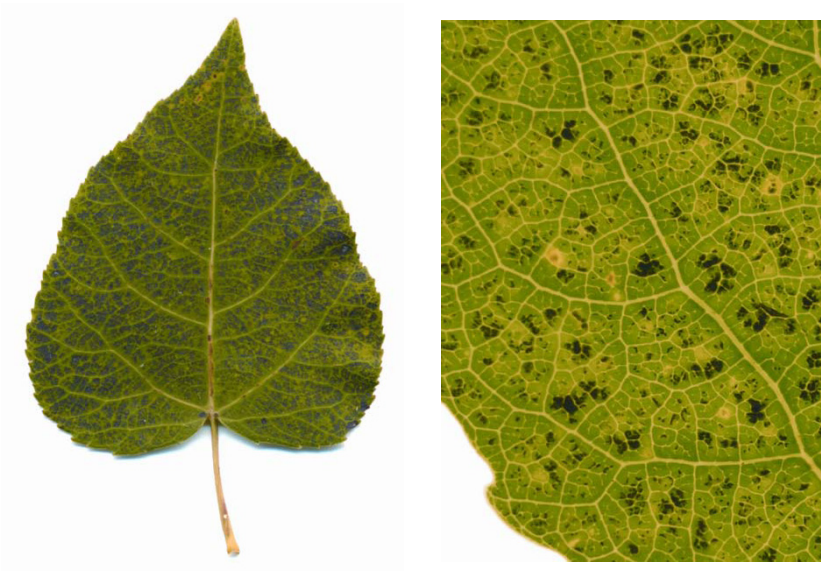

Fig. 8 Ozone-induced leaf injuries in the leaves of Clone 110 in 2003 and in 2005.

4.2 Is *Populus* an ozone-sensitive species?

In a meta-analysis by Wittig et al. (2009), *Populus* species were reported to be among the most ozone-sensitive forest trees with an average reduction in total biomass of 22 %. We did

not find very strong evidence of ozone sensitivity of *Populus* as a species in our short-term experiment with relatively low ozone concentrations, when the clones were pooled (I – III). We did find reduced biomass in ozone-sensitive hybrid aspen clones, especially in the coarse root system, but also enhanced growth in elevated ozone in some ozone-tolerant clones (I, II). The photosynthetic capacity was reduced in ozone-exposed plants early in the growing season but this could not explain the reduced growth in the ozone-sensitive group since no differences in the responses to ozone were found between the two groups (I, II). In long-term chronic ozone exposure, the competitive ability of ozone-sensitive clones may be reduced (Kubiske et al. 2007). Competition with other species may also affect the performance of trees in mixed communities (Novak et al. 2008). The largest ozone-induced decline in standing biomass was reported in a pure aspen community, whereas growth in mixed aspen–birch and aspen–maple communities became less sensitive to tropospheric O_3 over time (King et al. 2005). Also, elevated ozone in combination with elevated CO_2 may change species' importance in mixed forests as reported by Kubiske et al. (2007): elevated O_3 hastened the conversion of aspen-birch stands to birch stands whereas elevated CO_2 delayed it after seven years of elevated ozone exposure (50 ppb; Kubiske et al. 2007).

4.3 Can we find foliar characteristics that determine the ozone sensitivity of a tree?

After finding differences in growth responses to elevated ozone among the clones, we studied the differences in the physiology and foliar characteristics between the sensitivity groups. The sensitive group had more Rubisco and higher photosynthetic capacity, and also more starch and secondary compounds. However, there were no significant differences in the responses of the measured parameters to ozone between the two groups, as shown by the lack of O_3 x group interaction, except for F_v/F_m, which showed a significant decrease in elevated ozone in the sensitive group only (II). The higher proportion of total protein in Rubisco in the sensitive group might indicate less carbon allocation to enzymes related to redox reactions and regeneration of antioxidants, thereby leading to increased sensitivity to ozone (II). In our study, most flavonol glycosides were associated with the ozone-sensitive group and their concentrations were not affected by elevated ozone (II). High concentrations of salicylates were also found in the ozone-sensitive group whereas the tolerant clones allocated carbon to condensed tannins which have been reported to possess good radical-scavenging properties (Hagerman et al. 1998). Of the low-molecular weight flavonoids, chlorogenic acids may play a role in ozone tolerance (II). In agreement with our study, the concentrations of chlorogenic acid were higher in elevated ozone also in birch (Saleem et al. 2001, Peltonen et al. 2005). In our studies, the concentrations of chlorogenic acid and neochlorogenic acid were higher in ozone-exposed plants (II and III, respectively) but they were also negatively correlated with each other. Furthermore, neochlorogenic acid was associated with the ozone-sensitive clones (II, III), indicating that it may not be as effective as an antioxidant as chlorogenic acid, even though no differences in the antioxidative capacity between the isomers of chlorogenic acid were found by Nakatani et al. (2000). The glycosylation of flavonoids may reduce their activity compared to the corresponding aglycones (Rice-Evans et al. 1996), and especially the glycosylation of the C-3 hydroxyl group may result in loss of antioxidant activity (Burda and Oleszek 2001), which might explain the lack of ozone effects on flavonol glycosides in our study. High amounts of condensed tannins were correlated with ozone tolerance, but also resulted in poor growth (II,

