Interactive effect of springtime frost and elevated ozone on early growth, foliar injuries and leaf structure of birch (*Betula pendula*)

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Received: *27 February 2003*
Accepted: *9 May 2003*

doi: 10.1046/j.1469-8137.2003.00828.x

Summary

• Impacts of ozone and late frost on six birch (*Betula pendula*) genotypes from south-eastern Finland were studied in an 8-wk chamber experiment.

• The plants were measured for bud burst, growth, visible foliar injuries caused by ozone and frost, structural leaf properties and changes in chloroplasts.

• Ozone delayed bud burst but stimulated subsequent growth. Acute frost injuries were compensated by increased leaf production. Early bud burst predisposed to frost damage, whereas late bud burst increased the vulnerability to ozone. In combined ozone + frost treatment, freezing reduced visible ozone injuries, counteracted ozone-induced growth enhancement and stomatal changes, and exacerbated ozone-caused reduction in palisade cell, chloroplast and starch grain size. Rapid changes in epidermal cell differentiation towards stomata and/or glandular trichomes occurred to enhance ozone/frost tolerance.

• The results showed large genetic variation within birch population in response to frost and ozone. Generally, birch seem to recover from acute frost occurrence efficiently through compensating leaf production, but co-occurring ozone enhancement may disturb the recovery processes mechanistically through structural damage in photosynthetic tissue, especially in chloroplasts.

Key words: ozone, frost, birch (*Betula pendula*), bud burst, growth, foliar injury, leaf structure.

Introduction

Increasing concentrations of 'greenhouse gases' such as CO_2 and ozone, increasing global mean temperature through radiative forcing, increased frequency of extreme weather events and the depletion of stratospheric ozone leading to increases in UV-B radiation are the four features of climate change currently considered to be most important. Global atmospheric ozone concentrations have risen 36% since preindustrial times, and nearly 30% of global forests are currently exposed to damaging tropospheric ozone concentrations (Fowler *et al.*, 1999; IPCC, 2001). According to predictions by Fowler *et al.* (1999) the extent of temperate and subpolar forest regions exposed to damaging ozone concentrations will expand from 5.3×10^6 km^2 (1990) to 11×10^6 km^2 by the year 2100. Although prevailing ozone concentrations and the number of ozone episodes in northern European countries are lower than in central Europe, the results from controlled experiments indicate that the concentrations are high enough to impair growth of forest trees in field (Selldén *et al.*, 1997), and that there is a likelihood of higher spring-time ozone episodes in northern latitudes resulting from stratospheric ozone incursions (Finlayson-Pitts & Pitts, 1997; IPCC, 2001). Furthermore, the plants in northern latitudes are often more susceptible to ozone injuries than in southern Europe, because the nights in summertime become too short to recover from ozone injury through repair processes driven by dark respiration (De Temmerman *et al.*, 2002), and because lower vapour pressure deficit (VPD) conditions favour high ozone flux inside the leaves (Karlsson *et al.*, submitted).

Intensive monitoring of European forests (ICP Forests) has revealed that exposure to environmental stress factors such as deposition of nitrogen, acidity and heavy metals exceed critical loads over a large proportion of the monitoring plots resulting in enhanced risks for tree root damage, storm damage, crown damage by drought, frost and pests and changes in the plant diversity of ground vegetation (Executive Report, 2002). Lengthened growing seasons with increasing temperature variability including more frequent early spring frosts are predicted to occur in northern Europe, together with increased mean temperatures (IPCC, 2001). Timing of bud burst and frost damage risk of newly unfolded leaves of *Betula* spp. in response to climatic warming in Finland has been examined with two models of phenological timing of boreal trees (Linkosalo *et al.*, 2000). The first, referred to as the chilling-triggered model, describes a chilling requirement during dormancy that must be fulfilled before ontogenetic development towards bud burst can occur, regardless of environmental conditions (Linkosalo *et al.*, 2000). The second model, referred to as the light-climate-triggered model, assumes that regulatory mechanism connected to light conditions hinders ontogenetic development until spring although the chilling requirement is met (Linkosalo *et al.*, 2000). The chilling triggered-model forecast a significant and increasing risk with increasing warming, because ontogenetic development is possible during warm spells at any time following dormancy release. Such warm spells have been rather rare until today, but it is likely that they would become more frequent if climatic warming proceeds, causing bud burst to occur earlier than at present, hence exposing the newly unfolded leaves to frost damage (Hänninen, 1991, 1995). Seasonal changes in frost hardiness are strongly dependent on changes in photoperiod and temperature, but the underlying processes regulating frost tolerance, as well as relationships between environmental stress factors are still poorly understood (Leinonen *et al.*, 1995; Greer *et al.*, 2000; Kratsch & Wise, 2000; Li *et al.*, 2003). There are many factors such as light intensity, relative humidity, air pollutants and inherent sensitivity of the plant to low temperatures that interact and may either exacerbate or protect against frost injury (Li *et al.*, 2003).

Many tree species exposed to high ozone concentrations have shown decreased freezing tolerance (Skärby *et al.*, 1998). In conifers, it has been confirmed that ozone exposure increases the sensitivity of needles to photoinhibition and winter desiccation (Mikkelsen & Ro-Poulsen, 1995). In sugar maple, cold acclimation occurred earlier in ozone-treated seedlings, indicated by higher carbohydrate concentration of stem and earlier accumulation of (abscisic acid) ABA in the xylem sap (Bertrand *et al.*, 1999). However, high ozone concentration also shifted the seasonal cycle of de-acclimation in the following spring, leading to earlier swelling of buds and greater susceptibility to spring-time frosts. It has been demonstrated that ozone may accelerate budburst and thereby spring frost damage also in conifers (Skärby *et al.*, 1998). Detrimental influence of ozone on cold tolerance of forest trees has been suggested to be related to changes in membrane permeability, enzyme activity, antioxidants, photosynthetic carbon reduction and increased carbon demand for dark respiration, leading to reduced availability of carbon-based cryoprotectants, especially under chronic long-term ozone experiments (Waite *et al.*, 1994; Bertrand *et al.*, 1999). During freezing, cellular dehydration and destabilization of membrane are the key processes leading to frost damage (Pearce, 2001). Enzymatic reactions will be instantly slowed at freezing temperatures as a result of decreased substrate diffusion rates, and finally, transport processes across membranes will be interrupted (Kratsch & Wise, 2000). First and most severe freezing symptoms are found typically in chloroplasts, and frost-induced thylakoid injuries appears to be related to photo-oxidative conditions during illumination (Kratsch & Wise, 2000). Many of the freezing-caused ultrastructural injuries resemble those seen in programmed cell death (PCD) (Kratsch & Wise, 2000).

So far, interactive effects of ozone and cold stress have not been reported for birch, although in northern parts of Europe very low winter temperatures, as well as autumn and spring-time frosts are not rare. Furthermore, great susceptibility of European white birch (*Betula pendula* Roth) to elevated ozone has been indicated in several recent studies (Pääkkönen *et al.*, 1993, 1995a,b, 1996, 1998a,b; Oksanen & Saleem, 1999; Oksanen & Holopainen, 2001). Because there is obviously an increasing risk of concurrent exposure to increasing ozone concentrations and late frost in forest trees, we conducted a chamber experiment with both stress factors to simulate the interactive effect of ozone and low temperature in a number of birch genotypes at early growth stages in spring-time climatic conditions. The major aim was to study whether or not frost injuries are exacerbated by elevated ozone in birch. In this paper, we relate acute visible and ultrastructural foliar ozone and frost injuries with whole-plant growth responses, and discuss the recovery from acute frost injury under elevated ozone. Gas exchange and photosynthesis-related responses of these plants will be reported elsewhere (Prozherina *et al.* unpublished).

