

Physiological, stomatal and ultrastructural ozone responses in birch (*Betula pendula* Roth.) are modified by water stress

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ABSTRACT

The physiological, stomatal and ultrastructural responses to ozone and drought of ozone-sensitive and more ozone-tolerant birch (*Betula pendula* Roth.) clones were studied singly and in combination, in a high-stress chamber experiment and in a low-stress open-field experiment. In the chamber experiment, well watered (WW), moderately watered (MW) or drought-stressed (DS) saplings were exposed for 36 d to 0 or 130 nmol mol⁻¹ ozone. In the open-field experiment, well watered or drought-stressed saplings were grown for one growing season in ambient air or exposed to 1.8 × ambient ozone. Drought stress reduced growth rate, stomatal conductance, stomatal density and the proportion of starch and thylakoids in chloroplasts, but stimulated net photosynthesis, Rubisco and chlorophyll quantity at the end of the growing season, and increased the size and density of plastoglobuli. Ozone fumigations caused more variable, clone- and exposure-dependent responses in growth, decreased stomatal conductance and net photosynthesis, an increased number of stomata, visible and ultrastructural chloroplast injuries, and enhanced autumn yellowing of the leaves. Ozone-induced changes in plastoglobuli, starch and thylakoids resembled drought responses. The two experiments revealed that, depending on the experimental conditions and the variable, the response to drought and ozone stress can be independent, additive or interactive. Drought protected the plants from ozone injuries under high-stress conditions in the chamber experiment. In the low-stress, open-field experiment, however, enhanced ozone damage was observed in birch saplings grown under restricted water supply.

Key-words: *Betula pendula*; birch; clone; drought; growth; ozone; photosynthesis; ultrastructure.

INTRODUCTION

Forest trees are often exposed to air pollutants while experiencing natural stresses (Tingey & Taylor 1982; Krupa & Manning 1988; Darrall 1989; Kickert & Krupa 1990; Lee,

Chevone & Seiler 1990). During the warm periods of summer, elevated tropospheric ozone and soil-water deficit are environmental stresses likely to affect forest trees over widespread areas of Europe and the USA (Dobson, Taylor & Freer-Smith 1990; Greitner, Pell & Winner 1994). Plant responses to ozone may be greatly affected by environmental factors such as soil moisture, temperature, sunlight and nutrition (e.g. Krupa & Manning 1988; Pell *et al.* 1993).

Observations on the interactive effects of ozone and drought have been contradictory (Dobson *et al.* 1990). For example, soil-water deficit enhanced the ozone-induced yield loss in soybean (*Glycine max*) (Heggstad *et al.* 1985). In beech (*Fagus sylvatica*), impaired control of stomata under combined ozone and drought stress was reported to disturb the water status (Pearson & Mansfield 1993). In contrast, several investigations have indicated that drought-stressed plants are protected from ozone damage as a result of decreased stomatal conductance (Tingey & Hogsett 1985; Beyers, Riechers & Temple 1992; Pell *et al.* 1993; Le Thiec, Dixon & Garrec 1994; Reiner *et al.* 1996). In ponderosa pine (*Pinus ponderosa*), water stress reduced the ozone-induced loss of needle biomass (Beyers *et al.* 1992). Similarly, drought treatment protected Norway spruce (*Picea abies*) saplings from ozone-related growth reductions (Karlsson *et al.* 1995), ozone-induced visible foliar symptoms and ethylene production (Van den Driessche & Langebartels 1994). According to Le Thiec *et al.* (1994) ozone-treated and drought-stressed trees showed higher rates of photosynthesis and smaller reductions of chlorophyll content than well watered equivalents. In ash (*Fraxinus excelsior*), on the other hand, the smaller stomatal aperture and reduced ozone uptake under combined drought and ozone treatment caused reduced carbon assimilation and impaired stem radial growth (Reiner *et al.* 1996).

In several recent studies, birch (*Betula pendula*) has shown considerable sensitivity to ozone (Matyssek *et al.* 1991; Pääkkönen *et al.* 1993, 1995a,b, 1996, 1997; Pääkkönen 1996). Impaired growth and photosynthesis in sensitive birch clones under ozone stress have been found to be related to ultrastructural injuries, occurring especially in chloroplasts (Pääkkönen *et al.* 1996). In southern Finland, it is suspected that the accelerated yellowing and senescence of birch leaves in midsummer observed in recent years is related to drought. Coincident episodes of elevated ozone and possible interaction of these stresses

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may contribute to accelerated decline in birch. In the present study, we observed characteristic ozone- and drought-related physiological and ultrastructural changes in birch, and interactions of these two stresses in a high-stress chamber experiment and in a low-stress open-field experiment. Finding reliable indicators by which to separate drought-induced changes in birch from ozone-specific ones would allow more reliable assessment of the impact of these stresses on birch forests.

