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Modification of the Growth, Photosynthesis and Leaf Structure of Silver Birch by Elevated CO_2 and O_3

Doctoral dissertation

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ABSTRACT

Introduction As a result of human activities, the concentrations of atmospheric carbon dioxide (CO_2) and tropospheric ozone (O_3) have increased over the past few decades. The concentrations are expected to increase by 0.5-2% per year, which may result in major changes on the growth conditions of plants in future. Since the photosynthesis of the C_3 plants is limited by the current atmospheric CO_2 concentration, it is expected that increases in CO_2 concentration will have positive impact on plant growth. Ozone, on the other hand, is a highly phytotoxic pollutant, known to cause impaired growth and leaf injuries. Owing to the opposite effects of CO_2 and O_3, it has been questioned whether the stimulative effects of elevated CO_2 on growth and photosynthesis can compensate for the negative effects of O_3. The aims of the study were to examine: whether young silver birch trees benefit from increasing CO_2 in the atmosphere, whether elevated O_3 causes damage and growth losses, and whether CO_2 can compensate for the negative effects of O_3, if such losses exist.

Materials and methods Seasonal changes and differences in physiological characteristics between two European silver birch (*Betula pendula* Roth) clones were determined during the growing season of 1998. In 1999-2001, initially 7-year-old trees were exposed to the elevated concentrations of CO_2 and O_3, singly and in combination in open-top chambers, and the effects of elevated CO_2 and O_3 on growth, leaf structure, photosynthesis and related biochemistry were studied.

Results Elevated CO_2 increased photosynthesis and the enhancement was sustained over the three-year experiment. However, elevated CO_2 also resulted in an accumulation of starch in the leaves, followed by reduction in the amount and activity of Rubisco and N concentration, leading to downregulation of photosynthesis. Elevated CO_2 also affected the structure of the leaves by increasing the size of chloroplasts and starch grains, the number of mitochondria and hemicellulose concentrations and by decreasing SLA, α-cellulose, total lignin and nutrient concentrations. Growth was significantly increased by elevated CO_2 only in clone 80. The negative effect of elevated O_3 was small and found mainly in clone 4. Although O_3 had no effect on gas exchange, it reduced the apparent quantum yield of photosystem 2 photochemistry in light and photosynthetic leaf area by inducing earlier leaf abscission in the autumn and by reducing the mean leaf size and branching. Elevated O_3 decreased the root biomass and tended to decrease the total biomass in clone 4. Further, elevated O_3 increased the number of peroxisomes and mitochondria, and decreased the contents of Mn, Zn, Cu, hemicellulose and uronic acids. In most of the parameters related to the growth, photosynthesis and structure of the leaves, the negative effect of O_3 was smaller or absent in elevated CO_2 than in ambient CO_2. The counteracting effect of CO_2 may be due to decreased stomatal conductance or increased photosynthetic rate followed by increased activity of the enzymatic detoxification in elevated CO_2.

Conclusions The growth and physiology of the silver birch clones were modified by elevated CO_2 and O_3. In general, elevated CO_2 counteracted the negative effects of O_3. Clone 4 was not able to utilize the increased CO_2 concentration and was also shown to be more sensitive to O_3 than clone 80. Thus, the genetic differences modify the responses of the clones to the environmental changes. Owing to the long life span of the trees, this three-year study with young trees provides only suggestive information concerning the responses of trees to climate change. There is, therefore, a continuing need for long-term studies regarding the responses of forest trees to increasing CO_2 and O_3.

Universal Decimal Classification: 504.054, 546.264, 546.214, 582.632.1, 581.2, 632.151
CAB Thesaurus: forest trees; *Betula pendula*; carbon dioxide; ozone; growth; biomass; photosynthesis; photosystem II; chlorophyll; gas exchange; leaves; abscission; leaf area; cell ultrastructure; organelles; starch granules; chloroplasts; mitochondria; nutrients; nitrogen

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Kuopio, October 2004
Johanna Riikonen

ABBREVIATIONS

AOS	active oxygen species
AOT40	accumulated exposure over a threshold of 40 ppb
CC	chamber control
CFC	chlorofluorocarbon
CH_4	methane
C_i	intercellular CO_2 concentration
CO_2	carbon dioxide
CTC	closed-top chamber
EC	elevated CO_2
EC+EO	elevated CO_2 and O_3
EO	elevated O_3
FACE	free-air CO_2 enrichment
F_m	maximal fluorescence level
F_m'	maximal fluorescence at steady-state
F_0	minimal fluorescence level
F_s	steady state fluorescence
F_v	variable fluorescence (F_m-F_0)
F_v/F_m	maximum photochemical yield of PSII ((F_m-F_0)/F_m)
F_v'/F_m'	apparent quantum yield of PSII photochemistry in light ((F_m'-F_s)/F_m')
g_s	stomatal conductance
H_2O_2	hydrogen peroxide
LAR	leaf area ratio
LMR	leaf mass ratio
mRNA	messenger RNA
NO_x	nitrogen oxides
NPQ	non-photochemical fluorescence quenching
O_3	ozone
OC	outside control
OTC	open-top chamber
P_n	net CO_2 exchange (net photosynthesis)
ppb	parts per billion, nl l^{-1}
PSII	photosystem 2
RH	relative humidity
Rubisco	ribulose-1,5-bisphosphate carboxylase-oxygenase
SLA	specific leaf area (leaf area/leaf dry mass)
Transp	transpiration
WUE	water use efficiency (P_n/transp)

LIST OF ORIGINAL PAPERS

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

Chapter 2 Riikonen J, Oksanen E, Peltonen P, Holopainen T, Vapaavuori E (2003) Seasonal variation in physiological characteristics of two silver birch clones in the field. Canadian Journal of Forest Research 33: 2164-2176

Chapter 3 Riikonen J, Lindsberg M-M, Holopainen T, Oksanen E, Lappi J, Peltonen P, Vapaavuori E (2004) Silver birch and climate change: Variable growth and carbon allocation responses to elevated concentrations of carbon dioxide and ozone. Tree Physiology 24: 1227-1237

Chapter 4 Riikonen J, Holopainen T, Oksanen E, Vapaavuori E. Leaf photosynthetic characteristics of silver birch during three years of exposure to elevated CO_2 and O_3 in the field. Tree Physiology, in press.

Chapter 5 Oksanen E, Riikonen J, Kaakinen S, Holopainen T, Vapaavuori E. Structural characteristics and chemical composition of birch (*Betula pendula*) leaves are modified by increasing CO_2 and ozone. Manuscript.

CONTENTS

Chapter 1

General introduction

GENERAL INTRODUCTION

1.1 Climate change and forests

Since pre-industrial times the impact of human activities on atmospheric composition has been enormous. Mainly as a result of the burning of fossil fuels the concentrations of atmospheric CO_2 and tropospheric O_3 have increased by approximately 30% (IPCC 2001) and are expected to increase by 0.5-2% per year (IPCC 2001, Vingarzan 2004). Together with other greenhouse gases (e.g. CH_4 and CFCs) CO_2 and O_3 are contributing to climate change, including, for example, increases in temperature and changes in precipitation (IPCC 2001).

Photosynthesis is currently limited by the atmospheric CO_2 concentration and hence it is expected that the increase in CO_2 concentration will affect the growth and photosynthesis of the plants positively. Thus, it is of great importance whether the capacity of forest ecosystems to store part of the excess carbon in the biomass also increases (Saxe *et al.* 1998, Norby *et al.* 1999).

Ozone is a secondary pollutant that is formed photochemically in the presence of NO_x and hydrocarbons (Fowler *et al.* 1998). Ozone concentration varies widely in space and time. The highest O_3 concentrations are typically found in polluted urban regions in summer, but the long distance transport of the precursors and small traffic emissions of NO_x in rural areas can also cause large-scale production of O_3 far from the source regions (IPCC 2001). Tropospheric O_3 is considered to be the most harmful air pollutant in Europe (Fuhrer *et al.* 1997, Matyssek & Innes 1999). According to Fowler *et al.* (1999), around 30% of the world's forests are currently growing under damaging O_3 concentrations. In Finland the background O_3 concentrations are relatively low (<30 ppb), although during occasional O_3 episodes the O_3 concentration may reach 100 ppb (Laurila *et al.* 1999). However, forests in Scandinavia may be more susceptible to O_3 damage than forests in southern Europe. As a result of humid conditions, the stomatal conductance is high, resulting in higher O_3 uptake into the leaves. The short summer nights in Nordic countries may lead to an insufficient period of recovery from O_3 injury, since the length of the night may be important for repair processes related to dark respiration (De Temmerman *et al.* 2002).

In their natural environment, trees are exposed to many stress factors functioning at the same time. In the future, trees will be exposed simultaneously to elevated CO_2 and O_3. In response to the opposite effects of CO_2 and O_3, it has been questioned whether the positive effects of elevated CO_2 can compensate for the negative effects of O_3. However, the existing data is conflicting and more studies on this subject will be needed before any general conclusions can be made (Matyssek & Sandermann 2003).

Silver birch (*Betula pendula* Roth) covers large and ecologically diverse areas across the Northern Hemisphere and is a common and economically important deciduous tree species in Finland. According to Kellomäki *et al.* (1996), in altered environmental conditions the tree species with continuous growth pattern, such as birch, may be favoured over species with predetermined growth (e.g. conifers). Thus, the proportion of birch in the total forest area may increase, especially in Northern Scandinavia. However, silver birch is also known to be sensitive to O_3 (Matyssek 2001, Oksanen 2003) which may modify the competitive status of sensitive species and genotypes, as has been suggested on the basis of a free-air CO_2 enrichment experiment (FACE) with aspen clones (McDonald *et al.* 2002).

1.2 The impact of increasing CO_2 on tree growth and physiology

1.2.1 Carbon dioxide experiments

Despite numerous studies with elevated CO_2, it is extremely difficult to estimate the magnitude of the long-term CO_2 response, because, for example, of the long life-span of trees, and different experimental designs and exposure times (Saxe *et al.* 1998, Norby *et al.* 1999, Gielen & Ceulemans 2001). Most previous CO_2 experiments have been short-term growth chamber studies with seedlings. However, there are several methods available for also exposing plants to elevated CO_2 and to other air pollutants under field conditions. The most frequently used CO_2 exposure methods are open-top (OTC) and closed-top chambers (CTC), the branch-bag technique, free-air CO_2 enrichment (FACE), and natural CO_2 vents (Saxe *et al.* 1998, Norby *et al.* 1999). Inside OTCs and CTCs large trees can be grown on their natural growth site under conditions that can be maintained close to the ambient field conditions, CTCs being more expensive to construct than OTCs (Saxe *et al.* 1998, Gielen & Ceulemans 2001). In the case of the branch-bag technique, individual branches are exposed to treatments. This design is based on the assumption of the autonomy of branches in carbon metabolism (Sprugel *et al.* 1991). In the FACE approach and in natural CO_2 vents the trees can be exposed to CO_2 and other gases without the use of any type of enclosure. Each method has its advantages and disadvantages (Saxe *et al.* 1998, Norby *et al.* 1999). The advantages of OTCs are the relatively low costs of fumigation and maintenance, and the comparability of the growth conditions to the ambient field conditions. The disadvantage of OTCs is the changes in the microclimate caused by the chambers, e.g. altered temperature, light and humidity inside the chamber compared to the ambient air (Saxe *et al.* 1998). In the case of FACE and with natural CO_2 vents approaches, the responses of forest stands can be studied with no chamber effects. However, because of the high costs incurred in constructing and running the FACE experiments and the difficulty involved in finding adequate controls and replication for the natural CO_2 vent studies, only a few FACE and natural CO_2 vent studies have been or are currently being undertaken (Saxe *et al.* 1998).

Since the field exposure techniques became feasible, it has been possible to study whether the CO_2 responses found earlier in growth chamber studies with seedlings can also be found in long-term field experiments with saplings or with mature trees (Saxe *et al.* 1998, Norby *et al.* 1999, Gielen & Ceulemans 2001). According to Norby *et al.* (1999), the information gained from the short-term seedling experiments may be qualitatively correct but quantitatively inadequate to explain forest responses to elevated CO_2, since only the initial, exponential growth phase has been included in the experiments. The effect of elevated CO_2 on silver birch is not so well known, since only one field experiment (Rey & Jarvis 1997, 1998) and a few growth chamber studies (Petterson & McDonald 1992, Petterson *et al.* 1993, Juurola *et al.* 2003) on silver birch saplings have been conducted prior to this experiment.

1.2.2 Photosynthesis is modified by CO_2

The photosynthetic responses of forest trees to elevated CO_2 have been intensively studied and, according to review articles, the average enhancement in elevated CO_2 is around 50% for C_3 plants grown in the field in controlled environment or in OTCs (Saxe *et al.* 1998, Medlyn *et al.* 1999). In FACE experiments, the enhancement was around 26% (Nowak *et al.* 2004). The enhancement is mainly due to the kinetic properties of ribulose-1,5-bisphosphate carboxylase/-

oxygenase (Rubisco, CO_2 fixing enzyme). Since molecular oxygen is a competitive inhibitor in the carboxylation of Rubisco, in an elevated CO_2 concentration the ratio between the carboxylation and oxygenation of Rubisco increases, leading to decreased photorespiration and enhanced net photosynthesis (Drake *et al.* 1997).

However, in several experiments acclimation to an elevated CO_2 concentration has been found and observed as a downregulation of photosynthesis. The downregulation is seen in the form of lower photosynthetic rates in plants grown in elevated CO_2 than in plants grown in an ambient CO_2 concentration, when photosynthesis is measured in current ambient CO_2 (360 ppm). A downregulation of photosynthesis is often found under conditions where the sink capacity and the supply of nutrients and water are limited (Drake *et al.* 1997). In long-term field experiments downregulation has been both found (the average degree of downregulation around 10%) (Wang *et al.* 1998, Medlyn *et al.* 1999, Tissue *et al.* 1999, Griffin *et al.* 2000) and not found (Norby *et al.* 1999, Noormets *et al.* 2001, Sholtis *et al.* 2004). However, despite the acclimation processes related to the downregulation of photosynthesis, the photosynthetic capacity is usually higher in elevated CO_2 than in ambient CO_2 concentrations (Drake *et al.* 1997, Medlyn *et al.* 1999).

Acclimation processes involve a redistribution of resources towards the most limiting processes, resulting in optimal growth in the prevailing environmental conditions (Drake *et al.* 1997). In elevated CO_2, less Rubisco is required for a certain level of photosynthesis than in ambient CO_2. Since Rubisco may constitute 25% of leaf N, the released N may be reallocated to other plant parts where it can be used more efficiently (Drake *et al.* 1997, Cotrufo *et al.* 1998). This can often be seen in the form of a decreasing N concentration in the leaves in elevated CO_2 (Rey & Jarvis 1998; Pettersson & McDonald 1992) and may be considered as increased N use efficiency in elevated CO_2 (Drake *et al.* 1997). Alternatively, the reduction in N concentration in the leaves may simply be caused by increased growth, leading to a dilution of N in the leaves (Stitt & Krapp 1999).

Another process related to the downregulation of photosynthesis is an increased supply of carbohydrates in elevated CO_2. The accumulation of carbohydrates in the leaves reflects discrepancies between supply and demand of photosynthates, which is accentuated if the sink capacity is lowered, for instance, by an insufficient N supply, low temperature or restricted root growth. This may result in feedback inhibition of the photosynthetic capacity and suppression of several photosynthetic genes (Moore *et al.* 1999).

In addition to the dark reactions of photosynthesis, CO_2 may also affect the light reactions. The chlorophyll concentration of the leaves is often decreased in CO_2 experiments (Rey & Jarvis 1998, Wustman *et al.* 2001) and, in addition, the primary photochemistry of PSII and processes related to electron transport, measured as chlorophyll fluorescence, may be affected. However, the effects of elevated CO_2 on these parameters have not yet been well established (Ceulemans *et al.* 1995, Scarascia-Mugnozza *et al.* 1996, Hymus *et al.* 1999).

The CO_2-induced alterations in the leaf mesophyll structure and cell ultrastructure are important determinates of photosynthetic capacity, although cell ultrastructure has been less intensively studied. In elevated CO_2 the leaves are generally thicker than in ambient air as a result of increased cell size, cell number or the number of the cell layers and increased starch concentration (Pritchard *et al.* 1999, Taylor *et al.* 2001). In poplar and aspen elevated CO_2 increased the spongy mesophyll layer thickness and intercellular

air space (Radoglou & Jarvis 1990, Oksanen *et al.* 2001b). Elevated CO_2 may also modify the leaf cell wall chemistry, leading to alterations in the quality of the leaf tissue and the growth of the plants. Elevated CO_2 is found to alter the concentrations of, for instance, lignin, cellulose and hemicellulose, which, together with changes in the N concentration, can alter the carbon mineralization from the leaves (Poorter *et al.* 1997, Couteaux *et al.* 1999, Kaakinen *et al.* 2004).

1.2.3 Growth is increased by CO_2 under non-limiting growth conditions

In general, elevated CO_2 increases the growth of trees. However, since the growth is strongly affected by the environmental conditions (e.g. tree density, developmental stage of the plants, nutrients, light and water balance) and genetic differences, the magnitude of the long-term CO_2 effect cannot be predicted using existing data (Norby *et al.* 1999, Poorter & Navas 2003). On the basis of field experiments (1-9 growing seasons), the increase in above-ground biomass has been 4-242% (Medlyn *et al.* 2001a). Since most previous CO_2 studies have been conducted on young seedlings in growth chambers or in greenhouses, it has been questioned whether the growth stimulation by CO_2 will be due only to an acceleration of ontogenetic development in the early phase of exposure and hence will not be sustained in a forest after canopy and root closure (Centritto *et al.* 1999). Indeed, in many studies involving different tree species (holm oak, silver birch, sitka spruce, cherry, loblolly pine) the growth stimulation was found only in the early years of the experiments (Rey & Jarvis 1997, Centritto *et al.* 1999, Oren *et al.* 2001). In the long-term studies with trees growing near natural CO_2 vents the growth enhancement was surprisingly small or absent in mature trees, partly as a result of limited water and nutrient availability (Hättenschwiler *et al.* 1997, Tognetti *et al.* 2000). These results are an indication of uncertainty about whether the long-term C storage in wood significantly increases with a rising CO_2 concentration.

The leaf-level changes in photosynthesis are not always correlated with growth responses at tree-level (Wang *et al.* 1998, Noormets *et al.* 2001). In addition to the leaf photosynthetic rate, growth is also affected, for example, by the carbon allocation patterns of a tree, especially the development of the leaf area (Saxe *et al.* 1998, Wang *et al.* 1998, Noormets *et al.* 2001, Sigurdsson *et al.* 2001) and respiration rate (Anekonda *et al.* 1994, Norby *et al.* 1999, Davey *et al.* 2004). Elevated CO_2 generally increases the total leaf area, by increasing the individual leaf size or mean leaf number (Wang *et al.* 1998, Gielen & Ceulemans 2001, Wustman *et al.* 2001). However, according to Norby *et al.* (1999) there may not be a specific stimulatory effect of CO_2 on leaf production. The enhancement of leaf area may simply be connected to the increased size of the trees in elevated CO_2, since the leaf area ratio (LAR, leaf area/above-ground dry mass) is not always increased, at least after canopy closure (Centritto *et al.* 1999, Taylor *et al.* 2001).

The below-ground responses to elevated CO_2 have been studied less than the above-ground processes. According to Kubiske & Godbold (2001), root growth is stimulated by an enhanced carbohydrate supply to roots, although in many experiments the allometric relationship between roots and shoots was unaffected by elevated CO_2 (Curtis & Wang 1998, Norby *et al.* 1999, Kubiske & Godbold 2001). The below-ground responses are essential in determining the whole-plant response to elevated CO_2, since the uptake of nutrient and water must keep pace with the requirements set by the increased growth rate. However, in some experiments the

nutrient concentrations have been lower in elevated CO_2 than in control plants (Poorter *et al.* 1997, Roberntz & Linder 1999). This may be due to growth dilution or inadequate nutrient availability as a result of increased growth in elevated CO_2.

The growth of trees is also determined by the length of the growing season, which is expected to be prolonged by climate change. Research into the effect of elevated CO_2 regarding the timing of leaf abscission has produced contradictory results with earlier leaf abscission in some experiments (Stiling *et al.* 2002) and delayed leaf abscission in others (Tricker *et al.* 2004). According to the recent experiments with maple and aspen, the timing of budbreak was not affected by elevated CO_2 (Karnosky *et al.* 2003, Norby *et al.* 2003).

1.2.4 Stomatal conductance and water relations in elevated CO_2

The CO_2 effects on stomatal conductance and total leaf area may play an important role under conditions in which the water supply is limited. Stomatal conductance is dependent on the stomatal density and the opening responses of stomata to CO_2 (Pritchard *et al.* 1999). In a meta-analysis of 13 field-studies (>1 yr), elevated CO_2 decreased the stomatal conductance by 21% (Medlyn *et al.* 2001b), while in a review article including experiments conducted in field and controlled environments, Norby *et al.* (1999) found no effect or only moderate decreases. According to Woodward *et al.* (1987), in some temperate tree species the stomatal number has decreased since pre-industrial times, but the later studies have provided contradictory results (Drake *et al.* 1997, Saxe *et al.* 1998). Thus, it is believed that stomatal conductance is more often determined by partial stomatal closure than by the stomatal number (Drake *et al.* 1997, Saxe *et al.* 1998).

The increase in water use efficiency (WUE, photosynthesis/transpiration) is a common CO_2 response (Tognetti *et al.* 1998, Gunderson *et al.* 2002) and might improve the drought resistance of trees in the future. However, it is possible that the increase in the leaf-level WUE might be counteracted by an increase in the leaf area, and thus the whole-tree water use might not be changed (Poorter & Navas 2003).

1.3 Ozone and forest trees

1.3.1 Tropospheric O_3 and silver birch

The European critical levels for O_3 are expressed as the cumulative O_3 exposures using the AOT40 index (i.e. the sum of hourly O_3 concentrations above 40 ppb during daytime hours). Currently, the critical exposure AOT40 for forest trees is 10 ppm.h, accumulated over a six-month period, which has been found to cause significant growth reductions in beech (Fuhrer *et al.* 1997). Recently, it has been suggested that the critical level of 5 ppm.h would be more appropriate to protect the most sensitive species (Karlsson *et al.* 2004). Plant responses to O_3 depend on the concentration and length of the O_3 exposure, on the magnitude of O_3 uptake into the leaves, and also on the efficiency of the defence mechanisms of the plants (Matyssek & Sandermann 2003). Acute O_3 exposure (120-500 ppb for hours) causes the formation of leaf lesions, while chronic O_3 exposure (40-120 ppb for days) may cause alterations in biochemical processes without visible symptoms, even though the accumulating O_3 dose may be similar (Pell *et al.* 1997, Long & Naidu 2002).

After entering the leaf via stomata, O_3 reacts in the apoplast with the components of the cell wall and membranes, forming active oxygen species, AOS (hydroxyl radicals, singlet oxygen, superoxide anion radical, hydrogen peroxide) (Long & Naidu 2002).

In the case of acute exposure to a high O_3 concentration, AOS may induce unregulated cell death by damaging the plasmalemma and thus causing disturbances in the ion balance of the cells. Alternatively, in an acute exposure to lower O_3 concentration, the AOS may induce further oxidative stress, which may lead to changes in the gene expression, followed by programmed cell death that protects the cells near the injury area. In chronic exposure to O_3 AOS may trigger signal transduction pathways (related to ethylene, jasmonate, salisylic acid) leading to alterations in the gene expression, the induction of defence responses and changes in the biochemistry (Pell *et al.* 1997, Wustman *et al.* 2001, Long & Naidu 2002).

The defence responses against oxidative stress in the apoplast and within the cell include enzymatic (e.g. superoxide dismutase, ascorbate peroxidase, glutathione reductase) and non-enzymatic antioxidant molecules (ascorbate, glutathione, α-tocopherol) and secondary metabolites (phenols, flavonoids, carotenoids) (Long & Naidu 2002). The enhanced activity of detoxification and repair in elevated O_3 is indicated by increased dark respiration and altered primary metabolism of the leaves (Coleman *et al.* 1995, Maurer & Matyssek *et al.* 1997).

According to Pääkkönen *et al.* (1995), silver birch is sensitive to O_3, and may be negatively affected at AOT40 exposure below 5 ppm.h. However, the O_3-sensitivity varies, depending on clonal differences, growth conditions and tree age (Pääkkönen *et al.* 1997, 1998, Matyssek 2001, Oksanen 2003). Most of the information on the O_3 effects on silver birch derives from field and chamber studies with seedlings. These studies have revealed the principles of O_3 actions but, as a result of the differences in the physiology of juvenile and adult trees, the information may not be representative for forest sites (Kolb & Matyssek 2001, Matyssek 2001).

1.3.2 Ozone effects on leaf-level photosynthesis and stomatal conductance

Photosynthesis is commonly decreased by O_3 exposure. According to several studies, O_3 is capable of affecting almost all of the processes related to photosynthesis, including both carbon fixation and light-harvesting (Long & Naidu 2002). In general, the first change is a loss of the amount and activity of Rubisco (Oksanen & Saleem 1999, Noormets *et al.* 2001, Yamaji *et al.* 2003). Ozone may increase the protein degradation or decrease the protein synthesis by decreasing the mRNA for the large (rbcL) and small (rbcS) subunits of Rubisco (Pell *et al.* 1997), although the signal transduction from the apoplast to the chloroplasts is not known (Matyssek & Sandermann 2003).

Stomatal conductance is an important factor not only in determining the magnitude of O_3 uptake but also via its effects on the water use efficiency of the trees. The lower photosynthetic rate in elevated O_3 may be caused by the reduced carboxylation efficiency and/or decreased stomatal conductance (Long & Naidu 2002). In general, O_3 reduces the stomatal conductance directly by damaging the permeability of the guard cells. However, recent experiments show that the stomatal limitation of photosynthesis is less important than the processes related to carboxylation efficiency (Dizengremel 2001, Noormets *et al.* 2001). As a consequence, the CO_2 concentration in the intercellular space of the mesophyll may increase and cause stomatal closure (Long & Naidu 2002, Matyssek & Sandermann 2003). Stomatal conductance is found to be reduced in elevated O_3 (Long & Naidu 2002) even though in some experiments the stomatal density was increased simultaneously (Pääkkönen *et al.* 1995, 1998). The increase in the stomatal density may be related to the acclimation process associated with elevated O_3, resulting in a more even O_3 distribution

within the leaf tissue that facilitates more efficient detoxification processes (Pääkkönen *et al.* 1995).

Ozone may also affect the light reactions of photosynthesis. Elevated O_3 is known to decrease the chlorophyll concentration in leaves (Oksanen & Saleen 1999, Wustman *et al.* 2001). The functioning of PSII and the integrity of thylakoid membranes can be studied by measuring chlorophyll fluorescence (Long & Naidu 2002). The O_3-induced changes in PSII functioning may be related to disturbances in the cell metabolism or the direct effect of O_3 or AOS on the thylakoid functionality (Shavnin *et al.* 1999, Dizengremel 2001, Long & Naidu 2002). However, the results from different studies are not uniform: in most of the experiments conducted, elevated O_3 decreased the apparent quantum yield of PSII photochemistry in light (F_v'/F_m') and increased the NPQ, while the maximum photochemical yield of PSII (F_v/F_m) was either decreased or not affected (Maurer & Matyssek 1997; Grams *et al.* 1999, Shavnin *et al.* 1999 Lorenzini *et al.* 1999).

Ozone-induced changes in the anatomy and ultrastructure of leaves in deciduous forest trees are rather well known (e.g. Pääkkönen *et al.* 1995; Vollenweider *et al.* 2003). Generally, the O_3 effects are first found in the mesophyll tissue, where the size and structure of the chloroplasts, peroxisomes, mitochondia, cell wall and cytoplasm are often modified by elevated O_3 (Günthardt-Goerg *et al.* 2000, Oksanen *et al.* 2001b, Oksanen *et al.* 2004). Some of the changes are regarded to be related to the defense against O_3 stress (e.g. increased phenolic droplets and the proliferation of peroxisomes, and an increased mesophyll cell wall thickness), while other changes may indicate accelerated leaf senescence (e.g. an increase in the plastoglobuli and cytoplasmic lipids and initial chloroplast degeneration) (Pääkkönen *et al.* 1995, 1998, Günthardt-Goerg *et al.* 2000, Oksanen *et al.* 2004).

1.3.3 Whole-tree photosynthesis and growth in elevated O_3

Ozone also affects photosynthesis by reducing the photosynthetic leaf area, since O_3 is known to reduce the mean leaf size and induce accelerated senescence and early leaf-fall (Günthardt-Goerg *et al.* 1993, Karnosky *et al.* 1996, Bortier *et al.* 2000). Accelerated leaf senescence is caused by an increasing expression of senescence-associated genes and a decreased expression of genes-coding for photosynthetic proteins in elevated O_3 (Miller *et al.* 1999) and may be related to a remobilization of limiting reserves, such as nitrogen, from declining leaves before the leaf is killed by the stress (Long & Naidu 2002). This may be associated with compensatory growth processes in elevated O_3, for instance as increased height growth or the induction of new leaves, found in some experiments (Oksanen & Rousi 2001, Oksanen *et al.* 2001a, Matyssek & Sandermann 2003).

The decline in photosynthesis and photosynthetic leaf area, and the increased dark respiration associated with detoxification and repair may limit the biomass production and induce alterations in the carbon allocation (Skärby *et al.* 1998, Paludan-Müller *et al.* 1999). According to several studies with deciduous trees, the stem volume growth decreased by ca. 26% in elevated O_3 (Karnosky *et al.* 1996, Dickson *et al.* 1998, Isebrands *et al.* 2001). The stem diameter growth is generally more affected than stem height growth (e.g. Isebrands *et al.* 2001, Matyssek *et al.* 1992, Oksanen & Rousi 2001). However, data obtained with yellow poplar and silver birch shows that the negative effects of O_3 became evident only in the fifth year of O_3 exposure (Rebbeck & Scherzer 2002, Oksanen 2003), indicating that the O_3 effects may be cumulative, and

experiments of long duration are needed to reveal the actual O_3 effect in forest trees.

If the translocation of assimilates is inhibited, the growth of non-photosynthetic parts of the plants may be limited in elevated O_3 (Gunthardt-Goerg *et al.* 1993, Skärby *et al.* 1998, Matyssek 2001). Among the various tree species, a reduction in root growth is often found as a response to elevated O_3 (Coleman *et al.* 1995, Rebbeck & Loats 1997, Oksanen & Rousi 2001). Ozone particularly damages older leaves that provide most of the energy required for root growth (Coleman *et al.* 1995), which in some experiments has led to a reduced root:shoot ratio. As a consequence, the O_3-exposed trees might be predisposed to drought and nutrient deficiency (Oksanen & Saleem 1999, Yamaji *et al.* 2003). It is possible that in elevated O_3 the allocation of resources is more important for the survival of individual trees than the maintenance of high productivity (Matyssek & Sandermann 2003, Yamaji *et al.* 2003).

1.4 Interaction of CO_2 and O_3

Due to the opposite effects of CO_2 and O_3 on growth and physiology of the plants, it is logical to suggest that elevated CO_2 may reduce the detrimental effects of O_3. However, the field experiments on the interactive effect of elevated CO_2 and O_3 on deciduous trees have produced contradictory results. In long-term FACE and OTC studies with aspen and yellow poplar, elevated CO_2 and O_3 had a counteractive effect on the growth parameters: elevated CO_2 generally reduced the magnitude of the negative effect of O_3 (Isebrands *et al.* 2001, Percy *et al.* 2002, Rebbeck & Scherzer 2002), although in many experiments some of the positive effects of CO_2 were negated by elevated O_3 (Isebrands *et al.* 2001, Percy *et al.* 2002, Vanhatalo *et al.* 2003). In the FACE experiment with aspen, elevated CO_2 did not protect the trees from the negative effects of O_3 on the foliar gene expression and biochemistry (Wustman *et al.* 2001), while in terms of growth and gas exchange the ameliorating effect of CO_2 was found only in O_3-tolerant aspen clones (Dickson *et al.* 2001, Noormets *et al.* 2001). In some experiments, CO_2 even exacerbated the harmful effects of the O_3 (Kull *et al.* 1996, Karnosky *et al.* 1998).

It has been suggested that elevated CO_2 may ameliorate the O_3 effects by decreasing the stomatal conductance and thus decreasing the O_3 uptake in elevated CO_2 or by stimulating the photosynthesis, leading to higher nicotinamide adenine dinucleotide phosphate (NADPH) production and increased activity in enzymatic detoxification of AOS (Rao *et al.* 1995, Podila *et al.* 2001). In addition, the ameliorating effect of CO_2 may be related to decreased photorespiration, leading to reduced production of toxic H_2O_2 in elevated CO_2 (Eamus & Ceulemans 2001). The hypothesis that the elevated CO_2 may exacerbate the O_3 effects is based on the prediction that elevated CO_2 reduces the need for the cellular detoxification of AOS, for example, by inhibiting the photorespiration (Willekens *et al.* 1997). Thus, the ability to tolerate the oxidative stress caused by O_3 also decreases (Niewiadomska *et al.* 1999, Polle & Pell 1999, Wustman *et al.* 2001).

It is evident that the responses are strongly dependent on the length and growth conditions of the experiments and on the developmental stage of the plants used (Norby *et al.* 1999, Dickson *et al.* 2001). To clarify the interaction of the two gases involved in the climate change on the growth of forest trees, more long-term studies with large trees are needed.

Figure 1. Open-top chambers in Suonenjoki experimental field (photographed by Metla/ Erkki Oksanen).

Table 1. Summary of the research topics and the methods used in the present study

Research topic	Year	Methods used	Chapter
Seasonal variation and **clonal differences** in growth and physiological properties of silver birch clones 4 and 80	1998	**Growth:** height, stem diameter, dry mass, total leaf area, leaf area ratio **Photosynthesis:** gas exhange (P_n, g_s, Transp, C_i, WUE), chlorophyll fluorescence (F_v/F_m, F_v'/F_m', NPQ) **Biochemistry:** amount and activity of Rubisco, concentration of chlorophyll, soluble proteins, soluble sugars, starch, and foliar nutrients **Leaf structure:** cell ultrastructure, stomatal density, specific leaf area	2
Effects of **elevated CO_2 and O_3** on growth and carbon allocation of silver birch clones 4 and 80	1999-2001	**Growth:** height, stem diameter, stem volume, dry mass of different plant organs, mass-ratios, total leaf area, leaf area ratio, mean leaf size, leaf abscission	3
Effects of **elevated CO_2 and O_3** on photosynthesis and related biochemistry of silver birch clones 4 and 80	1999-2001	**Photosynthesis:** gas exhange (P_n, g_s, Transp, C_i, WUE), chlorophyll fluorescence (F_v/F_m, F_v'/F_m', NPQ), **Biochemistry:** amount and activity of Rubisco, concentration of N, C/N, concentration of chlorophyll, soluble proteins, soluble sugars and starch	4
Effects of **elevated CO_2 and O_3** on anatomy and ultrastucture of the leaves of silver birch clones 4 and 80	1999-2001	**Leaf structure:** mesophyll structure, cell ultrastructure, cell wall chemistry, foliar nutrients, stomatal density, specific leaf area	5

1.5 Aims and overview of the OTC-experiment and the present study

The aim of this open-top experiment was to simulate the growth conditions predicted to prevail by the end of this century by doubling the ambient concentrations of CO_2 and O_3 (IPCC 2001). This is the first long-term field experiment of the interactive effects of CO_2 and O_3 on silver birch. This study was designed in order to examine: 1) whether silver birch benefits from increasing CO_2 in the atmosphere; 2) whether elevated O_3 causes growth losses and damage in processes related to photosynthesis and leaf structure; 3) whether elevated CO_2 can compensate for the negative effects of O_3.

The experimental field (Figure 1) was located at the Suonenjoki Research Station in Central Finland. In the growing season 1998, prior the beginning of the fumigations, the two clones under study were characterised for their growth and physiology, as background information (Chapter 2).

At the beginning of the growing season 1999, forty trees representing clones 4 (V5952) and 80 (K1659) were selected for the three-year open-top chamber experiment (Vapaavuori *et al.* 2002). The O_3-sensitivity of the clones was previously determined in a two-year pot experiment with two-year-old saplings exposed to 1.7× ambient O_3 concentration; according to visible injuries, growth and leaf senescence clone 4 was classified as an O_3-tolerant genotype and clone 80 as an O_3-sensitive genotype, both representing mid-range responses rather than extremes in the O_3-sensitivity ranking (Pääkkönen *et al.* 1997).

Clones 4 and 80 were exposed to the following treatments: outside control (OC), chamber control (CC), 2× ambient CO_2 (EC), 2× ambient O_3 (EO), and 2× ambient CO_2 and 2× ambient O_3 together (EC+EO). The experimental conditions have been published in detail in Vapaavuori *et al.* (2002). During the three-year experiment, the biology of the clones was intensively studied, including, for instance, their growth and physiological and anatomical properties (Table 1). This study focuses on the annual effects of elevated CO_2 and O_3. The changes occurring during each of the three growing seasons are presented only in terms of the most important parameters, such as photosynthesis, stomatal conductance and the amount of Rubisco.

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Chapter 2

Seasonal variation in physiological characteristics of two silver birch clones in the field

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Seasonal variation in physiological characteristics of two silver birch clones in the field

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Abstract: Seasonal changes in growth, photosynthesis, and related biochemical properties and leaf structure were determined for two clones (4 and 80, 20 trees per clone) of 7-year-old *Betula pendula* Roth trees during the growing season of 1998. Differences between the two genotypes were determined to characterize the physiological traits that might affect growth and productivity and that might differ between the genotypes. Net photosynthesis of the short shoot leaves varied between 11 and 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and decreased only slightly towards the end of the summer. However, our results showed more marked decreases in the amount of Rubisco (ribulose biphosphate carboxylase/oxygenase) and leaf N and increases in the total leaf, palisade and spongy layer thickness, chloroplast and starch grain size, and diameter of plastoglobuli in both clones in response to leaf ageing and changes in growth environment. Height and biomass were greater in clone 80 than in clone 4. This was related to slightly more efficient net photosynthesis and higher stomatal conductance and density as well as higher activity of Rubisco and content of foliar nutrients (other than N). We conclude that clone 80 is characterized by faster gas exchange, higher Rubisco activity, stomatal conductance, and density, and earlier leaf ageing, which may be related to the higher ozone sensitivity determined previously in pot experiments with younger saplings.