Materials and Methods

Plant material and growth conditions

We have selected a naturally regenerated birch forest (mixed *Betula pendula* and *Betula pubescens*) from Punkaharju, south-eastern Finland (61°41′-N, 29°20′-E) for long-term studies (Laitinen *et al.*, 2000). To study the genetic differences among individual trees in several field and glasshouse experiments we took a random sample of 30 trees from this forest. The trees were micropropagated in 1997 for further field studies at Finnish Forest Research Institute, Punkaharju Research Station (61°41′-N, 29°20′-E). The material of this study is a

sample of open pollinated progenies of six parent trees (six half-sib families hereafter called genotypes); identification numbers of parent trees were 8, 12, 17, 18, 21, and 22 (Laitinen *et al.*, 2000, 2002). The parent trees were selected according to their secondary chemistry profiles and resistance to hare browsing (i.e. content of triterpenes and flavonoid aclygones). Two genotypes are resistant to hare browsing (17 and 18) and two susceptible (8 and 22) (Laitinen *et al.* unpublished). Two additional genotypes (12 and 21) are quite different in their phenolic compound profiles (Laitinen *et al.*, 2000).

Seeds from the parent trees were collected in late July 2000. The seeds were sown in May 2001, in EK-28 containers (volume 0.28 L/seedling) filled with Kekkilä prefertilized nursery peat and fertilized seven times (July 7–August 15) using Kekkilä Superex fertilization (NPK 12-5-27). In late autumn 40 winter-dormant plants of each genotype were selected randomly from all plant material for this study, and were replanted in 1.3 l pots filled with Kekkilä peat. The saplings were over-wintered in random order in field conditions at Punkaharju Research Station, covered with snow and were brought to the experiment chambers on 18 March 2002 at dormant stage. The plants were randomly divided into four treatments (10 plants/genotype/treatment): control (filtered air), frost stress, elevated ozone, and combined ozone + frost stress, and watered as needed with tap water and fertilized three times (week 16, 17 and 18) with 0.2% Kekkilä 9-Superex, NPK 19-5-20. The soil moisture was regularly controlled using Theta probe Soil moisture sensor (type ML 2).

The chamber experiment system

The experiment was conducted using four independent temperature, light and humidity controlled 2.6 m^3 Bioklim 2600T chambers, with air change of 250 l/min. Light was provided by eight lamps of the type Osram HQI-T and the energy emission of the lamps was quite similar to daylight in the visible range of the spectrum from 400 to 700 nm. All the inner walls of the chambers are white special painted aluminium, which scatters the light through multiple reflections. Ozone gas was produced by Fischer OZ 500 ozone generator (Meckenheim, Bonn, Germany), and ozone concentrations were continuously measured from the outlet air stream and analysed by an ozone analyser (Dasibi 1008-RS, Dasibi Environmental Corp., Glendale, CA, USA).

To simulate springtime conditions, the plants were grown from start (20 March) until 23 April, 2002, in May temperature profile (ranging from + 5°C in night to + 12°C in day), and thereafter in June temperature profile (ranging from 12°C in night to 19°C in day) until the end of the exposure (10 May) (Fig. 1a). Both profiles were based on 40-year weather data collected in Punkaharju Research Station. The light/dark cycle was 20/4 h in the May program, and 22/2 h in the June program with daylight illumination of 500 µmol m^{-2} s^{-1}, and relative air humidity was 60–75% (day) and 75–90% (night) in the May program, and 72–84% (day) and 80–90% (night) in the June program according to global radiation and weather data by Finnish Meteorological Institute in Jyväskylä, central Finland (62°14′-N, 25°20′-E). To avoid any inequality in growth conditions caused by the chamber, the position of the plants was changed twice a week within and between the chambers throughout the study. There was no shading among plants in fumigation chambers.

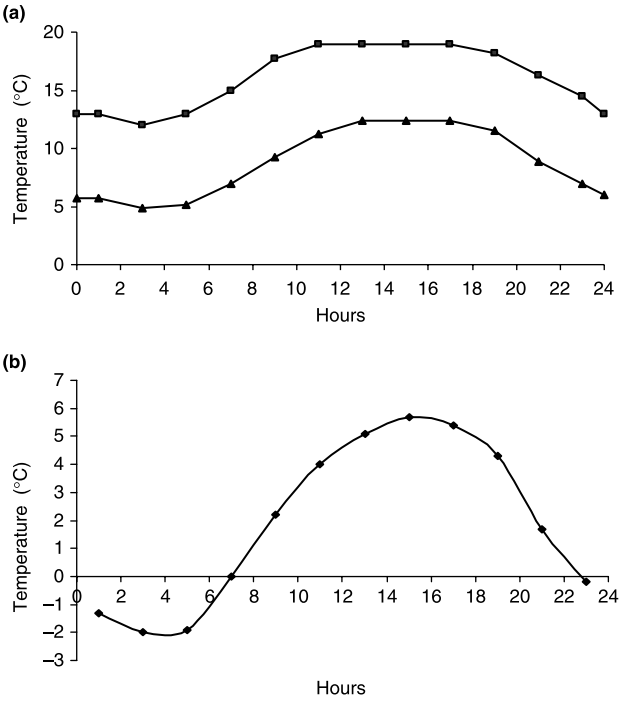


Fig. 1 Temperature profiles for (a) the May (closed triangles) and June (closed squares) programs, and (b) the frost treatment from 22 April until 23 April.

Control plants and frost-treated plants without elevated ozone were grown under filtered air (ozone concentration was close to zero) throughout the study, whereas ozone and ozone + frost treated plants were fumigated with ozone concentration of 65 ppb (10 hours/day/7d/week) over 8 wk, leading to total ozone exposure AOT40 (accumulated over a threshold of 40 ppb) of 10665 ppb-h (= 10.7 ppm-h) and 10602 ppb-h (= 10.6 ppm-h), respectively, which was equal to current critical ozone level for forest trees. We used ozone concentration of 65 ppb, because the field monitoring data by the Finnish Meteorological Institute indicated that average daytime concentration was 50 ppb for April–May over forested areas of central Finland, and as predicted by Stevenson *et al.* (1998), Fowler *et al.* (1999) and IPCC (2001) at least a 30% increase in background concentrations is thought likely in northern Europe over the next decades.

Frost treatment design was based on temperature records made in Punkaharju between the years 1961–2001. According to this data, the likelihood to have a temperature of −2°C was 25% in May after the bud burst of birch (temperature

sum > 33 degree day (dd), according to 6-yr data from Finnish Forest Research Institute, Punkaharju Research Station). The minimum night-time temperatures occurred from 2 AM to 5 AM. Based on this data, the profile for frost treatment was calculated as shown in Fig. 1(b). Under frost and ozone + frost treatments, the plants were exposed to frost treatment profile (temperature ranging from +5.7°C (day) to −2.0°C (night)) over two consecutive days on 22 and 23 April, while the control and ozone plants were growing under the May program.