III). Condensed tannins and chlorogenic acid seemed to protect the clones also from *Venturia* infection (Fig. 7), which is in line with the evidence of similar ROS signalling in the programmed cell death in ozone stress and the hypersensitive response in pathogen attack (Lamb and Dixon 1997, Kangasjärvi et al. 2005). However, although accumulation of flavonoids under oxidative stress and their effectiveness as antioxidants *in vitro* have been demonstrated, the importance of flavonoids as antioxidants *in planta* is still under debate (Hernández et al. 2009)

It has been proposed that when assessing the ozone sensitivity of plants, both the O_3 flux through stomata and the biochemical defences should be taken into account (Tausz et al. 2007). Ozone-induced injury is suggested to occur when the costs of synthesizing antioxidative defence compounds exceed the supply of photosynthate, letting the ozone-induced ROS escape the first line of defence, reach the plasmalemma and initiate stress signalling pathways (Wieser and Matyssek 2007). We found more H_2O_2 accumulation in the ozone-sensitive aspen clone than in the ozone-tolerant clones (IV), in agreement with Di Baccio et al. (2008) who found higher stomatal conductance and H_2O_2 accumulation and more pronounced membrane injury in an ozone-sensitive poplar clone than in a tolerant clone. In the tolerant clones of our study, H_2O_2 accumulation was found only on the outer surface of cell walls, indicating sufficient detoxification of ozone, whereas H_2O_2 accumulation was found next to the plasma membranes and extending to the cytoplasm and chloroplasts in the ozone-sensitive Clone 259 (IV). Also, the number of peroxisomes and transcripts of catalase were increased and the mesophyll cell walls were thicker in ozone-exposed trees only in the tolerant clones indicating effective defence against ozone-induced oxidative stress (IV).

4.4 Interactions of ozone and nitrogen

When considering the stress tolerance of a species in the changing climate, interactions with other factors have to be taken into account: ozone-induced reductions in the photosynthetic capacity or growth may be ameliorated by e.g. nitrogen fertilization (I) or elevated CO_2 concentrations (Noormets et al. 2001). In our pot experiment, nitrogen fertilization (140 kg N ha^{-1} yr^{-1} in the second growing season) resulted in surprisingly low foliar nitrogen concentrations (I) compared to the non-fertilized soil-grown trees (III). However, nitrogen amendment counteracted the minor adverse effects of elevated ozone on growth in the pot experiment (I, II). Nitrogen limitation is widespread and constrains net primary productivity in most ecosystems (LeBauer and Treseder 2008) and it has been argued that the net carbon sequestration by temperate and boreal forests is driven by anthropogenic nitrogen deposition (Magnani et al. 2007). In Scandinavia, the low atmospheric nitrogen deposition (2 – 4 kg N ha^{-1} yr^{-1}; Syri et al. 2004) does not exceed the critical loads of N for boreal forests (Nordin et al. 2005) but may have a fertilizing effect on the growth of trees. In short rotation poplar plantations, nitrogen fertilization regimes of up to 290 kg N ha^{-1} yr^{-1} may be used (Lagomarsino et al. 2008), even though doses of 120 kg N $ha^{-1}yr^{-1}$ are reported to be sufficient (Coleman et al. 2004). Excessive N fertilization may also lead to N leaching and contamination of surface and ground waters (Lee and Jose 2005). To conclude, the atmospheric N deposition may counteract the effects of ozone in native aspen stands, and reasonable nitrogen fertilization of hybrid aspen plantations could compensate for the decrease in growth caused by elevated ozone (I).

4.5 How to select for suitable cloning material?

For large-scale clonal plantations the stress tolerance or the responsiveness to fertilization or elevated CO_2 concentrations of the clones should be assessed beforehand. We found both high-yielding and low-yielding clones in both ozone-tolerant and ozone-sensitive groups in our experiments, indicating that the ozone-induced reduction of biomass must be weighed against the general growth potential of the clone (Fig. 4 vs. Fig. 9). High concentrations of condensed tannins were positively correlated with ozone tolerance and pathogen resistance, but negatively correlated with growth (II, III). Ozone-tolerant clones with low concentrations of salicylates (II) may also be susceptible to insect herbivores (Hwang and Lindroth 1997). Aspen shoot blight (*Venturia tremulae*) is a common disease that affects especially young aspen trees, and susceptible clones can lose all their young foliage in the spring (Newcombe 1996). Native aspen was found to be more susceptible to *Venturia* blight than hybrid aspen (Freiwald 2008), and when analyzed by PCA, the most severely infected native aspen clone 147 was associated with high concentrations of neochlorogenic acid and low concentrations of condensed tannins, whereas salicylate concentrations did not affect *Venturia* susceptibility (Fig. 7). Thus, selection for clonal material has to be based on a combination of traits and may prove difficult since e.g. selection for high fibre count for papermaking or high tannin concentration for ozone tolerance may result in a reduction in yield.