Résumé : Les changements saisonniers de la croissance, de la photosynthèse et des propriétés biochimiques apparentées ainsi que de la structure des feuilles ont été observés pendant la saison de croissance de 1998 chez deux clones (clones 4 et 80, 20 arbres par clone) de *Betula pendula* Roth âgés de 7 ans. Les différences entre les deux génotypes ont été estimées dans le but de caractériser les traits physiologiques qui peuvent affecter la croissance et la productivité et qui peuvent aussi être différents chez les deux génotypes. La photosynthèse nette des feuilles des rameaux courts variait entre 11 et 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ et diminuait seulement légèrement vers la fin de l'été. Nos résultats ont cependant montré chez les deux clones une diminution plus marquée dans la quantité de carboxydismutase et d'azote du feuillage, ainsi qu'une augmentation de l'épaisseur des feuilles, incluant celle des couches spongieuse et palissadique, de la taille des chloroplastes et des grains d'amidon et du diamètre des plastoglobules en réponse au vieillissement du feuillage et aux changements des conditions de croissance. La hauteur et la biomasse étaient plus élevées pour le clone 80 que pour le clone 4. Cela était dû à une photosynthèse nette légèrement plus efficiente, à une conductance et à une densité stomatiques plus élevées de même qu'à une activité plus forte de la carboxydismutase et à un contenu en nutriments foliaires (autres que N) plus élevé. Nous concluons que le clone 80 est caractérisé par des échanges gazeux plus rapides, une activité de la carboxydismutase, une conductance et une densité stomatiques plus élevées et un vieillissement plus précoce du feuillage qui pourrait être lié à une sensibilité plus élevée à l'ozone telle qu'établie précédemment dans des expériences en pots avec de plus jeunes semis.

[Traduit par la Rédaction]

Introduction

Silver birch (*Betula pendula* Roth) covers large and ecologically diverse areas across the Northern Hemisphere and is a common and economically important deciduous tree species in Finland. Earlier studies have shown that even in geographically small areas, a large variability exists amongst silver birch families and clones (e.g., in volume growth, biomass, allocation of growth, and phenological characteristics as well as in gas exchange, leaf chemical composition, and several other physiological properties) (Raulo and Koski 1977; Wang and Tigerstedt 1995; Pääkkönen et al. 1995, 1997; Jia et al. 1997; Keinänen et al. 1999; Mutikainen et al. 2000). The large variability between genotypes, which is also found in other tree species (Nelson and Ehlers 1984; Mebrahtu and Hanover 1991; Ledig and Clark 1977), has raised the interest in the physiological characteristics that could be used in determination of superior genotypes for tree improvement and in predicting the subsequent harvestable yield (Johnsen et al. 1999; Thomas et al. 1997*a*, 1997*b*).

Since photosynthesis is the primary factor for growth and biomass production, many studies have focused on photo-

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synthesis as an early indicator of growth vigour. However, there have been discrepancies in results between tree growth and net photosynthesis (Lapido et al. 1984; Ceulemans et al. 1987; Mebrahtu and Hanover 1991), partly because of differences in respiration rates and C allocation patterns, which may result in great differences in growth rate despite similar photosynthetic rates in the plants (Azcon-Bieto et al. 1983; Geider and Osborne 1989; Poorter et al. 1990; Barigah et al. 1994). In addition, net photosynthesis is affected by various environmental factors, making comparisons between experiments difficult. The discrepancies may also be due to seasonal changes that occur both in the structure and function of the leaves, which are dependent on the age and position of the leaves in the canopy (Vapaavuori and Vuorinen 1989; Vapaavuori et al. 1989). In many experiments, these factors causing temporal variation in gas exchange have been ignored, and thus, more long-term studies of physiological traits affecting growth and productivity are needed.

Within tall deciduous trees such as silver birch, a large number of short shoot leaves flush simultaneously in early spring from buds formed during the previous autumn, and later, a variety of new long shoot leaves appear along with shoot growth. Thus, irrespective of their position in the crown, the short shoot leaves are of the same age and are maintained in the tree over the whole growing season. In the present study, we have exploited this characteristic of short shoot leaves and our first aim was to investigate the various physiological and anatomical characteristics of silver birch, as affected by ageing of the leaves and by changing environmental conditions, during a growing season. For the study, two fast-growing silver birch clones (clones 4 (V5952) and 80 (K1659)) originating from southern and central Finland, Valkeakoski (61°08′N, 28°49′E) and Eno (62°48′N, 30°05′E), respectively, and growing under field conditions (Mutikainen et al. 2000; Anttonen et al. 2002) were selected. As a selection criteria, we used the results of a previous study with small potted plants showing differences in sensitivity to tropospheric ozone between these clones (Pääkkönen et al. 1997). Our second aim was to study whether there are differences in physiological traits between genotypes that may be of importance in determining the field performance, growth, and allocation.

Materials and methods

Experimental design

The experimental field, containing 15 *B. pendula* clones, is located at Suonenjoki Research Station in central Finland (62°40′N, 27°00′E). At the beginning of the growing season in 1998, clones 4 (V5952) (ozone tolerant) and 80 (K1659) (ozone sensitive) were selected according to their ozone sensitivity (Pääkkönen et al. 1997). Twenty trees of each clone were selected for the study. The trees were 7 years old, with a mean height of 4 m and a mean stand density of 4200 stems·ha^{-1}. The trees were fertilized four times during the growing season of 1998 with a commercial fertilizer (N–P–K 18:5:10, typpirikas Y-lannos; Kemira Ltd., Helsinki, Finland), receiving additional N, 24 kg N·ha^{-1} in total. More details about the field site and experimental design can be found in Mutikainen et al. (2000).

Growth analysis

Tree height, stem diameter (at ground level and at 130 cm), and length of the selected three biomass branches (at the middle section of the stem) were determined four times during the growing season. Also, three branches from the upper, middle, and lower sections of the tree were selected for gas exchange and chlorophyll fluorescence measurements and biochemical sampling. To estimate the area of single leaves, the length and maximum width of 10 representative short and long shoot leaves were measured in each biomass branch, and leaf area was calculated using an empirical equation (for clone 4: leaf area (square centimetres) = leaf width (centimetres) × leaf length (centimetres) × 0.630, r^2 = 0.961; for clone 80: leaf area = leaf width × leaf length × 0.623, r^2 = 0.952).

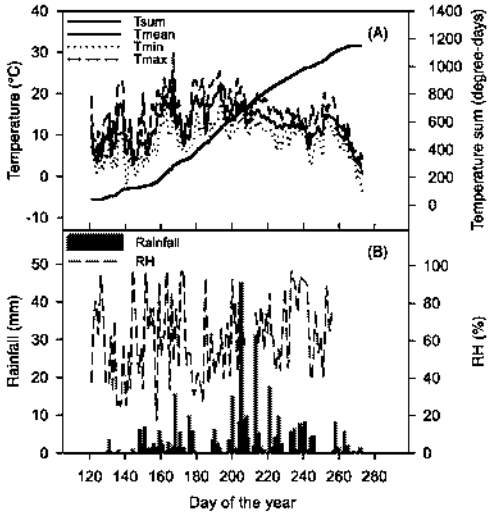
The total leaf area in each biomass branch was calculated by multiplying the mean area of a single leaf by the total number of leaves in each biomass branch. During the final harvest on 28 September 1998, the dry mass of the biomass branches was measured after drying at 80 °C for 4–11 days (until constant mass) and the mean leaf area ratio (square centimetres of leaf area per gram of branch dry mass) was calculated. After determining the total dry mass of all branches, the total leaf area in each tree was calculated using the leaf area ratio determined for each tree. During the final harvest, the dry mass of stems was determined after drying at 80 °C for 4–11 days.

Gas exchange and chlorophyll fluorescence measurements

Gas exchange measurements were made five times during the growing season from the middle section of each tree using a closed-system infrared gas analyzer (LI-6200; LI-COR Inc., Lincoln, Nebr.). Leaf photosynthetic rates were measured at a CO_2 concentration of 360 ppm in natural light (400–1400 μmol·m^{-2}·s^{-1} during the first four measurement periods and 200–400 μmol·m^{-2}·s^{-1} for the measurements made at the end of August). Relative humidity in the leaf chamber was adjusted close to the ambient relative humidity. Gas exchange properties including net photosynthesis, stomatal conductance, transpiration, and intercellular CO_2 concentration were measured on fully expanded short shoot leaves that had flushed from buds early in the spring. Thus, the leaves used in the measurements were of the same age throughout the study. The measurements were made between 1000 and 1600 hours. The mean temperature for the growing season of 1998 was lower and the rainfall higher than the long-term average at the site (Fig. 1).

Chlorophyll fluorescence was measured to assess the maximum photochemical efficiency and photosynthetic yield of PS II. The measurements were made on fully expanded short shoot leaves from branches at three different heights of the tree with a portable pulse amplitude modulated fluorometer (MINI-PAM; Walz, Effeltrich, Germany). After 10 min of dark adaptation of the leaves, the maximal fluorescence yield was obtained by exposing the leaf to a saturating pulse (9000 μmol·m^{-2}·s^{-1}). Thereafter, the leaf was illuminated with an external halogen lamp (600–1100 μmol photosynthetically active radiation·m^{-2}·s^{-1}) for 2 min. Then, a saturating pulse (9000 μmol·m^{-2}·s^{-1}) was applied and the effective quantum yield of PS II and the coefficient for

Fig. 1. (A) Maximum, minimum, and mean air temperature and the effective temperature sum in Suonenjoki (degree-days above a threshold of 5 °C) and (B) daily rainfall and mean values of relative humidity for the daytime hours (0800–1700) at the site during the growing season of 1998.



nonphotochemical fluorescence quenching were measured. Measurements from the three branches were averaged.

Amount and activity of Rubisco and amount of chlorophyll and soluble protein

After chlorophyll fluorescence measurements, the leaves were immediately detached and a 2-cm^2 disc was collected and frozen in liquid nitrogen. The samples from three different tree heights were pooled and stored at –80 °C. For determination of Rubisco (ribulose biphosphate carboxylase/oxygenase), soluble protein, and chlorophyll, the frozen leaf samples were homogenized in ice-cold extraction buffer containing 50 mmol/L morpholinoethanesulfonic acid (pH 6.8), 20 mmol/L $MgCl_2$, 50 mmol/L 2-mercaptoethanol, and 1% Tween-80. Aliquots of the crude extract was dissolved in 80% buffered acetone and analyzed for chlorophyll by the method of Porra et al. (1989). After centrifugation, the activity of Rubisco was determined as incorporation of ^{14}C into acid-stable products (Lorimer et al. 1977). The assay was performed at 25 °C in a reaction mixture containing 50 mmol/L EPPS–NaOH (pH 8.2), 20 mmol/L $MgCl_2$, 0.26 mmol/L EDTA, 10 mmol/L $NaH^{14}CO_3$ (0.1 Ci·mol^{-1}, 1 Ci = 37 GBq) (Amersham Pharmacia Biotech U.K. Ltd., Little Chalfort, U.K.), and 0.3 mmol/L RuBP (ribulose biphosphate) (Sigma Chemical Co., St. Louis, Mo.). Initial activity was measured immediately after the extraction by adding 200 µL of crude extract to the reaction mixture. To obtain total Rubisco activity, supernatant was preincubated in the reaction mixture without RuBP for 10 min at 25 °C before RuBP was added to start the reaction. The incorporation of ^{14}C into acid-stable products was determined by liquid scintillation spectrometry. Rubisco activation state was expressed as the ratio of initial activity to total activity (percent). The amount of Rubisco protein was determined by polyacrylamide gel electrophoresis (Ruuska et al. 1994) and the soluble protein content (Bradford 1976) after precipitation in cold ethanol (Ovaska et al. 1993).

Determination of soluble sugars, starch, C/N, and nutrients in the leaves

After Rubisco sampling, the rest of the leaves were measured for their fresh mass, leaf area (LI-3050A leaf area meter; LI-COR Inc., Lincoln, Nebr.), and dry mass (48 h at 60 °C). The analyses of soluble sugars, starch, and nutrients were conducted on the same pooled leaf material as used in the Rubisco analysis. Soluble sugars and starch were extracted and analyzed as described in Hansen and Møller (1975). Soluble sugars were extracted from a sample (30–40 mg dry mass) with a total volume of 15 mL of 80% aqueous ethanol. Three millilitres of ethanol was added to the sample, heated for 5 min at 60 °C, and centrifuged for 4 min at 1880*g*. The supernatant was collected and the extraction procedure was repeated four times. Total soluble sugars in the pooled supernatant were determined colorimetrically at 630 nm with anthrone using α-D-glucose (anhydrous, analytical grade; Serva, Heidelberg, Germany) as a standard. Starch was extracted from the residue with 20 mL of 30% perchloric acid. The residue was first shaken for 20 h at room temperature with 10 mL of 30% perchloric acid. After centrifugation for 13 min at 1880*g*, the supernatant was collected. The extraction was repeated twice with 5 mL of 30% perchloric acid. From the pooled supernatant, starch was determined colorimetrically at 625 nm with anthrone using soluble starch (pro analysi; Merck, Darmstadt, Germany) in 30% perchloric acid as a standard.

Total C and N from the same leaf samples was measured with a CHN-900 analyzer (Leco Co., St. Joseph, Mich.). For the analyses of other foliar nutrients, 12 extra leaves were collected from each tree on 28 July and 4 September 1998, and the concentrations were determined from an acidic solution (5 mL of HNO_3 and 3 mL of H_2O_2) of ammonium acetate (Halonen et al. 1983) using plasma emission spectrophotometry analysis (ICP, ARL 3800).

Leaf structure: morphological and ultrastructural differences

For electron and light microscopic studies, short shoot leaves were collected on 1 June, 27 July, and 7 September 1998 between 1000 and 1300 hours from the upper and the lower sample branches. Care was taken to select leaves of similar light environment and phenological stage. The samples were packed in plastic bags, placed in an ice box, and transported to the laboratory where sample strips were cut between the second and third leaf vein with a razorblade followed by cutting into 1.5-mm^2 square pieces under a drop of fixative solution. The samples were immediately placed in a 2.5% (v/v) glutaraldehyde fixative solution (in 0.1 mol/L phosphate buffer, pH 7.0), postfixed in 1% buffered OsO_4 solution, dehydrated with an ethanol series followed by a propylene oxide treatment, and embedded in LX 112 Epon. The thin sections for electron microscopy were stained with lead citrate and uranyl acetate and were studied with a JEOL

1200 EX electron microscope operating at 80 kV. The sections for light microscopy were stained with aqueous toluidine blue and were studied using a Nikon MicroPhot-FXA microscope.

The light microscopy samples were measured for total leaf thickness, spongy and palisade layer thickness, and proportion of intercellular space (percentage of mesophyll cross-sectional area) from digital micrographs using the Adobe Photoshop program (version 5.0). Thin sections were photographed with a Bioscan camera (Gatan Inc., Pleasanton, Calif.) (connected to the electron microscope) using the Digital Micrograph program for further image analysis with Adobe Photoshop 5.0. The samples were analyzed for section area of chloroplasts and starch grains, number and size (diameter) of plastoglobuli, and amount of cytoplasmic lipids and vacuolar tannin deposition by classification into four groups: 0, not detected; 1, small amount; 2, considerable amount; 3, abundant.

For stomatal analysis, the samples were collected on four different dates starting on 1 July and finishing on 7 September 1998. Pieces (1 cm^2) of leaf lamina between the second and third vein were taken and frozen in liquid nitrogen and then stored at –80 °C. The samples were examined at × 20 magnification in a light microscope (Zeiss; Axioplan, Oberkochen, Germany) with a camera attachment (Kodak DCS 460, resolution 2024 × 3048 pixels). Pictures were taken in five randomly chosen microscopic fields (0.135 mm^2) on the abaxial surface of the leaves and transferred from the digital images to Adobe Photoshop 5.0. The stomatal density was then measured and averaged for each leaf.

Statistical analyses

Analysis of variance for repeated measurements was used to determine the differences between clones and the effect of time and interactions between them. Pairwise comparisons were carried out using the independent sample t test. The data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variances. In analyses of growth, the initial values of height and diameter growth were used as covariates. The differences between the clones in allocating biomass to leaf area were examined with allometric analysis: the relative proportion of total leaf area to stem dry mass was tested using analysis of covariance, using stem dry mass as a covariate (Johnsen and Bongarten 1991). All statistical analyses were conducted using SPSS 9.0 for Windows (SPSS Inc. 1999), except the regression analysis, which was performed using Excel 5.0.

Results

Growth analysis

The trees representing clone 80 were initially taller and the stem diameter was greater than in clone 4 (Table 1). Also, in the final harvest, the total dry mass was significantly higher in clone 80. However, both height and diameter growth increment were similar in both clones when calculated over the whole growing season of 1998 (Table 1). The total leaf area tended to be higher in clone 80, whereas the leaf area ratio (the ratio of the total leaf area to the sum of the dry mass of stem and branches) and the specific leaf area were significantly higher in clone 4. The latter decreased towards the end of the growing season in both clones, while the leaf dry mass to fresh mass ratio increased significantly during the growing season similarly in both clones (Table 2). Allometric analysis between total leaf area and stem dry mass (Fig. 2) showed that neither the intercept (P = 0.811) nor the slope (P = 0.711) differed between the clones.

Gas exchange and chlorophyll fluorescence measurements

Net photosynthesis decreased significantly from mid-July towards the end of the growing season in both clones (Fig. 3A). Stomatal conductance increased significantly until the beginning of August and intercellular CO_2 increased until the end of August in both clones (Figs. 3B and 3D). Transpiration rate decreased significantly towards the end of August (Fig. 3C). Net photosynthesis was consistently higher in clone 80 (Fig. 3A), although the differences between clones were not significant. The other gas exchange properties measured were significantly higher in clone 80, and only water use efficiency was significantly higher (P = 0.006) in clone 4 (data not shown). The maximum photochemical efficiency of PS II (F_v/F_m) was stable between the clones (0.80–0.82) and during the growing season, except after a cold and rainy period at the end of June when F_v/F_m decreased (0.75 ± 0.008) (data not shown).

Total chlorophyll and soluble protein concentration and amount and activity of Rubisco

The mean values of total chlorophyll concentration, calculated on the basis of fresh mass (data not shown) and leaf area (Table 2), varied during the growing season and ranged between 0.21 and 0.24 $g \cdot m^{-2}$ without significant differences between the clones (Table 2). The concentration of total soluble protein was highest in June–July and decreased towards the end of the growing season. The mean values ranged from 5.11 to 6.28 $g \cdot m^{-2}$, and no significant difference between the clones was found (Table 2).

The amount of Rubisco, on both an area (Fig. 4A) and a fresh mass basis (data not shown), was highest in mid-July, and the significant decline towards the end of the growing season was similar in both clones. The amount of Rubisco was consistently higher in clone 80, although the differences between clones were not significant. The specific total activity of Rubisco increased significantly towards the end of the growing season similarly in both clones (Fig. 4B). Initial and total activities of Rubisco (Figs. 4C and 4D) were stable during the growing season, being significantly higher in clone 80. Rubisco activation state ranged from 44% to 55%, being highest on 24 June and lowest on 14 July, and increased again towards the end of the growing season. Values tended to be higher in clone 80, although neither the differences between clones nor those between sampling days were statistically significant (data not shown).

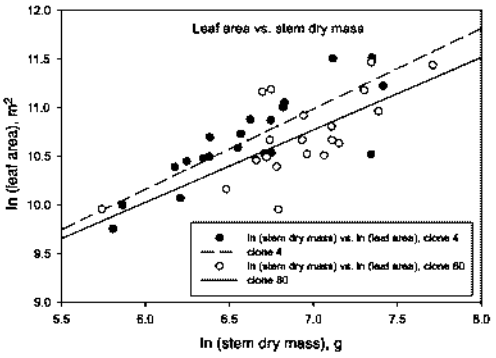
The relative changes between the dark and light reactions of photosynthesis were studied by calculating the amount of Rubisco on a chlorophyll basis (Fig. 4E). The Rubisco to chlorophyll ratio was slightly higher in clone 80 on 23 June and 14 July, and in both clones, after the leaves had fully expanded, the ratio decreased significantly towards the end of

Table 1. Height and diameter growth, total leaf area, and leaf area ratio of silver birch (*Betula pendula*) clones 4 and 80 during the growing season of 1998.

Parameter	Date	Clone 4	Clone 80
Height (cm)	8 May	376.0±12.2	417.0±13.0
	18 June	385.4±11.2	435.0±12.6
	4 Aug.	438.3±13.0	488.3±14.3
	7 Sept.	477.5±12.2	517.8±12.6
		Clone effect, P = 0.227	
Height growth increment (cm)		101.6±8.2	100.75±8.2
		Clone effect, P = 0.464	
Basal stem diameter (mm)	8 May	37.0±1.0	43.0±1.7
	18 June	37.6±1.2	44.4±1.7
	4 Aug.	44.8±1.7	51.6±2.2
	7 Sept.	48.9±1.3	54.2±2.3
		Clone effect, P = 0.987	
Basal stem diameter increment (mm)		11.9±0.87	11.1±1.5
		Clone effect, P = 0.381	
Stem diameter at 130 cm (mm)	8 May	18.5±0.9	22.4±1.2
	18 June	20.4±1.1	24.9±1.2
	4 Aug.	25.9±1.5	29.6±1.5
	7 Sept.	28.6±1.2	31.9±1.5
		Clone effect, P = 0.783	
Stem diameter increment at 130 cm (mm)		10.2±0.7	9.6±0.7
		Clone effect, P = 0.197	
Stem and branch dry mass (g)	28 Sept.	1086.11±116.0	1521.19±144.80
		Clone effect, P = 0.024	
Total leaf area (m^2)		4.74±0.51	4.90±0.48
		Clone effect, P = 0.813	
Leaf area ratio (leaf area/dry mass of stem and branches) ($cm^2 \cdot g^{-1}$)		44.80±2.06	32.89±1.48
		Clone effect, P = 0.000	

Note: Data are means (±SE) of 20 trees and were analyzed with repeated ANOVA; initial growth parameters were used as covariates.

Fig. 2. Relationship between total leaf area and stem dry mass in silver birch (*Betula pendula*) clones 4 and 80 at the end of the growing season of 1998. The lines show the linear regressions fitted to the data for each clone.



the growing season. The proportion of Rubisco of total soluble proteins (Fig. 4F) was 24%–62% and followed a similar pattern as the amount of Rubisco (Fig. 4A) and the Rubisco to chlorophyll ratio (Fig. 4E). The proportion of Rubisco N of total leaf N varied between 25% and 37%, being highest at the beginning of the growing season and decreasing significantly after June 24 towards the end of the growing season in both clones (P = 0.001). The proportion of Rubisco N was generally higher in clone 4, but the difference between clones was not significant (data not shown).

Soluble carbohydrates, starch, N concentration, and nutrients in leaves

The concentration of soluble sugars was relatively stable (9.0%–11.8%) during the summer, whereas the foliar starch concentration increased steadily until 12 August but declined markedly on 1 September similarly in both clones (Table 2). The N concentration of the leaves was highest in early summer and decreased significantly during the growing season. This was accompanied by a statistically significant increase in the C/N ratio, which tended to be slightly lower in clone 80 (Table 2). In addition, the concentration of structural N, calculated by excluding starch and soluble sug-

Table 2. Leaf dry mass to fresh mass ratio, specific leaf area, leaf chlorophyll, soluble protein, soluble sugars, starch and N concentrations, and the C/N ratio in silver birch clones 4 and 80 during the growing season of 1998.

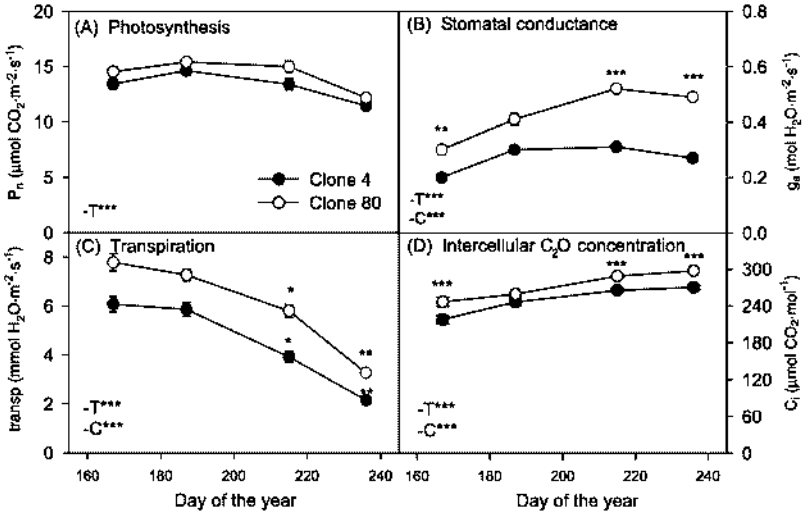
Parameter	Date	Clone 4	Clone 80
Leaf dry mass/leaf fresh mass (%)	10 June	30.2±0.61	29.0±0.18
	24 June	38.0±0.44	37.0±0.47
	14 July	41.0±0.48	40.5±0.60
	12 Aug.	42.7±0.37	42.3±0.29
	1 Sept.	41.3±0.47	41.4±0.48
		Time effect, $P = 0.000$	
		Clone effect, $P = 0.375$	
Specific leaf area ($cm^2 \cdot g$ fresh $mass^{-1}$)	10 June	65.71±0.48	65.34±0.57
	24 June	61.27±0.73	58.33±0.55
	14 July	59.65±0.77	58.58±0.92
	12 Aug.	60.08±0.71	58.12±0.62
	1 Sept.	61.18±0.71	58.68±0.75
		Time effect, $P = 0.000$	
		Clone effect, $P = 0.037$	
Chlorophyll ($g \cdot m^{-2}$)	10 June	0.21±0.007	0.22±0.007
	24 June	0.23±0.011	0.24±0.007
	14 July	0.23±0.009	0.23±0.010
	12 Aug.	0.21±0.008	0.22±0.009
	1 Sept.	0.22±0.008	0.23±0.011
		Time effect, $P = 0.055$	
		Clone effect, $P = 0.259$	
Soluble protein ($g \cdot m^{-2}$)	10 June	6.27±0.17	6.28±0.13
	24 June	5.44±0.33	5.86±0.34
	14 July	5.82±0.24	5.30±0.27
	12 Aug.	5.52±0.22	5.34±0.21
	1 Sept.	5.11±0.15	5.11±0.27
		Time effect, $P = 0.000$	
		Clone effect, $P = 0.749$	
Soluble sugars (% dry mass)	10 June	11.81±0.23	10.99±0.34
	24 June	9.88±0.36	10.28±0.48
	14 July	10.63±0.19	10.18±0.20
	12 Aug.	9.15±0.30	9.62±0.19
	1 Sept.	11.46±0.34	11.67±0.40
		Time effect, $P = 0.000$	
		Clone effect, $P = 0.981$	
Starch (% dry mass)	10 June	8.32±0.38	9.15±0.34
	24 June	10.81±0.54	10.56±0.48
	14 July	12.37±0.85	12.35±0.84
	12 Aug.	12.86±0.73	12.13±0.64
	1 Sept.	7.78±0.46	7.20±0.52
		Time effect, $P = 0.000$	
		Clone effect, $P = 0.898$	
N (% dry mass)	10 June	2.79±0.066	3.03±0.049
	24 June	2.02±0.048	2.12±0.052
	14 July	1.84±0.021	1.89±0.025
	12 Aug.	1.68±0.038	1.72±0.045
	1 Sept.	1.67±0.054	1.75±0.044
		Time effect, $P = 0.000$	
		Clone effect, $P = 0.100$	
C/N ratio	10 June	17.65±0.41	15.80±0.28
	24 June	24.16±0.56	22.59±0.58
	14 July	26.80±0.31	25.70±0.36

Table 2 (*concluded*).

Parameter	Date	Clone 4	Clone 80
	12 Aug.	28.81±0.57	27.79±0.90
	1 Sept.	29.35±0.99	27.14±0.69
		Time effect, $P = 0.000$	
		Clone effect, $P = 0.067$	
N (% structural dry mass)	10 June	3.50±0.078	3.80±0.078
	24 June	2.55±0.040	2.67±0.048
	14 July	2.49±0.047	2.49±0.045
	12 Aug.	2.11±0.050	2.23±0.038
	1 Sept.	2.10±0.056	2.16±0.040
		Time effect, $P = 0.000$	
		Clone effect, $P = 0.076$	

Note: Data are means (±SE) of 20 trees and were analyzed with repeated ANOVA.

Fig. 3. Gas exchange measured at ambient CO_2 during the growing season of 1998. (A) Net photosynthesis, (B) stomatal conductance, (C) transpiration, and (D) intercellular CO_2 concentration in the leaves of silver birch clones 4 and 80. Data are means (±SE) from 20 trees representing each clone. Statistical significances for the time (T) and clone (C) effect: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The asterisks above the lines indicate the differences between clones for each measurement period.



ars from the total dry mass, decreased significantly towards the end of the growing season and was consistently higher in clone 80, although the difference between clones was not significant (Table 2).

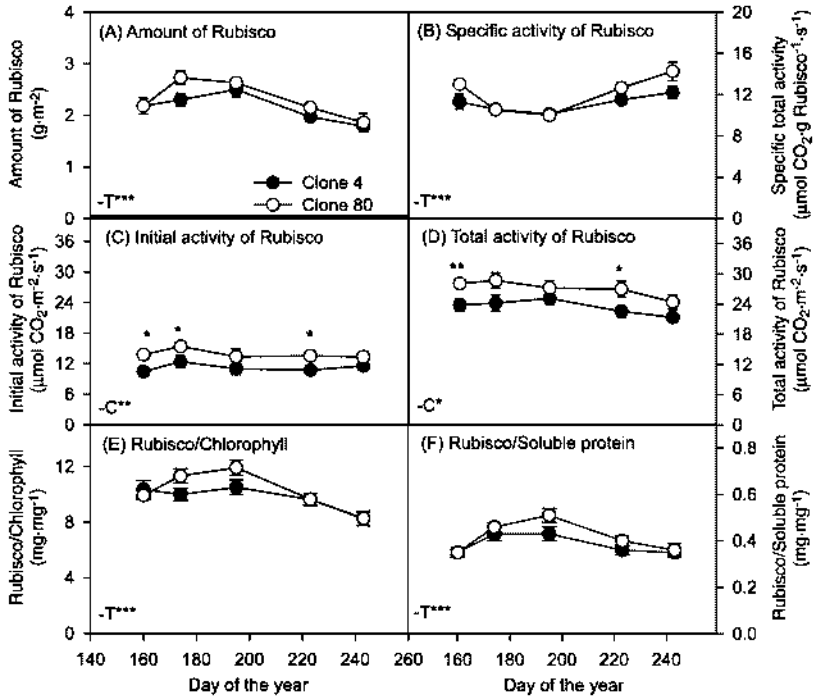
The concentrations of macro- and micro-nutrients (other than N) in the leaves expressed on a dry mass (Table 3) or residual dry mass basis (starch and soluble sugar free basis; data not shown) were stable in most cases during the growing season, with the exception of significantly increasing concentrations of P, Ca, and Zn. The concentrations of P, K, Ca, Mg, Fe, Zn, and B were significantly higher in clone 80. When expressed as an N ratio, the concentrations of mineral elements generally increased towards the end of the growing season and were higher in clone 80 (data not shown).

Leaf structure: morphological and ultrastructural differences

During leaf ageing, the palisade and spongy mesophyll layers increased significantly, and also, the ratio of palisade to spongy mesophyll increased similarly in both clones (data not shown). There were no significant differences between the clones in leaf thickness, palisade or spongy layer thickness, or proportion of intercellular space.

The size of chloroplasts and starch grains increased significantly towards the end of the growing season in the palisade and spongy mesophyll layers of both clones but did not differ between the clones (Table 4). The number of plastoglobuli declined significantly with time, which was accompanied by increased diameter of plastoglobuli in both clones.

Fig. 4. (A) Amount of Rubisco, (B) specific total activity of Rubisco, (C) initial activity of Rubisco, (D) total activity of Rubisco, (E) ratio of Rubisco to chlorophyll, and (F) ratio of Rubisco to total soluble protein in the leaves of silver birch clones 4 and 80 during the growing season of 1998. The data are means (±SE) from 20 trees representing each clone. Statistical significances for the time (T) and clone (C) effect: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The asterisks above the lines indicate the differences between clones for each measurement period.



On the other hand, the accumulation of cytoplasmic lipids and vacuolar tannin proceeded faster in clone 80 during foliar senescence (Table 5; Fig. 5).

The stomatal density was significantly higher in clone 80 (by 9%), but the number of stomata remained stable during the growing season in both clones (Fig. 6).

Discussion

The present results showed that net photosynthesis in both birch clones was relatively stable throughout the growing season and decreased only slightly towards the end of August, which is in accordance with previous studies on deciduous woody species (Ruuska et al. 1994; Vapaavuori et al. 1989). This is different from herbaceous species, where net photosynthesis starts to decrease soon after the leaves have reached full expansion (Makino et al. 1983). Our results showed increasing activation state of Rubisco towards the end of the growing season when the amount of Rubisco and soluble protein had started to decrease. A higher activation state of Rubisco, which was connected with increasing intercellular CO_2 concentration, could facilitate high rates of photosynthesis, even though degradation and reallocation of N from Rubisco are accelerating (Evans 1989). In contrast, stomatal conductance and intercellular CO_2 concentration were increasing, while transpiration was decreasing towards the end of the season in both clones. These changes were associated with increasing daytime relative humidity in the atmosphere (Fig. 1), which is known to affect the stomatal conductance of leaves (Grace 1983).

Decreasing net photosynthesis in the short shoot leaves towards the end of the summer was associated with simultaneous changes in leaf structure, such as increasing size of chloroplasts and starch grains, and finally accompanied by decreased starch concentration at the end of the growing season. Additionally, the palisade and spongy layer thickness of the mesophyll, total leaf thickness, dry matter content, and the diameter of plastoglobuli increased, while the number of plastoglobuli decreased. These changes are considered to be phenomena typical of developmental and ageing processes (Pääkkönen et al. 1995, 1996). At the end of the growing season, some differences between the clones were found. Clone 80, which is of a more northern origin than clone 4 (Mutikainen et al. 2000), was characterized by faster

Table 3. Foliar nutrient concentrations in silver birch clones 4 and 80 during the growing season of 1998.

	Date	Macronutrients (mg·g dry mass^{-1})				Micronutrients (μg·g dry mass^{-1})					
		N	P	K	Ca	Mg	Mn	Fe	Zn	Cu	B
Clone 4	28 July	18.4±0.21	2.83±0.12	7.14±0.27	5.64±0.21	2.81±0.08	111.65±8.91	42.46±1.23	27.85±1.93	4.56±0.22	12.42±0.82
	4 Sept.	16.7±0.54	3.89±0.15	7.80±0.27	6.58±0.30	2.85±0.09	127.63±9.30	46.08±2.21	32.47±1.89	4.07±0.14	11.55±0.60
Clone 80	28 July	18.9±0.25	3.41±0.14	7.98±0.18	8.77±0.34	3.67±0.09	130.52±12.94	56.54±3.65	37.38±2.71	4.34±0.18	16.80±1.62
	4 Sept.	17.5±0.44	4.61±0.23	8.25±0.26	9.40±0.43	3.55±0.10	149.31±14.05	54.71±2.17	42.88±2.82	4.30±0.18	17.52±1.17
Clone effect in ANOVA P		0.168	0.000	0.011	0.000	0.000	0.082	0.000	0.000	0.985	0.000
Time effect in ANOVA P		0.001	0.000	0.062	0.016	0.682	0.135	0.664	0.036	0.147	0.835

Note: The sample was composed of 12 short shoot leaves collected randomly throughout the whole crown. Data are means (±SE) of 20 trees.

senescence-related structural changes in the leaves, and this was seen as faster accumulation of cytoplasmic lipids and vacuolar tannins.

Data of the present study showed marked differences in several physiological traits between the two clones. Clone 80 had remarkably higher stomatal conductance and stomatal density than clone 4. These characteristics enabled a greater flux of CO_2 to the leaves and hence higher intercellular CO_2 concentration, net photosynthesis, and transpiration in clone 80 throughout the growing season. Such differences between the clones might be of importance under various environmental stress conditions and could make clone 80 more prone to, e.g., drought and air pollution. As a result of greater stomatal conductance in clone 80, intercellular CO_2 concentration and the carboxylation of RuBP by Rubisco increased. This is to be expected, since Rubisco is considered to be a limiting step in photosynthesis (Caemmerer and Farquhar 1981; Sage et al. 1989). Rubisco activity is regulated by Rubisco activase (Salvucci 1989), which modulates the activation state of Rubisco and thus balances the rate of CO_2 fixation with the rate of substrate regeneration. This ensures optimal levels of metabolites for photosynthesis under different environmental conditions. In this experiment, both the initial and the total activity of Rubisco were higher in clone 80, and the amount of Rubisco was also consistently slightly higher in clone 80. As a consequence, no differences were found in the specific activity of Rubisco between the two clones. Because there were no differences between the clones in the activation state of Rubisco, it appears that the higher Rubisco activity of clone 80 was caused both by a slightly higher amount of Rubisco and higher intercellular CO_2 concentration in the intercellular space. The concentration of total nonstructural carbohydrates varied between 18% and 22% but was similar in the two clones, indicating that carbohydrate accumulation was not causing lower Rubisco activity in clone 4.

Our data also showed no differences in chlorophyll concentration, chlorophyll *a* to chlorophyll *b* ratio, and F_v/F_m between the clones, indicating that photosynthetic light reactions had no significant role in different photosynthetic rates.

There were major differences in C fixation between the clones in favour of clone 80, which showed significantly higher aboveground biomass in the final harvest in September 1998, when the trees were 7 years old. In contrast, there were no differences in growth increments between the two clones in 1998, indicating season by season variation in growth and pinpointing the importance of long-term studies in assessing factors related to growth. The summer of 1998 was cool and rainy, which may have affected some other metabolic processes, such as respiration, more than photosynthesis. Indeed, a direct relationship between respiration rates and growth rates has been reported (Azcon-Bieto et al. 1983; Geider and Osborne 1989; Poorter et al. 1990), suggesting that respiration rate could serve as a better indicator in predicting long-term growth rate of a tree than net photosynthesis (Anekonda et al. 1993, 1994).