Growth measurements

The plants were measured for their initial height on 20 March 2002, before the start of exposure. Bud burst (open buds in relation to total number of buds) was recorded on 26 March, 28 March and on 3 April, when the majority of buds were already opened. At the final harvest on 10 May, five plants per genotype per treatment were measured for height increment and number of leaves, separating injured and intact leaves. For dry mass determination, wood, leaves shoot (wood + leaves) and roots were dried separately at 65°C in an oven for 5 d. The initial dry masses for each plant part were measured for additional five plants per genotype at the beginning of the experiment. The mean projected leaf area was measured for one fully enlarged leaf per plant from five plants per genotype and treatment (120 leaves in total) by scanning and calculating the area using a Logitech Photo Touch Colour program (Logitech Inc., Morges, Switzerland).

Visible foliar ozone and frost injuries

Early visible ozone injuries were determined on 23 April, after the first night of frost treatment. Ozone induced injuries were small light-green, yellowish or brown dots and incipient leaf chlorosis as described previously (Pääkkönen *et al.*, 1995b). The second ozone injury assessment was performed at the final harvest on 10 May. During assessments, the number of leaves showing ozone injuries was counted for each plant in relation to the total number of leaves.

Visible foliar frost injuries were determined on 24 April for each frost-treated plant. Frost injuries appeared as wilting of leaves (resembling drought stress) and dehydration of leaf margins, followed by inward folding.

Electron microscopy (TEM and ESEM) and light microscopy

For electron and light microscopical analyses, leaves were collected immediately after the frost treatment on 24 April (at 10.00 AM–13.00 PM) from fully enlarged first flush leaves (fifth leaf from the top) from three plants per genotype per treatment. Sample strips were cut between the second and third leaf vein, followed by cutting into 1.5 mm^2 square pieces under a drop of fixative solution with a razor blade. The samples were placed immediately in a 2.5% (v/v) glutaraldehyde fixative solution (in 0.1 M 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 122 Epon (Ladd Research Industries, Inc., Burlington, USA). The thin sections for electron microscopy were stained 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 Corporation, Tokyo, Japan).

Transmission electron microscope (TEM) samples were photographed with Bioscan camera (Gatan Inc., Pleasanton, CA, USA) (connected to electron microscope) using a Digital Micrograph program (Gatan Inc., Pleasanton, CA, USA) for further image analyses with Adobe Photoshop (Version 5.0) (Adobe Systems Incorporated, USA). Ultrastructural studies were focused on the chloroplast structure of mesophyll cells, because the chloroplasts are known to be the first and most severely impacted organelle during frost injury (Kratsch & Wise, 2000). The samples were analysed (separately for palisade and spongy cells) for section area of chloroplast and starch grain, number of plastoglobuli, and thylakoid membrane injuries such as dilation (swelling) of thylakoid interspaces and distortion (undulated shape) of thylakoids leading to unstacking of grana. 30 chloroplasts per genotype per treatment were studied (720 chloroplasts in total). The light microscopy samples were measured for total leaf thickness, spongy and palisade layer thickness, palisade cell length, and proportion of intercellular space (% of mesophyll cross-sectional area) from digital micrographs using Adobe Photoshop (Version 5.0) program.

The rest of the leaves collected for TEM analysis were used for surface structure studies with scanning electron microscopy (ESEM) (from three plants per genotype per treatment). The air-dried samples were coated with 34 nm of gold, using sputtering equipment (Polaron E 5100, Polaron Equipment Ltd., Watford, UK) and studied with a scanning electron microscope Philips XL 30 (FEI Company, Brno, Czech Republic). The leaf samples were measured for glandular trichome and stomatal density, length of guard cells, and width of stomatal apparatus (from 60 stomata per genotype per treatment, 1440 stomata in total) using a standard digital image analysis systems. To study the relationship between co-occurring changes in stomatal/glandular trichome density and average leaf size, the stomatal and trichome indexes were calculated as follows: stomatal or trichome density mm^{-2} per average leaf area cm^2.

Statistics

Multivariate ANOVA (SPSS-Win 9.0, GLM procedure) with ozone, frost and genotype as fixed factors was used to test differences in growth and structural parameters, and interactions between them. In addition to ozone × frost, ozone × genotype, and frost–genotype interactions, 3-way interactions between

ozone, frost and genotype were derived. Comparisons between the different treatments were carried out using Tukey's multiple range tests. The data were checked for normality and homogeneity of variances, and when necessary, the values were transformed to satisfy the assumptions of ANOVA. In growth analyses, the initial values of height and dry masses were used as covariates. Significance was accepted at the $P < 0.05$ level.

Results

Bud burst

There were great differences in the timing of bud burst among the genotypes (Fig. 2). Almost all the buds in genotype 18 were still under dormancy 8 d after the beginning of the experiment (on 26 March), whereas 34% of the buds were already opened in genotype 8 in control plants. The differences among the genotypes remained as before 10 d after the beginning of the experiment (on 28 March). The differences among the genotypes were less evident and the proportion of open buds ranged between 76% (genotype 18) and 92% (genotype 21) 17 d after the start of the experiment (on 3 April). On the basis of bud burst, the genotypes were denoted as early starting (genotype 8), late starting (genotype 18), or intermediate (12, 17, 21 and 22) genotypes. Ozone caused a significant delay in bud burst as compared with control plants in genotypes 8 and 12 (Fig. 2, $P = 0.045$ on 26 March; $P = 0.004$ on 28 March for pooled data).

Visible foliar ozone and frost injuries

The first ozone injury symptoms were found in the fully expanded first flush leaves, whereas later, injuries appeared also in the younger enlarging leaves. The first dots were found in leaf margins before the injuries were spread over the whole leaf area. According to early leaf injuries on 23 April, genotypes 8, 12 and 17 were the most sensitive to ozone. At the final harvest on 10 May, visible ozone injuries we found in all genotypes for ozone and ozone + frost treated plants (Fig. 3). In addition to dark spots and flecks on the upper leaf surface, chlorosis was a typical extended injury. The highest amount of ozone injured leaves (50% of all leaves) was now found in genotype 18, and the frost treatment reduced ozone injuries by 19–41% (significantly in genotype 18 and pooled data), except in genotype 22 (Fig. 3).