Trembling aspen is considered to be exceptionally tolerant to a wide range of natural stresses, especially compared to other *Populus* species (Lieffers et al. 2001). The hybrid aspen clones used in our study (I- III) were mostly crossings of Finnish female trees from latitudes of 60° to 62°, and Canadian male trees from latitudes 43° to 54°. The more southern origin of the *P. tremuloides* parents may account for the longer growing season of hybrid aspen compared to native aspen and result in the hybrid vigour of the hybrids (Yu et al. 2001a), even though the late bud set of the hybrids in the autumn may also result in poor winter hardening (Gang et al. 2007, I). Also, natural selection for ozone-tolerant genotypes may have already occurred in the aspen populations of North America (Berrang et al. 1989), where ozone concentrations are higher than in Finland. This suggests that ozone tolerance of sensitive northern populations could be improved by crossings with populations, where natural selection for ozone tolerance has already occurred.

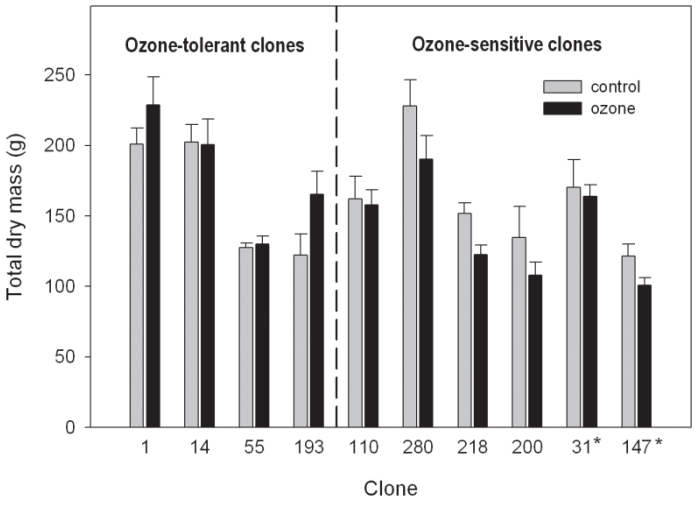

Fig. 9 The effects of ozone (with N levels pooled) on the total dry mass of potted three-year old ozone-sensitive and ozone-tolerant hybrid aspen saplings after the second growing season (I). The native aspen clones are marked with an asterisk.

4.6 Methodological considerations and limitations

- In the pot experiment we had only two native aspen clones as compared to the eight hybrid aspen clones. From the practical point of view, hybrid aspen clones were more interesting because they are favoured in commercial production, but the responses of natural native aspen clones (since aspen propagates mostly clonally in nature also) in the changing climate would have deserved more attention.
- The soil-grown saplings were planted 50 cm apart to study the effects of competition on the two hybrid aspen clones (III). In our experiment the competition for light and nutrients was extremely hard, because in normal forestry the planting densities of aspen range from 1200 trees per ha (2-3 m spacing) in the production of pulpwood (Tullus et al. 2007) to 5000 – 10000 trees per ha in very intense short rotation forestry with three year rotations (Liberloo et al. 2006).
- The foliar nitrogen concentrations of the potted trees were low (1.6 – 1.8 % N; I) compared to the non-fertilized soil-grown trees (3.3 % N; III) despite the copious amounts of ammonium nitrate applied. Especially during the second year of the experiment the potted trees were probably suffering from heat, drought and root restriction, and could not fully benefit from the nitrogen fertilization. The potted trees also had higher concentrations of foliar phenolics than the soil-grown trees of the same clones, indicating a shift in carbon allocation towards secondary compounds and a likely confounding effect of nutrient limitation and drought on the ozone responses of the potted saplings.
- The clones selected for planting in soil (III) were found to be intermediate in their ozone sensitivity (Fig. 9), even though in a previous experiment Clone 55 was reported to be more ozone-tolerant and Clone 110 more ozone-sensitive (Oksanen et al. 2001), and they were also grouped accordingly in the pot experiment (I). A better understanding of the mechanisms of ozone-induced responses could have been achieved by selecting more extreme clones, e.g. two fast-growing clones with contrasting ozone sensitivities (Clones 1 and 280, for example).
- We did not measure stomatal conductance of the potted trees during the second growing season, which might have been essential for explaining the ozone sensitivity of the clones considering the central role of stomatal conductance in the flux-based models of ozone effects on vegetation. Ozone has been reported to affect stomata directly, by inhibiting stomatal opening or by reducing the stomatal aperture (Torsethaugen et al. 1999, Zheng et al. 2002), and ozone sensitivity groups could possibly have been determined by stomatal openness. However, stomatal conductance is not always affected by ozone or related to ozone sensitivity (Woo and Hinckley 2005, Grulke et al. 2007).
- As the physiology of seedlings and mature trees may be very different, short-time studies with very young trees do not give a realistic picture of the ozone impacts on forest productivity in longer term (Karnosky et al. 2007). Extrapolating ozone sensitivity from young to mature trees may lead to substantial overestimations (Kolb and Matyssek 2001).