Our study also showed some differences between the clones in biomass allocation. The clones differed in leaf area ratio (relative proportion of total leaf area to woody biomass), and as a consequence, clone 80 produced more woody biomass (stem and branches) per unit leaf area than

Table 4. Ultrastructural differences between silver birch clones 4 and 80 during the growing season of 1998 in the Suonenjoki experiment.

		Palisade tissue		Spongy tissue	
Parameter	Date	Clone 4	Clone 80	Clone 4	Clone 80
Size of chloroplast (μm^2)	1 June	4.37±0.22	5.00±0.25	4.47±0.24	4.55±0.20
	27 July	8.03±0.43	8.93±0.44	8.81±0.68	9.66±2.44
	7 Sept.	10.03±0.42	9.50±0.50	9.32±0.45	9.21±0.61
	Time effect	$P = 0.000$		$P = 0.000$	
	Clone effect	$P = 0.075$		$P = 0.094$	
Size of starch grain (μm^2)	1 June	0.24±0.05	0.69±0.12	0.47±0.09	0.56±0.10
	27 July	2.72±0.32	3.05±0.32	4.24±0.61	3.57±0.40
	7 Sept.	4.48±0.53	3.09±0.33	3.89±0.47	3.89±0.41
	Time effect	$P = 0.000$		$P = 0.000$	
	Clone effect	$P = 0.150$		$P = 0.062$	
No. of plastoglobuli per chloroplast	1 June	7.59±0.38	6.76±0.36	7.33±0.40	6.25±0.28
	27 July	3.92±0.51	3.27±0.31	3.58±0.49	3.42±0.39
	7 Sept.	4.42±0.39	5.25±0.69	4.17±0.37	3.95±0.46
	Time effect	$P = 0.000$		$P = 0.000$	
	Clone effect	$P = 0.302$		$P = 0.301$	
Diameter of plastoglobuli (nm)	1 June	141.8±7.0	140.3±6.6	142.1±6.80	139.9±6.6
	27 July	543.4±20.5	484.2±21.9	478.2±20.9	452.7±23.6
	7 Sept.	594.9±36.2	611.1±28.3	626.5±31.7	570.1±36.4
	Time effect	$P = 0.000$		$P = 0.000$	
	Clone effect	$P = 0.927$		$P = 0.868$	

Note: Data are means (±SE) of 20 trees and were analyzed with repeated ANOVA.

Table 5. Differences between silver birch clones 4 and 80 in accumulation of cytoplasmic lipids and vacuolar tannin during the growing season of 1998.

		Palisade tissue						Spongy tissue					
		1 June		27 July		7 Sept.		1 June		27 July		7 Sept.	
Parameter	Group	4	80	4	80	4	80	4	80	4	80	4	80
Amount of cytoplasmic lipids	0	10.5	12.3	12.9	5.9	0	0	5.3	12.3	16.1	6.1	0	0
	1	81.6	63.0	58.1	55.9	26.3	0	75.0	68.5	54.8	57.6	21.1	0
	2	3.9	24.7	22.6	32.4	57.9	15.1	15.8	19.2	22.6	24.2	73.7	15.0
	3	3.9	0	6.5	5.9	15.8	84.9	3.9	0	6.5	12.1	5.3	85.0
Amount of vacuolar tannin	0	59.2	71.2	0	0	0	0	71.1	75.3	0	0	0	0
	1	22.4	20.5	45.2	35.3	0	2.5	21.1	24.7	51.6	36.4	0	0
	2	18.4	8.2	35.5	44.1	27.8	15.0	7.9	0	35.5	51.5	72.2	47.5
	3	0	0	19.4	20.6	72.2	80.0	0	0	12.9	12.1	27.8	52.5

Note: Data are percentages of samples classified into the following groups: 0, not detected; 1, small amount; 2, considerable amount; 3, abundant.

clone 4, partly due to slightly higher leaf level photosynthesis. Since stomatal conductance and transpiration were remarkably greater in clone 80 than in clone 4, it can be speculated that clone 80 had a higher capacity for water uptake and conductivity. In clone 4, the greater relative investment in leaf area, in terms of leaf area ratio, may serve as a necessary adjustment in maintaining high crown photosynthesis despite a lower rate of net photosynthesis when calculated on a leaf area basis.

In several studies, the N concentration of leaves has proven to be a good predictor of net photosynthesis in woody and other C_3 species (Evans 1989; Field and Mooney 1986; Reich et al. 1995), depending on the position of the leaf in the canopy and the prevailing light conditions (Kazda et al. 2000; Hollinger 1996). In our study, all of the trees received the same amount of fertilizer in 1998, as during previous years. Nevertheless, the leaves of clone 80 had a tendency to have slightly higher structural N concentration, which may partly explain the higher net photosynthesis in this clone. Moreover, several other macro- and micro-nutrients showed higher concentrations in clone 80. Since nutrients are transported to the leaves in the transpiration stream and since clone 80 had a higher rate of transpiration and lower water use efficiency than clone 4, accumulation of nutrients in the foliage would occur. However, the differences between clones in N concentration were small in comparison with

Fig. 5. Ultrastructural changes during leaf ageing in silver birch clone 80 grown under field conditions during the growing season of 1998. P, plastoglobuli; S, starch grain; T, tannin; L, lipid body. Scale bar = 1 μm. (A) Chloroplast of a young leaf sampled on 1 June 1998 showing numerous small plastoglobuli (P). Accumulation of starch is minimal, indicating high phloem loading and active transport of photosynthate. (B) Chloroplast of a mature leaf collected on 27 July 1998 with a larger starch grain (S) and incipient deposition of tannin (T) in the vacuole. Accumulation of tannin and cytoplasmic lipids (L) in senescing leaves collected on 7 September 1998 from (C) higher and (D) lower branches accompanied by increased size of plastoglobuli.

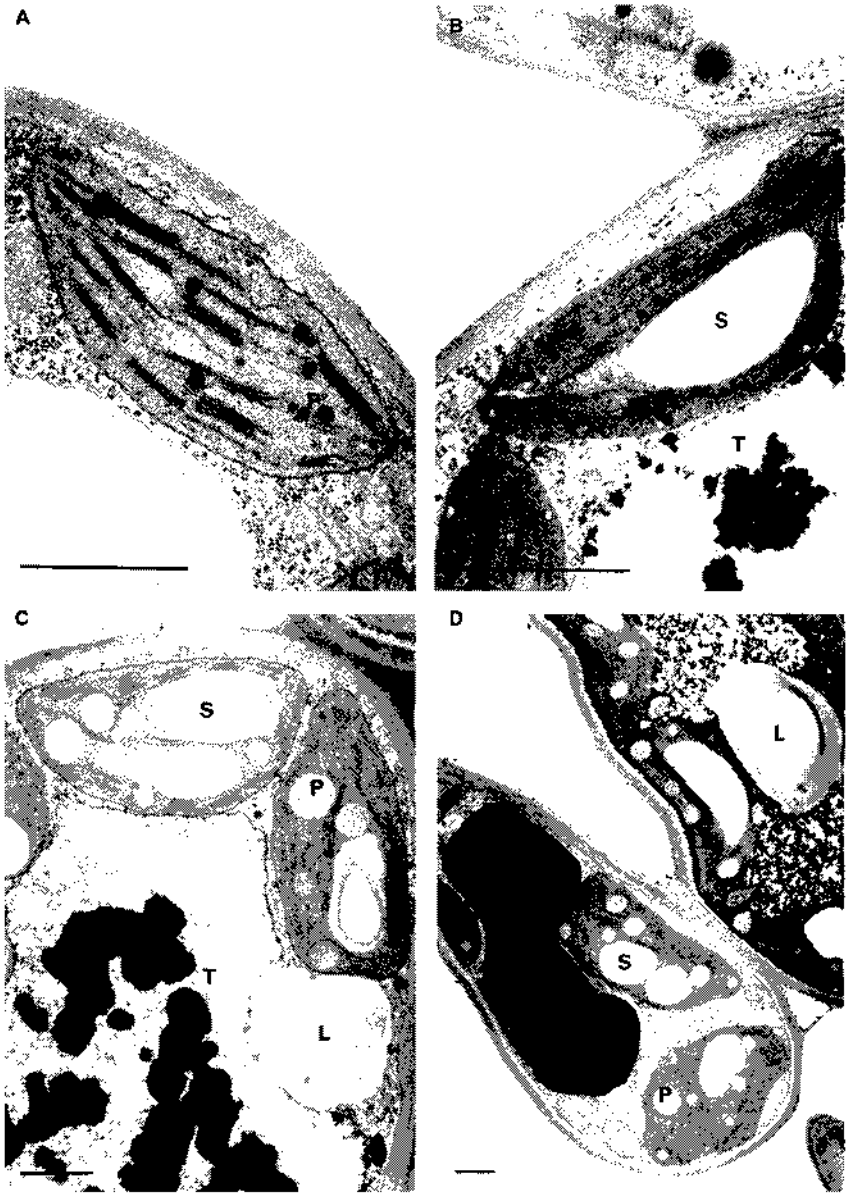
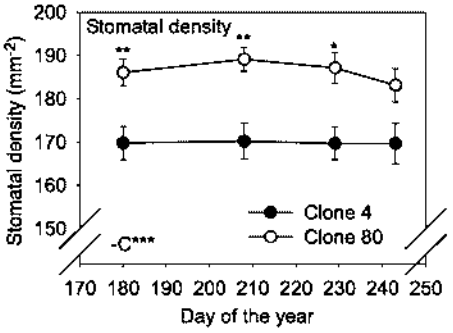


Fig. 6. Stomatal density in the leaves of silver birch clones 4 and 80. The data are means (±SE) from 20 trees representing each clone. Statistical significances for the clone effect (C): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The asterisks above the lines indicate the differences between clones for each measurement period.



differences in other macro- and micro-nutrient concentrations, leading to quite different N ratios of foliar nutrients in the two clones (data not shown). Since the N ratios were above the target values for woody species (Linder 1995), we assume that the trees were not suffering from nutrient imbalance. The greater accumulation of mineral nutrients in the leaf tissue of clone 80 is intriguing and would be expected to affect the cellular integrity in the leaves. All of the above phenomena indicate a more efficient root system for water and nutrient uptake in clone 80.

The main finding of this study was that clone 80 was physiologically more active compared with clone 4, which appeared as higher net photosynthesis and Rubisco activity. This was facilitated by remarkably higher stomatal conductance and stomatal density, which led to greater transpiration and nutrient uptake, but to lower water use efficiency, than in clone 4. Such differences would make clone 80 more fit for fertile sites with ample nutrient and water availability but more prone to drought. Also, our previous ozone-sensitivity ranking experiment with potted saplings over two growing seasons showed that clone 80 was more sensitive to ozone (Pääkkönen et al. 1997), probably due to a greater flux of ozone through the stomata. Thus, on the basis of the present results with larger soil-grown trees, it can be postulated that clone 80 is more sensitive to environmental stresses due to higher stomatal density and conductance. This hypothesis will be tested by us in further studies, where the same clones will be examined for ozone and CO_2 responses in a multi-year exposure.

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Chapter 3

Silver birch and climate change: variable growth and carbon allocation responses to elevated concentrations of carbon dioxide and ozone

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Silver birch and climate change: variable growth and carbon allocation responses to elevated concentrations of carbon dioxide and ozone

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Summary We studied the effects of elevated concentrations of carbon dioxide ($[CO_2]$) and ozone ($[O_3]$) on growth, biomass allocation and leaf area of field-grown O_3-tolerant (Clone 4) and O_3-sensitive clones (Clone 80) of European silver birch (*Betula pendula* Roth) trees during 1999–2001. Seven-year-old trees of Clones 4 and 80 growing outside in open-top chambers were exposed for 3 years to the following treatments: outside control (OC); chamber control (CC); 2 × ambient $[CO_2]$ (EC); 2 × ambient $[O_3]$ (EO); and 2 × ambient $[CO_2]$ + 2 × ambient $[O_3]$ (EC+EO). When the results for the two clones were analyzed together, elevated $[CO_2]$ increased tree growth and biomass, but had no effect on biomass allocation. Total leaf area increased and leaf abscission was delayed in response to elevated $[CO_2]$. Elevated $[O_3]$ decreased dry mass of roots and branches and mean leaf size and induced earlier leaf abscission in the autumn; otherwise, the effects of elevated $[O_3]$ were small across the clones. However, there were significant interactions between elevated $[CO_2]$ and elevated $[O_3]$. When results for the clones were analyzed separately, stem diameter, volume growth and total biomass of Clone 80 were increased by elevated $[CO_2]$ and the stimulatory effects of elevated $[CO_2]$ on stem volume growth and total leaf area increased during the 3-year study. Clone 80 was unaffected by elevated $[O_3]$. In Clone 4, elevated $[O_3]$ decreased root and branch biomass by 38 and 29%, respectively, whereas this clone showed few responses to elevated $[CO_2]$. Elevated $[CO_2]$ significantly increased total leaf area in Clone 80 only, which may partly explain the smaller growth responses to elevated $[CO_2]$ of Clone 4 compared with Clone 80. Although we observed responses to elevated $[O_3]$, the responses to the EC+EO and EC treatments were similar, indicating that the trees only responded to elevated $[O_3]$ under ambient $[CO_2]$ conditions, perhaps reflecting a greater quantity of carbohydrates available for detoxification and repair in elevated $[CO_2]$.

Keywords: Betula pendula, biomass, clone, field experiment, leaf abscission, leaf area, open-top chamber, stem volume.

Introduction

Concentrations of atmospheric carbon dioxide ($[CO_2]$) and ozone ($[O_3]$) are expected to double during this century (IPCC 2001). Generally, elevated $[CO_2]$ has a positive effect on tree growth (Saxe et al. 1998), whereas elevated $[O_3]$ has a negative effect (Skärby et al. 1998). Growth responses are mediated by changes in photosynthesis and photosynthesis-related biochemical properties of the leaves (Saxe et al. 1998, Skärby et al. 1998), changes in carbon allocation (Norby et al. 1999, Matyssek 2001) and leaf senescence and abscission (Karnosky et al. 1996, Gielen and Ceulemans 2001).

Although the effects of elevated $[CO_2]$ and $[O_3]$ have been studied extensively in seedlings and saplings, the responses of mature trees in closed forests are not well understood. In many short-term studies with elevated $[CO_2]$, tree growth has increased by nearly 50% (Saxe et al. 1998), whereas in long-term studies, growth stimulation was found only during the early years of the experiments (Rey and Jarvis 1997, Centritto et al. 1999). It has also been suggested that the results of short-term experiments with elevated $[O_3]$ underestimate the long-term negative effects (Oksanen 2003).

In their natural environment, trees are simultaneously exposed to many stresses that interact with each other. The few experiments that have focused on the interactive effects of elevated $[CO_2]$ and $[O_3]$ on deciduous trees have yielded inconsistent results. Elevated $[CO_2]$ protected the trees from the negative effects of elevated $[O_3]$ in some studies (Mortensen 1995, Volin and Reich 1996, Dickson et al. 1998, Loats and Rebbeck 1999, Rebbeck and Scherzer 2002), but not in others (Wustman et al. 2001, McDonald et al. 2002). In some experiments, elevated $[CO_2]$ exacerbated the harmful effect of elevated $[O_3]$ (Kull et al. 1996, Karnosky et al. 1998). Responses are strongly dependent on the length of the experiment, growth conditions and the developmental stage of the plants studied. To clarify the interaction of O_3 and CO_2 on growth of forest

trees, more long-term studies with large, mature trees are needed.

Silver birch is an economically important tree species in the Northern Hemisphere (Matyssek 2001) that shows great variability in O_3 sensitivity among genotypes (Matyssek 2001, Oksanen and Holopainen 2001, Oksanen and Rousi 2001).

In our study, we used open-top chambers (OTCs) to expose field-grown trees of an O_3-tolerant and an O_3-sensitive silver birch clone to elevated $[CO_2]$ and $[O_3]$, both alone and in combination, for 3 years. We monitored the effects of elevated $[CO_2]$ and $[O_3]$ on various growth parameters to test three hypotheses: (1) field-grown silver birch benefits from increasing $[CO_2]$ in the atmosphere; (2) elevated $[O_3]$ causes damage and growth losses; and (3) elevated $[CO_2]$ can compensate for the negative effects of elevated $[O_3]$. We also hypothesized that data from large field-grown silver birch trees are comparable with results obtained with small saplings.

Materials and methods

Field site and plant material

The experimental field is located at Suonenjoki Research Station in central Finland (62°39′ N, 27°03′ E, 120 m a.s.l.). The plantation was established in 1993 with 1-year-old cloned silver birch saplings, representing 15 clones originating from southern and central Finland as described by Mutikainen et al. (2000). The experimental plots in this study had the same design as described by Mutikainen et al. (2000). The study trees were fertilized annually, receiving 12, 12, 17, 17, 22 and 22 kg N ha^{-1} (18:5:10, N,P,K) in the years 1993–1998, respectively. Forty trees representing Clones 4 (V5952, O_3-tolerant) and 80 (K1659, O_3-sensitive) were selected for the 3-year OTC experiment. The O_3 sensitivity of the clones had been determined previously in a 2-year pot experiment with 2-year-old saplings that were exposed to low $[O_3]$ (1.6 × and 1.7 × ambient $[O_3]$) and had been ranked according to visible injuries, growth and leaf senescence (Pääkkönen et al. 1997).

At the beginning of the experiment, the trees were 7 years old with a mean height of about 5 m and a mean stand density of 4200 stems ha^{-1}. During the experiment, the trees were fertilized at 2- to 3-week intervals, receiving 22, 33 and 41 kg N ha^{-1} in 1999, 2000 and 2001, respectively. Soil water content was measured twice a week by time domain reflectometry (TDR) (TRIME-FM, IMKO Micromodultechnik GmbH, Ettlingen, Germany), and the trees were watered when soil water content was < 10% on a volume basis. The amount of water needed to maintain > 10% water content was estimated from the water retention characteristics of the soil, which were measured in 1999. Soil water content at field capacity was 11.8% (Vapaavuori et al. 2002).

Carbon dioxide and ozone exposure, and temperature and light conditions in open-top chambers

The trees were grown and fumigated for three growing seasons in cylindrical OTCs (one tree per OTC) that were 2.5 m in diameter and 7.8 m high. The treatments were: outside control (OC); chamber control in ambient air (CC); elevated $[CO_2]$ (2 × ambient, EC); elevated $[O_3]$ (2 × ambient, EO); and a combination of elevated $[CO_2]$ + elevated $[O_3]$ (both 2 × ambient, EC+EO). We aimed to double the ambient $[CO_2]$ in the EC treatments for 24 h per day, and to double the ambient $[O_3]$ in the EO treatments for 12 h per day between 0800 and 2000 h in 1999 and 2000, and for 14 h per day between 0800 and 2200 h in 2001. The fumigation periods were May 25 to October 4 in 1999, May 4 to September 29 in 2000 and May 2 to September 27 in 2001. During the 3-year exposure, $[CO_2]$ and $[O_3]$ were close to the target values: mean $[CO_2]$, AOT40 values for exposure hours (i.e., sum of hourly $[O_3]$ above 40 ppb during daytime hours) and AOT00 (i.e., sum of hourly $[O_3]$ above 0 ppb during daytime hours) values are given in Table 1. Ventilation and temperature were controlled by blowing air into the chambers at a flow rate of 0.1–0.6 $m^3 s^{-1}$ with computer-controlled blowers. The temperature sum for each year is presented in Table 1. Mean daily temperature was 1.7, 2.3 and

Table 1. Ozone exposure, mean values for carbon dioxide concentration ($[CO_2]$) and temperature sum (over the threshold of 5 °C) during the 1999, 2000 and 2001 growing seasons. Ozone and CO_2 exposures are based on hourly mean values. Ozone exposure is expressed as accumulated over a threshold of 0 ppb (AOT00) or 40 ppb (AOT40) during the exposure hours (12, 12 and 14 h in 1999, 2000 and 2001, respectively), and $[CO_2]$ is expressed as the mean of each growing season. For the EO and EC treatments, data are the means ± SD of 16 chambers and $n = 1$ for the CC treatment. Temperature sum is the mean of all 32 chambers ± SD, and temperature of ambient air was measured at one location within the canopy. Abbreviations: CC = chamber control; EC = elevated $[CO_2]$; and EO = elevated $[O_3]$.

Parameter	Treatment	1999	2000	2001
AOT00 (ppm h^{-1})	CC	49.4	66.2	66.3
	EO	73.6 ± 1.5	97.1 ± 1.8	106.8 ± 1.5
AOT40 (ppm h^{-1})	CC	2.8	2.5	1.7
	EO	20.6 ± 1.3	24.4 ± 1.5	30.9 ± 1.4
$[CO_2]$ (ppm)	CC	365	371	373
	EC	651 ± 85	720 ± 40	729 ± 38
Temperature sum (degree days)	Ambient	1390	1243	1303
	Chambers	1603 ± 52	1618 ± 65	1701 ± 77

2.4 °C higher in the chambers than in ambient air during the 1999, 2000 and 2001 growing seasons, respectively. The polyethylene film used to cover the chambers transmitted 91% of the incident light at wavelengths of 400–800 nm; transmittance decreased sharply at wavelengths < 400 nm and only 4.3% of the light at 300 nm was transmitted through the film.

Growth analysis

Height growth and stem diameter growth at 130 cm aboveground were measured each growing season. Timing of leaf fall was monitored by collecting the fallen leaves within the chambers weekly during each growing season from mid-July onward, which also enabled measurements of total leaf mass and area. These leaves were also used to determine specific leaf area (SLA): for which purpose, leaf area (LI-3050A leaf area meter, Li-Cor, Lincoln, NE) and leaf dry mass were measured weekly during September for 20 randomly sampled fallen leaves for each chamber tree. Total leaf area was obtained by multiplying mean SLA by the total dry mass of the leaf litter. The seeds produced by the trees in 2001 were collected manually at the beginning of August, dried at room temperature and weighed.

Final harvest

At the beginning of October 2001, the 40 study trees were harvested. Total stem length and yearly height increment in 1998, 1999, 2000 and 2001 were measured. Stem diameter was measured at the stem base, at 130 cm above ground and at 40% of the total stem height as well as at the base of the 1998, 1999, 2000 and 2001 stem sections. Total dry mass of the stem sections and branches was calculated from the fresh mass/dry mass ratio, measured in 1-cm-thick stem samples and a randomly selected branch from each stem section. Dry mass of each sample was determined after drying at 60 °C for 5 days. Coarse roots were excavated in May 2002 within a 1.5-m-diameter circle and to a depth of 20 cm using the stump as the central point. According to Laitakari (1935), the mean depth of a silver birch root system in sandy soil (stem diameter at 130 cm = 5–10 cm) is about 14 cm. Thus, most of the coarse roots were included in the excavated soil volume. After washing, the roots were dried at 80 °C and weighed. Biomass allocations to leaves (leaf mass ratio, LMR), branches (branch mass ratio, BMR), stems (stem mass ratio, SMR) and roots (root mass ratio, RMR) were determined as percentages of total dry mass of the tree.

To estimate the stem volume of trees, a stem curve was formulated from the height and stem diameter measurements at the final harvest based on the taut spline of de Boor (1978), which provides smooth and stable interpolating functions (stability parameter was given the value 6). Stem volume was computed separately for the stem between the base and 130 cm above ground and for the top section by integrating the cross-sectional area obtained from the stem curve. Stem curves were computed using the Jakta program (Lappi 2002). To estimate stem volumes during previous years, linear regression equations were estimated for the form factors (ratio of volume to the corresponding cone volume) of both stem sections with basal diameter, diameter at 130 cm above ground and total height as potential predictor variables. The lower form factor was dependent on the difference between basal diameter and diameter at 130 cm above ground, and the upper form factor was dependent on total height. Both regressions behaved logically for small stems. Previous stem volumes were computed using these form factor regressions.

Data analysis

Data were analyzed as the means of eight replicates per treatment when the clones were pooled and as the means of four replicates per treatment when the clones were analyzed separately. The chamber effect was studied by comparing CC and OC trees and the treatment effects were studied by excluding OC trees from the analysis. Initial values of height and stem diameter were the covariates in the analyses of stem height and diameter growth, and the initial stem volume was the covariate in the analysis of stem volume growth, biomass and leaf area. Mean values in the figures and tables are normalized with a covariate (estimated marginal means). Height, stem diameter and stem volume growth increments, total leaf area and leaf abscission data were subjected to analysis of variance (ANOVA) for repeated measurements, with [CO_2], [O_3], year and clone as fixed factors. To identify treatment differences in timing of leaf abscission, a cumulative leaf abscission curve was formed. From the curve, the date when about 50% of the leaves of CC trees had abscised was determined (September 27, September 29 and October 1 in 1999, 2000 and 2001, respectively). The date was determined from data where the clones were pooled. Differences in leaf abscission between treatments were compared on the same dates. Biomass and mass ratio data were analyzed by three-way ANOVA. All mass ratios and percentages were arcsine-transformed before analyses. Root biomass data were log-transformed to meet ANOVA requirements. For seed biomass and SLA data for the year 2001, the non-parametric Kruskal-Wallis test was used. Differences were considered to be significant at $P = 0.05$. All statistical analyses were performed with SPSS for Windows, Version 11.5.1 software (SPSS, Chicago, IL).

Results

Height, stem diameter and stem volume growth

In general, the two clones had similar height, stem diameter and stem volume growth (Figures 1 and 2, Table 2). When data for the clones were analyzed together, stem volume and stem diameter growth were increased by elevated [CO_2], whereas height growth was unaffected (Figures 1 and 2, Table 2). When results for the clones were analyzed separately, elevated [CO_2] increased stem diameter growth (Table 2). In Clone 80, stem volume growth was increased 56 and 54% by the EC and EC+EO treatments, respectively, compared with the CC treatment, and the elevated [CO_2] effect was enhanced during the experiment (Figure 2, Table 2).

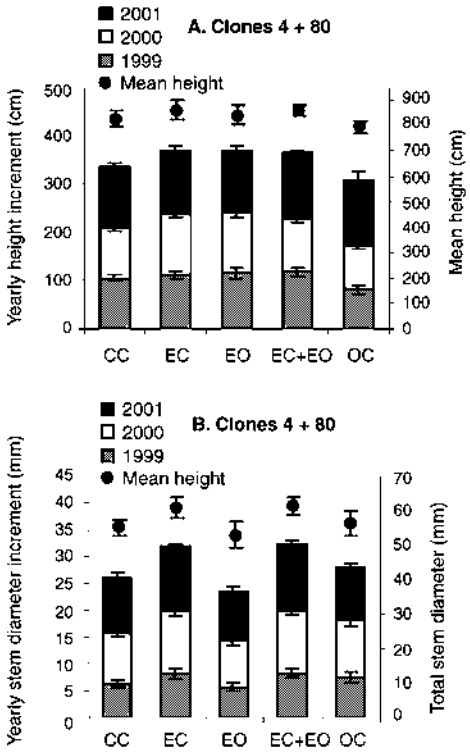

Figure 1. (A) Treatment effects on yearly height growth of silver birch Clones 4 and 80 in the 1999, 2000 and 2001 growing seasons and mean height at the end of the experiment. (B) Effects of treatments on yearly stem diameter growth measured at 130 cm above ground of Clones 4 and 80 and mean stem diameter at 130 cm above ground at the end of the experiment. Treatments were: chamber control (CC); elevated [CO_2] (EC); elevated [O_3] (EO); elevated [CO_2] + [O_3] (EC+EO); and outside control (OC). Data are estimated marginal means ± SE of eight replicates.

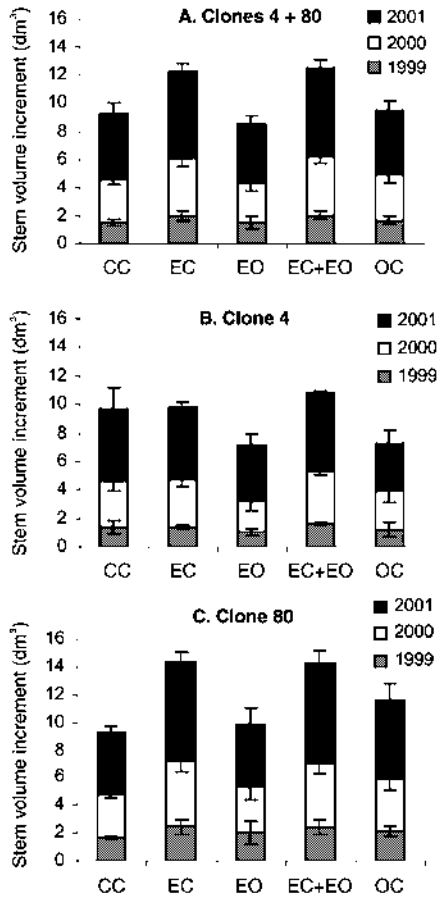

Figure 2. Effects of treatments on stem volume increment of silver birch Clones 4 and 80 in the 1999, 2000 and 2001 growing seasons, estimated from height and diameter data at the final harvest. Treatments were: chamber control (CC); elevated [CO_2] (EC); elevated [O_3] (EO); elevated [CO_2] + [O_3] (EC+EO); and outside control (OC). (A) Clones 4 and 80 together; (B) Clone 4; and (C) Clone 80. Data are estimated marginal means ± SE of eight (A) or four (B and C) replicates per treatment.

Leaf area

Total leaf area, calculated from fallen leaves at the end of each growing season, was similar in the clones (Figure 3, Table 2). Elevated [CO_2] increased total leaf area when results for both clones were analyzed together. This response to elevated [CO_2] was due solely to the response of Clone 80: the EC and EC+EO treatments increased total leaf area of Clone 80 by 28 and 34%, respectively, and the elevated [CO_2] effect was magnified during the experiment. In Clone 4, there were no main treatment effects, but elevated [O_3] tended to decrease total leaf area by 24% (Figure 3, Table 2). Leaf abscission occurred earlier in Clone 80 than in Clone 4. In both clones, leaf abscission was significantly delayed in elevated [CO_2]. When data for both clones were analyzed together, leaf abscission was accelerated by elevated [O_3] (Figure 4, Table 2).

Biomass and biomass allocation

Total dry mass was similar in Clones 4 and 80 (Tables 3 and 4). Elevated [CO_2] increased the dry mass of all plant parts when results for the clones were analyzed together. The increase was mainly attributable to increased growth in Clone 80, where dry mass of all plant parts increased and total dry mass was 40 and 49% greater in the EC and EC+EO treatments, respectively, than in the CC treatment. In Clone 4, elevated [CO_2] had a significant positive effect on root dry mass only. In the pooled data and in the data for Clone 4, there was a significant interac-

Table 2. The *P* values for the main effects of [CO_2], [O_3], clone, year and their interactions on height, stem diameter (measured at 130 cm above ground) and volume growth, total leaf area and the timing of leaf abscission. The data were subjected to ANOVA for repeated measurements. For height and stem diameter data, the initial values of height and stem diameter at 130 cm above ground were covariates, and for the stem volume and total leaf area data, the initial stem volume was the covariate. There were no significant chamber effects as determined by comparing data from the CC and OC treatments by one-way ANOVA. Abbreviations: CC = chamber control; OC = outside control; and nd = not detectable. Significant treatment effects and interactions ($P < 0.05$) are indicated with an asterisk.

Effect	Height growth	Stem diameter growth	Stem volume	Total leaf area	Leaf abscission
Clones 4 + 80					
[CO_2]	0.850	0.000 *	0.001 *	0.019 *	0.000 *
[O_3]	0.187	0.363	0.769	0.684	0.034 *
Clone	0.377	0.208	0.257	0.604	0.000 *
Year	0.002 *	0.000 *	0.000 *	0.000 *	0.000 *
[CO_2] × [O_3]	0.053	0.247	0.481	0.150	0.164
Clone × [CO_2]	0.130	0.927	0.149	0.131	0.155
Clone × [O_3]	0.221	0.775	0.595	0.820	0.793
Clone × [CO_2] × [O_3]	0.860	0.808	0.309	0.580	0.971
Year × [CO_2]	0.872	0.695	0.004 *	0.007 *	0.055
Year × [O_3]	0.910	0.922	0.772	0.927	0.296
Year × [CO_2] × [O_3]	0.205	0.690	0.385	0.127	0.962
Year × clone	0.423	0.004 *	0.830	0.197	0.525
Year × clone × [CO_2]	0.058	0.387	0.039 *	0.330	0.993
Year × clone × [O_3]	0.497	0.561	0.950	0.566	0.918
Year × clone × [CO_2] × [O_3]	0.238	0.607	0.819	0.935	0.888
CC versus OC	0.852	0.265	0.964	nd	nd
Clone 4					
[CO_2]	0.436	0.037 *	0.305	0.649	0.017 *
[O_3]	0.152	0.451	0.603	0.629	0.170
Year	0.013 *	0.000 *	0.000 *	0.000 *	0.000 *
[CO_2] × [O_3]	0.235	0.274	0.228	0.110	0.300
Year × [CO_2]	0.168	0.266	0.775	0.307	0.209
Year × [O_3]	0.599	0.776	0.834	0.724	0.539
Year × [CO_2] × [O_3]	0.082	0.445	0.524	0.127	0.847
CC versus OC	0.515	0.814	0.500	nd	nd
Clone 80					
[CO_2]	0.145	0.011 *	0.002 *	0.024 *	0.001 *
[O_3]	0.934	0.631	0.841	0.918	0.112
Year	0.151	0.000 *	0.000 *	0.000 *	0.004 *
[CO_2] × [O_3]	0.119	0.520	0.776	0.577	0.359
Year × [CO_2]	0.353	0.914	0.000 *	0.025 *	0.254
Year × [O_3]	0.821	0.678	0.906	0.726	0.511
Year × [CO_2] × [O_3]	0.971	0.885	0.660	0.587	0.986
CC versus OC	0.903	0.434	0.405	nd	nd

tive effect of elevated [CO_2] and elevated [O_3] on root and branch dry mass that was associated with decreased growth in elevated [O_3] under ambient [CO_2] conditions (Tables 3 and 4).

Seed production, measured in 2001, was significantly higher in Clone 80 than in Clone 4. In Clone 80, elevated [CO_2] significantly increased seed dry mass, whereas seed dry mass was low in the other treatments (Tables 3 and 4).

Although total biomass was similar in the two clones, there were clonal differences in some biomass allocation parameters. For example, SMR and mean leaf size (MLS) were greater in Clone 80 than in Clone 4, whereas BMR, LMR, leaf area ratio (LAR) and SLA were greater in Clone 4 than in Clone 80 (Tables 5 and 6). Clonal responses to the treatments varied (Tables 5 and 6). When the clones were analyzed together, elevated [O_3] decreased mean leaf size in ambient [CO_2]. In both clones, elevated [CO_2] decreased SLA (Tables 5 and 6).

Discussion

Experimental conditions

In this experiment, we simulated growth conditions predicted to prevail by the end of this century by doubling ambient [CO_2] and [O_3] (IPCC 2001) in OTCs. In Suonenjoki, mean daily [O_3] in ambient air varied between 10 and 60 ppb during the experiment, indicating that the elevated [O_3] treatment was moderate and realistic (Vapaavuori et al. 2002). In the OTCs,

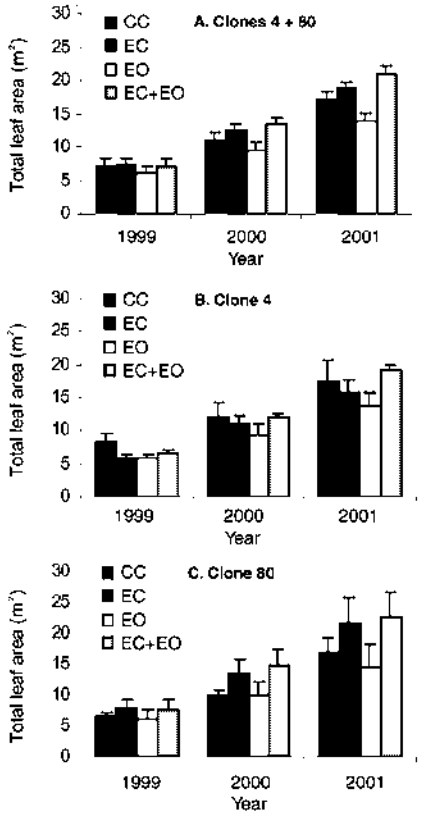

Figure 3. Effects of the treatments on total leaf area (m^2) of silver birch Clones 4 and 80 at the end of the 1999, 2000 and 2001 growing seasons. Treatments were: chamber control (CC); elevated $[CO_2]$ (EC); elevated $[O_3]$ (EO); and elevated $[CO_2] + [O_3]$ (EC+EO). Leaf abscission from the chamberless outside control treatment (OC) could not be determined. (A) Clones 4 and 80 together; (B) Clone 4; and (C) Clone 80. Data are estimated marginal means ± SE of eight (A) or four (B and C) replicates per treatment.