The first visible foliar symptoms of frost treatment (wilting, incipient drying of leaf margins) emerged on 23 April, after the first night of frost temperatures. Considerable differences in frost injury between the genotypes were found in assessment on 24 April. The highest amount of injuries appeared in genotypes 8, 12 and 22 (60–70% of plants showing injuries),

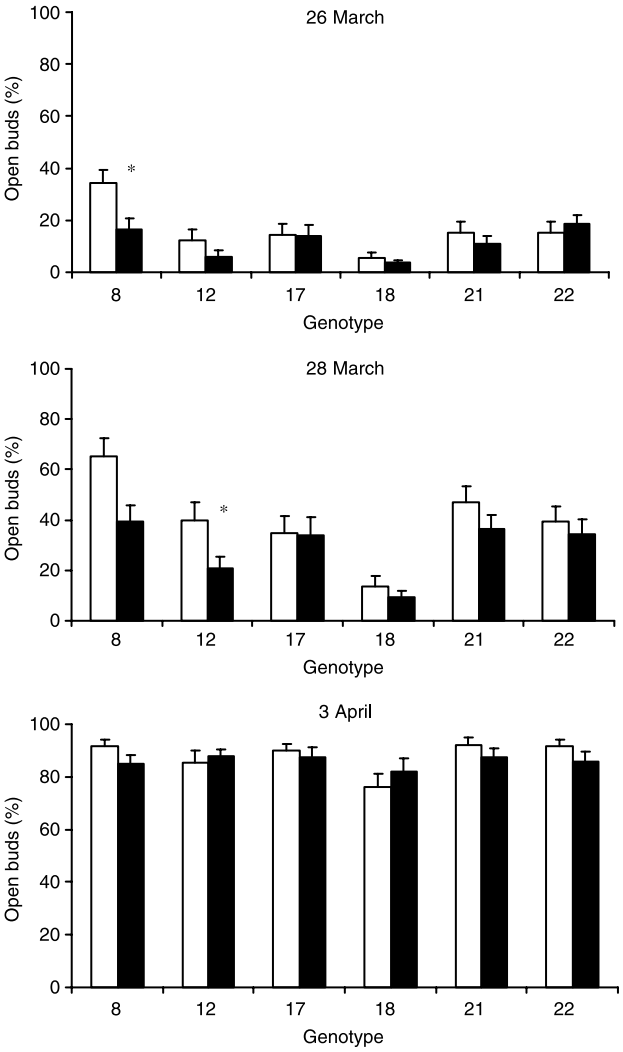


Fig. 2 Bud break as percentage of open buds in relation to all buds on 26 March, 28 March and 3 April, 2002, in six *Betula pendula* genotypes. Values are means ± SD ($n = 10$). Bars: open, control plants; closed, ozone plants. Multivariate ANOVA, $P < 0.05$; * = significant at 5% level.

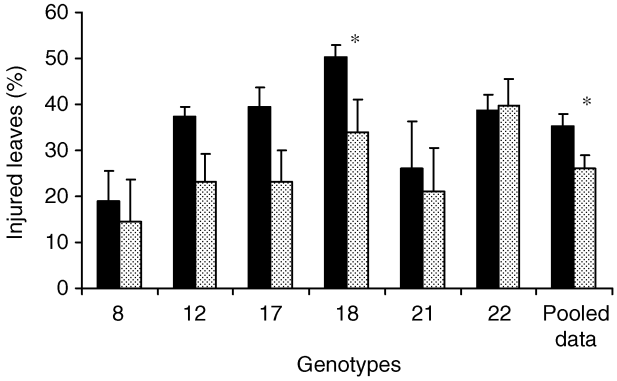


Fig. 3 Percentage of ozone-injured leaves of total number of leaves in ozone and ozone + frost treated plants of six *Betula pendula* genotypes. Values are means ± SD ($n = 10$). Bars: closed, ozone plants; dotted, ozone + frost plants. Multivariate ANOVA, $P < 0.05$, * = significant at 5% level.

Table 1 Statistical significances (*P*-values) of main effects of ozone, frost and genotypes and their 2- and 3-way interactions in leaf growth parameters and dry masses of wood, shoot, root and total plant of six *Betula pendula* genotypes

Parameter	O_3	Frost	Genotype	O_3 × frost	O_3 × genotype	Frost × genotype	O_3 × frost × genotype
Leaf area	0.405	0.419	0.001	0.634	0.562	0.234	0.251
Number of leaves	0.067	0.825	0.012	0.188	0.186	0.260	0.690
Leaf dry mass	0.685	0.079	0.026	0.001	0.175	0.022	0.107
Wood dry mass	0.012	0.600	0.211	0.876	0.242	0.510	0.237
Shoot dry mass	0.868	0.712	0.180	0.433	0.827	0.541	0.413
Root dry mass	0.049	0.260	0.354	0.158	0.181	0.154	0.134
Total dry mass	0.376	0.401	0.693	0.191	0.405	0.186	0.383

Multivariate ANOVA (n = 10).

whilst genotypes 18 and 21 were less affected by frost treatment (< 10% of plants showing injuries). The relationship between the timing of bud burst and the appearance of frost injuries was evident; the early starting genotype 8 showed a great amount of injuries, whereas the late starting genotype 18 was practically unaffected. In the combined stress treatment, ozone did not affect the amount of frost injuries in genotypes 8, 12, 17 and 18, whilst a synergistic effect was found in genotype 21 and an antagonistic effect in genotype 22 (data not shown).

Growth parameters

The main effects of ozone, frost and genotype on growth and interactions among them are shown in Table 1. When all genotypes were pooled, the main effect of ozone was found as a significantly increased wood and root dry mass (Table 1; Fig. 4). Ozone fumigation increased the total dry mass by 4–18%, except in genotype 22, where a 5% decrease was found. There were no significant main effects of frost on growth as a result of highly variable responses among the genotypes (Table 1; Fig. 4). However, the dry mass of leaves increased by 9%, 25%, 13%, 4% and 4% in genotypes 8, 12, 17, 18 and 22, respectively (significantly in genotype 12), whereas a 20% decrease was found in genotype 21. Although the leaf area was not significantly affected by ozone or frost, there were significant main effects of genotype in leaf area, number of leaves and leaf dry mass (Table 1). A significant ozone × frost and frost–genotype interaction was found in dry mass of leaves, indicating that responses in leaf growth differed between stress factors and genotype (Table 1; Fig. 4). Height increment was not significantly affected by ozone or frost (data not shown). When the total dry mass accumulation of control plants during the exposure was compared, the genotypes could be ranked according to growth rate as follows: 22 (15.9 mg) > 21 (13.9 mg) > 8 (13.7 mg) > 18 (13.5 mg) > 17 (13.0 mg) > 12 (12.4 mg).

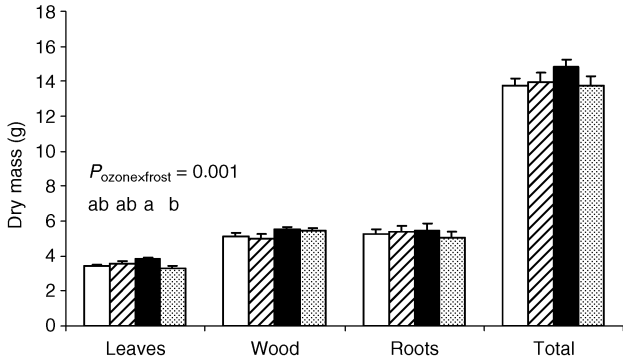


Fig. 4 Impacts of ozone and frost on dry mass of leaves, wood, roots and total plant in six *Betula pendula* genotypes (pooled data). Values are means ± SD (n = 5). Multivariate ANOVA, Tukey's multiple range test, $P < 0.05$. Significant differences between the treatments are indicated by different letters above the bars. Bars: open, control plants; /–hatched, frost plants; closed, ozone plants; dotted, ozone + frost plants.