MATERIALS AND METHODS

Chamber experiment (high stress)

Two-year-old saplings of the ozone-sensitive clone KL-5-M (clone 5) and the more ozone-tolerant clone KL-2-M (clone 2) of European white birch (*Betula pendula* Roth.) were used. In our earlier experiments (Pääkkönen *et al.* 1993, 1995a, 1996), clone 5 showed high ozone sensitivity and clone 2 low ozone sensitivity, when growth, visible and ultrastructural leaf injuries, contents of Rubisco and chlorophyll, and net photosynthesis were used as criteria. Four weeks before the chamber exposure, 120 saplings (60 per clone) were transplanted into 3.5 dm³ pots. The pots contained a 3:1 (v/v) mixture of fertilized sphagnum peat and sand. The saplings were grown in the controlled growth chamber until exposure to the experimental conditions. The exposure period was from 16 May to 19 June 1995. Ten plants per clone per treatment were randomly distributed into two fumigation and two control chambers, in which which temperature, humidity and light were controlled. The control plants were grown in filtered air (ozone concentration 0 nmol mol⁻¹ ± 1.5, SD), whereas the ozone-fumigated plants received 130 nmol mol⁻¹ (± 23, SD) ozone for 36 d, 12 h d⁻¹ (0600–1800 h). For fumigated plants, the total ozone exposure AOT00 (cumulative exposure accumulated over threshold 0 nmol mol⁻¹) was 34.8 μmol mol⁻¹ h, and AOT40 (cumulative exposure accumulated over threshold 40 nmol mol⁻¹) was 21.8 μmol mol⁻¹ h. During the experiment, the plants were treated with three different rates of water supply, designated as well watered (WW), moderately watered (MW) and drought-stressed (DS). The WW plants received 0.1 (until 1 June) or 0.2 dm³ (later) water every other day to reach field capacity; the same amount of water was given at 4 d intervals to the MW plants, and once a week to the DS plants. Before watering, the soil moisture was monitored daily using golden electrode-containing gypsum blocks, embedded in the bottom of each container. The electrical conductivity was then related to the water potential of the soil in the container (Percy *et al.* 1989). The predawn leaf water potential was measured with a thermocouple psychrometer (Wescor, model L-51) from five plants per treatment. Until 24 May, the temperature was 12 °C (night) to 19 °C (day) and air humidity was 60–85%. From 25 May to the end of the experiment the temperature was 16–23 °C and air humidity was 40–60%. The light/dark cycle was 22 h/2 h and daylight photon flux

densities (PPFD) at crown-level 490 μmol m⁻² s⁻¹. To minimize the effect of chamber on the plant response, the saplings were rotated every 4 d between the four chambers, and the location of the plants within the chambers was randomized each time. In addition to water, all plants received 0.2% 9-Superex fertilizer (19:5:20 N:P:K) once a week.

Open-field experiment (low stress)

Two-year-old saplings of clones 2 and 5 (120 saplings, 60 per clone) were transplanted into 5 dm³ pots 4 weeks before being transferred to open-air exposure sites in the field. The plants were grown in the controlled growth chamber until 1 June. Thereafter, they were acclimated to open-air conditions over 1 week, and were randomly divided into two control (ambient air) and two elevated-ozone fields on 8 June 1995. To minimize the effect of exposure position on plant response, the position of the plants was rotated and randomized within the sites throughout the experiment. The pots were covered with plastic rain exclusion caps. The trees were well watered (WW) or drought stressed (DS). The WW plants received 0.25–0.5 dm³ water (depending on weather conditions) twice a week to reach field capacity; DS plants received the same amount of water once a week. The leaf water potentials and soil moisture in pots were determined as in the chamber experiment. Fifteen trees per clone per treatment were used for the field experiment. All the plants were fertilized similarly with 0.2% 9-Superex once a week until the end of July.

In the elevated ozone concentration treatment areas, the plants were surrounded by perforated, gas-releasing tubes in a natural microclimate (for details, see Wulff *et al.* 1992). The computer-controlled system maintained the elevated ozone concentration, on average, 1.8 times higher than the ambient concentration, following the natural ozone fluctuation. The total ozone exposures (AOT00), the AOT40 values, and the mean and maximum 24 h concentrations for ozone until the harvest, on 15 August, are shown in Table 1. Five trees per clone and treatment were fumigated until 25 September to follow the autumn senescence of leaves. In both experiments, ozone was generated from pure oxygen (Fischer OZ 500) and ozone concentrations were continuously monitored (model 1008-RS, Dasibi Environmental Corp.).