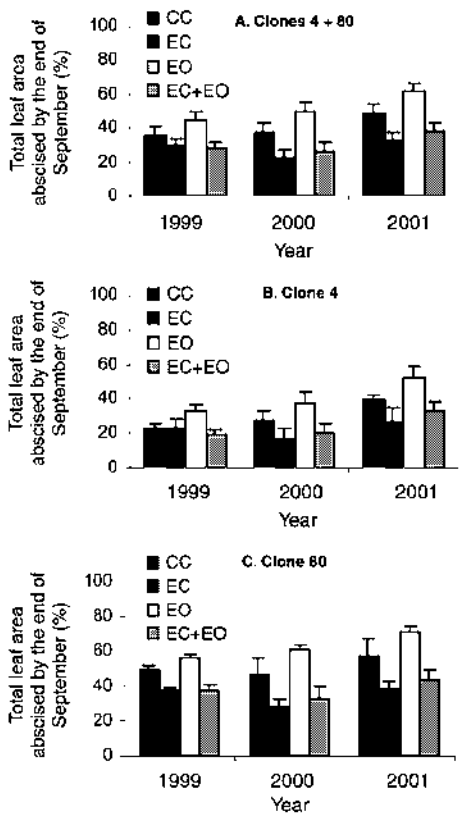

Figure 4. Effects of treatments on timing of leaf abscission in silver birch Clones 4 and 80 during the 1999, 2000 and 2001 growing seasons. To show treatment differences in timing of leaf abscission, a cumulative leaf abscission curve was developed. From the curve, we determined the date of leaf collection closest to the time when about 50% of the leaves of CC trees of both clones were abscised (September 27 in 1999, September 29 in 2000 and October 1 in 2001). Because leaf abscission occurred earlier in Clone 80 than in Clone 4, the percentage of leaves abscised from CC trees of Clone 4 on the dates mentioned above was less than 50%. Differences between treatments in cumulative leaf abscission were compared on the above dates in each clone. Treatments were: chamber control (CC); elevated $[CO_2]$ (EC); elevated O_3 concentration ($[O_3]$) (EO); and elevated $[CO_2] + [O_3]$ (EC+EO). Leaf abscission from trees in the chamberless outside control treatment (OC) could not be determined. (A) Clones 4 and 80 together; (B) Clone 4; and (C) Clone 80. The data are means ± SE of eight (A) or four (B and C) replicates per treatment.

mean daily temperature was 2–3 °C higher, and light and relative humidity were slightly lower than in ambient air (Vapaavuori et al. 2002). The clones responded similarly to the altered microclimate, and there were no significant chamber effects on the measured growth parameters despite the genetic differences in growth patterns and physiological properties of the clones (Riikonen et al. 2003). However, biomass allocation differed between clones: Clone 80 preferentially invested carbon in stem growth, whereas Clone 4 primarily invested carbon in the growth of branches and leaves.

Ozone sensitivity of silver birch clones

The response of our 10-year-old field-grown trees to elevated $[O_3]$ was opposite to that previously reported in an O_3-sensitivity test on potted seedlings (Pääkkönen et al. 1997). Contrary to the study of Pääkkönen et al. (1997), Clone 80 was not sensitive to elevated $[O_3]$, whereas total biomass of Clone 4 tended to decline in response to elevated $[O_3]$ in ambient $[CO_2]$ (360 ppm). This observation is in accordance with data from a

Table 3. Effects of the treatments on dry mass of plant organs of silver birch Clones 4 and 80 after final harvest in 2001. In the OC treatment, the total dry mass data could not be calculated because no leaf mass data were obtained. Treatments were: chamber control (CC); elevated [CO_2] (EC); elevated [O_3] (EO); elevated [CO_2] + [O_3] (EC+EO); and outside control (OC). Data are estimated marginal means ± SE of four replicates for each clone and treatment. Abbreviation: nd = not detectable.

Dry mass	Clone	CC	EC	EO	EC+EO	OC
Total (kg)	4	10.38 ± 1.93	10.14 ± 0.93	7.65 ± 1.21	11.50 ± 0.08	nd
	80	10.56 ± 0.65	14.76 ± 1.92	10.52 ± 2.83	15.71 ± 2.08	nd
	4 + 80	10.47 ± 0.95	12.45 ± 1.45	9.08 ± 1.48	13.60 ± 1.31	nd
Root (kg)	4	2.25 ± 0.37	2.14 ± 0.22	1.40 ± 0.12	2.29 ± 0.08	2.21 ± 0.48
	80	2.03 ± 0.14	2.92 ± 0.42	1.87 ± 0.40	3.10 ± 0.41	2.18 ± 0.23
	4 + 80	2.14 ± 0.19	2.53 ± 0.28	1.64 ± 0.20	2.70 ± 0.26	2.19 ± 0.26
Stem (kg)	4	5.46 ± 1.16	5.66 ± 0.54	4.26 ± 0.85	6.31 ± 0.02	4.80 ± 1.25
	80	6.06 ± 0.36	8.52 ± 1.14	6.28 ± 1.79	8.80 ± 1.10	5.93 ± 1.02
	4 + 80	5.76 ± 0.56	7.09 ± 0.88	5.27 ± 0.96	7.56 ± 0.73	5.37 ± 0.76
Branch (kg)	4	1.64 ± 0.28	1.35 ± 0.08	1.16 ± 0.16	1.70 ± 0.06	1.41 ± 0.46
	80	1.44 ± 0.07	1.87 ± 0.19	1.46 ± 0.43	2.30 ± 0.40	1.36 ± 0.27
	4 + 80	1.54 ± 0.15	1.61 ± 0.15	1.31 ± 0.22	2.00 ± 0.23	1.39 ± 0.25
Leaf (kg)	4	1.03 ± 0.18	0.99 ± 0.11	0.82 ± 0.11	1.20 ± 0.04	nd
	80	1.05 ± 0.14	1.45 ± 0.26	0.90 ± 0.23	1.51 ± 0.24	nd
	4 + 80	1.04 ± 0.10	1.22 ± 0.16	0.86 ± 0.12	1.35 ± 0.13	nd
Seed (g)	4	0	0.11 ± 0.08	0.11 ± 0.08	0.03 ± 0.08	0
	80	0	13.83 ± 15.05	1.74 ± 15.11	6.97 ± 15.07	0
	4 + 80	0	6.97 ± 3.51	0.93 ± 0.40	23.5 ± 16.14	0

Table 4. The *P* values for the main effects of [CO_2], [O_3] and clone and their interactions on dry mass of different organs of silver birch Clones 4 and 80 after final harvest in 2001. Data were subjected to ANOVA. The initial stem volume values were the covariate. There were no significant chamber effects as determined by comparing data from the CC and OC treatments by one-way ANOVA. Dry mass of seeds was analyzed with the Kruskal-Wallis test. Abbreviations: CC = chamber control; OC = outside control; and nd = not detectable. Significant treatment effects and interactions ($P < 0.05$) are indicated by an asterisk.

Effect	Dry mass					
	Total	Root	Stem	Branch	Leaf	Seed
Clones 4 + 80						
[CO_2]	0.000 *	0.000 *	0.000 *	0.004 *	0.001 *	0.020 *
[O_3]	0.867	0.258	0.972	0.533	0.790	0.819
Clone	0.128	0.309	0.054	0.610	0.616	0.001 *
[CO_2] × [O_3]	0.108	0.041 *	0.305	0.035 *	0.157	
Clone × [CO_2]	0.074	0.043 *	0.145	0.087	0.137	
Clone × [O_3]	0.421	0.230	0.529	0.261	0.814	
Clone × [CO_2] × [O_3]	0.379	0.327	0.356	0.539	0.780	
CC versus OC	nd	0.649	0.706	0.762	nd	0.144
Clone 4						
[CO_2]	0.099	0.025 *	0.091	0.450	0.180	0.644
[O_3]	0.477	0.082	0.639	0.661	0.982	0.538
[CO_2] × [O_3]	0.060	0.021 *	0.147	0.020 *	0.098	
CC versus OC	nd	0.877	0.482	0.594	nd	1.000
Clone 80						
[CO_2]	0.001	0.001 *	0.002 *	0.011 *	0.006 *	0.003 *
[O_3]	0.663	0.605	0.680	0.295	0.771	0.749
[CO_2] × [O_3]	0.635	0.293	0.963	0.358	0.503	
CC versus OC	nd	0.654	0.885	0.997	nd	0.131

Table 5. Root mass ratio (RMR), stem mass ratio (SMR), branch mass ratio (BMR), leaf mass ratio (LMR), leaf area ratio (LAR), mean leaf size (MLS), root:shoot dry mass ratio and specific leaf area (SLA) after final harvest in 2001 of silver birch Clones 4 and 80. Leaf data for the chamber trees were calculated from leaf litter. Mass ratios were not calculated for OC because no leaf mass data were obtained. Treatments were: chamber control (CC); elevated [CO_2] (EC); elevated [O_3] (EO); elevated [CO_2] + [O_3] (EC+EO); and outside control (OC). Data are means ± SE of four replicates for each clone and treatment.

Parameter	Clone	CC	EC	EO	EC+EO
RMR	4	0.22 ± 0.01	0.22 ± 0.01	0.19 ± 0.01	0.20 ± 0.01
	80	0.19 ± 0.01	0.20 ± 0.02	0.19 ± 0.01	0.20 ± 0.01
	4 + 80	0.21 ± 0.01	0.021 ± 0.01	0.19 ± 0.01	0.20 ± 0.0
SMR	4	0.54 ± 0.02	0.55 ± 0.01	0.56 ± 0.03	0.56 ± 0.01
	80	0.58 ± 0.01	0.58 ± 0.01	0.60 ± 0.01	0.57 ± 0.02
	4 + 80	0.56 ± 0.01	0.57 ± 0.01	0.58 ± 0.02	0.57 ± 0.01
BMR	4	0.16 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.00
	80	0.14 ± 0.00	0.13 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
	4 + 80	0.15 ± 0.00	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.00
LMR	4	0.098 ± 0.005	0.101 ± 0.006	0.109 ± 0.010	0.104 ± 0.004
	80	0.098 ± 0.007	0.096 ± 0.005	0.084 ± 0.005	0.095 ± 0.003
	4 + 80	0.098 ± 0.004	0.099 ± 0.004	0.097 ± 0.007	0.100 ± 0.003
LAR ($cm^2\ g_{DW}^{-1}$)	4	16.4 ± 0.7	15.6 ± 1.0	18.3 ± 1.7	16.8 ± 0.6
	80	15.7 ± 1.2	14.4 ± 1.1	13.7 ± 0.7	14.1 ± 0.8
	4 + 80	16.1 ± 0.6	15.0 ± 0.7	16.0 ± 1.2	15.5 ± 0.7
MLS (cm^2)	4	15.9 ± 1.0	15.1 ± 0.6	14.1 ± 0.7	15.5 ± 0.2
	80	17.2 ± 0.6	16.1 ± 0.5	15.6 ± 0.7	16.8 ± 1.1
	4 + 80	16.6 ± 0.6	15.6 ± 0.4	14.9 ± 0.5	16.2 ± 0.6
Root:shoot dry mass ratio	4	0.28 ± 0.02	0.28 ± 0.01	0.23 ± 0.02	0.25 ± 0.01
	80	0.24 ± 0.01	0.25 ± 0.02	0.23 ± 0.02	0.25 ± 0.01
	4 + 80	0.26 ± 0.00	0.27 ± 0.00	0.23 ± 0.00	0.25 ± 0.00
SLA ($cm^2\ g_{(l)w}^{-1}$)	4	167.9 ± 3.4	155.0 ± 6.3	167.7 ± 1.9	160.8 ± 1.6
	80	160.7 ± 0.8	149.5 ± 6.2	161.7 ± 0.8	149.2 ± 2.9
	4 + 80	164.3 ± 2.1	152.3 ± 4.2	164.7 ± 1.5	155.0 ± 2.7

recent, multi-year O_3-exposure study with Clone 4, showing increased O_3 sensitivity with exposure period and tree growth (Oksanen 2003). Our data pinpoint the difficulties in assessing O_3 sensitivity of genotypes at the seedling or sapling stage, because O_3 sensitivity may increase or decrease during growth and maturation. Such changes in O_3 sensitivity may be partly explained by higher respiratory costs and lower photosynthetic rates and stomatal conductances in mature trees compared with saplings (Oksanen 2003).

Elevated [O_3] often reduces root growth of tree species (Coleman et al. 1995, Rebbeck and Loats 1997, Oksanen and Rousi 2001). In our study, root growth was inhibited by elevated [O_3] in Clone 4 in ambient [CO_2] conditions only. Decreased root growth may be associated with decreased translocation of assimilates from shoots to roots (see review by Skärby et al. 1998) or decreased C available from photosynthesis (Paludan-Müller et al. 1999). Coleman et al. (1995) concluded that O_3 preferentially damages older leaves that provide most of the energy required for root growth. When we analyzed the pooled data for both clones, elevated [O_3] reduced mean leaf size in ambient [CO_2] and accelerated leaf abscission. Furthermore, in Clone 4, total leaf area and total leaf mass tended to decrease in response to elevated [O_3] in ambient [CO_2]. These responses would result in a shorter period of active photosynthesis and a decreased photosynthetic surface area, leading to decreased net photosynthesis and growth at the tree level. However, elevated [O_3] had no effect on photosynthesis at the leaf level in either Clone 4 or Clone 80 (Riikonen et al., unpublished observations). Nevertheless, the reduction in root biomass in Clone 4 in response to elevated [O_3] was reflected in reductions in total biomass and branch biomass, but only under ambient, not elevated, [CO_2] conditions.

The reason for the clonal differences in O_3 sensitivity is unclear, because the gas exchange parameters were unaffected by elevated [O_3] in either of the clones (Riikonen et al., unpublished observations). Increased O_3 tolerance could be related to thick leaves and cell walls (Oksanen et al. 2001), investment in foliage mass (Pääkkönen et al. 1996), low stomatal density and conductance (Pääkkönen et al. 1997) and low growth rate (Skärby et al. 1998). The thinner leaves of the O_3-sensitive Clone 4 (cf. SLA of Clones 4 and 80) may enable greater flux of O_3 into the leaves compared with leaves of Clone 80. On the other hand, stomatal conductance and density were lower in Clone 4 than in Clone 80 (Riikonen et al. 2003), which would

Table 6. The P values for root mass ratio (RMR), stem mass ratio (SMR), branch mass ratio (BMR), leaf mass ratio (LMR), leaf area ratio (LAR), mean leaf size (MLS), root:shoot ratio and specific leaf area (SLA) after final harvest in 2001 of silver birch Clones 4 and 80. Leaf data for the chamber trees were calculated from leaf litter. Mass ratios were not calculated for the outside control because no leaf mass data were obtained. Main effects and interactions were tested by ANOVA. The SLA 2001 data were analyzed with the Kruskal-Wallis test. Significant treatment effects and interactions ($P < 0.05$) are indicated with an asterisk.

Effect	RMR	SMR	BMR	LMR	LAR	MLS	Root: shoot	SLA 2001
Clones 4 + 80								
[CO_2]	0.372	0.740	0.235	0.691	0.288	0.750	0.377	0.001 *
[O_3]	0.121	0.500	0.246	0.978	0.792	0.282	0.120	0.821
Clone	0.141	0.007 *	0.019 *	0.029 *	0.004 *	0.017 *	0.142	0.022 *
[CO_2] × [O_3]	0.715	0.303	0.134	0.810	0.704	0.033 *	0.732	
Clone × [CO_2]	0.852	0.344	0.230	0.547	0.616	0.826	0.799	
Clone × [O_3]	0.277	0.676	0.718	0.093	0.076	0.819	0.268	
Clone × [CO_2] × [O_3]	0.813	0.757	0.943	0.259	0.383	0.998	0.811	
Clone 4								
[CO_2]	0.621	0.688	0.064	0.898	0.311	0.691	0.661	0.012 *
[O_3]	0.080	0.483	0.505	0.283	0.184	0.338	0.079	0.916
[CO_2] × [O_3]	0.675	0.638	0.242	0.575	0.744	0.116	0.689	
Clone 80								
[CO_2]	0.451	0.332	0.993	0.422	0.669	0.947	0.424	0.012 *
[O_3]	0.732	0.842	0.350	0.183	0.250	0.571	0.737	1.000
[CO_2] × [O_3]	0.928	0.310	0.331	0.274	0.353	0.155	0.941	

decrease the flux of [O_3] to the leaves. In turn, the higher O_3 tolerance of Clone 80 may be related to more efficient detoxification of reactive oxygen species by the leaves, which is an important determinant of O_3 sensitivity (Rao et al. 1995).

Elevated [CO_2] and growth

When data for both clones were analyzed together, elevated [CO_2] had a positive effect on the growth parameters measured and, in accordance with earlier studies, stem diameter growth was accelerated more than height growth (Kubiske et al. 1998, Isebrands et al. 2001, Sigurdsson et al. 2001). In Clone 80, the elevated [CO_2] treatments increased total biomass by 40–49%, which is comparable with the average increase of 49% reported for deciduous trees (Saxe et al. 1998). Elevated [CO_2] increased the stem volume of Clone 80 by 44%, which is considerably more than the 28% reported for aspen in FACE and OTC studies (Zak et al. 2000, Isebrands et al. 2001). Because most CO_2 studies have been conducted on young seedlings in which leaf area has not stabilized, it is not known if the growth stimulation by elevated [CO_2] will be sustained after canopy and root closure (Rey and Jarvis 1997, Centritto et al. 1999, Norby et al. 1999). In our stand, which was approaching canopy closure, the growth enhancement in Clone 80 was sustained over the 3 years and the stimulation by elevated [CO_2] of stem volume growth was enhanced toward the end of the experiment. According to Centritto et al. (1999), the main effect of elevated [CO_2] is acceleration of ontogenetic development in the early phase of exposure. However, Zak et al. (2000) found that plants grew larger and faster in elevated [CO_2], at both low and high concentrations of soil nitrogen. In our experiment, elevated [CO_2] significantly increased seed production in Clone 80, indicating ontogenic advancement in addition to increased growth.

There are few studies in which the growth response to elevated [CO_2] has been insignificant (Volin and Reich 1996, Kubiske et al. 1998, Sigurdsson et al. 2001). We found no significant increase in total dry mass, stem volume or total leaf area of Clone 4 in response to elevated [CO_2], although net photosynthesis increased in elevated [CO_2] in both clones (Riikonen et al., unpublished data). In our study, the difference in growth response of the clones was unrelated to differences in nutrient availability at the site, because trees of both clones experienced the same fertilizer regimes and the soil nutrient concentrations (Vapaavuori et al. 2002) were comparable with those of forest and nursery soils (Luoranen 2000). It is possible that the clones differ in growth requirements, reflecting their different origins. Clone 4 may have been subject to greater environmental stress in Suonenjoki than Clone 80, which was growing closer to its latitude of origin (Vapaavuori et al. 2002). If so, the overall capacity to increase sink size may have been limited in Clone 4. Alternatively, Clone 4 may have invested more in processes other than growth. This notion is supported by other data from this site showing a higher leaf phenolic content in Clone 4 than in Clone 80, indicating increased investment in chemical defence (Peltonen et al., unpublished observations).

Combined effects of elevated [CO_2] and [O_3]

The negative effects of elevated [O_3] were found mainly in ambient [CO_2] (360 ppm), not in elevated [CO_2] (720 ppm). The ameliorating effect of elevated [CO_2] is in accordance with the

results of single-season OTC and growth chamber studies on small saplings of various deciduous tree species (Mortensen 1995, Volin and Reich 1996, Dickson et al. 1998, Loats and Rebbeck 1999) and long-term open-field and OTC studies with aspen and yellow-poplar (Percy et al. 2002, Rebbeck and Scherzer 2002). The ameliorating effect of elevated [CO_2] may be associated with either increased detoxification capacity as a consequence of higher carbohydrate concentrations in leaves grown in elevated [CO_2], or decreased stomatal conductance and thus decreasing [O_3] uptake in elevated [CO_2] conditions (e.g., Rao et al. 1995).

Leaf area

Leaf area is often increased by elevated [CO_2] (Norby et al. 1999) and decreased by elevated [O_3] (Günthardt-Goerg et al. 1993). Elevated [O_3] tended to decrease total leaf area in ambient [CO_2] in Clone 4. The positive effect of elevated [CO_2] on leaf area was found only in Clone 80 and the magnitude of the effect increased during the experiment. The increase resulted from enhanced leaf production, as mean leaf size was unaffected. In the elevated [CO_2] treatments, SLA was significantly reduced in both clones, indicating adjustments and acclimation in leaf structure and thus light interception (see review by Norby et al. 1999).

Tree growth is determined by the length of the growing season, which may be affected by the predicted climate change. Elevated [O_3] resulted in earlier leaf abscission, (observed when the clones were pooled), which is a typical response to O_3 in many deciduous species (Karnosky et al. 1996, Bortier et al. 2000) and may be related to O_3-induced ethylene production (Kangasjärvi et al. 1994). The studies of the effect of elevated [CO_2] on the timing of leaf abscission have yielded contradictory results (Gielen and Ceulemans 2001). In our study, the treatments with elevated [CO_2] delayed leaf abscission.

Seed production

The effects of elevated [CO_2] and [O_3] on seed production are not well known, because most studies have been made with juvenile trees. As reviewed by Black et al. (2000), elevated [O_3] reduces seed yield in a wide range of species, either directly by affecting the reproductive structures or indirectly by altering assimilate production and partitioning. In contrast to [O_3], elevated [CO_2] usually stimulates plant reproduction, although there is great variability in the effect among species (Jablonski et al. 2002). Seed production in Clone 4 was negligible in all treatments. In Clone 80, the elevated [CO_2] treatments significantly increased seed production, perhaps indicating accelerated ontogenetic development in elevated [CO_2], as suggested by Centritto et al. (1999). Elevated [O_3] alone had no effect on seed production, but in combination with elevated [CO_2], seed production was 3.5-fold greater than with elevated [CO_2] alone. Ozone is known to alter the synthesis and distribution of plant hormones and induce chemical defence genes (Kangasjärvi et al. 1994), which may be involved in the mechanism underlying the interaction we observed.

In conclusion, elevated [CO_2] increased stem diameter and volume growth, total biomass and total leaf area when results for both clones were analyzed together. The effect of elevated [O_3] alone was small, causing earlier leaf abscission in the autumn. There were, however, significant interactions between [CO_2] and [O_3]. Elevated [O_3] decreased the dry mass of roots and branches and mean leaf size in ambient, but not in elevated [CO_2]. When results for the clones were analyzed separately, growth, biomass and total leaf area of Clone 80 increased in elevated [CO_2], but were unaffected by elevated [O_3]. In Clone 4, root and branch biomass decreased in elevated [O_3] in ambient [CO_2], but the response to elevated [CO_2] was small. Leaf area increased significantly in elevated [CO_2] in Clone 80 only, which may partly explain the smaller growth responses to elevated [CO_2] in Clone 4 compared with Clone 80. The negative effects of elevated [O_3] were smaller in elevated [CO_2] than in ambient [CO_2], possibly because greater amounts of carbohydrates were available for detoxification and repair in elevated [CO_2]. The response of our 10-year-old field-grown trees to elevated [O_3] was opposite to that previously reported in an [O_3]-sensitivity test on potted saplings. This finding indicates that O_3 sensitivity changes with exposure period and tree growth and highlights the need for long-term field experiments with mature trees to assess the effects of climate change on natural forests.

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Chapter 4

Leaf photosynthetic characteristics of silver birch during three years of exposure to elevated CO_2 and O_3 in the field

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Tree Physiology, in press

Leaf photosynthetic characteristics of silver birch during three years of exposure to elevated CO_2 and O_3 in the field

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Abstract The effects of elevated concentrations of CO_2 and O_3 on photosynthesis and related biochemistry of two European silver birch (*Betula pendula* Roth) clones were studied under field conditions during 1999-2001. Initially seven-year-old trees of clones 4 and 80 were exposed to the following treatments in an open-top chamber experiment: outside control (OC), chamber control (CC), 2× ambient CO_2 (EC), 2× ambient O_3 (EO), and 2× ambient CO_2 and 2× ambient O_3 together (EC+EO). During the experiment, gas exchange, chlorophyll fluorescence, amount and activity of Rubisco, concentration of chlorophyll, soluble protein, soluble sugars, starch, N and C/N were determined from short- and long-shoot leaves. Elevated CO_2 increased photosynthetic rate by around 30% when measurements were made in their respective growth CO_2. In ambient CO_2, photosynthesis was around 15% lower in plants grown in elevated CO_2 than in CC trees. This was due to decreased concentration of total leaf N (around 10%) and amount and activity of Rubisco (26% and 20%, respectively), and increased concentration of starch (49%). Elevated O_3 had no significant effect on gas exchange parameters and also the effect on biochemistry was small in both clones. However, elevated O_3 decreased the proportion of Rubisco of total soluble proteins and apparent quantum yield of PSII photochemistry in light and increased non-photochemical quenching in 2000. The interactive effect of CO_2 and O_3 was variable. Elevated O_3 decreased the chlorophyll concentration only in ambient CO_2, while elevated CO_2 and O_3 together decreased the total activity of Rubisco and increased the C/N ratio more than elevated O_3 alone. In conclusion, photosynthesis of silver birch clones was stimulated in elevated CO_2 although downregulation of photosynthesis was evident. The small effect of elevated O_3 on photosynthsesis and related biochemistry of the clones indicates that these young silver birches were fairly tolerant to the annual O_3 exposures that were 2-3 times higher than the AOT40-value of 10 ppm.h. set as a critical dose for forest trees.

Keywords *Betula pendula*, clone, chlorophyll, chlorophyll fluorescence, field experiment, gas exchange, nitrogen, open-top chamber, Rubisco

Introduction

The rising concentrations of CO_2 and O_3 are predicted to have a remarkable effect on the growth conditions of plants in future (IPCC 2001). The mechanisms and effects of the single gases on the physiology of the woody species are relatively well known (Medlyn et al. 1999; Norby et al. 1999; Matyssek 2001; Matyssek and Sandermann 2003). However, only a few field experiments with silver birch (*Betula pendula* Roth) in elevated CO_2 (Rey and Jarvis 1998) or in elevated O_3 (Matyssek 2001, Oksanen 2003) have been conducted despite of its wide distribution and economic importance in Northern Hemisphere. According to Kellomäki et al. (1996) in altered environmental conditions tree species with continuous growth pattern such as birch, may be favoured over the species with deterministic growth (e.g. conifers). However, silver birch is also known to be sensitive to O_3 (Matyssek 2001, Oksanen 2003) which may modify the competitive status of sensitive genotypes, as has been is suggested from a free-air CO_2 enrichment-experiment (FACE) on aspen clones (McDonald et al. 2002).

Photosynthetic responses of forest trees to elevated CO_2 have been intensively studied and the average enhancement in elevated CO_2 in field experiments has been 30-50% (Saxe et al. 1998, Medlyn et al. 1999, Nowak et al. 2004). Enhancement is mainly due to kinetic properties of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco): in elevated CO_2 concentration the ratio between carboxylation and oxygenation of Rubisco increases, leading to enhanced net photosynthesis and decreased photorespiration (Drake et al. 1997). However, in several long-term experiments acclimation to elevated CO_2 concentration (downregulation of photosynthesis) has been detected, especially under conditions where the sink capacity and the supply of nutrients and water are limited (Drake et al. 1997), but also in some long-term field experiments with non-limited rooting volume (Rey and Jarvis 1998, Medlyn et al. 1999, Nowak et al. 2004). This is exhibited as lower photosynthetic rates in plants grown in elevated CO_2 than in plants grown in ambient CO_2 concentration when all the plants are measured in current ambient CO_2 (360 ppm). Despite acclimation photosynthetic capacity is generally higher in elevated CO_2 than in ambient CO_2 concentration (Drake et al. 1997, Medlyn et al. 1999). In general, acclimation involves a redistribution of resources towards the most limiting processes, resulting in optimal growth in the prevailing environmental conditions, leading to increased resource use efficiency (e.g. N use efficiency, water use efficiency) (Drake et al. 1997). In elevated CO_2 leaf carbohydrate concentration often increases, reflecting discrepancies between supply and demand of photosynthates if the sink capacity is lowered by e.g. insufficient N supply, low temperature or restricted root growth. This may result in feedback inhibition of photosynthetic capacity and suppression of several photosynthetic genes (Thomas and Strain 1991, Drake et al. 1997).

Photosynthesis and stomatal conductance are commonly decreased by O_3 exposure (Long and Naidu 2002, Matyssek and Sandermann 2003). Ozone is capable of affecting almost all processes related to photosynthesis, including carbon fixation and light harvesting (Long and Naidu 2002). In general, the first change is a loss of amount and activity of Rubisco (Oksanen and Saleem 1999, Noormets et al. 2001, Yamaji et al. 2003): Ozone may increase the proteolysis of Rubisco or decrease the protein synthesis (Pell et al. 1997). Ozone may reduce stomatal conductance either directly by damaging the permeability of the guard cells or indirectly by reducing the photosynthetic capacity within the mesophyll (Long and Naidu 2002, Matyssek and Sandermann 2003). Elevated O_3 has also

been shown to decrease chlorophyll concentration (Oksanen and Saleem 1999, Wustman et al. 2001) and maximum photochemical yield of photosystem II (PSII) and apparent quantum yield of PSII photochemistry in light (F_v/F_m and F_v'/F_m', respectively) and to increase the non-photochemical fluorescence quenching (NPQ) (Lorenzini et al. 1999, Shavnin et al. 1999). Changes in these photosynthetic parameters may indicate direct O_3 effect to the thylakoid membrane and inactivation of active reaction centres leading to the dissipation of excitation energy into heat to avoid overexcitation of PSII (Lorenzini et al. 1999, Long and Naidu 2002).

It has been questioned whether the positive effects of elevated CO_2 on growth and photosynthesis can compensate for the negative effects of O_3 (Matyssek and Sandermann 2003). Field studies with CO_2 and O_3 have given conflicting results. In long-term FACE and open-top chamber (OTC) studies with aspen and yellow-poplar, elevated CO_2 and O_3 had counteractive effect on growth: elevated CO_2 generally reduced the negative effects of O_3 (Isebrands et al. 2001, Percy et al. 2002, Rebbeck and Scherzer 2002) although in many parameters the positive effect of CO_2 was negated by elevated O_3 (Isebrands et al. 2001, Percy et al. 2002). However, in Aspen FACE Project the counteractive effect of elevated CO_2 was not found in all response variables (Wustman et al. 2001) or was found only in O_3-tolerant aspen clones (Noormets et al. 2001). In some experiments elevated CO_2 even exacerbated the harmful effects of O_3 (Kull et al. 1996, Karnosky et al. 1998). It has been suggested that elevated CO_2 may counteract the O_3 effects by decreasing stomatal conductance and thus decreasing O_3 uptake in elevated CO_2 (Long and Naidu 2002). Alternatively, the effect might be due to enhanced photosynthesis leading to higher carbohydrate concentration in leaves and greater detoxification capacity in leaves grown in elevated CO_2 (e.g. Rao et al. 1995). The hypothesis that elevated CO_2 may exacerbate O_3 effects is based on the prediction that elevated CO_2 reduces the need for cellular detoxification of active oxygen species (AOS) and thus the trees growing in elevated CO_2 are also more susceptible to oxidative stress generated by O_3 (Niewiadomska et al. 1999, Polle and Pell 1999).

For this OTC study, two fast growing silver birch clones (clones 4 and 80, O_3-tolerant and –sensitive, respectively) were selected according to previous O_3-sensitivity test with potted seedlings (Pääkkönen et al. 1997). The silver birch clones were exposed to elevated CO_2 and O_3 both alone and in combination, using open-top chambers. By measuring photosynthesis and related parameters during the three-year exposure, the hypotheses tested were: 1) elevated CO_2 increases photosynthesis and induces acclimation processes (downregulation of photosynthesis) in the field conditions; 2) elevated O_3 decreases photosynthesis and related biochemistry in the field in both silver birch clones and 3) elevated CO_2 can compensate for the negative effects of O_3, if such effects are found.

Materials and methods

Field site and plant material

The experimental field was located at Suonenjoki Research Station in central Finland (62°39′N, 27°03′E, 120 m a.s.l.). At the beginning of the growing season in 1999, 20 trees of each clone (clones 4, V5952 and 80, K1659) were selected for the 3-year open-top chamber experiment. At the beginning of this experiment the trees were 7-years-old with a mean height of about 5 m and a mean stand density of 4200 stems per hectare. The O_3-sensitivity of the clones was previously determined in a 2-year pot experiment with 2-year old saplings exposed

to low O_3 concentrations (1.6× and 1.7× ambient O_3 concentration). According to visible injuries, growth and leaf senescence clone 4 was classified as O_3-tolerant and clone 80 as O_3-sensitive, both representing mid-range responses rather than extremes in the O_3-sensitivity ranking (Pääkkönen et al. 1997).

The trees were fertilised at two to three-week intervals receiving 22 kg N ha^{-1} in 1999 (Typpirikas Y-lannos, N-P-K 18-5-10, Kemira Ltd), 33 kg N ha^{-1} in 2000 (two times with Pellon Y-lannos, N-P-K 20-3-9, Kemira Ltd. and four times with Puutarhan Y-lannos 1, 10-7-14, Kemira Ltd.), and 41 kg N ha^{-1} in 2001 (Puutarhan Y-lannos 1, 10-7-14, Kemira Ltd.). The soil water content was measured twice a week (Time Domain Reflectometry, TDR, TRIME-FM, IMKO Micromodultechnik GMBH, Germany) and the trees were watered whenever the soil water content was <10%. More details about the field site, experimental design and technical details can be found in Vapaavuori et al. (2002).

Chamber conditions

The trees were grown and fumigated for three growing seasons in cylindrical open-top chambers (one tree in each chamber). The chambers were 2.5 m in diameter and 6.0 m high in 1999, and extended to 7.8 m high in 2000 and 2001, to account for tree growth. The treatments were: outside control (OC), chamber control in ambient air (CC), elevated CO_2 (2× ambient, EC), elevated O_3 (2× ambient, EO), and elevated CO_2 and elevated O_3 together (both 2× ambient, EC+EO). The treatments were set up in randomized incomplete block design (two of the treatments for both clones appeared in each block with each other) with 4 independent replicates for both clones and thus there were altogether 20 trees of each clone (Vapaavuori et al. 2002). Our aim was to double the CO_2 concentration in the EC treatments for 24 hours/day and the ambient O_3 concentration in the EO treatments for 12 h/day between 0800-2000 h in 1999 and

Table 1. Ozone exposure (AOT00, AOT40), mean values for CO_2 concentration and temperature sum (over the threshold of 5°C) during the 1999, 2000 and 2001 growing seasons in Suonenjoki OTC experiment. Ozone and CO_2 exposures are based on hourly mean values. Ozone exposure is expressed as accumulated over a threshold of 0 ppb (AOT00) or 40 ppb (AOT40) during the exposure hours (12h, 12h and 14h in 1999, 2000 and 2001, respectively), and CO_2 concentration is expressed as the mean of each growing season.

Parameter	Treatment	1999	2000	2001
AOT00 (ppm h^{-1})	CC	49.4	66.2	66.3
	EO	73.6±1.5	97.1±1.8	106.8±1.5
AOT40 (ppm.h^{-1})	CC	2.8	2.5	1.7
	EO	20.6±1.3	24.4±1.5	30.9±1.4
CO_2, (ppm)	CC	365	371	373
	EC	651±85	720±40 7	29±38
Temperature sum (degree days)	Ambient	1390	1243	1303
	OTCs	1603±52	1618±65	1701±77

In EO and EC, n=16±SD and the values for CC were determined from one OTC, *n*=1.Temperature sum (with 5 °C threshold) is the mean of all chambers (*n*=32±SD), and ambient air was measured at one location within the canopy. Abbreviations: CC = chamber control, EC = elevated CO_2 and EO = elevated O_3.

2000, and for 14h/day between 0800-2200 h in 2001. The fumigation periods for CO_2 and O_3 were from May 25 to October 4 in 1999, from May 4 to September 29 in 2000, and from May 2 to September 27 in 2001. When the fumigation was started in spring 1999, the leaves had already expanded and in 2000 and 2001 the buds were still developing. At the time of termination of the fumigations in autumn, 40-60% of the leaves had abscised. During the 3-year exposure the CO_2 and O_3 concentrations were close to the target (Table 1). In 1999 the temperature sum in the chambers was measured from June 28 to October 20. In 2000 and 2001 the temperature sum was measured from April 1 to September 29 (Table 1). The daily mean temperature in the chambers was on an average 1.7, 2.3 and 2.4 °C higher than the daily mean temperature of the ambient air during the growing seasons 1999, 2000 and 2001, respectively. The OTCs, covered by the polyethene film, transmitted 91% of the incident light at wavelengths of 400-800 nm; but at wavelengths <400 nm the transmittance decreased sharply, and at 300 nm only 4.3 % of the light was transmitted through the film.

Gas exchange and chlorophyll fluorescence measurements

Gas exchange and chlorophyll fluorescence measurements were made in June, July, August and September each growing season at three different tree heights (lower, middle and upper third of the stem) in south- or west-facing branches of each experimental tree (n= 40). In June and July the measurements were made on fully expanded short-shoot leaves that had burst in the spring from buds formed during the previous autumn. In August and September the measurements were made both on short- and long-shoot leaves (from branches in the middle and upper third of the stem). The gas exchange measurements (net photosynthesis (P_n), stomatal conductance (g_s), transpiration (transp) and intercellular CO_2 concentration (C_i)) were completed in one week's time, except for rainy periods, when the measurements were interrupted. The measurements were made between 10 a.m. and 4 p.m. To minimize the effect of variable weather conditions during each measurement round, the trees were divided into four groups (each including one tree for each treatment and clone), which could be measured within one day. The gas exchange measurements were carried out using a portable gas exchange apparatus Li-6400 (LI-COR Inc., Lincoln, NE., U.S.A), using either ambient (360 ppm) or growth CO_2 concentrations (360 ppm for CC, EO and OC treatments and 720 ppm for EC and EC+EO treatments) in the leaf chamber, under saturating light (450-700 µmol m^{-2} s^{-1}) and at leaf temperature of 20 °C. Relative humidity (RH) in the leaf chamber was adjusted similar to that of ambient air. The measurements were done after steady state conditions of P_n and g_s were reached. The data from the three different branches were averaged for each tree.

Chlorophyll fluorescence measurements were made with a portable pulse amplitude-modulated fluorometer (MINI-PAM, Walz, Effeltrich, Germany) and the same measurement protocol was used as in gas exchange measurements. The measurements were made between 10 a.m. and 4 p.m. After 10 min dark-adaptation of the leaves, the minimal fluorescence level (F_0) was determined. Maximal fluorescence level (F_m) was obtained by exposing the leaf to light pulse (9000 µmol m^{-2} s^{-1}). The maximum photochemical yield of PSII in a dark-adapted state (F_v/F_m) was calculated as $(F_m-F_0)/F_m$ (Genty et al. 1989). Thereafter the leaf was illuminated with external halogen lamp (700 µmol m^{-2} s^{-1} PAR in 1999 and 450 µmol m^{-2} s^{-1} PAR in 2000 and 2001) for a minimum time of 2 min until fluorescence was stabilised, the steady-state

fluorescence (F_s) was determined, after which a light pulse (9000 µmol m^{-2} s^{-1}) was applied and the maximal fluorescence at steady-state (F_m') was determined. Apparent quantum yield of PSII photochemistry in light (F_v'/F_m') was calculated as $(F_m'-F_s)/F_m'$ (Genty et al. 1989). Non-photochemical fluorescence quenching (NPQ) was determined according to the Stern-Volmer equation as $(F_m-F_m')/F_m'$ (Bilger and Björkman 1990). Mean values of the three branches were used for further analysis.