Leaf structure

The main effects of ozone, frost and genotype on leaf and chloroplast structure, and interactions among them are shown in Table 2. In the mesophyll structure, the main effect of ozone was found as significantly thinner leaves, lower palisade and spongy layer thickness, smaller proportions of intercellular space, and shorter palisade cell lengths (Tables 2 and 3). The significant main effects of frost appeared as smaller proportions of intercellular space, thinner palisade layers, and shorter palisade cell lengths leading to increased spongy to palisade layer ratio (Tables 2 and 3). One of the most frost sensitive, genotype 12 (according to visible injuries), was characterized by the smallest proportion of intercellular space, while the most frost tolerant and ozone sensitive genotype 18 had the largest proportion of intercellular space. The main effect of genotype was significant in palisade and spongy layer thickness and palisade cell length (Table 2). Significant antagonistic ozone–frost interaction was found in spongy/palisade layer ratio and palisade layer thickness (Table 2). Significant frost × genotype and ozone × frost–genotype interactions appeared in spongy/palisade layer ratio and spongy layer thickness, indicating that structural responses to these stress factors were genotype-dependent (Table 2).

Table 2 Statistical significances (*P*-values) of main effects of ozone, frost and genotypes and their 2–3-way interactions on leaf and chloroplast structure of six *Betula pendula* genotypes

Parameter	O_3	Frost	Genotype	O_3 × frost	O_3 × genotype	Frost × genotype	O_3 × frost × genotype
Leaf thickness	0.001	0.546	0.175	0.535	0.866	0.294	0.499
Proportion of intercellular space	0.002	0.001	0.758	0.238	0.672	0.075	0.329
Spongy/palisade parenchyma ratio	0.054	0.003	0.894	0.010	0.092	0.002	0.015
Palisade parenchyma:	< 0.001	< 0.001	< 0.001	< 0.001	0.222	0.897	0.584
Layer thickness	< 0.001	< 0.001	< 0.001	< 0.001	0.222	0.897	0.584
Cell length	0.001	< 0.001	0.034	< 0.001	0.125	0.191	0.198
Chloroplast size	< 0.001	< 0.001	0.019	< 0.001	< 0.001	< 0.001	< 0.001
Starch grain size	< 0.001	< 0.001	< 0.001	< 0.001	0.014	< 0.001	< 0.001
Spongy parenchyma:	< 0.001	0.815	< 0.001	0.847	0.094	< 0.001	< 0.001
Layer thickness	< 0.001	0.815	< 0.001	0.847	0.094	< 0.001	< 0.001
Chloroplast size	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.114
Starch grain size	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Guard cell length	< 0.001	0.625	< 0.001	< 0.001	0.373	0.054	0.191
Guard cell width	< 0.001	< 0.001	0.003	0.049	0.012	0.004	0.474
Stomatal index	0.024	0.805	< 0.001	0.162	0.836	0.031	0.056
Trichome index	0.983	0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.001

For statistics, see Table 1.

Table 3 Significant effects of frost and ozone treatment on leaf structural and ultrastructural parameters of six *Betula pendula* genotypes (pooled data). Multivariate ANOVA followed by Tukey's multiple range test

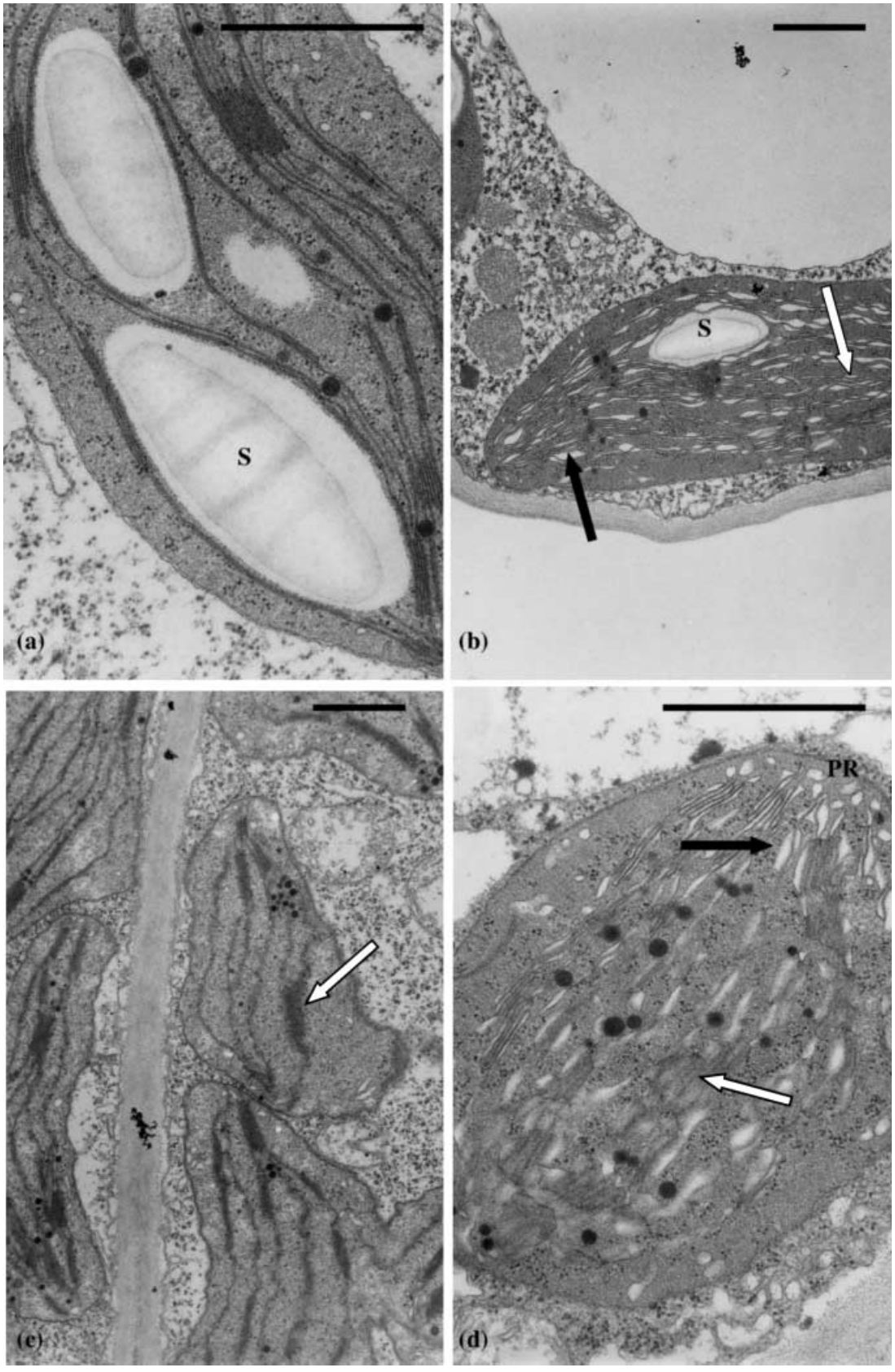
Parameter	Control	Frost	Ozone	Ozone + frost
Leaf thickness (μm)	143.5 ± 7.5a	139.4 ± 2.4ab	130.0 ± 4.0b	130.1 ± 2.7b
Proportion of intercellular space, % of mesophyll area	41.7 ± 2.1a	32.3 ± 2.1a	33.8 ± 1.3a	28.6 ± 1.7a
Spongy/palisade parenchyma	1.7 ± 0.04a	2.0 ± 0.1b	1.7 ± 0.1a	1.8 ± 0.04a
Palisade parenchyma:				
Layer thickness (μm)	42.7 ± 0.6a	37.6 ± 0.6b	38.1 ± 0.7b	38.2 ± 0.6b
Cell length (μm)	27.9 ± 0.4a	24.5 ± 0.4b	23.8 ± 0.3b	23.6 ± 0.4b
Size of chloroplast (μm^2)	6.48 ± 0.17a	3.53 ± 0.10b	3.93 ± 0.10bc	4.00 ± 0.11c
Size of starch grain (μm^2)	1.50 ± 0.09a	0.11 ± 0.02c	0.39 ± 0.07b	0.11 ± 0.03c
Spongy parenchyma:				
Layer thickness (μm)	72.0 ± 1.9a	72.1 ± 1.6a	65.1 ± 1.7b	66.0 ± 1.2b
Size of chloroplast (μm^2)	6.91 ± 0.18a	4.41 ± 0.12bc	4.48 ± 0.12b	3.94 ± 0.10c
Size of starch grain (μm^2)	1.67 ± 0.10a	0.25 ± 0.03b	0.51 ± 0.06c	0.11 ± 0.02b
Stomatal index	6.55 ± 0.36a	6.99 ± 0.45a	7.97 ± 0.65a	7.33 ± 0.36a
Trichome index	0.37 ± 0.02a	0.47 ± 0.02b	0.43 ± 0.03ab	0.41 ± 0.01ab