5. MAIN RESULTS AND CONCLUSIONS

We defined ozone sensitivity in our experiments as reduced growth in the presence of elevated ozone. Tree growth is mainly affected by the production of assimilates (photosynthesis) and by the allocation of the assimilates to growth or production of secondary compounds (defence). Growth is also affected by turgor pressure (water relations through the action of stomata), hormone levels, nutrient uptake etc., all of which can be affected by ozone and nitrogen. In our experiments, we mainly concentrated on the impacts of ozone and nitrogen on carbon assimilation and allocation.

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- Hybrid aspen was superior in growth compared to native aspen when eight hybrid aspen clones were compared to two native aspen clones. Native aspen suffered from severe *Venturia tremulae* infection, whereas hybrid aspen showed higher mortality and shoot-tip dieback in the winter following the planting.
- We found small (statistically non-significant) growth reductions in native aspen and hybrid aspen in elevated ozone when the clones were pooled. Nitrogen amendment counteracted the adverse ozone effects and significantly increased growth through higher photosynthetic capacity. However, when the clones were placed into two groups based on the extent of growth reduction in elevated ozone, we found significant growth reductions in the ozone-sensitive group, whereas the ozone-tolerant group had no effect or increased growth in elevated ozone. Both native aspen clones were grouped among the ozone-sensitive clones.
- We found both ozone-sensitive and ozone-tolerant clones among the good growers, which does not support the hypothesis that fast-growing species/clones would be more sensitive to ozone stress. Also, no differences in the ozone responses between the two native aspen clones were found even though Clone 31 was a significantly better grower than Clone 147. Accordingly, enhanced soil nitrogen levels promoting the growth of the trees did not increase the ozone sensitivity of the trees.
- We found no changes in the competitive ability of two hybrid aspen clones after three years of elevated ozone exposure. Also, no differences in the ozone responses of the clones were found when potted trees were compared to soil-grown trees of the same clones.
- Nitrogen amendment did not affect the concentrations of phenolics in hybrid aspen. However, the higher resource availability resulted in lower concentrations of phenolics of the soil-grown trees compared to the potted trees of the same clones.
- Ozone induced the synthesis of chlorogenic acids, and to some extent catechins and condensed tannins. Condensed tannins but not salicylates or flavonol glycosides may play a role in ozone tolerance, since the ozone-tolerant group had higher concentrations of condensed tannins and catechins, whereas the ozone-sensitive clones allocated carbon to salicylates.

- Ozone-sensitive clones had higher photosynthetic capacity and more Rubisco and phenolics. However, differences in assimilate accumulation and allocation between ozone-sensitive and ozone-tolerant groups did not explain the decreased growth of the ozone-sensitive clones in elevated ozone, since we found no differences in the responses to elevated ozone between the sensitivity groups except for F_v/F_m, which was significantly reduced only in the ozone-sensitive group. Direct effects of ozone on growth rate or differences in stomatal conductance and ozone uptake may be involved.
- We found H_2O_2 accumulation next to the plasma membrane and extending to the cytoplasm and chloroplasts in an ozone-sensitive aspen clone, whereas in the tolerant clones, H_2O_2 accumulation was found only on the outer surface of cell walls, indicating sufficient detoxification of ozone before entry into the leaf cell. The number of peroxisomes and transcripts of catalase were increased and the mesophyll cell walls were thicker in ozone-exposed trees only in the tolerant clones indicating effective defense against ozone-induced oxidative stress.

To conclude, single variables predicting plant responses to ozone are difficult to find since different physiological or biochemical attributes vary independently within an individual plant resulting in varying degrees of ozone sensitivity or tolerance. The high intraspecific genetic variation in aspen and hybrid aspen suggests that natural aspen populations will adapt to changes in the environment through selection of tolerant genotypes. However, for large-scale clonal plantations the stress tolerance or responsiveness to fertilization of the selected clones should be assessed beforehand and weighed against the desirable wood properties or growth potential of the clones.

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