Amount and activity of Rubisco, and amount of chlorophyll and soluble protein

Immediately after gas exchange measurements, the leaves were detached and a 2 cm^2 disc was frozen in liquid nitrogen. The samples from different tree heights were pooled and stored at –80°C. For determination of Rubisco, soluble protein and chlorophyll, the frozen leaf samples were homogenized in ice-cold extraction buffer containing 50 mM morpholinoethanesulfonic acid (MES, pH 6.8), 20 mM $MgCl_2$, 50 mM 2-mercaptoethanol and 1% Tween-80. Aliquots of the crude extract were dissolved in 80% buffered acetone and analysed for chlorophyll by the method of Porra et al. (1989). After centrifugation, the activity of Rubisco was determined as incorporation of ^{14}C into acid-stable products (Lorimer et al. 1977). The incorporation of ^{14}C into acid-stable products was determined by liquid scintillation spectrometry. Rubisco activation state was expressed as the ratio of initial activity (data not shown) to total activity (%). The amount of Rubisco protein was determined by polyacrylamide gel electrophoresis (Ruuska et al. 1994) and the soluble protein content (Bradford 1976) after precipitation in cold ethanol (Ovaska et al. 1993).

Determination of soluble sugars, starch, N and C/N in the leaves

After Rubisco samples had been taken, the rest of the leaves were measured for their fresh mass, leaf area (LI-3050A leaf area meter, Li-Cor, Inc., Lincoln, NE) and dry mass (48h at 60°C). Thereafter, leaves from one tree in each sampling time were pooled and analysed for soluble sugars and starch (Hansen and Møller 1975, Riikonen et al. 2003). The same samples were further analysed for total carbon and nitrogen with a CHN-900 Analyzer (Leco Co., St. Joseph, MI). The concentration of structural N was calculated by excluding starch and soluble sugars from total dry mass. The proportion of N bound to Rubisco was calculated as a percentage of total leaf N concentration, assuming that Rubisco contains 16.67% N (Evans 1989).

Statistical analyses

Data were analysed and are represented as the means of four replicates per treatment and clone. The chamber effect was studied separately by comparing CC to OC trees and the treatment effects by excluding OC trees from the data. The chamber effect was similar in both clones in all the parameters analysed and thus in Tables 3 and 5, only the significance of the chamber effect is presented. All the parameters were measured from short- and long-shoot leaves but the presentation of data concentrates on short-shoots, since the treatment effects were similar in the two leaf types. Four-way analysis of variance for repeated measurements with year as repeated measures factor was used to test the main effects and interaction of CO_2, O_3, clone and year. Analysis for repeated measurement with sampling date as repeated measures factor was performed for each year separately for data of net photosynthesis, stomatal conductance and amount of

Rubisco to examine the treatment effects during each growing season. The chlorophyll fluorescence parameters were arcsine-transformed prior to statistical analysis. Logarithmic or square root transformations were performed to assure the homogeneity of variances and normal distribution of data. All the statistical analyses were conducted using SPSS 9.0 for Windows (SPPS Inc. 1999).

Results

Effects of clones, leaf types and OTCs

In general, P_n and g_s (Tables 2 and 3) and the amount and total activity of Rubisco (Tables 4 and 5) were higher in clone 80 than in clone 4, whereas WUE was higher in clone 4 (Tables 2 and 3). The apparent quantum yield of PSII photochemistry in light (Table 3) and most biochemical parameters (Tables 4 and 5) were similar in the clones. In general, the treatments affected the short- and long-shoot leaves similarly. In short-shoot leaves, that burst in the spring from buds formed during the previous autumn, P_n and the other parameters were 5-25% lower than in long-shoot leaves that started to appear in long-shoots in July.

The clones were affected similarly by the OTCs. Stomatal conductance (Tables 2 and 3) and starch concentration (Tables 4 and 5) were higher in OC than in CC, while WUE, F_v'/F_m' (Tables 2 and 3), amount and activity of Rubisco, Rubisco N/total N-ratio and total chlorophyll were lower in OC than in CC (Tables 4 and 5, Figures 1 and 3).

Effects of elevated CO_2 and O_3

Gas exchange and chlorophyll fluorescence
When the gas exchange measurements were made at the actual growth CO_2 concentrations (360 or 720 ppm) the treatments with elevated CO_2 increased P_n in clone 4 by 19-58% and in clone 80 by 17-34% (Tables 2 and 3). When gas exchange was measured at ambient CO_2 concentration (360 ppm) P_n was significantly decreased in EC trees on an average by 15% in clones 4 and 80 (Tables 2 and 3). Net photosynthesis and the magnitude of the CO_2 response varied between the growing seasons (Tables 2 and 3, Figure 1). When gas exchange was measured either at ambient or growth CO_2 concentration, g_s (Tables 2 and 3) and transpiration (data not shown) were around 23% lower in EC and EC+EO than in CC in both clones. Intercellular CO_2 (C_i) (data not shown) and WUE, when measured at growth CO_2 concentration (Tables 2 and 3), increased significantly in elevated CO_2, especially in clone 4, but were similar in the treatments when measured at ambient CO_2 concentration. The CO_2 response on WUE was found each year, although as in P_n, the increase was smaller in 2000 than in 1999 and 2001 (Tables 2 and 3). Elevated O_3 had no effect on gas exchange (Tables 2 and 3, Figure 1).

Maximum photochemical yield of PSII (F_v/F_m) varied between the years (P= 0.001) and fluctuated between 0.799-0.850, but was not affected by the treatments (data not shown). Apparent quantum yield of PSII photochemistry in light (F_v'/F_m') increased and NPQ decreased in elevated CO_2 in both clones (Table 3, Figure 2). Elevated O_3 decreased apparent quantum yield of PSII photochemistry in light (F_v'/F_m') and increased NPQ in 2000 (Table 3, Figure 2).

Rubisco, chlorophyll and soluble proteins
Elevated CO_2 decreased the amount of Rubisco more in clone 80 than in clone 4, and the magnitude of the downregulation of Rubisco decreased during the experiment in both clones (Tables 4 and 5, Fig. 1). Total activity of Rubisco (Tables 4 and 5, Figure 3) decreased in elevated CO_2, by 14% in clone 4 and by 26% in clone 80. The specific activity of Rubisco was significantly increased and Rubisco N/total N decreased

Table 2. Net photosynthesis (P_n), stomatal conductance (g_s) and water use efficiency (WUE) in short-shoot leaves of silver birch clones 4 and 80 in Suonenjoki OTC experiment during 1999-2001. Gas exchange measurements were made using growth (360 or 720 ppm) and ambient (360 ppm) CO_2 concentration. The clones and years are pooled when significant main effects or interactions of year, clone and treatments were not found (Table 3) and average values over each growing season are presented for P_n and WUE and averages over the experiment for g_s. Absolute values are given for CC trees and the treatments are presented relative to CC trees (%).

Parameter	Clone	year	CC	EC	EO	EC+EO	OC	EC, EC+EO
				measured at growth CO_2 concentration				measured at ambient CO_2
P_n, µmol CO_2 $m^{-2}s^{-1}$	**4+80**	1999	13.4±0.6	39	-2	38	12	-17
		2000	10.5±0.3	17	-2	17	-4	-19
		2001	10.4±0.5	39	0	35	-4	-19
		99-01	**11.5±0.3**	**32**	**-1**	**30**	1	**-15**
	4	1999	12.3±0.8	44	-2	53	15	-16
		2000	10.3±0.4	19	-3	23	-5	-18
		2001	9.5±0.7	58	7	44	0	-16
		99-01	**10.7±0.4**	**40**	1	**40**	**5**	**-14**
	80	1999	14.5±0.8	34	-2	25	10	-17
		2000	10.7±0.6	17	0	10	-4	-19
		2001	11.3±0.5	24	-6	27	-8	-22
		99-01	**12.2±0.6**	**25**	**-3**	**21**	**-1**	**-16**
g_s, mol H_2O $m^{-2}s^{-1}$	**4+80**	**99-01**	**0.22±0.01**	**-23**	4	**-21**	17	**-22**
	4	**99-01**	**0.18±0.02**	**-26**	7	**-20**	14	**-25**
	80	**99-01**	**0.25±0.03**	**-18**	2	**-21**	20	**-19**
WUE, mmol CO_2 mol^{-1} H_2O	**4+80**	1999	5.8±0.2	59	-8	61	-8	-5
		2000	5.4±0.5	43	-1	60	-16	5
		2001	6.5±0.3	75	-12	75	-9	-3
		99-01	**5.9±0.2**	**59**	**-7**	**65**	**-11**	**-1**
	4	1999	6.2±0.4	63	-13	55	-12	-7
		2000	5.9±0.8	46	-5	84	-13	13
		2001	6.7±0.4	95	-8	92	-8	2
		99-01	**6.3±0.5**	**68**	**-9**	**77**	**-11**	**3**
	80	1999	5.4±0.2	53	-2	67	-4	-2
		2000	5.0±0.6	35	3	29	-19	-8
		2001	6.3±0.4	50	-17	54	-9	-8
		99-01	**5.6±0.4**	**46**	**-5**	**50**	**-11**	**-6**

Data are means of the measurements made on June, July, August and September each growing season. The treatments were chamber control (CC), elevated CO_2 (EC), elevated O_3 (EO), elevated CO_2 and O_3 (EC+EO), and outside control (OC).

by elevated CO_2, although the CO_2 effect was decreasing towards the end of the experiment (Tables 4 and 5). Elevated CO_2 did not significantly affect the total soluble protein (data not shown) or chlorophyll concentration (Tables 4 and 5) whereas the proportion of Rubisco relative to total soluble proteins and to total chlorophyll (Tables 4 and 5) decreased especially in clone 80. The biochemistry of the clones was only slightly affected by elevated O_3, mainly in clone 4. Elevated O_3 decreased chlorophyll concentration in clone 4 by 19% (in ambient CO_2). The amount and activity of Rubisco tended to be lower in EO than in CC trees (Tables 4 and 5, Figure 3), especially in clone 4, leading to 9% lower proportion of Rubisco of total soluble proteins (Table 4). In clone 4, elevated O_3 decreased the Rubisco N/ total N-ratio

Table 3. *P*-values for the main effects of CO_2, O_3, clone and year and their interaction on net photosynthesis (P_n, μmol CO_2 $m^{-2}s^{-1}$), stomatal conductance (g_s, mol H_2O $m^{-2}s^{-1}$) and water use efficiency (WUE, mmol CO_2 mol^{-1} H_2O) (measured either at growth or at ambient CO_2 concentration), and for the apparent quantum yield of PSII photochemistry in light (F_v'/F_m') and non-photochemical quenching (NPQ) when differences were found (P<0.5). The parameters were determined in the leaves of silver birch clones 4 and 80 during 1999-2001 in Suonenjoki OTC experiment.

	Growth CO_2			Ambient CO_2				
Effect	P_n	g_s	WUE	P_n	g_s	WUE	F_v'/F_m'	NPQ
CO_2	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.537	**0.000**	**0.000**
O_3	0.530	0.554	0.954	0.151	0.571	0.229	**0.030**	0.056
Clone	**0.006**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.601	**0.000**
Clone×CO_2	**0.006**	0.930	**0.002**	0.273	0.953	0.124	0.284	0.581
Year	**0.000**	0.121	**0.000**	**0.000**	0.190	**0.011**	**0.000**	**0.000**
Year×CO_2	**0.005**	0.722	**0.004**	0.948	0.641	0.858	0.199	0.199
Year×O_3	0.961	0.575	0.400	0.477	0.500	0.245	0.418	**0.029**
CC-OC	0.230	**0.010**	**0.023**				**0.001**	0.150

Data were analysed using ANOVA for repeated measurements, with year as repeated measures factor. The data are means of 16 measurements of short-shoot leaves for each clone and growing season. The chamber effect was analysed separately by comparing CC and OC treatments using one-way ANOVA. Abbreviations: CC = chamber control and OC = outside control. Significant treatment effects and interactions (*P*<0.05) are shown in bold.

significantly in 1999 and 2001. When the clones were pooled, the combination of elevated CO_2 and O_3 (EC+EO) decreased the amount and activity of Rubisco (amount of Rubisco only in 1999) more than O_3 alone (EO), while EC and EC+EO were similar (Tables 4 and 5, Figures 1 and 3).

Starch and N concentration In both clones the starch concentration increased in elevated CO_2 (by 64% and 43% in clones 4 and 80, respectively) (Tables 4 and 5) but no effect on soluble sugar concentration was found (data not shown). Total leaf N concentration on a leaf area basis decreased only in clone 80 (by 9%) (Figure 3) but the structural N concentration decreased in both clones by 10%. The C/N-ratio increased by around 17% when the clones were analysed together (Tables 4 and 5). Elevated O_3 decreased total leaf N concentration significantly in 1999 and 2000 in clone 4 (Tables 4 and 5, Figure 3). In 1999 elevated CO_2 decreased the total leaf N concentration only in ambient O_3 concentration (Table 5, Figure 3). The combination of elevated CO_2 and O_3 (EC+EO) increased C/N-ratio more than O_3 alone (EO), while EC and EC+EO were similar (Tables 4 and 5).

Seasonal variation

The seasonal variation of the effect of CO_2 on P_n, g_s and amount of Rubisco was studied during the three growing seasons (Figure 1). Net photosynthesis was constantly higher and g_s and amount of Rubisco lower in elevated CO_2 than in CC trees. Interactions between CO_2 and time were found in P_n and amount of Rubisco, indicating that the changes in the CO_2 response were not consistent during the growing seasons (Figure 1). Net photosynthesis was slightly lower in EO than in CC only at the end of the growing seasons 2000 and 2001. Stomatal conductance was similar in EO than in CC, although higher g_s in EO was found in mid-summer 2000 and 2001. Amount of Rubisco was consistently lower in EO than in CC, except in 2000, when O_3 decreased the amount of Rubisco only at the end of the growing season (Figure 1).

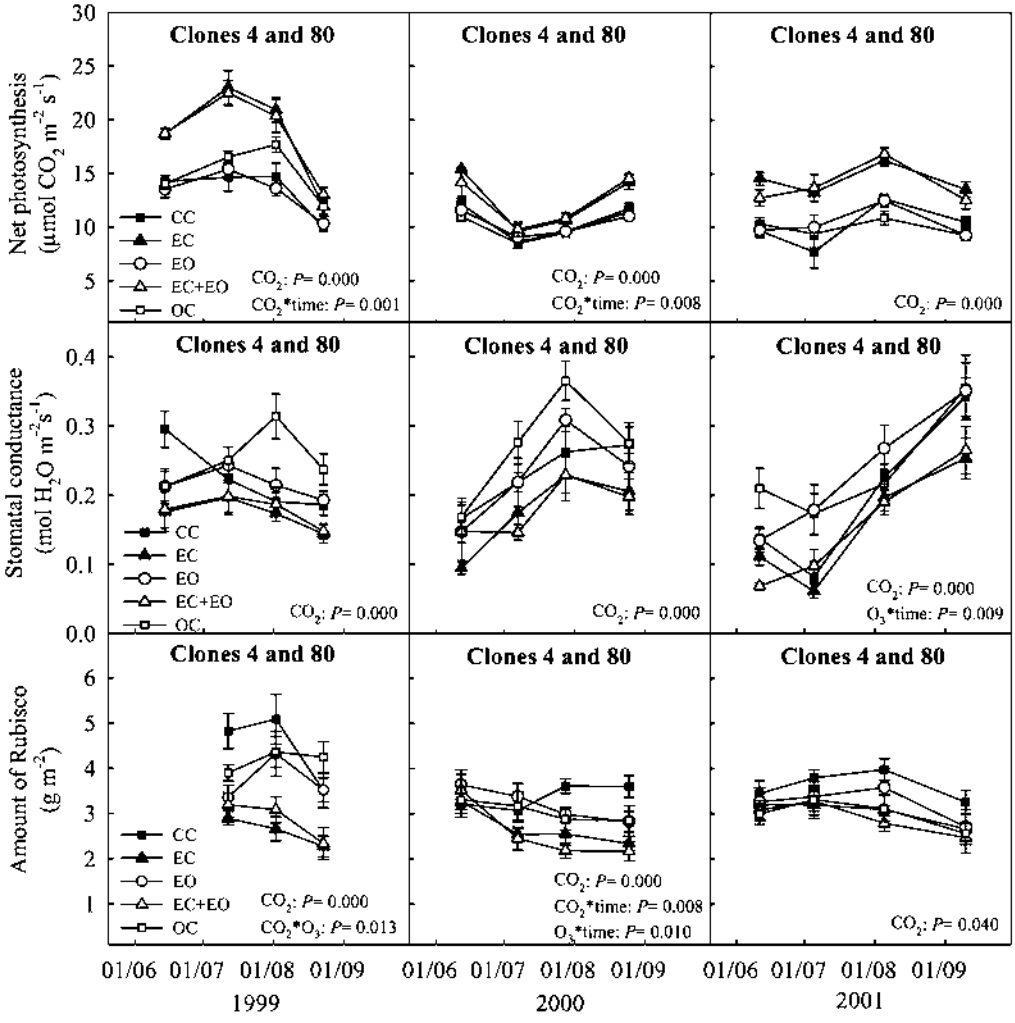

Figure 1. Net photosynthesis, stomatal conductance and amount of Rubisco in short-shoot leaves of silver birch clones 4 and 80 measured in Suonenjoki. Net photosynthesis and stomatal conductance were measured at growth CO_2 concentration during the growing seasons 1999-2001. The treatments were chamber control (CC), elevated CO_2 (EC), elevated O_3 (EO), elevated CO_2 and O_3 (EC+EO), and outside control (OC). To examine the treatment effects during the growing seasons the data were analysed using ANOVA for repeated measures, with sampling date as repeated measures factor. Time effect was significant (P= 0.000) in all parameters. Otherwise the *P*-values for CO_2 and O_3 and their interaction with time are presented in the figures when significant differences were found ($P< 0.05$). Data are means (±SE) of 4 replicates representing each treatment and clone.

Table 4. Biochemical parameters in short-shoot leaves of silver birch clones 4 and 80 in Suonenjoki OTC experiment during 1999-2001. For CC trees, the mean values over a growing season or over the experiment ± SE are presented, and the treatment effects are expressed as a difference between the CC trees and treatments (%). The clones and years are pooled when significant main effects or interactions of year, clone and treatments were not found (Table 5).

Parameter	Clone	year	CC	EC	EO	EC+EO	OC
Amount of Rubisco, g m^{-2}	**4+80**	1999	4.5±0.3	-42	-18	-35	-7
		2000	3.4±0.1	-21	-5	-24	-10
		2001	3.6±0.1	-16	-11	-20	-17
		99-01	**3.8±0.1**	**-26**	**-11**	**-26**	**-11**
	4	1999	4.1±0.4	-41	-22	-31	-5
		2000	3.1±0.2	-10	-12	-11	-5
		2001	3.4±0.2	3	-16	-16	-13
		99-01	**3.5±0.2**	**-16**	**-17**	**-19**	**-8**
	80	1999	4.8±0.4	-42	-15	-38	-7
		2000	3.6±0.1	-29	1	-34	-14
		2001	3.9±0.2	-33	-7	-23	-21
		99-01	**4.1±0.2**	**-35**	**-7**	**-32**	**-14**
Total chlorophyll, g m^{-2}	**4+80**	**99-01**	**0.41±0.01**	**-7**	**-10**	**-5**	**-7**
	4		**0.43±0.01**	**-14**	**-19**	**-7**	**-12**
	80		**0.40±0.01**	**-3**	**-5**	**-5**	**-3**
Rubisco/chlorophyll, mg mg^{-1}	**4+80**	**99-01**	**9.3±0.3**	**-18**	**-1**	**-20**	**-2**
	4		**8.4±0.7**	**-5**	**1**	**-13**	**8**
	80		**10.3±0.7**	**-30**	**-3**	**-27**	**-9**
Rubisco/soluble protein, %	**4+80**	**99-01**	**60.1±1.5**	**-20**	**-9**	**-23**	**-10**
	4		**55.6±1.9**	**-10**	**-9**	**-18**	**-6**
	80		**64.3±2.0**	**-28**	**-8**	**-27**	**-13**
Specific activity of Rubisco, µmol CO_2 s^{-1} g^{-1}	**4+80**	1999	11.2±0.5	19	4	17	1
		2000	12.4±0.5	3	-1	10	3
		2001	10.4±0.3	-2	7	2	-3
		99-01	**11.3±0.3**	**7**	**3**	**10**	**0**
Rubisco N, % of total N	**4+80**	1999	42.9±2.6	-33	-12	-32	-10
		2000	35.5±1.1	-18	1	-21	-3
		2001	45.0±1.3	-12	-9	-16	-14
		99-01	**41.0±1.0**	**-21**	**-7**	**-23**	**-9**
	4	1999	39.5±2.5	-31	-8	-24	-3
		2000	33.3±1.8	-12	-2	-11	5
		2001	44.0±2.0	-2	-15	-18	-12
		99-01	**39.0±1.4**	**-15**	**-8**	**-18**	**-3**
	80	1999	46.7±4.6	-35	-17	-40	-16
		2000	37.6±1.1	-24	5	-30	-9
		2001	46.0±1.8	-20	-3	-15	-16
		99-01	**43.1±1.5**	**-26**	**-5**	**-28**	**-14**
C:N ratio	**4+80**	**99-01**	**24.1±0.25**	**17**	**7**	**16**	**5**
Structural N, % dry mass	**4+80**	**99-01**	**2.62±0.03**	**-10**	**-5**	**-9**	**-1**
Starch, % dry mass	**4+80**	**99-01**	**8.4±0.3**	**49**	**7**	**56**	**20**

The data are means of 16 measurements of short-shoot leaves for each clone and growing season. The treatments were chamber control (CC), elevated CO_2 (EC), elevated O_3 (EO), elevated CO_2 and O_3 (EC+EO), and outside control (OC).

Table 5. The *P*-values for the main effects of CO_2, O_3, clone and year and their interaction on biochemical parameters in short-shoot leaves in silver birch clones 4 and 80 when significant differences ($P<0.5$) were found.

Effect	Amount of Rubisco of Rubisco	Total activity of Rubisco	Specific activity	Rubisco N/ Total N	Rubisco/ protein	Rubisco/ Chl	Total Chl	Total leaf N	Structural N	C/N-ratio	Starch
CO_2	**0.000**	**0.000**	**0.023**	**0.000**	**0.000**	**0.000**	0.270	**0.042**	**0.001**	**0.000**	**0.000**
O_3	0.137	0.196	0.258	0.103	**0.037**	0.864	0.056	0.944	0.272	0.199	0.143
$CO_2 \times O_3$	**0.040**	**0.008**	0.894	0.320	0.158	0.836	**0.003**	**0.012**	0.071	**0.038**	0.375
Clone	**0.029**	**0.015**	0.487	0.077	**0.009**	0.098	0.950	0.203	0.452	0.200	0.647
Clone×CO_2	**0.002**	**0.001**	0.151	**0.009**	**0.006**	0.011	0.660	**0.038**	0.256	0.226	0.825
Clone×CO_2×O_3	0.984	0.978	0.712	0.991	0.265	0.394	**0.013**	0.677	0.568	0.469	0.233
Year	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.011**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Year×CO_2	**0.001**	0.074	**0.017**	**0.009**	0.135	0.207	0.096	0.486	0.486	0.595	0.501
Year×CO_2×O_3	**0.047**	0.113	0.337	0.384	0.143	0.445	0.562	0.053	0.661	0.406	0.711
Year×Clone	0.091	**0.000**	**0.014**	0.851	0.299	0.842	**0.000**	**0.000**	0.486	**0.001**	**0.002**
Year×Clone×O_3	0.310	0.553	0.318	**0.025**	0.372	0.245	0.537	**0.036**	0.614	0.496	0.642
CC-OC	**0.003**	**0.016**	0.622	**0.030**	0.118	0.278	**0.018**	0.619	0.689	0.254	**0.017**

Data were analysed using ANOVA for repeated measurements, with year as repeated measures factor. The chamber effect was analysed separately by comparing CC and OC treatments using one-way ANOVA. Abbreviations: CC = chamber control and OC = outside control. Significant treatment effects and interactions ($P<0.05$) are shown in bold.

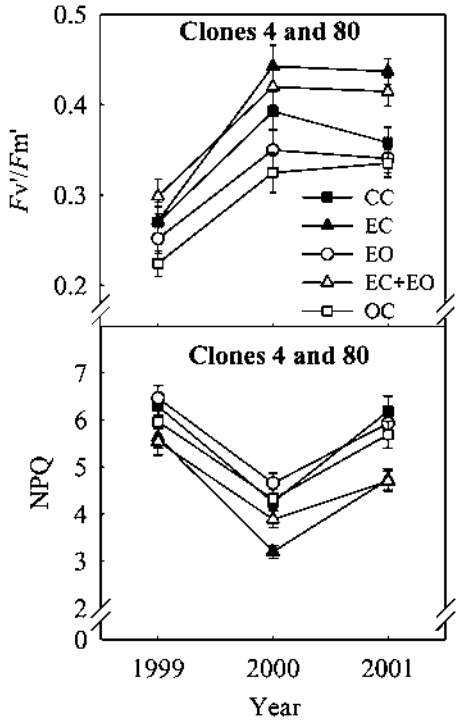

Figure 2. The apparent quantum yield of PSII photochemistry in light (F_v'/F_m') and non-photochemical quenching (NPQ) in short-shoot leaves of silver birch clones 4 and 80 during the growing seasons 1999-2001 in Suonenjoki. Data are means (±SE) over each growing season (16 measurements in each clone and year). The treatments were chamber control (CC), elevated CO_2 (EC), elevated O_3 (EO), elevated CO_2 and O_3 (EC+EO), and outside control (OC).

Discussion

Clonal differences, chamber effect and the importance of the leaf types

In accordance with the earlier field study with the two clones (Riikonen et al. 2003), clone 80 was physiologically more active than clone 4 in terms of gas exchange and amount and activity of Rubisco. However, the chamber effect was similar in the clones. Despite the 2-3°C higher daily mean temperature and slightly lower light and RH in the OTCs than in the ambient air, net photosynthesis was not affected by the chambers, while stomatal conductance and transpiration were lower in CC trees than in OC trees, leading to higher WUE in the chambers. In addition, the amount and activity of Rubisco and the concentration of chlorophyll were higher in the chambers, which may be related to higher temperature and slightly lower light which may have changed the N partitioning among photosynthetic components. Due to lower stomatal conductance it is likely that the uptake of CO_2/O_3 was lower in the OTCs than outside. Thus the treatment responses could have been greater in free air exposures.

The treatments affected the short- and long-shoot leaves similarly, although the absolute value of almost all measured parameters was higher in long-shoot leaves than in short-shoot leaves. However, it has been estimated that in birch (Kauppi et al. 1988) and aspen (Pollard 1970) the short-shoot leaves are more important in carbon fixation than the long-shoot leaves, and the importance of long-shoot leaves still decreases during the ageing of the trees. Thus the data from short-shoot leaves may give more representative information of the treatment effects on tree performance during the experiment.

Elevated CO_2 stimulated photosynthesis

In this experiment, the trees grown at elevated CO_2 sustained higher photosynthetic rate compared to those grown in ambient CO_2, when measurements were made at their respective growth CO_2. This increase was 20-60% depending on the clone and the year. The higher photosynthetic rate was maintained over the three growing seasons, although the magnitude of CO_2 response differed between the years,

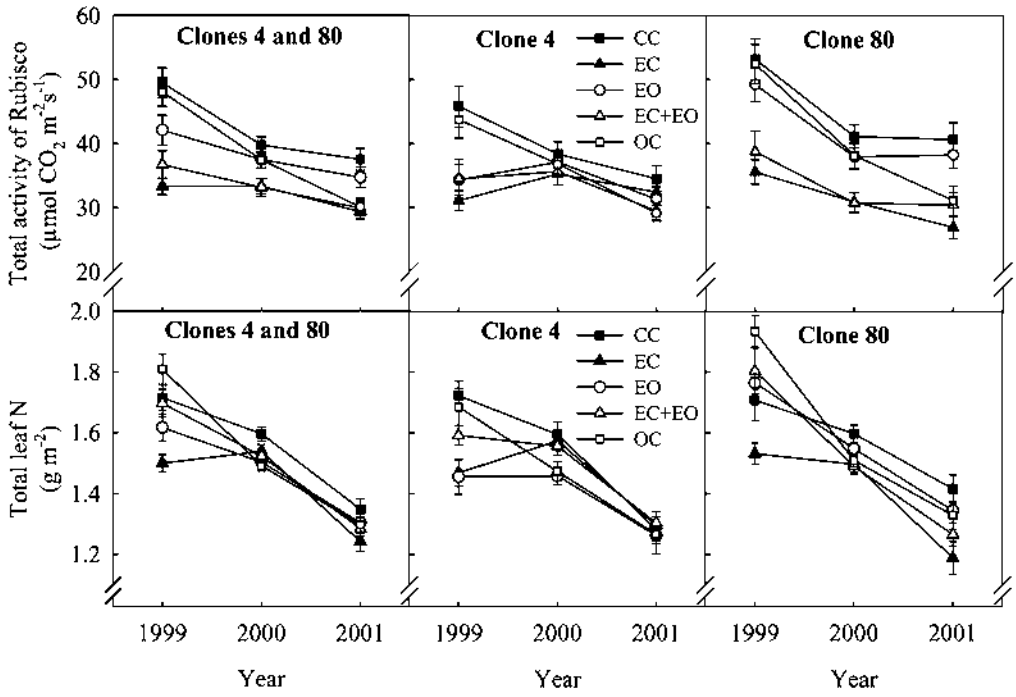

Figure 3. Total activity of Rubisco and nitrogen concentration in short-shoot leaves of silver birch clones 4 and 80 during the growing seasons 1999-2001 in Suonenjoki. Data are means (±SE) over each growing season (16 measurements in each clone and year). The treatments were chamber control (CC), elevated CO_2 (EC), elevated O_3 (EO), elevated CO_2 and O_3 (EC+EO), and outside control (OC).

depending on the weather conditions (Vapaavuori et al. 2002). The stomatal conductance was around 20% lower and WUE around 70% higher in trees grown under elevated CO_2 concentration than in CC trees. The increase in WUE is a common CO_2 response (Saxe et al. 1998) and might improve the drought resistance of the trees in the future. However, since in clone 80 elevated CO_2 increased total leaf area on an average by 30% during the experiment (Riikonen et al. 2004), it is possible that the increase in leaf-level WUE was counteracted by an increase in leaf area and thus the whole tree water use might not have changed.

When measured at ambient CO_2 (360 ppm), photosynthesis was around 15% lower in plants grown in elevated CO_2 than in CC trees. The downregulation of photosynthesis was consistent with accumulation of starch in the leaves (about 49%), indicating imbalance between supply and demand for carbohydrates (end-product inhibition), which may lead to sugar-signalling feedback inhibition in photosynthetic capacity and further to suppression of several photosynthetic genes (Drake et al. 1997, Stitt & Krapp 1999). In elevated CO_2 the concentration of nitrogen, calculated both on an area and on structural dry mass basis, was reduced despite the N fertilisation during the growing season. Reductions in amount and activity of Rubisco and in ratio of Rubisco and total N concentration, chlorophyll and soluble proteins indicate smaller investment of N to Rubisco, resulting in increased N use efficiency in elevated CO_2, especially in clone 80. Reduced N concentration may have been partly caused by growth dilution and increased demand of N (Stitt & Krapp 1999).

In addition, elevated CO_2 may change the N allocation of the plants to optimize the energy costs: less N is allocated to photosynthetic apparatus and proportionally more N is allocated to the root system (Cotrufo et al. 1998). According to Drake et al. (1997) the average reduction in the amount and activity of Rubisco is 15% and 24%, respectively. In our study the amount and activity of Rubisco responded differently in the two clones and the downregulation of Rubisco was greater in clone 80 (-34%) than in clone 4 (-20%). As a consequence, elevated CO_2 stimulated P_n more in clone 4 than in clone 80. However, the magnitude of downregulation in terms of P_n was similar in the clones. This may be due to greater g_s and C_i in clone 80 than in clone 4. The magnitude of the CO_2 response on the amount of Rubisco and g_s was consistent during each growing season but the photosynthetic enhancement in elevated CO_2 varied during the growing seasons.

The downregulation of photosynthesis was maintained over the experiment, although the rooting volume was not limited and the soil nutrient concentrations were comparable to those in forest and nursery soils (Luoranen 2000). This is consistent with experiments with unlimited rooting volume, using silver birch (Rey and Jarvis 1998) and other species (Nowak et al. 2004). However, downregulation in amount and specific activity of Rubisco and Rubisco N/total N-ratio was strongest in 1999 and decreased towards end of the experiment.

In long-term field experiments downregulation has been both found (the average degree of downregulation around 10%) (Medlyn et al. 1999, Griffin et al. 2000) and not found (Norby et al. 1999, Noormets et al. 2001, Sholtis et al. 2004) and at present, there is no general agreement of the significance of downregulation of photosyntesis in the field (Drake et al. 1997, Saxe et al. 1998, Nowak et al. 2004).

Chlorophyll and total soluble protein concentration were not significantly affected by elevated CO_2, unlike in some experiments with silver birch (Rey and Jarvis 1998, Juurola 2003). The Rubisco/chlorophyll-ratio decreased, especially in clone 80, which means that in elevated CO_2 more N was allocated to light than to dark reactions of photosynthesis (Evans 1989). The effects of elevated CO_2 on photochemistry are not well established (Saxe et al. 1998), since the results from studies with different forest tree species have been variable, showing increased (Ceulemans et al. 1995), decreased (Scarascia-Mugnozza et al. 1996) or unchanged (Hymus et al. 1999) efficiency of photochemistry in elevated CO_2. In this experiment F_v/F_m was unaffected but F_v'/F_m' was increased and NPQ decreased by elevated CO_2, indicating increases in the use of absorbed light energy in photochemistry and possibly inhibition of photorespiration in elevated CO_2 (Long and Drake 1991). From the above observations it can be concluded that adjustments had occurred both in light and dark reactions to maximise photosynthetic capacity in response to elevated CO_2.

Elevated CO_2 increased biomass growth significantly only in clone 80 (Riikonen et al. 2004). The reason for the discrepancy between photosynthesis and growth in clone 4 may be the different growth requirements of the clones, since clone 80 is originating from eastern Finland and clone 4 from southern Finland, where the climate is less continental (Vapaavuori et al. 2002). Alternatively, lower stomatal density and conductance in clone 4 than in clone 80 (Riikonen et al. 2003) may indicate water stress in clone 4 (Pääkkönen et al. 1998).

Elevated O_3 had a small effect on photosynthesis and related biochemistry

According to many experiments silver birch is sensitive to O_3 (Oksanen and Saleem 1999, Oksanen 2003). However, in this study O_3 had no significant effect on gas exchange parameters and also the biochemistry was only slightly affected. Elevated O_3 decreased F_v'/F_m' and increased NPQ (in 2000). Similar O_3-effects have been found in silver birch (Shavnin et al. 1999) and in poplar (Lorenzini et al. 1999). This indicates that O_3 affected the electron flow and increased the dispersal of energy as heat to avoid overexcitation of PSII, which are supported by the laboratory analyses of the same trees (Eichelmann et al. 2004). Elevated O_3 decreased the chlorophyll concentration by 19% in ambient CO_2 in clone 4. The slightly decreased amount of Rubisco led to significant reduction in the proportion of Rubisco of total soluble proteins in each clone and decreased Rubisco N/ total N-ratio and N concentration in clone 4. However, the O_3 responses discussed above were apparently too weak to affect the net photosynthesis.

The small effect of O_3 on photosynthesis and biochemistry of the clones is interesting since the biomass production was affected by elevated O_3 in clone 4, where the dry mass of roots and branches was reduced and also total dry mass tended to be lower in elevated O_3 (P= 0.060) (Riikonen et al. 2004).

The interaction of $CO_2 \times O_3$ was variable

In the few parameters related to photosynthesis, where O_3 effect was found, such as chlorophyll concentration and chlorophyll fluorescence, the effect of O_3 was smaller in elevated CO_2 than in ambient CO_2. Also the negative effect of O_3 on growth parameters was mainly found in ambient CO_2 (in EO treatment) and the EC and EC+EO treatments were similar in both clones (Riikonen et al. 2004). The compensation effect of CO_2 was probably related to decreased stomatal conductance and thus decreased O_3 uptake in elevated CO_2 or to higher photosynthesis in elevated CO_2 leading to higher carbohydrate concentration and greater detoxification capacity in leaves grown in elevated CO_2 (e.g. Rao et al. 1995). However, we also found that elevated CO_2 and O_3 together decreased the total activity of Rubisco and increased the C/N-ratio more than O_3 alone while the treatments with elevated CO_2 were similar. The synergistic effect of CO_2 and O_3 may be caused by simultaneous negative effects of CO_2 and O_3 on these parameters. It can be concluded that the protective effect of CO_2 against one negative effect is not necessarily reflected to other parameters, as suggested by Polle and Pell (1999).

Conclusions

In this experiment with two silver birch clones, elevated CO_2 increased the photosynthetic rate by around 30%, when the gas exchange measurements were made at their respective growth CO_2. When measured at ambient CO_2, photosynthesis was around 15% lower in plants grown in elevated CO_2 than in CC trees. The downregulation of photosynthesis was maintained during the experiment, although in some parameters related to Rubisco the magnitude of CO_2 effect decreased towards the end of the experiment. Elevated O_3 had no effect on the gas exchange parameters and also the effect on biochemistry was small. The few interactive effects of CO_2 and O_3 found in this experiment were variable. Generally the effects of O_3 were smaller or absent in elevated CO_2. The photosynthesis and related biochemistry of these young silver birch trees were relatively tolerant to elevated O_3 although the annual AOT40-

values were 2-3 times higher that the current critical dose of 10 ppm.h for forest trees.