Values are means ± SD (n = 3). Means followed by different letters are significantly different, $P < 0.05$.

Chloroplast structure

The significant main effects of ozone and frost on chloroplast structure were found as smaller sizes of chloroplast and starch grain (Tables 2 and 3). In the whole mesophyll (palisade and spongy layer pooled) the size of the chloroplast was 37–41% smaller in ozone and frost treated plants (respectively) than in control plants (Table 3), which was linked with 72–89% reductions in starch grain size and similar decreases in proportion of starch grain in relation to total chloroplast cross-sectional area. Significant main effects of the genotype was also found in chloroplast and starch grain size. Frost treatment exacerbated ozone-caused reduction in chloroplast and starch grain size, as indicated by significant ozone–frost interaction (Tables 2 and 3). Reductions in chloroplast and starch grain size were genotype-specific, because significant ozone × genotype, frost × genotype, and ozone × frost–genotype interactions were found in both tissues (Tables 2 and 3).

Significant difference ($P < 0.004$) in ultrastructural responses between palisade and spongy mesophyll cells to each treatment was found, because the changes in chloroplasts were generally more pronounced in spongy parenchyma. However, the changes were basically similar. Ozone induced chloroplast injuries were similar, such as dilation and distortion of


S
(a)
S
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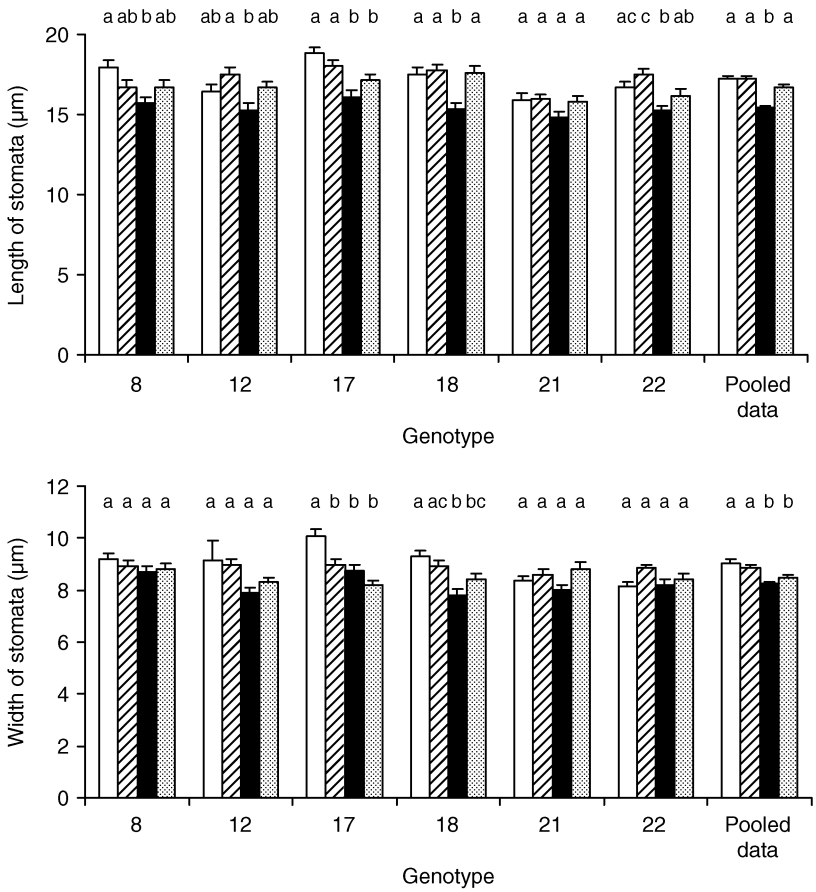

Fig. 6 Ozone- and frost induced changes in length and width of stomata in six *Betula pendula* genotypes and pooled data. Values are means ± SD (n = 3). Multivariate ANOVA, Tukey's multiple range test, $P < 0.05$. Significant differences between the treatments are indicated by different letters above the bars. Bars: open, control plants; hatched, frost plants; closed, ozone plants, dotted, ozone + frost plants.

thylakoids, and smaller starch grains (Fig. 5b), as previously reported for birch by Pääkkönen *et al.* (1995a,b). Frost treatment caused shrinkage of chloroplasts (as a result of dehydration) leading to disintegration of chloroplast envelopes, disappearance of starch granules, peripheral reticulum (vesicles arising from inner membrane of chloroplast envelope) and destabilization of thylakoid membranes (Fig. 5c). In ozone + frost treated plants, both ozone and frost-induced injuries were found within the same chloroplasts (Fig. 5d). There were no significant changes in number of plastoglobuli in any treatments (data not shown), and no structural chloroplast injures were found in any control plants.

Responses of stomatal complex and glandular trichomes

The significant main effects of ozone were found as reduced length of guard cells and width of the stomatal apparatus (indicating reduced turgor and stomatal closure) and as a higher stomatal index (Tables 2 and 3; Fig. 6). The greatest ozone-induced decrease in guard cell lengths was found in ozone-sensitive genotype 18 (13%, P = 0.001, Fig. 6a). The significant main effects of frost appeared as reduced width of guard cells and increased density of glandular trichomes (Tables 2 and 3; Fig. 6b). The significant main effects of the genotype were found in guard cell lengths and widths, and stomatal and trichome indexes (Table 2). Significant antagonistic ozone–frost interactions were found in guard cell length and width and trichome index, indicating that ozone counteracted the frost-induced increase in trichome density in combined treatment. Significant ozone–genotype interactions appeared in guard cell width and trichome index, while significant frost–genotype interaction was observed also in stomatal index (Table 2). A significant ozone × frost–genotype interaction appeared in trichome index (Table 2). These interactions revealed that frost and ozone induced responses in stomata and trichomes were strongly regulated by genotype.