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Chapter 5

Structural characteristics and chemical composition of birch (*Betula pendula*) leaves are modified by increasing CO_2 and ozone

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Manuscript

Structural characteristics and chemical composition of birch (*Betula pendula*) leaves are modified by increasing CO_2 and ozone

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Abstract

Impacts of ozone and CO_2 enrichment, alone and in combination, on leaf anatomical and ultrastructural characteristics, nutrient status and cell wall chemistry in two European silver birch (*Betula pendula* Roth) clones were studied. The young soil-growing trees were exposed in open-top chambers over three growing seasons to 2 × ambient CO_2 and/or ozone concentrations in central Finland. The trees were measured for changes in altogether 35 variables of leaf structure, nutrients and cell wall chemistry of green leaves, and 20 of the measured variables were affected by CO_2 and/or O_3. Elevated CO_2 increased the size of chloroplasts and starch grains, number of mitochondria, P:N ratio, and contents of cell wall hemicellulose, and decreased the total leaf thickness, SLA, concentrations of N, K, Cu, S and Fe, and contents of cell wall α-cellulose, uronic acids, acid-soluble lignin and acetone-soluble extractives. Elevated ozone led to thinner leaves, higher palisade to spongy ratio, increased number of peroxisomes and mitochondria, reduced content of Mn, Zn, Cu, hemicellulose and uronic acids, and lower Mn:N- and Zn:N-ratios. In the combined exposure, interactions were antagonistic. Ultrastructural changes became more evident towards the end of the exposure. Young leaves were tolerant against ozone-caused oxidative stress, whereas oxidative H_2O_2 accumulation was found in older leaves. CO_2 enrichment improved ozone tolerance not only through increased photosynthesis rates, but also through changes in cell wall chemistry (hemicellulose, in particular). However, nutrient imbalances due to ozone and/or CO_2 may predispose the trees to other biotic and abiotic stresses. Down-regulation and up-regulation of photosynthesis under elevated CO_2 through anatomical changes is discussed.

Keywords: CO_2, ozone, interaction, birch, *Betula pendula*, ultrastructure, anatomy, cell wall chemistry, nutrient content

Introduction

The impacts of increasing atmospheric CO_2 and ozone on forest trees have attracted considerable attention since early 1990's (e.g. Saxe *et al.*, 1998; Karnosky *et al.*, 2003; Matyssek & Sandemann, 2003). Recent long-term studies indicate stimulation of photosynthesis and enhancement of tree growth in CO_2 enriched environment, whereas adverse effect of ozone is well documented (Karnosky *et al.*, 2003). Although CO_2 enhancement may increase the capacity of ozone detoxification and repair, and decrease the stomatal uptake (and thereby ozone influx) resulting in ameliorative effects during interaction, beneficial CO_2 effects are unlikely to occur throughout the entire lifespan of trees due to multiple and complex internal and external influences (Matyssek & Sandermann, 2003). Understanding the CO_2- and/or ozone-caused changes in whole trees and ecosystems ultimately requires information about functioning of leaves, cells and molecules. Therefore, structural and morphological markers provide valuable information for more profound understanding of the intra-leaf processes under increasing ozone and/or CO_2.

Ozone-caused changes in anatomy and ultrastructure of leaves in deciduous forest trees have been intensively studied (e.g. Pääkkönen *et al.*, 1995; Vollenweider *et al.*, 2003). Typical structural ozone responses in birch (*Betula pendula*) have appeared as increased stomatal density, reduced intercellular air space, smaller size of chloroplasts and starch grain, precipitation of chloroplast stroma, thylakoid injuries, translucency of the mitochondrial matrix, increased amount of cytoplasmic lipids (Pääkkönen *et al.*, 1995, 1997), increased vacuolar tannin and phenolic depositions, and as pectinaceous cell-wall protrusions (Pääkkönen *et al.*, 1998). Some of these changes have been regarded as signs of activated defence against ozone stress (e.g. increased phenolic droplets), while some may rather indicate accelerated leaf senescence (e.g. increase in plastoglobuli and cytoplasmic lipids). In addition to above-listed symptoms, disruption of cell structure, collapse of cell walls, increase in vacuoles, incomplete degeneration of cell organelles following rapid cell death, chromatin condensation, nucleus degeneration, and accumulation of antioxidants have been regarded as cellular markers for ozone stress in deciduous forest trees (Vollenweider *et al.*, 2003). In birch, ozone tolerance is related to thicker leaves and higher stomatal density as compared to more sensitive clones (Pääkkönen *et al.*, 1997). Similarly, ozone sensitive aspen (*Populus tremuloides*) clones were characterized by thinner leaves and mesophyll cell walls, lower proportion of cell wall volume, lower palisade to spongy mesophyll ratio, and higher volume for vacuoles as compared to tolerant clones (Oksanen *et al.*, 2001, 2004).

CO_2 enrichment has been reported to result in thicker leaves, enlargement of leaf cells, and increase in number of leaf cells or cell layers within mesophyll, together with an increase in starch content (Saxe *et al.*, 1998). In *Alnus glutinosa*, increased stomatal index was reported in plants grown under 2 × ambient CO_2 (Poole *et al.*, 2000). Impacts of CO_2 enrichment on leaf ultrastructural parameters of broadleaved trees have been less intensively studied. In aspen, elevated CO_2 increased chloroplast cover index, leaf and spongy mesophyll layer thickness, intercellular air space, amount of cytoplasmic lipids and starch in chloroplasts, whereas the number of plastoglobuli declined (Oksanen *et al.*, 2001). These changes were contrasting to ozone responses, and chloroplastic ozone injuries were ameliorated by simultaneous exposure to elevated CO_2 (Oksanen *et al.*, 2001).

Reports on responses of leaf cell wall chemistry to elevated CO_2 in deciduous trees

are variable (Couteaux *et al.*, 1999). Increase in lignin and cellulose (Staudt *et al.*, 2001), decrease in lignin (Blaschke *et al.*, 2002) or no change in lignins and total structural carbohydrates, cellulose, hemicellulose and pectin (Poorter *et al.*, 1997) have been reported. Reductions in the quality of plant tissue at elevated CO_2 have been reported to occur through reduced nitrogen concentration (Cotrufo *et al.*, 1998). Ozone has been reported to increase the synthesis of stress lignin in poplar (*Populus tremula* x *alba*) and beech (*Fagus sylvatica*) leaves (Baumgarten *et al.*, 2000; Cabané *et al.*, 2004), and increase the transcript levels and enzymatic activities of phenylpropanoid pathway by which lignin is synthesised (Eckey-Kaltenbach *et al.*, 1994).

Silver birch (*Betula pendula* Roth) has been reported to be sensitive to increasing ozone according to numerous chamber and open-field experiments (e.g. Pääkkönen *et al.*, 1997; Oksanen, 2003; Yamaji *et al.*, 2003). Birch trees in the present experiment responded to ozone by earlier leaf abscission (Riikonen *et al.*, 2004), lower contents of Rubisco and by reduced photochemical efficiency, while CO_2 enrichment led to increased growth, total leaf area, delayed leaf abscission, increased photosynthesis at their growth CO_2 concentration (720 ppm), down-regulation of photosynthesis at ambient CO_2 concentration (360 ppm), and lower stomatal conductance (Eichelmann *et al.*, 2004, Riikonen *et al.*, in press). CO_2 enhancement fully compensated the negative ozone effects on growth by increasing the carbohydrate pool for detoxification and repair processes, and ozone had less effect on photosynthetic parameters in the presence of elevated CO_2 than alone (Eichelmann *et al.*, 2004). Results from these physiological studies also showed that these two birch clones differed in their responsiveness to ozone and CO_2; clone 4 was more sensitive to ozone whereas CO_2 enrichment was more efficiently utilized by clone 80.

Despite the large number of experiments that have been performed aiming to define the physiological and biochemical changes in forest trees under ozone and CO_2 exposure, the structural aspects have been largely ignored although structural and chemical properties of leaves may interfere biological processes at various trophic levels. Therefore, the purpose of this study was primarily to detect anatomical and ultrastructural changes caused by concomitant CO_2 and ozone enrichment in leaves of European silver birch (*Betula pendula*) trees, in relation to nutrient composition. Secondly, we focus on changes in cell wall chemistry where information of climate change effects is lacking. It was hypothesized, that increased C gain at elevated CO_2 would lead to increased accumulation of storage compounds (starch) and to alterations in cell wall structure, that ozone exposure would lead to structural adaptations to oxidative stress, and that the effects of ozone and CO_2 are counteracting. Third, we aimed to demonstrate the sites for ozone-caused oxidative stress within leaf mesophyll by *in situ* H_2O_2 detection. Finally, we link these structural and chemical alterations to physiological responses of these trees such as growth, photosynthesis and stomatal conductance.

Materials and Methods

Experimental site and plant material

The study was conducted at the silver birch experimental field of Suonenjoki Research Station in central Finland (62°39´N, 27°03´E, 120 m above the sea level), established in 1993 (Mutikainen *et al.*, 2000). From that study area, consisting of 15 clones, forty trees representing clones 4 (V5952, O_3-tolerant in previous testing by Pääkkönen *et al.*, 1997) and 80 (K1659, O_3-sensitive in previous testing by Pääkkönen *et al.*, 1997) were selected in April 1999 for the

experiment. The selected trees started their eighth growing season with a mean height of about 5 m and a mean stand density of 4200 stems per hectare at the beginning of the experiment. The fertilization regimes have been explained in details in Vapaavuori *et al.*, (2002). The soil water content was measured twice a week (Time Domain Reflectometry, TDR, TRIME-FM, IMKO Micromodultechnik GMBH, Germany) and the trees were watered when the soil water content fell below 10%. More information about the exposure area, experimental design, technical constructions and devices are available in Mutikainen *et al.* (2000) and Vapaavuori *et al.* (2002).

CO_2 and ozone enrichment and chamber conditions

The exposure trees were fumigated over three growing seasons (from May 1999 to September 2001) in cylindrical open-top chambers that were 2.5 m in diameter and 7.8 m high. The treatments were: outside control (OC), chamber control in ambient air (CC), elevated CO_2 (2× ambient, EC), elevated O_3 (2× ambient, EO), and elevated CO_2 and elevated O_3 together (both 2× ambient, EC + EO). The treatments were set up in randomized incomplete blocks design with four independent replicates per clone (Vapaavuori *et al.*, 2002). We targeted to double the prevailing CO_2 concentration in the EC treatments for 24 hours/day throughout the experiment, and to double the ambient O_3 concentration in the EO treatments for 12 h/day between 0800-2000 h in 1999 and 2000, and for 14h/day between 0800-2200 h in 2001. The exact fumigation periods were from May 25 to October 4 in 1999, from May 4 to September 29 in 2000 and from May 2 to September 27 in 2001. Data for the average CO_2 and ozone concentrations, total ozone exposure (AOT00), AOT40 values (Accumulated Over the Threshold of 40 ppb) for exposure hours, and temperature sums are given in Table 1. The daily mean temperature in the chambers was on an average 1.7, 2.3 and 2.4 °C higher than that of the ambient air during the growing seasons 1999, 2000 and 2001, respectively. The polyethene film used in the chamber walls transmitted 91% of the incident light at wavelengths of 400-800 nm. At wavelengths below 400 nm the transmittance decreased sharply, and at 300 nm only 4.3 % of the light was transmitted through the film.

Light and transmission electron microscopy

For ultrastructural study, fully-expanded short-shoot leaves were collected from four exposure trees per clone per treatment (n = 4) four times (June, July, August and September) during each growing season from two different tree heights, one from the lowest and the other from the highest third of the tree. The sampled leaves were selected from branch sections that had formed during the previous year and thus were located in the outer layer of the canopy, facing from south to west. In the laboratory, leaf sections were cut between the second and third leaf vein from leaf base, followed by cutting into 1.5 mm^2 square pieces. Aiming to indicate oxidative stress within the leaf mesophyll *in situ* by localizing subcellular accumulation of H_2O_2 (from 2 August 1999 onwards), half of the cut leaf pieces were incubated under vacuum with 5 mM $CeCl_3$ solution (in 0.1 M phosphate buffer, pH 7.0) for 1h before pre-fixation. During incubation Ce^{3+} ions react with H_2O_2 forming electron dense cerium perhydroxide precipitates in reaction: $H_2O_2 + CeCl_3 \rightarrow Ce(OH_2)OOH$ (Bestwick *et al.*, 1997). The $CeCl_3$ treated samples and control samples (without $CeCl_3$ incubation) were pre-fixed with 2.5% (v/v) glutaraldehyde solution (in 0.1M phosphate buffer, pH 7.0) at +4°C overnight. Thereafter, the samples were post-fixed in 1% buffered OsO_4 solution, dehydrated with

Table 1. Ozone, CO_2 and temperature data calculated for each growing season. Values are mean hourly concentrations for the eight control chambers (CC), elevated ozone chambers (EO), and elevated CO_2 chambers (EC). The cumulative ozone exposures AOT00 and AOT40 (ppm.h, Accumulated Over the Threshold of 0 and 40 ppb) were calculated for exposure hours (12h, 12h and 14h in 1999, 2000 and 2001, respectively). Temperature sum (over the threshold of 5°C) is the mean value for all (32) chambers.

Parameter	Treatment	1999	2000	2001
Mean 24-h ozone concentration	CC	29	28	29
(ppb, max)	EO	36	34	40
AOT00 (ppm h^{-1})	CC	49.4	66.2	66.3
	EO	73.6	97.1	106.8
AOT40 (ppm h^{-1})	CC	2.8	2.5	1.7
	EO	20.6	24.4	30.9
24-h CO_2 (ppm)	CC	365	371	373
	EC	651	720	729
Temperature sum (degree days)	Ambient	1390	1243	1303
	Chamber	1603	1618	1701

an ethanol series followed by a propylene-oxide treatment, and embedded in LX 122 Epon (Ladd Research Industries Inc., Burlington, USA). The thin sections for electron microscopy were stained on grids with lead citrate and uranyl acetate, and were studied with an electron microscope JEOL 1200 EX (JEOL LTD, Tokyo, Japan) operating at 80 kV. The sections for light microscopy were stained with aqueous Toluidine blue, and studied with a Nikon MicroPhot-FXA microscope (Nikon Corp., Tokyo, Japan). The light microscopy samples were measured for total leaf thickness, palisade and spongy layer thickness, and volume of vacuoles, chloroplasts, cytosol, cell walls, and intercellular space (% of mesophyll section area) from digital micrographs using Adobe Photoshop (Version 5.0, Adobe Systems Incorporated, USA) program. Results from two randomly selected microscopic fields (section length 0.154 mm) were averaged for each leaf. Thin sections were photographed with Bioscan camera (Gatan Inc, Pleasanton, CA, USA) connected to electron microscope using Digital Micrograph program (Gatan Inc, Pleasanton, CA, USA) for further image analyses. The samples were analysed for sectional area of chloroplasts and starch grains, cell wall thickness in mesophyll cells, number of plastoglobuli per chloroplast, number of mitochondria and peroxisomes per unit cytoplasm (rectangular area of 4.0 μm^2 lining the cell wall), thylakoid membrane injuries, and localization of H_2O_2 accumulation from 10 cells per tissue per sample. Each year a total number of 6400 cells were examined (4 exposure trees × 5 treatments × 2 clones × 2 leaves × 2 tissues × 10 cell × 4 sampling days/year).

Stomatal density

The samples for stomatal analyses were collected from fully expanded short-shoot leaves on 2 August and 6 September 1999, on 21 July 2000, and on 27 June 2001 from three different tree heights in south- to west-facing branches. In 1999, 0.5 cm^2 pieces of leaf lamina between the second and third vein were first frozen in liquid nitrogen, and then stored at -80°C until examination. During 2000 and 2001 the measurements were done using fresh leaf samples. The samples were examined at a magnification of 10-20 × using a light microscope (Zeiss, Axioplan, Oberkochen, Germany) with a

camera attachment (Kodak DCS 460, resolution: 2024 × 3048 pixels). Photographs were taken from four randomly chosen microscopic fields (0.135 mm^2 or 0.54 mm^2) on the abaxial surface of the leaves, and the digital images were analysed with Adobe Photoshop. The stomatal density was averaged for each leaf and tree. In 2001, stomatal number per whole leaf area was calculated to study whether co-occurring changes exist between stomatal density and total leaf size.

Nutrient analyses and SLA

For the analyses of foliar nutrients other than N, ten short-shoot leaves were randomly collected throughout the whole crown on July and September in 1999 and on June, July, August and September in 2000 and 2001. The leaves were dried at 60 °C for 48 hrs and ground to fine powder. The concentrations of mineral nutrients were determined from an acidic solution of ammonium acetate (Halonen *et al.*, 1983) using plasma emission spectrophotometry analysis (ICP, ARL 3800). The samples for analysing total nitrogen were collected from three different tree heights four times during each growing season, and were measured with a CHN-900 Analyzer (Leco Co., St. Joseph, MI). The same leaves were used for determining leaf area (LI-3050A, LI-COR Inc., Lincoln, NE., U.S.A) and dry mass (48 hrs at 60 °C) to calculate the specific leaf area (SLA; cm^2 per g dry mass).

Cell wall chemistry

Leaf cell wall composition was analysed in 2001 using the same leaf powder as for nutrient analyses in June (day 161), July (day 186), August (day 220), and September (day 248). Milled leaf powder (0.5 g) was extracted in acetone (150 ml) by Soxhlet method according to the SCAN-CM (1994) standard for gravimetric measurement of acetone-soluble extractives and to yield extractive-free samples. Total lignin, α-cellulose and hemicellulose were analysed from the acetone-extractive-free samples as described in Anttonen *et al.* (2002). In hardwood leaves a large amount of polyphenolics and suberin exist, that form an insoluble complex with lignin and precipitate with lignin in the analysis we have used. Consequently, we call this fraction as total lignin + polyphenolics.

Statistics

The experimental design was a randomized incomplete block design with five treatments in four replicate trees. The exposure tree was the experimental unit for all statistical testing (n = 4). The chamber effect was studied separately by comparing CC to OC trees and the treatment effects by excluding OC trees from the data. Four-way analysis of variance for repeated measurements (with year and sampling date as repeated measures factor) was used to test the main effects and interaction of CO_2, ozone, clone, year and/or sampling date. Before analyses, the data were checked for normality and homogeneity of variance, and when necessary the values were transformed to satisfy the assumptions of ANOVA. To study correlations between ultrastructural changes, Pearson's correlation test was used. All the statistical analyses were conducted using SPSS 9.0 for Windows (SPPS Inc., 1999). Differences were considered significant at $P < 0.05$.

Results

Effects of genotype, year, sampling day and chamber

There were significant differences between the clones in spongy layer thickness, palisade to spongy ratio, volume of cell walls, number of plastoglobuli, number of stomata, foliar nutrients (P, Ca, Mg, Fe, B,

P:N, B:N), and the cell wall components (Table 2, 3, 4). When the treatments, exposure years and sampling dates were pooled together, clone 80 showed 6% higher palisade to spongy ratio, 6% higher volume of cell walls, 7% higher number of plastoglobuli, 17 % higher number of stomata, 9-37 % higher nutrient (P, Ca, Mg, Fe, B) concentrations, 20% less acetone-soluble extractives, 8% less α-cellulose, 9% more hemicellulose, and 11% more uronic acids than clone 4. However, there was no significant ozone × clone or CO_2 × clone interactions, except for number of mitochondria, showing that both clones responded similarly to ozone and/or CO_2 exposures (Tables 2-4).

Significant year × ozone interaction was found only in the number of peroxisomes and in Cu concentration, while significant year × CO_2 interaction was found only in the size of starch grain and in Fe concentration, indicating that responses to both CO_2 and ozone treatments were mostly similar between the different growing seasons (Tables 2, 3). Significant CO_2 × sampling day or ozone × sampling day interactions were not found for any anatomical, ultrastructural or nutrient parameters in any year, showing that these responses to CO_2 and ozone were also similar throughout the growing seasons (data not shown). Therefore, data are averaged over the years in Table 5 and 8, and over the growing seasons in Table 6 and Figure 1. In cell wall chemistry, however, significant CO_2 × sampling date interactions were found in the concentration of α-cellulose, total lignin + polyphenols, acid-soluble lignin and acetone-soluble extractives, whereas a significant ozone × sampling date interaction appeared in the concentration of uronic acids, acid-soluble lignin and acetone-soluble extractives (Table 4; Fig 3).

Significant chamber effect was found as 5% thicker leaves, 20% lower volume of chloroplasts, 9% increased volume of intercellular air space, 10% lower stomatal density, 17% lower concentration of K, 8-25% higher concentration Fe and Zn, 19% higher Zn:N ratio and 6% higher concentration of uronic acids in the chamber trees (Tables 2-5, 8).

Effects of elevated CO_2 and ozone on leaf anatomy and ultrastructure

Significant 'net effects' of elevated ozone and/or CO_2 on anatomy and ultrastructure were rare, when the data were pooled over the three growing seasons (Table 2, 5). The main effect of elevated CO_2 was found only as significantly larger size of starch grains, as an increased number of mitochondria, and as lower SLA (Table 2, 3, 5, Fig 1a). The main effect of elevated ozone appeared as significantly thinner spongy mesophyll layer, leading to higher palisade to spongy ratio, and as an increased number of peroxisomes and mitochondria (clone 80) (Table 2, 5). In the combined exposure, significant antagonistic interactions between ozone and CO_2 were found, when the ozone-caused thinning of spongy layer tissue and the increase of the number of peroxisomes were counteracted by CO_2, whereas the CO_2-caused increase in starch grain size was nullified by simultaneous ozone (Table 2, 5). Although stomatal density was not affected by single CO_2 and ozone treatment, there was a significant CO_2 × ozone interaction that led to 8% lowered density in the EC + EO treatment as compared to chamber controls (Table 3; Fig. 1b,c). Calculations of stomatal number per whole leaf area confirmed that changes in individual leaf size did not affect the stomatal density (data not shown).

When the exposure years were analysed separately, the only significant ultrastructural effects in 1999 and 2000 were found as an ozone-caused increase in peroxisomes and a CO_2-caused increase in

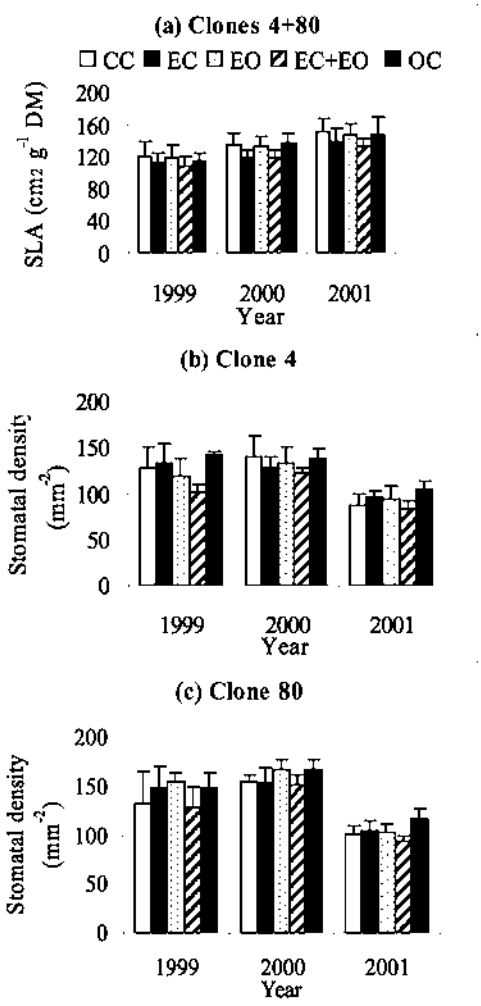

Figure 1. (a) Specific leaf area (SLA cm^2 g^{-1}) in short shoot leaves of *Betula pendula* clones 4 and 80, averaged over each growing season ± SD; (b,c) mean stomatal density (cm^{-2}) ± SD in clones 4 (b) and 80 (c).

mitochondria in both clones (data not shown). In the third exposure year, however, significant ultrastructural changes were more evident (Table 6): Elevated CO_2 decreased the total leaf thickness as well as the palisade and spongy layer thicknesses, and increased the size of chloroplasts and starch grain, and the number of mitochondria (Table 6; Fig 2b). Elevated ozone also decreased the total leaf thickness and the palisade and spongy layer thicknesses, but increased the number of peroxisomes and mitochondria (the latter only in clone 80) (Table 6). In the combined exposure, significant changes were seen as thinner spongy mesophyll layer (only in clone 80), bigger starch grain size, and as increased number of mitochondria and peroxisomes as compared to control trees (Table 6; Fig 2d). The interactions on starch grain size, mitochondria and peroxisomes were, again, antagonistic (Table 6).

Effects of elevated CO_2 and ozone on chemical structure of leaves

Nutrient analyses indicated that the trees received sufficient amount of macro- and micronutrients for adequate growth in all treatments, and were well below toxicity levels according to definition by Marschner (1995). Elevated CO_2 decreased significantly the foliar concentration of N, K, Cu, S and Fe in 2000 while P:N-ratio increased (Table 3, 8). Elevated O_3 decreased significantly the concentrations of Mn, Zn and Cu (only in year 2001), as well as Mn:N- and Zn:N-ratios (Table 3, 8). Significant CO_2 × ozone interaction in N concentration indicated that CO_2 decreased N concentration in both ozone levels while ozone decreased N concentration significantly only in combination with elevated CO_2 (Table 3, 8). In addition, CO_2-caused increase in B:N ratio was significantly counteracted by elevated ozone (Table 3, 8).

The significant main effect of elevated CO_2 were found as increased concentration of hemicellulose and decreased concentration of α-cellulose, uronic acids, acid-soluble lignin and acetone-soluble extractives (Table 4; Fig 3). Concentration of acid-soluble lignin decreased significantly under elevated CO_2 throughout the growing season (P = 0.000, 0.004 and 0.005 for days 161, 186 and 220, respectively) except for the last sampling day 248 (P = 0.166) (Fig 3e).

Table 2. *P*-values for the main effects of clone, CO_2, ozone, and year, and their interactions (when significant differences were found) on anatomical and ultrastructural parameters of birch (*Betula pendula*). Data were analysed using ANOVA for repeated measurements, with year as repeated measures factor. The chamber effect was analysed separately by comparing CC and OC treatments using one-way ANOVA. Abbreviations: CC = chamber control, OC = outside control. Significant treatment effects and interactions ($P<0.05$) are shown in bold. Arrows after *P*-value indicates increasing (↑) or decreasing (↓) effect of CO_2 or ozone.

Response	Clone		CO_2	O_3	$CO_2 \times O_3$	$CO_2 \times$ clone	$O_3 \times$ clone	$CO_2 \times$ year	$O_3 \times$ year	CC-OC
Total leaf thickness, µm	0.676		0.713	0.059	0.062	0.528	0.417	0.357	0.417	**0.021**
Spongy layer thickness, µm	**0.043**	80>4*	0.996	**0.001**↓	**0.048**	0.955	0.578	0.051	0.578	0.310
Palisade to spongy ratio	**0.023**	80>4	0.676	**0.009**↑	0.885	0.394	0.165	0.470	0.165	0.705
Cell wall thickness in mesophyll, nm	0.229		0.905	0.155	0.661	0.990	0.062	0.108	0.990	0.921
Volume of chloroplasts, % of mesophyll	0.600		0.241	0.051	0.383	0.301	0.155	0.161	0.301	**0.047**
Volume of cell walls, % of mesophyll	**0.022**	80>4	0.517	0.905	0.584	0.695	0.681	0.995	0.695	0.153
Volume of intercellular space, % of mesophyll	0.321		0.888	0.117	0.527	0.898	0.547	0.757	0.898	**0.042**
Size of chloroplast, μm^2	0.742		0.103	0.273	0.334	0.847	0.117	0.210	0.117	0.147
Size of starch grain μm^2	0.492		**0.020**↑	0.222	**0.043**	0.539	0.161	**0.000**	0.161	0.149
Number of plastoglobuli	**0.021**	80>4	0.974	0.223	0.466	0.361	0.738	0.173	0.738	0.855
Number of mitochondria	0.328		**0.000**↑	**0.000**↑	0.056	**0.000**	**0.015**	0.726	0.419	0.768
Number of peroxisomes	0.102		0.076	**0.000**↑	**0.000**	0.999	0.419	0.189	**0.015**	0.456

* value of parameter was higher in clone 80 as compared to clone 4

Table 3. *P*-values for the main effects of clone, CO_2, O_3, and year and their interaction (when significant differences were found) on stomatal density, specific leaf area (SLA), foliar nutrients and nitrogen ratios. Data were analysed using ANOVA for repeated measurements, with year as repeated measures factor. The chamber effect was analysed separately by comparing CC and OC treatments using one-way ANOVA. Abbreviations: CC = chamber control and OC = outside control. Significant treatment effects and interactions ($P<0.05$) are shown in bold. Arrows after *P*-value indicates increasing (↑) or decreasing (↓) effect of CO_2 or ozone.

Response	Clone		CO_2	O_3	Year	$CO_2 \times O_3$	$CO_2 \times$ year	$O_3 \times$ year	$CO_2 \times$ clone × year	CC-OC
Stomata, mm^{-2}	**0.000**	80>4*	0.108	0.177	**0.000**	**0.012**	0.526	0.445	0.952	**0.015**
SLA, $cm^2 g^{-1}$ dw	0.454		**0.000**↓	0.222	**0.000**	0.527	0.206	0.819	0.393	0.074
N, mg g^{-1} dw	0.620		**0.000**↓	0.322	**0.000**	**0.008**	0.348	0.297	**0.005**	0.182
P, mg g^{-1} dw	**0.023**	80>4	0.280	0.086	0.358	0.256	0.399	0.798	0.416	0.558
K, mg g^{-1} dw	0.120		**0.000**↓	0.917	**0.000**	0.919	0.107	0.658	0.215	**0.009**
Ca, mg g^{-1} dw	**0.000**	80>4	0.440	0.086	**0.000**	0.240	0.373	0.855	0.844	0.142
Mg, mg g^{-1} dw	**0.001**	80>4	0.271	0.240	**0.000**	0.477	0.426	0.726	0.517	0.794
Mn, mg g^{-1} dw	0.411		0.851	**0.029**↓	**0.000**	0.676	0.336	0.853	**0.042**	0.083
Fe, µg g^{-1} dw	**0.001**	80>4	0.079	0.344	**0.000**	0.067	**0.005**	0.943	**0.020**	**0.006**
Zn, µg g^{-1} dw	0.106		0.978	**0.011**↓	**0.004**	0.942	0.714	0.847	0.308	**0.007**
Cu, µg g^{-1} dw	0.762		**0.000**↓	0.330	**0.000**	0.405	0.556	**0.020**	0.833	0.120
B, µg g^{-1} dw	**0.000**	80>4	0.116	0.278	**0.000**	0.157	0.862	0.684	0.805	0.655
S, mg g^{-1} dw	0.155		**0.000**↓	0.112	**0.000**	0.059	0.515	0.953	0.556	0.295
P:N, %	**0.037**	80>4	**0.000**↑	0.156	0.113	0.925	0.874	0.951	0.921	0.835
Mn:N, %	0.510		0.119	**0.046**↓	**0.000**	0.426	0.184	0.981	0.268	0.161
Zn:N, %	0.172		0.093	**0.027**↓	**0.001**	0.574	0.664	0.959	0.928	**0.027**
B:N, %	**0.001**	80>4	0.826	0.300	**0.000**	**0.041**	0.675	0.931	0.971	0.989

* value of parameter was higher in clone 80 as compared to clone 4

Table 4. Summary of *P*- values for the effects of treatments on cell wall chemistry (% of dry mass) in silver birch leaves. The chamber effect was analysed separately by comparing CC and OC treatments using one-way ANOVA. Abbreviations: CC = chamber control and OC = outside control. Significant treatment effects and interactions ($P<0.05$) are shown in bold. Arrows after *P*-value indicate increasing (↑) or decreasing (↓) effect of CO_2 or ozone.

Response	Clone	CO_2	O_3	Date	$CO_2 \times O_3$	$CO_2 \times$ date	$O_3 \times$ date	Date × clone	$CO_2 \times O_3 \times$ date	CC - OC
α-cellulose	**0.000** 80<4*	**0.000**↓	0.298	**0.000**	**0.036**	**0.002**	0.408	**0.003**	**0.022**	0.318
Hemicellulose	**0.002** 80>4	**0.005**↑	**0.048**↓	**0.000**	0.502	0.281	0.450	0.818	0.894	0.113
Uronic acids	**0.000** 80>4	**0.005**↓	**0.022**↓	**0.000**	0.481	0.620	**0.000**	0.755	0.895	**0.012**
Total lignin + polyphenolics	0.063	0.168	0.605	**0.000**	0.424	**0.029**	0.577	0.974	0.582	0.422
Acid-soluble lignin	0.368	**0.000**↓	0.768	**0.000**	0.516	**0.008**	**0.044**	0.832	0.486	0.817
Acetone-soluble extractives	**0.000** 80<4	**0.000**↓	0.643	**0.000**	0.128	**0.006**	**0.013**	**0.000**	0.328	0.107

* value of parameter was higher (>) or lower (<) in clone 80 as compared to clone 4

Similarly, elevated CO_2 decreased significantly the concentration of acetone-soluble extractives (P = 0.000, 0.023, 0.000 and 0.001 for days 161, 186, 220 and 248, respectively) (Fig 3f). The significant main effect of ozone appeared as lower content of hemicellulose and uronic acids (Table 4; Fig 3b-c). Significant decreases in uronic acids were found only in the first sampling days 161 and 186 (P = 0.038 and 0.004, respectively) (Fig 3c).

In situ localization of H_2O_2 accumulation and thylakoid injuries

Visible H_2O_2 accumulation was not found in any OC, CC or EC trees, indicating that H_2O_2 precipitation was due to ozone-caused oxidative stress. H_2O_2 accumulation was mainly found in EO and EC + EO samples collected during July and August in each year, suggesting that ozone-derived oxidative stress was strongest in mid-aged leaves (Table 7; Fig 4). Trees showing visible H_2O_2 accumulation within leaf mesophyll are given in Table 7. In 1999 and 2001 both clones showed H_2O_2 accumulation, whereas in 2000 all the positive samples were from clone 80 (Table 7). According to Table 7, four ozone-exposed trees (namely 6, 8, 28 and 40) were particularly sensitive to ozone stress, and clone 80 seemed to be more sensitive as compared to clone 4. In average 21% of palisade cells and 24% of spongy cells of all affected EO and EC + EO trees listed in Table 7 showed excess H_2O_2 accumulation. In all cases, the cell wall was the primary site of H_2O_2 accumulation (Fig 4). Cytosolic and chloroplastic precipitation was found only in three EO trees of clone 80 in August 1999 and 2001 (Table 7; Fig 4c,d). Ozone-caused structural thylakoid injuries indicating oxidative damage were present in 83% of positively stained cells, and there were no differences between the clones in amount of injuries (Fig 2c). Thylakoid injuries were similar as reported previously for birch by Pääkkönen *et al.* (1995) and Oksanen *et al.* (2001), e.g. dilation (swelling of thylakoid membrane interspaces) and distortion (disorganised shape of thylakoid membranes) (Fig 2c).

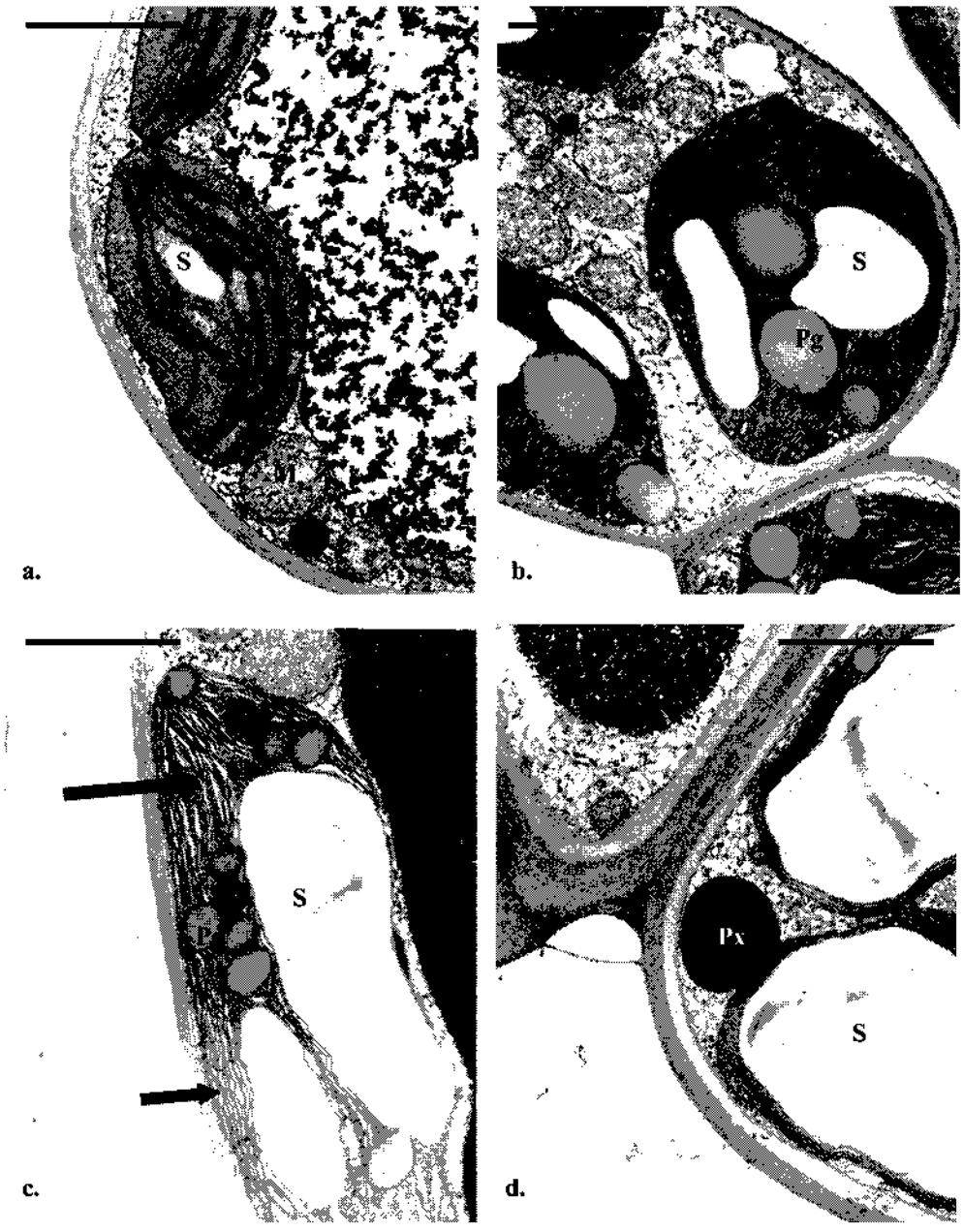

Figure 2. Ultrastructural changes in leaves of birch (*Betula pendula*) grown under elevated CO_2 and/or ozone over three growing seasons. The samples were taken in 6 August, 2001. (M) mitochondria; (Pg) plastoglobuli; (Px) peroxisome; (S) starch grain; (T) tannin; Scale = 500 nm. (a) Chloroplast and mitochondria in of control leaf of clone 80; (b) CO_2 caused increase in size of chloroplast and starch grain and number of mitochondria in clone 80; (c) ozone caused dilation (long arrow) and distortion (short arrow) of thylakoid membranes in clone 80; (d) combined action of elevated CO_2 and ozone appearing as increased accumulation of starch and presence of peroxisomes.