Fig. 5 Ozone and frost caused changes in ultrastructure of chloroplasts in birch (*Betula pendula*) mesophyll cells. Bar, 1 µm. (a) Chloroplast of control plant showing large starch grains and intact thylakoid membranes; (b) ozone-caused dilation (black arrow) and distortion (white arrow) of thylakoid membranes, accompanied by smaller starch grain; (c) frost-caused shrinkage of chloroplast, disintegration of chloroplast envelope, destabilization of thylakoid membranes (white arrow), and lack of starch; (d) ozone and frost-caused dilation (black arrow) and destabilization (white arrow) of thylakoid membranes, and formation of peripheral reticulum (PR).

Discussion

Frost and ozone exposure

Freezing of plants and plant parts occur when they cannot avoid nucleation (water molecules come together to form a stable ice nucleus) and are not able to prevent the expansion of ice (Pearce, 2001). Normally, over-wintering temperate woody species freeze between −1.2 and −2.1°C in field conditions (Ashworth & Davis, 1986; Yamada *et al.*, 2002). Therefore, freezing temperatures (minimum −2.0°C) causing potential cell damage were obviously reached in our experiment. Because lower nucleation temperatures have been observed in laboratory tests than in natural conditions in several species, the chamber experiment results may underestimate the severity of frost damage in the field (Ashworth *et al.*, 1985; Flinn & Ashworth, 1994). Together with freezing temperatures, we were able to expose the plants to a critical ozone level, exceeding AOT40 of 10 ppb-h, which is expected to cause biomass reductions in forest trees. Although the critical ozone exposure is generally accumulated over a 6 month period, 80–90% of cumulative exposure index AOT40 is accumulated during April and May in northern latitudes, concomitantly with growth initiation, as reported by Laurila & Tuovinen (1996) based on Finnish observations.

Ozone exposure delays bud burst but stimulates growth

Our results indicate that there is a large variation in bud burst within a population of birches as suggested also by Rousi (unpublished) using the same birch genotypes. This high variability among birch genotypes was also in accordance with previous studies by Häkkinen (1999) and Linkosalo *et al.* (2000) with other Finnish birch origins. It was evident that early starting genotypes were more susceptible to acute frost injury as compared to late starting genotypes. In the earliest genotype (genotype 8) visible foliar frost injuries were accompanied by 9% reduction in total dry mass, whereas the latest genotype (genotype 18) was resistant against acute frost injury and showed an 8% increase in total dry mass under frost stress. On the other hand, in intermediate genotype 12 with extensive foliar frost injuries, photosynthetic area loss was efficiently compensated by enhanced leaf growth after the frost stress resulting in 33% increased total dry mass. The results from microscopical assessment suggested, that in the frost sensitive genotypes (genotype 12) small proportions of intercellular space in the mesophyll tissue may be predisposed to freezing injury probably as a result of more extensive extracellular ice formation and cellular dehydration than to genotypes (genotype 18) characterized by large proportions of intercellular space. Thereby, this experiment revealed that both bud burst timing and leaf anatomical properties are important factors affecting the vulnerability to frost and ozone stress in birch.

Earlier, Bertrand *et al.* (1999) found that previous season ozone exposure of 2-yr-old-sugar maple seedlings resulted in 5 d premature bud swelling in the following spring. However, our experiment revealed that springtime ozone exposure caused a delay in bud burst of birch, which has not been reported elsewhere. Delayed bud burst may reduce the risk of frost injury, but on the other hand, it could lead to yield reductions in the long term, therefore representing a critical ecological and evolutionary tradeoff between survival and growth. The mechanism for ozone-caused delay needs further investigations, and the role of changed hormonal balance, particularly alterations in ABA, should be necessarily examined (Li *et al.*, 2003).

An increased dry mass production under ozone exposure was found for most genotypes, which is in accordance with our previous short-term and low-ozone experiments with birch (Oksanen & Saleem, 1999; Oksanen & Rousi, 2001; Oksanen & Holopainen, 2001), and with studies on *Populus* species (Brendley & Pell, 1998; Bielenberg *et al.*, 2001) and oak (*Quercus rubra*) (Samuelson *et al.*, 1996). In hybrid poplar, compensatory responses of young leaves to ozone exposure were related to decline in N availability, and accelerated leaf senescence (Bielenberg *et al.*, 2001), or ozone-induced reductions in leaf-level photosynthesis, pigments and Rubisco, as reported by Brendley & Pell (1998). Compensatory growth as a result of ozone may indicate a shift in carbon allocation, which has been discussed by Laurence *et al.* (1994). In this experiment, stimulatory growth was also observed in some genotypes under frost and ozone + frost treatment. Because significant main effects of genotype were found in the leaf area, number of leaves and leaf dry mass, different growth responses among the genotypes occurred mainly through leaf growth.

Frost protects against visible ozone injury

In most genotypes, simultaneous ozone exposure did not protect the plants against acute visible frost injuries, which is in accordance with Waite *et al.* (1994), who reported that cold tolerance was not affected by elevated ozone concentrations during spring in red spruce seedlings. However, because there were three times more frost injuries after the interactive ozone and frost treatment in genotype 21 (synergistic effect) than single frost stress, some genotypes may be particularly sensitive to joint action. Furthermore, some evidence of improved frost tolerance as a result of ozone was found in genotype 22, suggesting that within a birch population, there is a wide range of interactions between ozone and frost stress among genotypes. There is evidence that freeze-induced production of reactive oxygen species (ROS) contributes to membrane damage and enhancement of antioxidative mechanisms plays a major role in frost defence (McKersie & Bowley, 1997). Brüggemann *et al.* (1999) suggested that improved frost tolerance in *Lycopersikon* was not a direct

consequence of a better detoxification system by ROS, but rather a result of a more efficient photochemistry at suboptimal temperatures and higher Calvin-cycle turnover rates, leading to reduced ROS production. On the other hand, Aroca *et al.* (2001) proposed a better co-ordination of the antioxidative enzymes (like superoxide dismutase, ascorbate peroxidase and glutathione reductase) with each other and a faster recovery of the photosynthetic activity in maize to be responsible for a better acclimation to low temperatures.

The visible injury assessment at the final harvest clearly indicated that frost treatment generally protected the leaves from ozone injury. Because it is well known that both ozone and chilling stress cause an increase of ROS formation inside the leaf leading to oxidative stress (e.g. Noctor & Foyer, 1998), exposure to frost may have activated defence mechanisms that also protect against simultaneous ozone fumigation, as discussed above. Alternatively, exposure to frost resulted in dehydration of leaves and closure of stomata leading to reduced stomatal conductance and hence lower ozone uptake (Prozherina, unpublished).

Contradictory results of foliar ozone injuries between the two assessments were probably caused by differences in timing of bud burst and hence the developmental stage of leaves between the genotypes. Previously, we have demonstrated that newly expanded leaves of birch are more sensitive to ozone than developing ones (Pääkkönen *et al.*, 1995b), which explains the lack on injuries in the late starting genotype 18 with premature leaves in the first assessment. The results from the injury assessment at harvest suggest that the late starting genotype 18 was the most sensitive to ozone, and the early starting genotype 8 was the most tolerant genotype to ozone. This is also in accordance with the recent finding with birch (Oksanen & Rousi, 2001), where differences of *Betula* origins in ozone sensitivity were investigated. However, in this experiment there were no clear correlations between foliar ozone injuries and biomass responses, probably as a result of short-term duration of exposure and compensation reactions through increased leaf production under ozone. According to biomass data, the most fast-growing genotype 22 showed the greatest sensitivity to ozone, which was accompanied by the highest amount of visible foliar frost injury. Because in earlier tests this genotype proved to be vulnerable to hare browsing as well (Laitinen *et al.* unpublished), we suggest that high growth rate may increase the risk for abiotic and biotic stress factors.