Table 5. Mean values ± SD for leaf structural and anatomical parameters in two clones (4 and 80) of birch (*Betula pendula*) exposed to elevated CO_2 (EC), elevated ozone (EO) and their combination (EC + EO) over the 3-year exposure. CC = chamber control, OC = outside control. Statistical differences are given in Table 2.

Parameter	Clone	CC	EC	EO	EC+EO	OC
Total leaf thickness,	4	173.3 ± 24.5	170.3 ± 15.9	164.4 ± 20.7	168.9 ± 23.4	165.4 ± 24.0
µm	80	173.1 ± 22.1	167.0 ± 19.0	166.7 ± 19.3	169.4 ± 18.2	165.4 ± 30.1
	4+80	173.2 ± 23.2	168.6 ± 17.5	165.5 ± 19.9	169.1 ± 20.9	165.4 ± 27.1
Palisade/spongy thickness ratio	4	0.62 ± 0.23	0.58 ± 0.20	0.65 ± 0.23	0.65 ± 0.18	0.63 ± 0.20
	80	0.62 ± 0.19	0.67 ± 0.22	0.71 ± 0.24	0.64 ± 0.18	0.68 ± 0.21
	4+80	0.62 ± 0.21	0.62 ± 0.22	0.68 ± 0.23	0.65 ± 0.18	0.65 ± 0.21
Palisade layer thickness,	4	52.9 ± 8.9	50.7 ± 10.5	52.5 ± 11.5	54.1 ± 10.1	51.7 ± 11.0
µm	80	53.3 ± 9.6	54.1 ± 9.5	54.8 ± 10.8	53.3 ± 10.6	53.6 ± 12.0
	4+80	53.1 ± 9.2	52.4 ± 10.1	53.6 ± 11.2	53.7 ± 10.3	52.6 ± 11.5
Spongy layer thickness,	4	92.1 ± 23.5	91.1 ± 14.3	85.3 ± 19.3	86.4 ± 15.9	87.2 ± 20.2
µm	80	91.0 ± 21.4	86.3 ± 18.7	81.6 ± 17.4	86.5 ± 14.3	83.2 ± 21.8
	4+80	91.6 ± 22.4	88.7 ± 16.7	83.5 ± 18.4	86.4 ± 15.0	85.2 ± 21.0
Cell wall thickness	4	306 ± 62	312 ± 70	285 ± 69	304 ± 70	309.6 ± 58.0
of mesophyll cell, nm	80	293 ± 58	309 ± 60	295 ± 63	297 ± 59	289.3 ± 41.5
	4+80	300 ± 60	310 ± 65	290 ± 65	300 ± 65	299.4 ± 51.1
Volume of chloroplasts,	4	8.0 ± 3.4	9.7 ± 4.4	9.9 ± 5.6	10.5 ± 5.7	10.3 ± 4.8
% of mesophyll	80	9.3 ± 4.6	10.0 ± 4.2	10.6 ± 4.6	9.6 ± 4.9	11.4 ± 5.2
	4+80	8.7 ± 4.1	9.9 ± 4.3	10.2 ± 5.1	10.0 ± 5.3	10.9 ± 5.0
Volume of intercellular	4	40.5 ± 10.3	41.4 ± 11.7	39.5 ± 10.1	39.5 ± 11.7	37.8 ± 12.3
space, % of mesophyll	80	41.1 ± 10.6	38.9 ± 10.6	38.6 ± 10.5	38.6 ± 10.8	36.3 ± 11.9
	4+80	40.8 ± 10.4	40.1 ± 11.2	39.0 ± 10.3	39.1 ± 11.2	37.1 ± 12.0
Size of chloroplasts, μm^2	4	9.5 ± 2.0	10.6 ± 2.3	10.4 ± 2.3	9.9 ± 2.0	9.9 ± 1.6
	80	10.5 ± 2.5	10.7 ± 2.1	9.4 ± 1.9	10.3 ± 2.5	9.4 ± 2.2
	4+80	10.0 ± 2.3	10.6 ± 2.2	9.9 ± 2.1	10.1 ± 2.2	9.6 ± 1.9
Size of starch grain, μm^2	4	3.0 ± 1.5	3.8 ± 2.0	3.5 ± 1.7	3.3 ± 1.6	3.2 ± 1.3
	80	3.4 ± 2.3	4.2 ± 2.0	3.0 ± 1.2	3.6 ± 2.0	2.9 ± 1.5
	4+80	3.2 ± 1.9	4.0 ± 2.0	3.2 ± 1.5	3.5 ± 1.8	3.1 ± 1.4
Number of mitochondria	4	3.2 ± 0.9	6.2 ± 1.1	3.9 ± 1.1	6.1± 1.0	3.1 ± 1.0
per cell unit cytoplasm[1]	80	3.3 ± 1.1	6.2 ± 1.0	5.9 ± 0.9	6.1 ± 1.3	3.2 ± 0.8
	4+80	3.2 ± 1.0	6.2 ± 1.0	4.9 ± 1.4	6.1 ± 1.1	3.2 ± 0.9
Number of peroxisomes	4	1.9 ± 0.9	2.2 ± 1.1	5.6 ± 1.0	4.3 ± 0.7	2.0 ± 1.0
per cell unit cytoplasm	80	2.2 ± 1.1	2.5 ± 0.9	5.7 ± 1.1	4.5 ± 1.1	2.1 ± 1.1
	4+80	2.0 ± 1.0	2.3 ± 1.0	5.6 ± 1.1	4.4 ± 0.9	2.1 ± 1.1

[1]Cell unit cytoplasm = rectangular area of 4.0 μm^2 lining the cell wall.

Table 6. Mean values ± SD for leaf structural and anatomical parameters in clones 4 and 80 of birch (*Betula pendula*) exposed to elevated CO_2 (EC), elevated ozone (EO) and their combination (EC + EO) during the third exposure year 2001. CC = chamber control, OC = outside control. Statistical differences are given in Table 2.

Parameter	Clone	CC	EC	EO	EC+EO	OC
Total leaf thickness, µm	4	188.2 ± 21.8	171.3 ± 11.1	172.1 ± 23.1	182.0 ± 20.2	173.9 ± 14.0
	80	192.9 ± 17.0	178.7 ± 18.3	179.5 ± 13.7	183.8 ± 10.5	187.5 ± 28.1
	4+80	190.5 ± 19.4	175.0 ± 15.3	175.8 ± 19.0	182.9 ± 15.9	180.7 ± 22.9
Palisade layer thickness, µm	4	50.9 ± 6.4	45.3 ± 3.5	44.0 ± 3.4	48.6 ± 6.7	43.7 ± 4.0
	80	49.8 ± 10.7	50.2 ± 7.5	46.7 ± 6.3	48.6 ± 10.8	54.3 ± 14.5
	4+80	50.3 ± 8.7	47.7 ± 6.3	45.4 ± 5.1	48.6 ± 8.8	48.9 ± 11.7
Spongy layer thickness, µm	4	107.3 ± 16.9	91.8 ± 6.1	99.5 ± 19.5	99.0 ± 14.5	99.2 ± 12.3
	80	112.5 ± 19.5	101.6 ± 15.0	97.9 ± 15.2	98.0 ± 9.8	100.6 ± 19.9
	4+80	109.9 ± 18.1	96.7 ± 12.3	98.7 ± 17.2	98.5 ± 12.2	99.9 ± 16.3
Size of chloroplasts, μm^2	4	9.2 ± 1.6	10.6 ± 2.2	10.7 ± 2.4	9.1 ± 1.4	9.5 ± 1.8
	80	9.8 ± 2.2	11.7 ± 2.3	8.6 ± 1.8	10.7 ± 2.4	8.9 ± 1.5
	4+80	9.5 ± 1.9	11.1 ± 2.3	9.6 ± 2.3	9.9 ± 2.1	9.2 ± 1.6
Size of starch grain, μm^2	4	2.7 ± 1.0	4.3 ± 1.3	3.3 ± 1.3	3.8 ± 1.1	3.5 ± 1.6
	80	2.6 ± 1.6	5.2 ± 2.5	2.7 ± 1.0	4.4 ± 1.6	2.7 ± 1.1
	4+80	2.7 ± 1.3	4.8 ± 2.0	3.0 ± 1.2	4.1 ± 1.4	3.1 ± 1.4
Number of mitochondria	4	2.8 ± 0.8	5.6 ± 1.2	3.4 ± 1.1	5.6 ± 1.3	3.0 ± 1.0
per cell unit cytoplasm	80	3.0 ± 1.0	5.9 ± 1.1	5.6 ± 1.2	5.4 ± 1.1	3.2 ± 0.9
	4+80	2.9 ± 0.9	5.8 ± 1.2	4.5 ± 1.6	5.5 ± 1.2	3.1 ± 1.0
Number of peroxisomes	4	1.8 ± 0.7	2.6 ± 1.0	5.5 ± 1.3c	4.4 ± 0.8	2.1 ± 1.0
per cell unit cytoplasm	80	2.0 ± 0.9	2.3 ± 0.9	5.9 ± 1.0c	4.8 ± 0.9	2.0 ± 1.2
	4+80	1.9 ± 0.8	2.5 ± 1.0	5.7 ± 1.1c	4.6 ± 0.9	2.1 ± 1.1

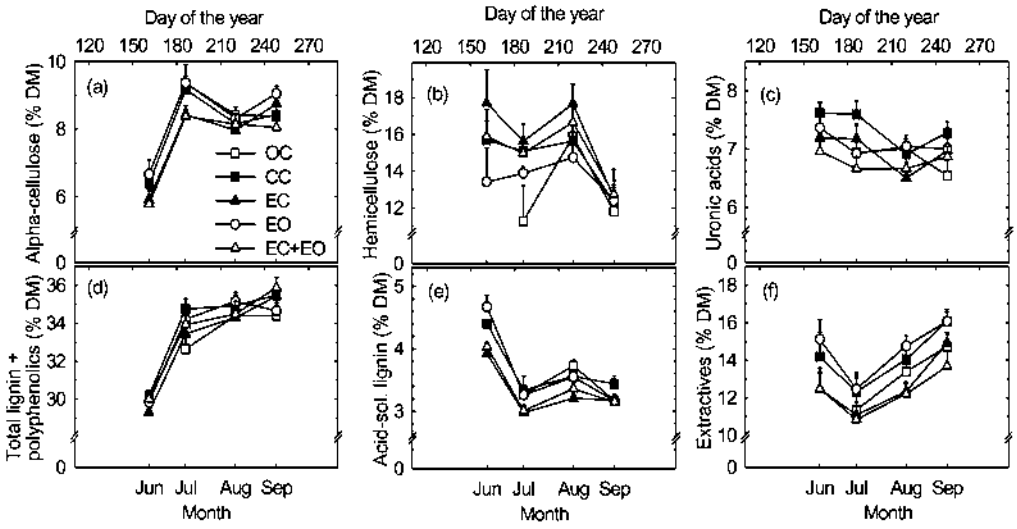

Figure 3. Overall responses on (a) α-cellulose, (b) hemicellulose, (c) uronic acids, (d) total lignin + polyphenolics, (e) acid-soluble lignin, and (f) acetone-soluble extractives in silver birch short-shoot leaves for each treatment, summarizing clone averages in the third treatment year. Values are means (% of dry mass) ± SE.

Table 7. Accumulation of H_2O_2 in palisade and spongy mesophyll cells, cell walls, cytosol and chloroplasts in positively stained samples. Identification number of each tree showing H_2O_2 accumulation is given in parentheses after the clone number. Values are percentage numbers of cells showing H_2O_2 precipitation (± SD), when 640 cells/treatment/clone/year were examined.

Year	Sampling day	Clone (tree)	Treatment	Palisade cells	Spongy cells	Cell wall	Cytosol	Chloroplast
1999	August 2	4 (6)	EO	30 ± 3	40 ± 3	35 ± 3	0	0
		4 (18)	EC+EO	25 ± 4	30 ± 3	28 ± 4	0	0
		80 (19)	EO	10 ± 2	25 ± 4	18 ± 3	10 ± 2	10 ± 4
		4 (30)	EC+EO	25 ± 3	20 ± 3	23 ± 3	20 ± 4	0
		80 (32)	EC+EO	20 ± 4	35 ± 5	28 ± 5	0	0
		80 (40)	EC+EO	10 ± 2	0	5 ± 1	0	0
2000	July 3	80 (8)	EO	20 ± 3	15 ± 3	18 ± 3	0	0
		80 (28)	EO	30 ± 4	25 ± 5	28 ± 5	0	0
	July 31	80 (8)	EO	25 ± 3	30 ± 4	28 ± 4	0	0
		80 (28)	EO	40 ± 5	25 ± 4	33 ± 5	0	0
		80 (36)	EO	15 ± 3	45 ± 5	30 ± 4	0	0
		80 (40)	EC+EO	20 ± 4	35 ± 3	28 ± 4	0	0
2001	June 4	4 (6)	EO	0	10 ± 2	5 ± 1	0	0
	August 6	80 (8)	EO	25 ± 4	20 ± 3	28 ± 4	10 ± 3	5 ± 2
		4 (10)	EC+EO	10 ± 2	15 ± 2	13 ± 2	0	0
		80 (28)	EO	30 ± 5	20 ± 3	25 ± 4	10 ± 3	10 ± 2
		80 (40)	EC+EO	15 ± 3	10 ± 3	18 ± 3	0	0
	September 3	80 (8)	EO	10 ± 2	5 ± 1	8 ± 2	0	0

Table 8. Foliar nutrient concentrations (mg g^{-1} dry mass) in short-shoot leaves of silver birch clones 4 and 80. The values are averages of the data collected at the end of July 2000 and 2001 ± SD. The treatments were chamber control (CC), elevated CO_2 (EC), elevated O_3 (EO), elevated CO_2 and O_3 (EC+EO), and outside control (OC).

Parameter	Clone	CC	EC	EO	EC+EO	OC
N	4	20.4 ± 2.4	18.1 ± 2.0	19.1 ± 1.5	18.1 ± 1.6	19.0 ± 3.2
mg g^{-1} dw	80	21.0 ± 1.5	17.1 ± 1.7	20.6 ± 1.1	17.4 ± 1.4	19.6 ± 2.2
	Pooled	20.7 ± 2.0	17.6 ± 1.8	19.9 ± 1.5	17.8 ± 1.5	19.3 ± 2.7
P	4	3.25 ± 0.60	3.27 ± 0.34	2.84 ± 0.47	3.29 ± 0.57	3.04 ± 0.33
mg g^{-1} dw	80	3.80 ± 0.25	3.51 ± 0.46	3.20 ± 0.48	3.40 ± 0.71	3.66 ± 0.72
	Pooled	3.51 ± 0.53	3.38 ± 0.40	3.02 ± 0.49	3.35 ± 0.62	3.37 ± 0.64
K	4	8.24 ± 1.14	7.45 ± 1.02	7.73 ± 0.79	7.48 ± 0.92	8.93 ± 1.59
mg g^{-1} dw	80	8.32 ± 0.98	6.80 ± 1.12	8.66 ± 1.09	7.11 ± 1.20	10.78 ± 1.90
	Pooled	8.27 ± 1.03	7.15 ± 1.08	8.20 ± 1.04	7.29 ± 1.05	9.91 ± 1.95
Ca	4	6.56 ± 1.14	5.78 ± 1.38	6.18 ± 1.11	6.06 ± 1.27	6.22 ± 1.33
mg g^{-1} dw	80	9.58 ± 1.60	8.41 ± 1.59	8.01 ± 2.76	8.37 ± 2.30	8.82 ± 2.22
	Pooled	7.97 ± 2.05	7.00 ± 1.97	7.09 ± 2.24	7.22 ± 2.16	7.61 ± 2.24
Mg	4	2.88 ± 0.58	2.61 ± 0.66	2.87± 0.49	2.78 ± 0.54	3.09 ± 0.53
mg g^{-1} dw	80	3.75 ± 0.55	3.45 ± 0.65	3.35± 0.85	3.33 ± 0.82	3.53 ± 0.64
	Pooled	3.29 ± 0.71	3.00 ± 0.77	3.11± 0.71	3.05± 0.73	3.32 ± 0.61
Mn	4	1.15 ± 0.46	1.26 ± 0.31	0.91 ± 0.45	0.99 ± 0.38	0.85 ± 0.36
mg g^{-1} dw	80	1.31 ± 0.35	1.23 ± 0.28	1.12 ± 0.51	1.04 ± 0.34	1.15 ± 0.37
	Pooled	1.22 ± 0.40	1.25 ± 0.29	1.01 ± 0.48	1.02 ± 0.35	1.01 ± 0.38
Fe	4	52.2 ± 8.6	45.0 ± 9.6	49.2 ± 11.4	45.5 ± 8.3	46.0 ± 8.1
µg g^{-1} dw	80	61.4 ± 12.3	50.6 ± 10.4	55.6 ± 15.2	60.8 ± 15.6	56.9 ± 11.1
	Pooled	56.5 ± 11.2	47.6 ± 10.1	52.4 ± 13.4	53.2 ± 14.4	51.9 ± 11.0
Zn	4	320.4 ± 79.8	368.8 ± 119.7	296.0 ± 74.1	294.6 ± 20.0	255.9 ± 67.7
µg g^{-1} dw	80	415.6 ± 105.5	329.4 ± 65.6	335.1 ± 55.6	322.5 ± 27.4	291.4 ± 31.8
	Pooled	364.8 ± 101.8	350.4 ± 97.1	315.6 ± 66.4	308.6 ± 27.3	274.8 ± 53.0
Cu	4	5.63 ± 0.96	4.92 ± 1.59	6.09 ± 2.34	5.15 ± 1.39	5.76 ± 1.50
µg g^{-1} dw	80	5.42 ± 1.31	4.10 ± 1.15	5.75 ± 0.94	4.89 ± 1.24	5.25 ± 1.19
	Pooled	5.53 ± 1.10	4.54 ± 1.42	5.92 ± 1.73	5.02 ± 1.28	5.48 ± 1.32
B	4	41.8 ± 15.9	48.0 ± 11.8	42.7 ± 11.4	33.8± 8.0	34.0 ± 11.0
µg g^{-1} dw	80	59.2 ± 19.2	50.9 ± 18.3	56.0 ± 22.9	46.4 ± 18.4	63.9 ± 23.6
	Pooled	49.9 ± 19.1	49.3 ± 14.7	49.4 ± 18.8	40.1 ± 15.2	50.0 ± 23.8
S	4	1.46 ± 0.08	1.22 ± 0.06	1.37 ± 0.11	1.25 ± 0.12	1.36 ± 0.19
mg g^{-1} dw	80	1.38 ± 0.13	1.12 ± 0.09	1.35 ± 0.04	1.16 ± 0.14	1.42 ± 0.10
	Pooled	1.42 ± 0.11	1.17 ± 0.09	1.20 ± 0.13	1.20 ± 0.13	1.39 ± 0.15
P:N	4	16.1 ± 3.1	17.7 ± 1.2	15.0 ± 3.3	18.1 ± 2.1	16.1 ± 3.6
(%)	80	18.4 ± 1.9	20.5 ± 1.9	15.4 ± 1.7	19.5 ± 3.4	18.7 ± 3.4
	Pooled	17.2 ± 2.8	19.1 ± 2.1	15.2 ± 2.6	18.8 ± 2.8	17.4 ± 3.7
Mn:N	4	5.73 ± 2.45	6.70 ± 1.43	4.79 ± 2.36	5.47 ± 1.97	4.56 ± 1.18
(%)	80	6.17 ± 1.57	7.18 ± 1.47	5.39 ± 2.37	5.92 ± 1.62	5.85 ± 1.56
	Pooled	5.95 ± 2.00	6.94 ± 1.42	5.09 ± 2.31	5.69 ± 1.76	5.21 ± 1.49
Zn:N	4	1.59 ± 0.39	1.90 ± 0.61	1.55 ± 0.38	1.64± 0.19	1.36 ± 0.40
(%)	80	1.94 ± 0.42	1.94 ± 0.46	1.63 ± 0.27	1.87 ± 0.24	1.49 ± 0.11
	Pooled	1.76 ± 0.43	1.92 ± 0.52	1.59 ± 0.32	1.75 ± 0.24	1.42 ± 0.29
B:N	4	0.21 ± 0.08	0.28 ± 0.09	0.23 ± 0.07	0.19 ± 0.06	0.20 ± 0.08
(%)	80	0.28 ± 0.09	0.30 ± 0.11	0.27 ± 0.12	0.27 ± 0.11	0.33 ± 0.13
	Pooled	0.24 ± 0.09	0.29 ± 0.10	0.25 ± 0.10	0.23 ± 0.09	0.27 ± 0.12

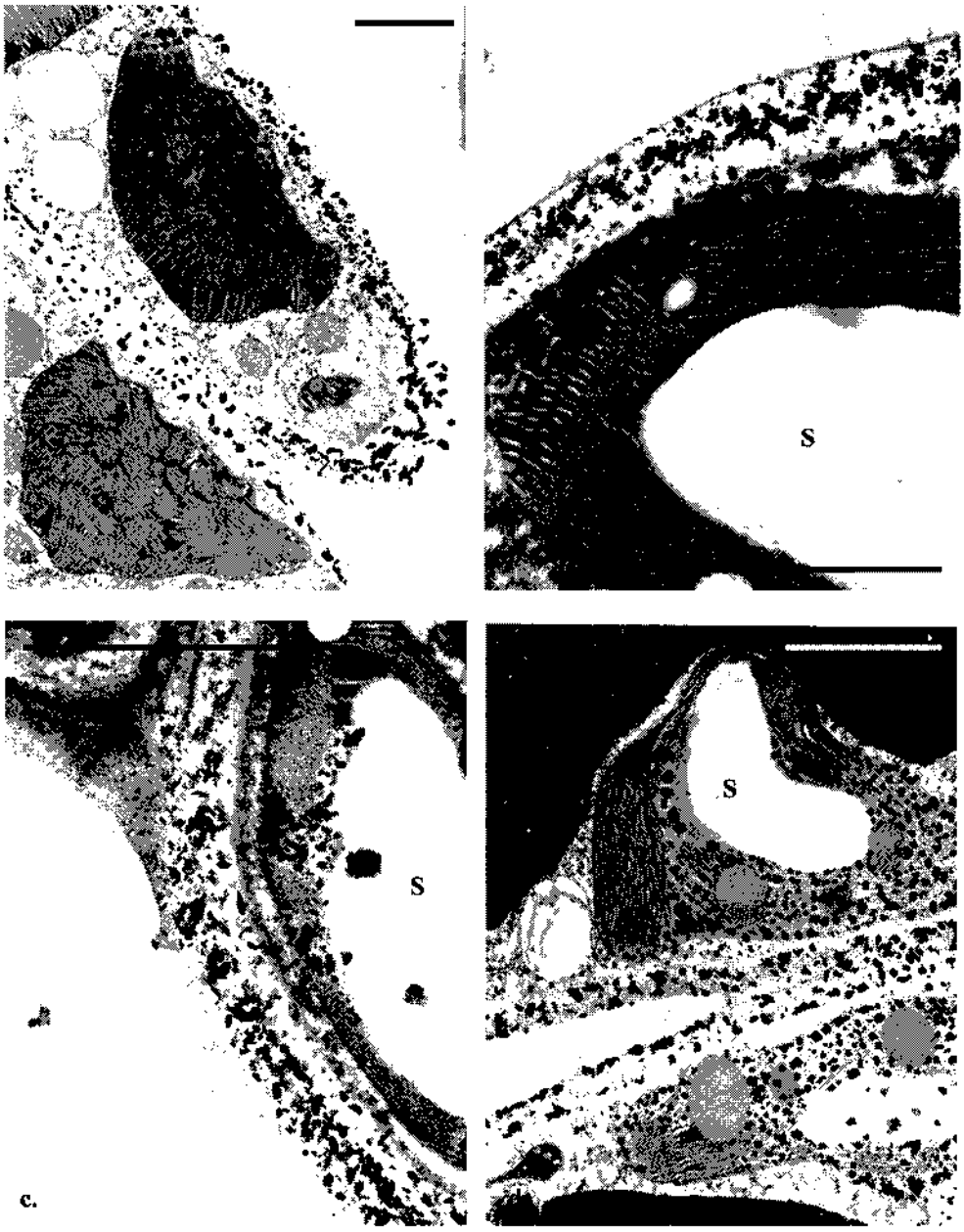

Figure 4. Ozone caused accumulation of H_2O_2 in birch (*Betula pendula*) leaves visualized as electron dense (black) cerium perhydroxide precipitates. The samples were taken in 6 August, 2001, from EO treated clone 80. Scale = 500 nm. (a) and (b) H_2O_2 precipitation in palisade cell walls of clone 80; (c) excess H_2O_2 accumulation extending from cell wall to cytosol and adjacent chloroplast; (d) abundant H_2O_2 accumulation indicating severe oxidative stress in chloroplasts of ozone exposed clone 80. For legends, see Fig 2.

Discussion

In this 3-year experiment we demonstrated the effects of ozone and/or CO_2 enrichment on anatomy, ultrastructure, cell wall chemistry and nutrient status of leaves in two soil-growing birch clones. In general, the structural responses to ozone and/or CO_2 over the whole exposure period were relatively small, but they became gradually more evident in the third exposure year, indicating that long-term experiments are necessary to reveal the true responses in forest trees. In this experiment also higher AOT00 and AOT40 values in the third exposure year due to longer duration of ozone exposure (12 to 14 hrs) may have contributed to ozone effects. Chloroplasts, mitochondria and peroxisomes were the most responsive organelles to ozone and/or CO_2, reflecting the changes in energy and detoxification demands. In addition to basic structural study, we were able to visualize and locate ozone-induced H_2O_2 accumulation within mesophyll cells, and to relate the oxidative stress with structural thylakoid injuries. Changes in nutrient status of leaves were connected to structural alterations and down-regulation of photosynthesis (Eichelmann *et al.*, 2004). In addition, CO_2- and/or ozone-caused changes in cell wall chemistry were demonstrated to play a role in tolerance against oxidative stress.

CO_2 affects mitochondria, chloroplasts and SLA

Number of mitochondria almost doubled in EC + EO and EC exposed leaves as compared to CC leaves, which may indicate increased respiratory activity under CO_2 enhancement. Increased number of mitochondria and rate of respiration has been previously observed in *Pinus radiata* needles, exposed to elevated CO_2 partial pressure (Griffin *et al.*, 2003) and are suggested to be related to increased cellular energy demands under elevated CO_2. In addition to respiration, plant mitochondria participate in many other metabolic processes including the supply of carbon skeletons for e.g. amino acid and sugar biosynthesis (Logan, 2003), alternative respiratory pathway, and ascorbate-glutathione cycle (Mackenzie & McIntosh, 1999). One needs to be aware that mitochondria are highly dynamic organelles, and there is a delicate balance of mitochondrial fusion and fission, controlling the number, size and shape of these organelles (Logan, 2003). Increase in mitochondria in this study was correlated with a higher volume of chloroplasts (correlation coefficient = 0.145, P = 0.006) and bigger size of starch grain (correlation coefficient = 0.108, P = 0.042) under CO_2 enrichment, which was consistent with the increased starch concentrations in these EC and EC + EO trees (Riikonen *et al.*, in press). Accumulation of starch was regarded as an indication of down-regulation of photosynthesis under elevated CO_2 (Riikonen *et al.*, in press).

CO_2-caused decrease in SLA was in accordance with several other studies (Saxe *et al.*, 1998). Previously, reduced SLA under CO_2 enrichment has been explained by increased leaf thickness due to increased cell size, cell number, or the number of cell layers, and was found to correlate with increase in starch content (Lee & Overdieck, 1996). In our study, however, decreased SLA could not be explained by changes in leaf thickness, but rather by subtle increases in average mesophyll cell wall thickness, small decrease in intercellular air space, increase in starch, and mitochondria, and by increased palisade to spongy ratio (EC + EO plants). Although the trees under elevated CO_2 tended to increase the total leaf area (Riikonen *et al.*, 2004) and the relative proportion of palisade tissue, this anatomical up-regulation of photosynthesis was likely

counterbalanced through increased cell wall thickness and reduced intercellular air space leading to greater limitations to internal CO_2 diffusion. In addition, stomatal density tended to decrease in EC + EO trees. As a result, a potential down-regulation of the rate of carboxylation per unit leaf area was followed. Previously, also Peterson *et al.* (1999) reported that in some tree species (e.g. *Betula pendula*, *Acer rubrum*) increase in leaf mass per area together with reduced biochemical capacity resulted in inhibition of leaf-level photosynthesis at elevated CO_2. Thereby, interactions between biochemical and morphological responses to elevated CO_2 may have important effects on photosynthesis.

Ozone affects peroxisomes and mitochondria

Elevated ozone increased the number of peroxisomes 2.2-2.8 times in EC+EO and EO trees, demonstrating a clear ozone-induced proliferation of peroxisomes. This is in accordance with the recent study with aspen and paper birch (*Betula papyrifera*) exposed to 1.5 × ambient ozone in a free-air experiment (Oksanen *et al.*, 2004). Ozone-caused increase in peroxisomes was also reported in needles of Norway spruce (*Picea abies*) by Morré *et al.* (1990) and Kivimäenpää *et al.* (2003). Peroxisomes play a significant role in defence against ozone stress because they contain catalase-enzyme (Morré *et al.*, 1990; Willekens *et al.* 1997; del Rio *et al.* 2002), which converts excess H_2O_2 to water and molecular oxygen resulting in ozone detoxification (Podila *et al.*, 2001). Besides catalase, many other antioxidative systems were found to be located in plant peroxisomes, including the ascorbate-glutathione cycle and different superoxide dismutases (del Rio *et al.*, 2002). In addition to antioxidative systems, increased number of peroxisomes may reflect activation of photorespiration (Tolbert, 1997), which is a balancing route to eliminate excess reducing power produced by light reactions when CO_2 fixation is low, e.g. when content of Rubisco and photochemical efficiency (densities of PSI and PSII, F_v'/F_m') are declined as reported for the present trees (Eichelmann *et al.*, 2004, Riikonen *et al.*, in press). Close connections to photosynthesis were also indicated by the significant negative correlations (correlation coefficient = -0.110, $P = 0.038$) between the number of peroxisomes and the size of chloroplasts.

Increased need for photorespiration under oxidative stress was also supported by increased number of mitochondria (in clone 80), operating at close connections to peroxisomes. Increase in the number of mitochondria could be linked to mitochondrial alternative respiratory pathway keeping electron transfer chain adequately oxidised (avoidance of ROS), to detoxification of ROS through ascorbate-glutathione cycle, MnSOD, catalase, glutathione peroxidase system or thioredoxin system, or to repair of ROS damage (Møller, 2001). This study suggests, that increase in mitochondria was connected to oxidative stress (accumulation of H_2O_2), which was also more evident in clone 80, probably due to higher ozone flux. Ozone-caused increase in mitochondria could partly explain the increased transcript levels for the mitochondrial phosphate translocator (*Mpt1*) found in ozone-stressed birch by Kiiskinen *et al.* (1997). Recent studies have also recognised mitochondria as active participants in signal transduction pathway, particularly during programmed cell death (PCD) processes and in cytoplasmic calcium signalling (e.g. Logan, 2003). Recently, evidence of ozone-induced increase in number of mitochondria was also reported in needles of Norway spruce (Kivimäenpää *et al.*, 2003).

H_2O_2 accumulation in ageing leaves

Excess H_2O_2 accumulation in leaf mesophyll indicating ozone-caused oxidative stress was found mainly in the cell walls, suggesting that apoplastic ozone detoxification was efficient, especially in young leaves. Chloroplastic H_2O_2 precipitation was found only in mature and senescing leaves of EO trees of clone 80, but not in the presence of elevated CO_2. Greater H_2O_2 precipitation in clone 80 is likely explained by the higher stomatal conductance rates leading to higher ozone uptake as compared to clone 4 (Riikonen *et al.*, 2003). Previously, ozone-caused H_2O_2 accumulation has been detected in the apoplast of sunflower (Ranieri *et al.*, 2003) and in cell walls, plasma membranes, mitochondria, peroxisomes and cytosol of birch leaves (Pellinen *et al.*, 1999) after acute exposure to high concentrations. In more realistic field conditions, ozone-sensitive aspen clones showed H_2O_2 accumulation in the inner side of cell wall adjacent to plasma membrane, while ozone-tolerant clones showed precipitation only on the outer surface of the cell wall indicating also efficient decomposition of ozone before reaching the plasma membrane (Oksanen *et al.*, 2004). Studies with sunflower (*Heliantus annuus*) have indicated that ozone derived H_2O_2 accumulation is a complex and highly regulated event requiring the time-dependent stimulation and down-regulation of differently located enzymes involved in H_2O_2 generation and degradation (Ranieri *et al.*, 2003).

Chloroplastic accumulation of H_2O_2 precipitates was eliminated by elevated CO_2, as reported previously by Oksanen *et al.* (2004). This antagonistic effect may be explained by increased rate of photosynthesis at the presence of elevated CO_2 (Eichelmann *et al.*, 2004), leading to higher NADPH production and increased activity of enzymatic detoxification, e.g. through ascorbate-glutathione cycle (Podila *et al.*, 2001). This was supported by the increased number of mitochondria and bigger chloroplasts and starch grains as discussed above. In addition, changes in cell wall chemistry (discussed below) and increased mesophyll cell wall thickness under elevated CO_2 is likely to improve the ozone scavenging efficiency, as suggested by Moldau (1998). Furthermore, counteracting effects of CO_2 enrichment on chloroplastic H_2O_2 accumulation could also be related to lower production of toxic H_2O_2 from photorespiration due to increased CO_2 to O_2 ratio (Willekens *et al.*, 1997).

Cell wall chemistry is responsive to CO_2 and/or ozone

The present study showed that both CO_2 and ozone can change the chemical properties of mesophyll cell wall. In general, effects of CO_2 were more marked as compared to ozone. CO_2-caused decline in the concentrations of α-cellulose and acid-soluble lignin agreed with Poorter *et al.* (1997), suggesting that trees rather invested to growth than to cell walls under elevated CO_2. Our results also suggest that hemicellulose could have a possible role in CO_2-caused protection of mesophyll cells against ozone through barrier effect, because components of hemicellulose cross-link cellulose microfibrils to each other more tightly (Brett and Waldron, 1996). CO_2-caused increase in hemicellulose in our study was evident especially in young leaves which were tolerant against oxidative stress as judged by H_2O_2 accumulation. In older leaves, however, concentration of hemicellulose decreased in all treatments, which was accompanied by increased sensitivity to oxidative stress. In addition, ozone-caused decrease in hemicellulose could further predispose the mesophyll cells to oxidative stress. Recently, Cabané *et al.* (2004) reported that ozone-induced stress-lignin formation in leaf mesophyll cells

might contribute to ozone tolerance in poplar through barrier or antioxidant effect. In our study, acid-soluble lignin concentration declined rapidly in ozone-exposed trees during leaf senescence, which may be linked to increased H_2O_2 accumulation therein.

Changes in nutrient status is linked to photosynthesis

Ozone and CO_2 enrichment led to reductions in different foliar nutrients. CO_2-caused reductions in N, K, Cu, S and Fe could be partially explained by dilution effect since SLA decreased indicating increased dry mass of leaves. This was partly due to accumulation of starch (Riikonen *et al.*, in press); however, true decrease of N had occurred because N expressed on a structural dry mass basis (thus omitting the influence of starch) also decreased by about 10%. Ozone-caused decreases in Mn, Zn and Cu, in the other hand, may reflect the 23% decrease in growth of roots reducing the uptake area (Riikonen *et al.*, 2004). This CO_2-caused decline in nutrients may be linked to reduced stomatal conductance and thereby, transpiration flow, leading to less efficient uptake of these minerals (Riikonen *et al.*, in press) to cope with the increased growth under elevated CO_2. Reduced foliar content of N is well-documented for several plant species growing at elevated CO_2 (Cotrufo *et al.*, 1998), and was connected to reduced amount and activity of Rubisco and down-regulation of photosynthesis in this experiment (Eichelmann *et al.*, 2004). CO_2-caused decline in K content could also be linked with down-regulation of photosynthesis, because K has a dominant role in the light-induced H^+ flux across the thylakoid membranes and in the establishment of the transmembrane pH gradient, necessary for the synthesis of ATP (Marschner, 1995). Furthermore, declines in Fe and S concentrations likely contributed to down-regulation of photosynthesis under elevated CO_2, because these components are integral parts of the photosynthetic electron transport chain, e.g. in ferredoxin.

Ozone-caused decline in Mn may be linked to reduced photochemical efficiency (activity of PS II) (Eichelmann *et al.*, 2004) as well as to injuries in thylakoid membranes, because Mn reduction is reported to cause loss of functional particles in PS II (Marschner, 1995). In addition, ozone-caused decline in Mn might have contributed the accelerated leaf abscission through degradation of chloroplasts (Eichelmann *et al.*, 2004; Riikonen *et al.*, in press). Ozone-caused disintegration of thylakoid membranes and H_2O_2 accumulation in chloroplasts was likely linked to lower Zn content, because Zn is required for maintenance of integrity of membranes, and it is essential in protecting membrane lipids and proteins against oxidative damage e.g. through activity of CuZnSOD (Marschner, 1995). In ozone trees, reductions in Mn and Zn resulted also in nutrient imbalances, which were manifested as increased N to other nutrient ratios. Previously, accumulation of excess N and unbalanced nutrient status of ozone-stressed birch saplings were reported also in Yamaji *et al.* (2003). Nutrient imbalances may result in predisposition of trees to infestations with insect herbivores, as reported in beech and Norway spruce by Flückiger and Braun (1999), and have indirect effect on fundamental ecosystem processes such as litter decomposition (Cotrufo *et al.*, 1998).