Acute frost injury in chloroplasts

Previously, formation of vesicles of the peripheral reticulum has been found in chloroplasts of chilling-treated cotton (*Gossypium hirsutum*) and bean (*Phaseolus vulgaris*), which also showed dilation and distortion of thylakoids (Kratsch & Wise, 2000). In addition, lack of starch or reduction of starch granules was reported in chilling-treated collard (*Brassica oleracea*) and cucumber (*Cucumis sativus*) (Kratsch & Wise, 2000). Thereby, acute freezing injuries in chloroplasts of birch resemble those reported for several crop species. However, we were not able to find any chloroplast swelling, which has been a universal symptom in crop plants (Kratsch & Wise, 2000), but rather, chloroplast shrinkage, partly caused by reduced size of starch grain. Although it is well documented that irradiance during chilling/frost greatly exacerbates the chloroplast injury caused by ROS formation (Kratsch & Wise, 2000), our experiment demonstrated that light is not a prerequisite for structural damage, because the freezing temperatures co-occurred with the 4-h dark period. It is not possible to say whether the observed chloroplast injuries were strictly the result of a direct effect of freezing temperature, or caused indirectly by programmed cell death mechanisms that are triggered by frost. The initiation and propagation of PCD during low temperature stress has been proposed to proceed from ROS formation in the chloroplast to increased free Ca^{2+}, changes in protein phosphorylation and cascade of proteolytic enzymes finally leading to digestion of chloroplasts and other organelles. Despite extensive chloroplast-level injuries and local leaf dehydration damage in our study, recovery from frost treatment was evident in some genotypes (namely 12 and 17), but not in others, suggesting, again, a variable inherent frost sensitivity of birch.

Both ozone and frost stress affect the differentiation of epidermal cells

This experiment indicated that both ozone and frost stress may affect the performance of stomatal complex, that frost treatment tended to counteract ozone-triggered changes in stomata, and that there was a large variation among the genotypes in the way of response. Ozone caused increase in stomatal density is a well known acclimation mechanism for birch to reduce ozone load per single stoma resulting in more even ozone distribution within leaf tissue and a better detoxification processes (Pääkkönen *et al.*, 1993, 1995b). Because the precisely regulated patterning of stomatal complexes is a way to achieve the optimal stomatal density in regard to gas exchange and transpiration, we believe that ozone-caused decline in photosynthesis may be compensated through increased stomatal density in young birch leaves. It is known that the series of cell divisions which differentiate the guard cells and the stomatal subsidiary cells occurs late in epidermal development, during cell expansion of the pavement cells (unspecialized epidermal cells) (Glover, 2000). Therefore, ozone-caused change in stomatal density is likely to occur during the springtime leaf expansion. It is well known that the differentiation of plant epidermal cells is a complex process, where signalling between differentiating cells is important (Glover, 2000). The precisely regulated pattern of stomatal complexes generated form precursor cells and guard mother cells is achieved through a cell lineage

mechanism (ordered system of cell divisions), but there is also a large body of evidence that the prepattern of stomatal guard cells is modified by interactions between developing stomatal complexes, interactions between stomata and other epidermal cells (such as trichomes) and by environmental inputs (Glover, 2000).

The frost induced a rapid increase in glandular trichome density and this may indicate a structural acclimation to low temperatures through altered change in regulation of epidermal cell development. As found for resin glands of birch shoot, the glandular trichomes in developing leaves serve as a glue and moisture eliminator to keep water droplets off the leaf surface and the stomata, which helps to protect the leaves from freezing and to maintain leaf gas exchange (Lapinjoki *et al.*, 1991; Gutschik, 1999). In addition, the resin (triterpenoids in particular) may also serve to deter birds and insects early in the spring, when food availability is limited (Lapinjoki *et al.*, 1991). As far as we know, ozone-induced increase in glandular trichomes has not been reported elsewhere before. High developmental plasticity in birch leaves in regard to trichomes has been previously demonstrated by Rautio *et al.* (2002), reporting that previous-year defoliation induced a shift from glandular to nonglandular leaf trichomes. In our experiment, competitive interactions between stomatal complexes and trichomes were not found, although this has been demonstrated in other studies (Glover, 2000). Unlike stomatal guard cells, the regulation of trichome development in *Arabidopsis* requires a balance of cell proliferation, differentiation, intercellular communication and morphogenesis control, and is therefore integrated with leaf development, hormone levels and vegetative developmental stage (Glover, 2000; Szymanski *et al.*, 2000). According to *Arabidopsis* studies, the developmental window during which cells have the potential to acquire trichome cell fate is limited; cells beyond a certain developmental stage lose their ability to respond to trichome differentiation signals (Szymanski *et al.*, 2000). Our results indicate that in birch leaves, frost-induced glandular trichome inititation (exceeding a threshold of trichome-promoting activity) may occur in fully enlarged leaves, within a few days after the frost stress. Important in active research on mutant plants and genes involved in pattering of *Arabidopsis* stomata and trichomes (Geisler *et al.*, 1998; Glover, 2000; Szymanski *et al.*, 2000), the molecular basis for ozone and frost induced alteration in stomata and trichome pathways may become better understood in the next few years.

Conclusions

This study indicates that increasing ozone delayed bud burst in birch, whereas subsequent growth was stimulated leading to increased biomass (in average 6.7%) in short-term in most genotypes. Frost induced acute injuries were compensated by increased leaf production in most genotypes resulting in 4–25% enhanced leaf dry mass. Frost injury was most severe in the early starting genotype (genotype 8), whereas the late starting genotype (genotype 18) was resistant against frost stress but most susceptible to ozone according to foliar injuries. On the other hand, the most fast-growing genotype (genotype 22) showed the greatest vulnerability to both ozone and frost damage, and interaction of them, on the basis of biomass response and acute frost injury in leaves. In a joint action of ozone and frost stress, co-occurring ozone enhancement did not protect the plants from acute frost damage, whereas frost stress clearly reduced the appearance of visible foliar ozone injuries and counteracted the ozone-induced changes in stomatal size and density. However, negative interactions were evident as well, because frost treatment nullified ozone-induced growth enhancement, led to antagonistic responses in leaf dry mass, and exacerbated ozone-caused reduction in palisade cell, chloroplast and starch grain size. Overall, our results indicate very large genetic variation in response to abiotic factors. Taken together, the results showed that generally birch recover from acute, short-term frost occurrence efficiently through compensating leaf production, but co-occurring ozone enhancement may disturb the recovery processes mechanistically through structural damage in photosynthetic tissue, especially in chloroplasts. In addition, rapid changes in epidermal cell differentiation towards stomatal apparatus and/or glandular trichomes occurred as a result of both stress factors to enhance stress tolerance. Because earlier experiments have indicated that the effects of abiotic stress in glasshouse or pot experiments is neglible compared to field conditions, long-term studies in field environment are now needed to verify the present results and to estimate their implications to ecosystems and practical forestry under current climate change.

Acknowledgements

This study was funded by Academy of Finland (projects 51758, 51661) and CIMO (HA1144). We thank Timo Oksanen for ozone fumigations and frost treatments, and Hanni Sikanen for general help.

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