Chamber trees vs. free-air trees

Effect of chamber needs a special attention when interpreting the present results. As indicated by our structural analysis, the trees inside the chambers formed thicker leaves, which were characterized by lower volume of chloroplasts, higher volume of intercellular air space, lower stomatal

density, and higher concentration of uronic acids. Previously, studies with Southern beech (*Nothofagus cunninghamii*) also indicated that environmental factors caused significant variation in all gross leaf morphological variables, such as leaf thickness, leaf area, SLA and stomatal density (Hovenden & Vader Schoor, 2003). Contrasting to our results, stomatal density was found to increase in glass-house growing beech trees because of reduced leaf expansion (Hovenden & Vader Schoor, 2003). Increased leaf thickness is generally expected to be accompanied by higher carbon assimilation rates, as long as light is not a limiting factor (Körner & Diemer, 1994). In our study, a higher chlorophyll fluorescence (F_v'/F_m'), amount and activity of Rubisco and WUE, but lower stomatal conductance and concentration of starch were detected in the chamber trees as compared to outside controls, although net photosynthesis was unaffected (Riikonen *et al.*, in press). Thereby, we assume that the resposes of trees to CO_2 and/or ozone enhancement in a more stressful free air environment would be even stronger through greater ozone/CO_2 gas uptake, which is in agreement with the study by Eichelmann *et al.* (2004). Reduced stomatal conductance within the chambers may also have affected the nutrient uptake as indicated by significant changes in K, Fe and Zn.

Clonal differences

On the basis of growth, gas exchange, and Rubisco activity measurements clone 80 is physiologically more active as compared to clone 4 (Riikonen *et al.*, 2003). This was supported by the present structural study, revealing that higher photosynthetic activity of clone 80 was partially explained by higher palisade to spongy ratio. In addition, higher stomatal density of clone 80 enabled a greater CO_2 flux into the leaf mesophyll, which was, on the other hand, accompanied by a greater ozone uptake and therefore, need for detoxification. Greater physiological activity of clone 80 was also supported by the higher nutrient concentration of leaves. Although the differences between the clones in growth were minor, clone 4 allocated proportionally less carbon to roots and branches under ozone exposure as compared to clone 80 (Riikonen *et al.*, 2004). Greater ozone sensitivity of clone 4 became evident also in the photosynthesis study by Eichelmann *et al.* (2004). However, data from the current study suppose that clone 80 was more sensitive to oxidative stress over clone 4, since H_2O_2 accumulation was more marked and frequent in this clone, especially in chloroplasts. Therefore, this study indicates that H_2O_2 accumulation within the leaves is not a direct measure of the ozone sensitivity of plant, but rather, reflects rates of ozone uptake. Furthermore, there were great differences between the individual trees in appearance of H_2O_2 accumulation. This study also indicated that the cell wall chemistry was clone-specific similarly as in wood (Pot *et al.*, 2002; Kaakinen *et al.*, 2004).

Conclusions

The present results indicate that structural changes in birch leaf mesophyll are likely to occur in the long-term exposure to increasing CO_2 and/or ozone level. Increased demand for energy production and detoxification processes in trees exposed to ozone (and CO_2) was manifested particularly by increased number of mitochondria and peroxisomes. CO_2-caused effects on photosynthesis processes (Eichelmann *et al.*, 2004) seem to be affected by interplay between down-regulating and up-regulating anatomical changes, such as mesophyll density and proportion of palisade tissue. In ozone-exposed trees, excess accumulation of H_2O_2 was restricted to apoplast in young leaves, and chloroplastic H_2O_2 accumulation

was found only in physiologically more active clone 80 when the ageing of the leaves proceeded. Ozone scavenging capacity was improved by simultaneous CO_2 enrichment, likely through CO_2-caused increases in photosynthetic and mitochondrial activity, and cell wall chemistry, hemicellulose in particular. Similarly, ozone-caused changes in cell wall chemistry may play a role in tolerance against oxidative stress. These structural alterations under elevating CO_2 and/or ozone are underlying mechanisms for responses of photosynthesis and respiration and thus may have a significant influence on the leaf, tree, and ecosystem carbon balance, and ultimately to global carbon cycle under changing atmospheric conditions.

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Chapter 6

General discussion

GENERAL DISCUSSION

6.1 Experimental conditions

In Suonenjoki the daily mean O_3 concentration in the ambient air varied between 10 and 60 ppb during the experiment. For around 40% of the daytime hours in 1999-2001, the O_3 concentration in the ambient air was about 30 ppb, and thus the elevated O_3 concentrations used in this experiment were moderate and realistic (1.6× ambient O_3 concentration) (Vapaavuori *et al.* 2002). The AOT40 value for elevated O_3 was 20-30 ppm.h at the end of each growing season, and thus the threshold level of 10 ppm.h for trees was exceeded during each of the growing seasons (Vapaavuori *et al.* 2002).

In the course of this OTC study, some changes in the microclimate inside the chambers were found. In the open-top chambers, the daily mean temperature was 2-3°C higher, and the light and relative humidity rates were slightly lower than in the ambient air (Vapaavuori *et al.* 2002). These changes may have modified the responses of the trees to the treatments, and hence the chamber effect needs to be taken into account when interpreting these results. The trees representing clone 4 tended to grow better in the chambers than outside, although in the other parameters measured the clones responded similarly to the altered microclimate. However, the stomatal conductance and density were lower in the chamber-grown trees of both clones. Hence, we assume that the responses to elevated CO_2 and/or O_3 would have been stronger without the chambers, owing to the greater CO_2 and O_3 flux into the leaves (Chapters 3-5).

The weather conditions during the four growing seasons were variable, summer of 1998 being the coolest and most rainy and summer of 1999 being the warmest and driest (Table 1). The different backround weather conditions may complicate the comparison of the data between the different growing seasons. However, in the course of this experiment it was possible to assess the treatment effects and the differences between the clones in weather conditions representing the average long-term variation in Finnish climate (Drebs *et al.* 2002).

6.2 Differences between the clones

According to this experiment, clone 80 was physiologically more active compared to clone 4, which appeared, for example, as 14% higher net photosynthesis and 7% higher Rubisco activity. Interestingly, the chlorophyll fluorescence patterns were similar in the clones, and thus played no significant role in the different photosynthetic rates between the clones. Clone 80 had 40% higher stomatal conductance and 17% higher stomatal density than clone 4, which may have led to

Table 1. Rainfall in the growing seasons 1998-2001 (mm) and mean monthly air temperature (°C) in Suonenjoki site during the experiment.

	1998	1999	2000	2001
Rainfall (mm)	339	239	344	339
Mean monthly air temperature (°C)				
May	8.1	6.4	9.2	7.6
June	13.4	18.4	14.1	14.2
July	16.1	17.3	16.6	18.7
August	12.7	13.4	13.9	14.6
September	9.6	10.6	8.2	10.4

greater transpiration, but also to lower WUE in clone 80 than in clone 4. In addition, the nutrient concentrations in the leaves were 3-46% higher in clone 80 than in clone 4 (Chapters 2, 4 & 5).

Major differences in the carbon fixation in the clones were found, in favour of clone 80, which displayed 40% higher above-ground biomass in the final harvest in September 1998, when the trees were seven years old. However, there were no differences in growth increments between the two clones thereafter (1999-2001), indicating that the growth in clone 80 was higher than in clone 4 in the early years of the growth, highlighting the importance of long-term studies in assessing factors related to growth (Chapters 2 & 3). The biomass allocation also differed between the clones: clone 80 invested more carbon in the growth of the stem, while clone 4 did so in the growth of its branches and leaves (Chapters 2 & 3). These differences may have compensated for the lower leaf-level photosynthesis in clone 4 than in clone 80, resulting in similar biomass growth in the two clones during the experiment.

According to the physiological characteristics, and also on the basis of the visual assessment of the appearance of the trees, clone 80 was more suitable for the Suonenjoki experimental field than clone 4. Clone 80 originates from Eno, in eastern Finland, and clone 4 from Valkeakoski, in southern Finland, where the climate is less continental. It can be speculated whether clone 4 was more stressed by the environmental factors (e.g. lower temperature and temperature sum) in Suonenjoki than clone 80, which was growing closer to its original latitude (Vapaavuori *et al.* 2002). However, this theory is difficult to prove. Alternatively, the differences between the clones may have been caused by their genetic differences, resulting in different responses to environmental conditions. Clone 4 allocated more biomass in the leaves than clone 80, leading to a relatively greater transpiratory leaf area. Despite the larger stomatal number and conductance in clone 80 the control of stomata may have been better in clone 80, than in clone 4. In the chambers, where the RH was lower and temperature higher than outside, g_s was reduced more in clone 80 than in clone 4. It is possible that clone 4 had suffered from drought, although the trees were watered regularly during the experiment (Vapaavuori *et al.* 2002). A reduction in the g_s and in the number of stomata is the general response to drought stress (Pääkkönen *et al.* 1998a,b) (Chapters 2-5).

The different origins of the clones were reflected in their different phenology. Clone 80, which was of a more northern/eastern origin than clone 4 (Mutikainen *et al.* 2000), was characterized by faster senescence-related structural changes in its leaves. This was seen as a faster accumulation of cytoplasmic lipids and vacuolar tannins (Chapter 2), and by earlier leaf abscission in the autumn (Chapter 3) in clone 80 than in clone 4. In addition, the bud burst occurred earlier in clone 4 than in clone 80 (data not shown). In conclusion, clone 80 was adapted to a shorter active growing season than clone 4.

6.3 Effects of elevated CO_2 on the silver birch clones

6.3.1 Elevated CO_2 stimulated the photosynthesis

The trees grown in elevated CO_2 over the three growing seasons sustained a higher photosynthetic rate than those grown in ambient CO_2 (Table 2), when the measurements were made under the conditions of their respective growth CO_2 environments (360 ppm or 720 ppm). The photosynthetic rate was 20-60% greater, although the magnitude of the CO_2 response

differed between the years, depending on the weather conditions (Table 1, Chapter 3, Vapaavuori *et al.* 2002). The stomatal conductance was around 20% lower and the leaf level WUE around 60% higher in the trees grown under elevated CO_2 concentration than in the CC trees (Chapter 3), although stomatal density was unaffected by elevated CO_2 (Chapter 4). It has been suggested that increased WUE may impact on the water use and drought resistance of trees in the future (Rey & Jarvis 1998, Gunderson *et al.* 2002). However, since in clone 80 the elevated CO_2 also increased the total leaf area by around 30% during the experiment, it is possible that the increase in leaf-level WUE may be counteracted by an increase in the leaf area, and thus the whole-tree water use might not be changed in this clone (Chapters 3 & 4).

When measured in ambient CO_2 (360 ppm), photosynthesis was around 15% lower in the plants grown in elevated CO_2 than in the CC trees (Table 2). This conforms with many experiments using several tree species (e.g. Rey & Jarvis 1998, Medlyn *et al.* 1999, Tissue *et al.* 1999, Griffin *et al.* 2000) but contrasts with others (Herrick & Thomas 2001, Noormets *et al.* 2001, Sholtis *et al.* 2004). In a review article, Nowak *et al.* (2004) discovered the downregulation of photosynthesis in many FACE experiments, depending on species and growth conditions, and especially on the N concentration. At present, there is no general agreement about the significance of the downregulation of photosynthesis in the field (Drake *et al.* 1997, Saxe *et al.* 1998, Nowak *et al.* 2004) (Chapter 4).

In the present experiment, the downregulation of photosynthesis was maintained throughout the experiment and was connected to an increased starch concentration (about 50%) and larger starch grains (25%), indicating discrepancies between supply and demand for carbohydrates (end-product inhibition) (Chapters 4 & 5). Carbohydrate accumulation may have led to a signal transduction response, causing feedback inhibition in the photosynthetic capacity and to the suppression of several photosynthetic genes (Drake *et al.* 1997, Moore *et al.* 1999). In elevated CO_2 the concentration of total leaf nitrogen was reduced despite N fertilization during the growing season. Reductions in the amount and activity of Rubisco and in the ratio of Rubisco and total N concentration, chlorophyll and soluble proteins indicate a smaller investment of N to Rubisco, resulting in increased N use efficiency in elevated CO_2, especially in clone 80. On the other hand, the reduced N concentration, and also the reduced concentrations of other nutrients, may have been caused by growth dilution and increased demand for nutrients (Stitt & Krapp 1999). According to Drake *et al.* (1997), the average reduction in the amount and activity of Rubisco is 15% and 24%, respectively. In this study the amount of Rubisco reduced by 35% in clone 80 and by 16% in clone 4. Despite these differences in the mechanisms of downregulation between the clones, the magnitude of downregulation in terms of net photosynthesis was similar in the clones. This may be due to greater stomatal conductance and C_i in clone 80 than in clone 4 (Chapters 2, 4 & 5).

In the present experiment, some adjustments had occurred in both light and dark reactions of photosynthesis in order to maximize the photosynthetic capacity in response to the elevated CO_2 (Table 2). The chlorophyll and the total soluble protein concentration were not significantly affected by the elevated CO_2, despite a 14% higher volume of individual chloroplasts (Chapters 4 & 5). In clone 80, the Rubisco/chlorophyll-ratio decreased, which means that in elevated CO_2 more nitrogen was allocated to the light than to the dark reactions of the photosynthesis (Evans 1989). A higher F_v'/F_m' indicates increase in the use of

absorbed light energy in photochemistry, and a decrease in the NPQ may indicate the inhibition of photorespiration in elevated CO_2 (Long & Drake 1991) (Chapter 4).

The SLA in green leaves was reduced in elevated CO_2, an occurrence that was related to a slightly increased mesophyll cell wall thickness and to a subtle decrease in the intercellular air space, as well as to an increase in the starch concentration (Chapter 5). Also the structure of the leaf cell walls was altered by elevated CO_2 (Table 2). Amongst other changes in leaf chemical composition, such as reduced N concentration, an increased concentration of hemicellulose, together with an increased concentration of starch, led to an enhanced C:N ratio (Chapters 4 & 5). An increase in the C:N ratio is a common CO_2 response in various tree species, suggesting a slower rate of litter decomposition and alterations in the nutrient dynamics (Saxe *et al.* 1998). In addition, elevated CO_2 reduced the concentrations of α-cellulose and lignin, suggesting that trees rather invested to growth than to cell walls under elevated CO_2 (Chapter 5). Blaschke *et al.* (2002) found that in beech seedlings the leaf lignin concentration was decreased by elevated CO_2 only under conditions of a high N concentration. They concluded that an increased growth in response to elevated CO_2 and N resulted in a transient shortage of C for lignin production. However, Poorter *et al.* (1997) found no effect resulting from elevated CO_2 on the tissue lignin content when a range of plant species was studied.

The number of mitochondria doubled in the trees exposed to elevated CO_2, which may indicate increased respiratory activity under CO_2 enhancement (Chapter 5). This is supported by our gas-exchange measurements made in June and July 2001, which revealed that the dark respiration rate increased under conditions of elevated CO_2 in both clones (n= 2 trees for each clone, unpublished results). This is in accordance with a recent study with nine different tree species (Davey *et al.* 2004) and may be related to increased cellular energy demands in elevated CO_2.

6.3.2 Carbon dioxide and growth: variable responses in the clones

In the present experiment, elevated CO_2 had a positive effect on the growth of the trees, especially in clone 80. In accordance with earlier studies, the stem diameter growth was more affected than the height growth (Kubiske *et al.* 1998, Isebrands *et al.* 2001, Sigurdsson *et al.* 2001). According to previous experiments, the average increase under elevated CO_2 in the total biomass is 49% (Saxe *et al.* 1998) and in stem volume around 28% (Zak *et al.* 2000, Isebrands *et al.* 2001) for deciduous trees. In the present experiment, elevated CO_2 increased the total biomass by 45% in clone 80, but by only 7% in clone 4. Similarly, elevated CO_2 increased the stem volume by 44% in clone 80, and by only 2% in clone 4. This difference between the clones is difficult to explain, and only a few suggestions can be presented on the basis of the existing data gained from this experiment. In this study, the trees of both clones were irrigated and fertilised in the same way (Vapaavuori *et al.* 2002), and the soil nutrient concentrations were comparable to those in forest and nursery soils (Luoranen 2000). Thus, the different growth responses were not due to differences in the nutrient availability at the site. The stomatal conductance was lower in clone 4 than in clone 80, which may indicate water stress in clone 4. Alternatively, clone 4 may have invested more in other processes than growth. According to Poorter *et al.* (1997), a high investment of C for example in secondary chemistry may decrease the growth response of a tree to elevated CO_2 concentration.

In the present experiment, where the stand was approaching canopy closure, the

growth enhancement in clone 80 was sustained over the three-year period and the positive CO_2 effect on stem volume growth was magnified towards the end of the experiment. Elevated CO_2 also significantly increased the seed production in clone 80, which may indicate ontogenic drift in addition to increased growth (Centritto *et al.* 1999) (Chapter 3). The leaf area development is known to be an important determinant of growth response in elevated CO_2 (Saxe *et al.* 1998). Since the canopy was not completely closed in this experiment and the leaf area was still increasing in clone 80, it is possible that the growth stimulation observed may represent the maximum response to elevated CO_2. In a closed forest stand leaf area production is generally constrained, for example, by nutrient and water deficiency, or by a low light level in dense canopies (Norby *et al.* 1999). After canopy closure, the growth response is determined, for instance, by the canopy architecture and respiratory costs, while the leaf-area development is of less importance (Saxe *et al.* 1998).

At the end of the experiment the total leaf area of clone 80 had increased in elevated CO_2 conditions by 29%, while in clone 4 the total leaf area tended to be reduced (by 10%). The increase in the leaf area in clone 80 was magnified during the experiment, and was caused by enhanced leaf production, since the mean leaf size was not affected. However, the LAR and LMR were unaffected by elevated CO_2 in either clone. Hence, it can be concluded that elevated CO_2 had no specific stimulatory effect on the leaf area, and thus larger trees simply had more leaves than smaller ones. However, elevated CO_2 delayed leaf abscission, indicating a prolonged seasonal growing period (Chapter 3), which is in accordance with Tricker *et al.* (2004), who found delayed autumnal senescence in poplar.

6.4 Effects of elevated O_3 on the silver birch clones

6.4.1 Ozone and photosynthesis

According to numerous experiments, silver birch is sensitive to O_3 (Oksanen & Saleem 1999; Matyssek 2001, Oksanen 2003a,b). However, in the present study O_3 had no effect on the gas exchange parameters. The non-responsiveness of the net photosynthesis and stomatal conductance to elevated O_3 (Chapter 4) is surprising since, in general, both processes are reduced by elevated O_3 (Long & Naidu 2002, Matyssek & Sandermann 2003). This is in accordance with Eichelmann *et al.* (2004), who also found only a small decrease in the net photosynthesis in elevated O_3 in laboratory measurements of the detached leaves of the same trees, although some other parameters related to photosynthesis were affected, especially in clone 4.

Despite the lack of response of the gas exchange parameters to O_3, the amount and activity of Rubisco tended to be lower in elevated O_3 than in the control plants (17% and 11% in clone 4, and 7% and 2% in clone 80, respectively). This resulted in a significant decrease in the proportion of Rubisco in relation to the total soluble proteins in both clones, since the amount of total soluble proteins was unaffected. The decreased Rubisco N/ total N-ratio and N concentration (on an area basis) in clone 4 may be related to N redistribution in the leaves in the case of elevated O_3.

The chlorophyll fluorescence parameters (F_v'/F_m' and NPQ) were affected by elevated O_3, indicating a reduction in the use of absorbed light energy in photochemistry and an increase in the dispersal of energy as heat to avoid overexcitation of the PSII (Chapter 4). These results, together with the O_3-induced disintegration of the thylakoid membranes (Chapter 5) and the reduced density of PSI

and PSII in clone 4 (Eichelmann *et al.* 2004) suggest that elevated O_3 may have affected the thylakoid functionality. However, in this experiment the maximum photochemical yield of the PSII (F_v/F_m), which is highly dependent on membrane integrity (Long & Naidu 2002), was not altered by elevated O_3. In clone 4 the chlorophyll concentration also decreased by 19%. However, the O_3 effects mentioned above were apparently not severe enough to affect the net photosynthesis.

Elevated O_3 increased the number of mitochondria in clone 80 by 80% and the number of peroxisomes by 60% in both clones (Chapter 5). The increased number of mitochondria may indicate increased photorespiration under oxidative stress (Kellomäki & Wang 1998). Peroxisomes are related to defence processes acting against the ozone stress, since they contain the catalase-enzyme (Willekens *et al.* 1997, del Rio *et al.* 2002). Catalase converts excess H_2O_2 into water and molecular oxygen, resulting in ozone detoxification (Podila *et al.* 2001).

6.4.2 Ozone and growth

Elevated O_3 affected growth negatively mainly in clone 4. However, most responses were found only in the ambient CO_2 concentration, and some of the effects were not statistically significant, owing to the limited number of replicates in this experiment. Elevated O_3 induced leaf abscission at an earlier stage in the autumn, which is a typical O_3 response in many deciduous species (Karnosky *et al.* 1996, Bortier *et al.* 2000, Novak *et al.* 2003) (Chapter 3).

Elevated O_3 reduced the dry mass of roots and branches and the mean leaf size, but only in ambient CO_2 (Chapter 3). Elevated O_3 also reduced the branching (measured as shoot ramification) of the same birches (Kull *et al.* 2003). The total dry mass in clone 4 was 26% lower in elevated O_3 than for the control trees, although the difference was not statistically significant owing to the large variation between the replicates. These responses were found primarily in clone 4, while the response of clone 80 to elevated O_3 was small (Chapter 3).

The O_3 effects on the nutrient status in silver birch have been variable. In some studies, the O_3 effect has been small or negligible (Maurer & Matyssek 1997, Karlsson *et al.* 2003), while in some experiments reductions in nutrient concentrations have been found (Oksanen & Saleem 1999, Yamaji *et al.* 2003). The lower nutrient concentrations in elevated O_3 may be connected to a tendency towards a reduction in the root:shoot ratio (Oksanen & Saleem 1999, Yamaji *et al.* 2003). In the present experiment, the reduced root growth and the tendency towards a lower root:shoot ratio in elevated O_3 led to a reduced root surface area for nutrient uptake that was connected with significantly reduced concentrations of Mn (by 17%) and of Zn (by 13%), and slightly lower concentrations of other nutrients in the leaves of the O_3 trees. The reduction in the concentrations of Mn and Zn may be linked with the O_3-induced disintegration of the thylakoid membranes, since Mn ions are essential for the PS II function and Zn is required for the maintenance of the integrity of membranes, and it is also essential in protecting membrane lipids and proteins against oxidative damage through the activity of the CuZn-superoxide dismutase (Marschner, 1995) (Chapter 5).

The O_3 effect on the cell wall chemistry is not well known. In some experiments with aspen, birch, maple, poplar, elevated O_3 induced a lignin synthesis, which has been suggested to be connected with enhanced activity of the phenylpropanoid biosynthetic pathway and O_3 resistance (Wustman *et al.* 2001, Cabane *et al.* 2004, Kaakinen *et al.* 2004). In the

present study the only O_3 effect found on cell wall structure was the reduced concentrations of hemicellulose and its components, uronic acids (Chapter 5). This may be an indication of changes in the rigidity and elasticity of the cell walls.

The O_3-induced earlier leaf abscission in the autumn and the decreased mean leaf size suggest not only a shorter period of active photosynthesis but also a decreased photosynthetic surface area, thus causing decreased net photosynthesis and growth at tree-level. According to Matyssek & Sandermann (2003), reduced branching and leaf size with premature leaf loss may limit the biomass production more than the decline in leaf-level photosynthesis.

These changes were detected after three years of exposure to elevated O_3. It is evident that even minor declines in the annual growth increment could lead to major cumulative growth losses after long-term O_3-exposures (Rebbeck & Scherzer 2002, Oksanen 2003a).

6.4.3 Ozone sensitivity of the clones

The O_3-sensitivity of the clones was previously determined in a 2-year pot experiment with 2-year old saplings exposed to a slightly elevated O_3 concentration (around 1.7× ambient O_3 concentration). According to the visible injuries, the growth and leaf senescence clone 4 was classified as O_3-tolerant and clone 80 as an O_3-sensitive genotype, both representing mid-range responses rather than extremes in the O_3-sensitivity ranking (Pääkkönen *et al.* 1997). The data from this experiment shows that the response of the two clones to O_3 was different from the earlier O_3-sensitivity test. In the present study, clone 4 appeared to be clearly more sensitive to O_3 than clone 80 (Chapters 3 & 4, Eichelmann *et al.* 2004). This is in accordance with recent data by Oksanen (2003a), who found that the O_3-sensitivity of clone 4 increased with the exposure period and tree growth. Changes in O_3-sensitivity were explained by a change in growth form (relatively reduced foliage mass) and a lower net photosynthesis to stomatal conductance ratio that promoted O_3 uptake and decreased photosynthetic gain (Oksanen 2003a). However, also the different growth conditions of the plants in the O_3-sensitivity test (potted saplings) and in the present study (field-grown young trees) and the different response parameters measured might have affected the O_3-sensitivity of the clones.

The reason for the different O_3-sensitivity of the two clones is not clear. It has been suggested that better O_3-tolerance could be related to thicker leaves (Oksanen *et al.* 2001), an investment in foliage mass (Pääkkönen *et al.* 1996), lower stomatal density and conductance (Pääkkönen *et al.* 1997), higher peroxisomal activity (Oksanen *et al.* 2004), and a slower growth rate (Skärby *et al.* 1998, Bortier *et al.* 2000). However, many of the physiological properties connected with O_3 tolerance, as mentioned above, were found in the O_3-sensitive clone 4 (with a lower stomatal conductance and density and higher LAR and LMR in clone 4 than in clone 80). In addition, the peroxisomal activity, SLA and the growth rate were similar in the clones and thus could not explain the O_3-sensitivity of the clones in this experiment.

The different phenology of the clones might have affected the O_3-sensitivity. Since the bud burst occurred earlier and leaf abscission later in clone 4 than in clone 80, the cumulative O_3 dose might have been higher in clone 4 than in clone 80. However, according to Padu *et al.* (in press), in mid-summer 2001, the higher stomatal conductance in clone 80 led to a greater O_3-flux into the leaves in clone 80 than in clone 4. The better O_3-tolerance in clone 80 was related to the more efficient apoplastic O_3 scavenging resulting from the higher ascorbate concentration in clone 80 (Padu *et*

al. in press). The higher O_3-flux into the leaves and better detoxification capacity in clone 80 than in clone 4 were also demonstrated by the chloroplastic H_2O_2 accumulation in some O_3 trees in clone 80, and by the increased number of mitochondria in clone 80 in elevated O_3, indicating higher respiratory activity in clone 80 than in clone 4 (Chapter 5).

In the present experiment the growth conditions of the clones were similar (Vapaavuori *et al.* 2002). If clone 4 was more stressed in the experimental field due to water stress and/or due to different growth requirements, the O_3-sensitivity of this clone should also have been affected. Drought stress may either protect a plant from O_3 damage by reducing the stomatal conductance (Pääkkönen *et al.* 1998a) or it may enhance the O_3 effect by causing disturbances in the water regulation capacity of the stomata. However, the interactions are highly dependent on other environmental factors and on the genotype (Pääkkönen *et al.* 1998a,b). In this experiment no net effect of O_3 on the g_s was found when the values were averaged over each growing season. However, a higher g_s was found in elevated O_3 than in the CC trees in some of the measurements made during each growing season. These changes may indicate impaired control of stomata in elevated O_3, especially in clone 4 (Chapter 4).

6.5 Interaction of CO_2 and O_3

In the present study, the negative effect of elevated O_3 on the growth parameters was mainly found in the ambient CO_2, while the EC and EC+EO treatments were similar in both clones (Chapter 3). In addition, in the few parameters related to photosynthesis and leaf structure, where O_3 effect was found, such as chlorophyll concentration, chlorophyll fluorescence and chloroplastic H_2O_2 precipitation in clone 80 and in the size of the peroxisomes, the effect of the O_3 was smaller or absent in elevated CO_2. The compensation effect of CO_2 was probably related to reduced stomatal conductance, and hence reduced O_3 uptake in elevated CO_2, and/or also to the increased photosynthetic rate followed by the increased activity of enzymatic detoxification in elevated CO_2 (Rao et al. 1995, Podila *et al.* 2001). However, the size of the starch grains was increased in the EC, while in the EC+EO these responses were counteracted by the elevated O_3. Further, elevated CO_2 and O_3 together decreased the total activity of Rubisco and increased the C/N-ratio more than O_3 alone in both clones, while the EC and EC+EO treatment were similar. The synergistic effect of elevated CO_2 and O_3 may be caused by the simultaneous negative effects of CO_2 and O_3 on the parameters.

It can be concluded that the protective effect of CO_2 against one negative effect is not necessarily reflected in the other parameters, as suggested by Polle & Pell (1999). In addition, since the responses of the trees to CO_2 and O_3 are strongly modified by environmental conditions and by the internal factors of a particular tree, the interaction of CO_2 and O_3 may not be uniform throughout the entire lifespan of a tree (Matyssek & Sandermann 2003).

6.6 Conclusions

The genetic differences of silver birch clones modify the responses of the clones to the environmental changes. Depending on the genotype, silver birch was able to utilize the increasing CO_2 concentration in the atmosphere, and this positive effect was maintained throughout the three-year experiment. Elevated O_3 had a small effect on the growth and physiology of the silver birches although the annual O_3 exposures were 2-3 times higher than the AOT40-value of 10 ppm.h., set as a critical dose for forest trees. However, even minor reductions in the

Table 2. Summary of the responses of silver birch clones 4 and 80 to elevated CO_2, O_3 and CO_2+O_3 in Suonenjoki open-top chamber experiment during 1999-2001. The treatments were compared with control trees (CC) Abbreviations: ↑ = significant increase; (↑) = tendency towards increasing values; ↓ = significant decrease; (↓) = tendency towards decreasing values; - = no treatment effect. The data are presented in detail in chapters 3-5.

Parameter	CO_2			O_3			CO_2+O_3		
	4	80	4+80	4	80	4+80	4	80	4+80
Growth parameters (3)									
Height growth[a]	-	-	-	-	-	-	-	-	-
Stem diameter growth[a]	↑	↑	↑	-	-	-	↑	↑	↑
Stem volume[b]	-	↑	↑	-	-	-	-	↑	↑
Total leaf area[b]	-	↑	↑	(↓)	-	(↓)	-	↑	↑
Leaf abscission[b]	↓	↓	↓	(↑)	(↑)	↑	↓	↓	↓
Total dry mass[c]	-	↑	↑	(↓)	-	-	-	↑	↑
Dry mass of roots[c]	-	↑	↑	↓	-	↓	-	↑	↑
Dry mass of stem[c]	-	↑	↑	(↓)	-	-	-	↑	↑
Dry mass of branch[c]	-	↑	↑	↓	-	-	-	↑	↑
Dry mass of leaves[c]	-	↑	↑	(↓)	-	-	-	↑	↑
Dry mass of seeds[c]	-	↑	↑	-	-	-	-	↑	↑
Mass-ratios[c]	-	-	-	-	-	-	-	-	-
Leaf area ratio[c]	-	-	-	-	-	-	-	-	-
Mean leaf size[c]	-	-	-	(↓)	(↓)	↓	-	-	-
Root:shoot ratio[c]	-	-	-	(↓)	-	(↓)	-	-	-
SLA_{litter}[c]	↓	↓	↓	-	-	-	↓	↓	↓
Gas exchange[a] (4)									
P_n, growth CO_2	↑	↑	↑	-	-	-	↑	↑	↑
P_n, ambient CO_2	↓	↓	↓	-	-	-	↓	↓	↓
g_s, growth CO_2	↓	↓	↓	-	-	-	↓	↓	↓
Transp, growth CO_2	↓	↓	↓	-	-	-	↓	↓	↓
C_i, growth CO_2	↑	↑	↑	-	-	-	↑	↑	↑
WUE, growth CO_2	↑	↑	↑	-	-	-	↑	↑	↑
Chlorophyll fluorescence[a] (4)									
F_v/F_m	-	-	-	-	-	-	-	-	-
F_v'/F_m'	↑	↑	↑	-	-	↓	↑	↑	↑
NPQ	↓	↓	↓	-	-	(↑)	↓	↓	↓
Biochemistry of leaves[a] (4)									
Amount of Rubisco	↓	↓	↓	(↓)	-	(↓)	↓	↓	↓
Total activity of Rubisco	↓	↓	↓	(↓)	-	(↓)	↓	↓	↓
Specific activity of Rubisco	-	(↑)	↑	-	-	-	-	(↑)	↑
Activation state of Rubisco	-	-	-	-	-	-	-	-	-
Rubisco/chlorophyll	-	↓	↓	-	-	-	-	↓	↓
Rubisco/protein	↓	↓	↓	↓	↓	↓	↓	↓	↓
Rubisco N/ total N	↓	↓	↓	(↓)	(↓)	(↓)	↓	↓	↓
Total chlorophyll	(↓)	-	(↓)	↓	-	↓	(↓)	-	(↓)
Total soluble protein	-	-	-	(↓)	-	-	-	-	-
Total leaf N, g m^{-2}	-	↓	↓	(↓)	-	(↓)	-	(↓)	(↓)
Structural N	↓	↓	↓	(↓)	-	(↓)	↓	↓	↓
C/N ratio	↑	↑	↑	(↑)	-	(↑)	↑	↑	↑
Starch	↑	↑	↑	(↑)	-	-	↑	↑	↑
Soluble sugars	-	-	-	-	-	-	-	-	-

Table 2. Continued

Parameter	CO_2			O_3			CO_2+O_3		
	4	80	4+80	4	80	4+80	4	80	4+80
Cell anatomy and ultrastructure[a] (5)									
Total leaf thickness	-	-	-	(↓)	(↓)	(↓)	-	-	-
Spongy layer thickness	-	-	-	↓	↓	↓	-	-	-
Palisade to spongy ratio	-	-	-	↑	↑	↑	-	-	-
Cell wall thickness in mesophyll	-	-	-	(↓)	-	-	-	-	-
Volume of chloroplasts, % mesophyll	-	-	-	(↑)	(↑)	(↑)	-	-	-
Volume of cell walls, % mesophyll	-	-	-	-	-	-	-	-	-
Volume of intercellular space	-	-	-	(↓)	(↓)	(↓)	(↓)	(↓)	(↓)
Size of chloroplast	(↑)	(↑)	(↑)	-	-	-	-	-	-
Size of starch grain	↑	↑	↑	-	-	-	↑	↑	↑
Number of plastoglobuli	-	-	-	-	-	-	-	-	-
Number of mitochondria	↑	↑	↑	-	↑	↑	↑	↑	↑
Number of peroxisomes	(↑)	(↑)	(↑)	↑	↑	↑	↑	↑	↑
Leaf structure (5)									
Stomatal density[d]	-	-	-	-	-	-	↓	(↓)	↓
SLA_{fresh}[a]	↓	↓	↓	-	-	-	↓	↓	↓
N[e]	↓	↓	↓	(↓)	-	(↓)	↓	↓	↓
P[e]	-	-	-	(↓)	(↓)	(↓)	-	-	-
K[e]	↓	↓	↓	-	-	-	↓	↓	↓
Ca[e]	-	-	-	(↓)	(↓)	(↓)	-	-	-
Mg[e]	-	-	-	-	-	-	-	-	-
Mn[e]	-	-	-	↓	↓	↓	↓	↓	↓
Fe[e]	(↓)	(↓)	(↓)	-	-	-	(↓)	-	-
Zn[e]	-	↓	-	↓	↓	↓	↓	↓	↓
Cu[e]	↓	↓	↓	-	-	-	↓	↓	↓
B[e]	-	-	-	-	-	-	(↓)	(↓)	(↓)
S[e]	↓	↓	↓	-	-	-	↓	↓	↓
Cell wall chemistry in leaves[f] (5)									
α-cellulose	↓	-	↓	-	-	-	↓	↓	↓
Hemicellulose	↑	↑	↑	↓	↓	↓	-	-	-
Uronic acids	↓	↓	↓	↓	↓	↓	↓	↓	↓
Total lignin + polyphenolics	-	-	-	-	-	-	-	-	-
Acid-soluble lignin	↓	↓	↓	-	-	-	↓	↓	↓
Acetone-soluble extractives	↓	↓	↓	-	-	-	↓	↓	↓

[a]measurements were made in June, July, August and September each growing season. The gas exchange measurements were carried out using either ambient (360 ppm) or growth CO_2 concentrations (360 ppm for CC, EO and OC treatments and 720 ppm for EC and EC+EO treatments) in the leaf chamber.

[b]measured at the end of each growing season

[c]measured after final harvest 2001

[d]stomatal density was determined on fully expanded short-shoot leaves on 2 August and 6 September 1999, on 21 July 2000, and on 27 June 2001.

[e]data are averages of the measurements made in July 2000 and July 2001. Nutrient concentrations are expressed as mg g^{-1} dry mass (macronutrients) or as μg g^{-1} dry mass (micronutrients).

[f]determined in June, July, August and Spetember of the third year of experiment (2001)

yearly growth increment may lead to major growth losses in long-term exposures.

In the present study the interactive effect of the CO_2 and O_3 was complex. However, in most of the parameters related to the growth, photosynthesis and structure of the leaves, the negative effect of O_3 was smaller or absent in elevated CO_2 compared with ambient CO_2. The counteracting effect of CO_2 may be due to decreased stomatal conductance and to an increased photosynthetic rate followed by the increased activity of the enzymatic detoxification in elevated CO_2.

Owing to the great genetic variation in the responses to CO_2 and O_3, it is difficult to draw general conclusions about the success of silver birch in the future on the basis of only two clones. Considering the long life-span of the trees, this three-year study conducted with young trees may have provided only suggestive information about the tree responses to climate change. Nevertheless, clone 4 was unable to utilize the increased CO_2 concentration and was also proven to be more sensitive to O_3 than clone 80, indicating a poor competitive status in relation to clone 80. Therefore, the present results indicate that not all individuals within the Finnish birch population are able to adapt to a changing environment.

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