Table 1. Total cumulative ozone exposures [over a threshold of 0 nmol mol⁻¹ (AOT00) and over a threshold of 40 nmol mol⁻¹ (AOT40)], and the 24 h mean and maximum ozone concentrations for the field experiment with birch saplings from 8 June to 15 August. Values are the means for the two ambient-ozone/elevated-ozone blocks

	Ambient	Elevated
AOT00 (μmol mol ⁻¹ h)	37.9	66.9
AOT40 (μmol mol ⁻¹ h)	0.3	11.6
24 h mean (nmol mol ⁻¹)	24	42
24 h maximum (nmol mol ⁻¹)	40	77

Growth

At the final destructive harvest in the chamber experiment on 19 June and in the field experiment on 15 August, 10 plants per clone per treatment were measured for height, number of leaves, mean leaf size and foliage area (total leaf area). The mean projected leaf area was measured for five fully grown leaves (5th to 10th leaf from the top on the main stem) per plant by scanning and calculating the area using a Locitech Photo Touch Color program. The total foliage area was estimated by multiplying the number of leaves by the mean leaf size. At the harvest, the dry mass of stem, leaves and roots was determined for all plants in the chamber experiment and for 10 plants per clone per treatment in the field experiment. For the field experiment, mean relative growth rates (RGR) were calculated for each plant component $\{= [\ln(\text{final dry mass}) - \ln(\text{mean initial dry mass})]/\text{number of weeks}\}$ (e.g. Hunt 1990).

Visible injuries and leaf senescence

In both experiments, the proportion of leaves showing visible ozone injuries was calculated for each group of 15 plants in relation to the total number of leaves at harvest. Visible injuries were small, light-green, yellowish or brown dots, necrotic flecks and chlorotic leaves. The proportion of yellowed leaves of the total number of leaves was determined on 25 September for five plants per clone per treatment in the field experiment.

Stomatal responses

For the determination of stomatal density, fully grown leaves (one leaf per plant), attached to the main stem, were frozen in liquid nitrogen at harvest. In the laboratory, stomatal density was counted on the abaxial surface of the leaves, under a light microscope, from 3×3 mm square pieces (four pieces per leaf) using systematic uniform random sampling according to Kub'nová (1994). Stomatal density was averaged for each leaf.

Stomatal conductance was measured on 16 June in the chamber experiment, and five times between 3 July and 2 August in the field experiment. Three fully grown leaves, attached to the main stem, from 10 plants per clone per treatment were measured between 1000 and 1500 h using a porometer (LI-1600, Li-Cor Inc., NE, USA).

Net photosynthesis, transpiration, Rubisco and chlorophyll analyses

Net photosynthesis and transpiration were measured on 15 June in the chamber experiment and on 8 August, 5 September and 19 September in the field experiment. The sixth emergent leaf was selected from five to six plants per clone per treatment. Measurements were made with a closed-loop photosynthesis system (LI-6200, Li-Cor Inc., NE, USA). All the measurements were made

under saturating sunlight and a supplementary halogen light (Sylvania Professional, FTY/50 W/8°) was used if the natural PPFD was below $900 \mu\text{mol m}^{-2} \text{s}^{-1}$. Leaf areas were determined with a portable area meter (LI-3000 A, Li-Cor Inc. NE, USA).

The leaves used for gas-exchange measurements were excised, weighed and then immediately frozen and stored in liquid nitrogen until analysed under saturating PPFD. Leaf tissue was preground under liquid nitrogen, 0.1 g weighed on to 3 cm^3 frozen ground extraction buffer and homogenized with pestle and mortar until melted. The CO_2 -free extraction buffer contained 50 mmol m^{-3} Tricine-NaOH pH 8.2, 10 mmol m^{-3} MgCl_2 , 1 mmol m^{-3} Na_2EDTA , 10 mmol m^{-3} β -mercaptoethanol and 1% TWEEN-80. The crude homogenate was centrifuged at $12\,000 \text{ g}$ for 2 min and the supernatant immediately used for the spectrophotometric (Perkin Elmer, Lambda Bio) Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) activity assays according to Lilley & Walker (1974) (with slight modifications) in a final volume of 470 mm^3 . Initial Rubisco activity measurement was carried out in the reaction medium containing 100 mmol m^{-3} Tricine-NaOH pH 8.2, 10 mmol m^{-3} NaHCO_3 , 20 mmol m^{-3} MgCl_2 , 3.13 U glyceraldehyde phosphate dehydrogenase phosphoglycerate phosphokinase, 3.13 U creatine phosphokinase, 5 mmol m^{-3} ATP, 0.2 mmol m^{-3} NADH, 5 mmol m^{-3} phosphocreatine, 0.66 mmol m^{-3} RuBP and 10 mm^3 extract. Total activity was measured by incubating the extract in the presence of 10 mmol m^{-3} NaHCO_3 and 20 mmol m^{-3} MgCl_2 for 10 min. Measurements were conducted at 23°C .

For the Rubisco and soluble protein studies, 200 mm^3 aliquots of the supernatant, as described in the last section, were precipitated with ethanol (67% v/v) and stored at -20°C until analysed. Each Rubisco sample was centrifuged at $12\,000 \text{ g}$ for 5 min and the pellet was resuspended in 200 mm^3 buffer containing 1 mmol m^{-3} Na_2EDTA , 50 mmol m^{-3} Tris-HCl pH 6.8 and 1% TWEEN-20. Three volumes of sample buffer containing 62 mmol m^{-3} Tris-HCl pH 6.8, 2% SDS, 0.71 mol m^{-3} β -mercaptoethanol, 0.05% bromophenol blue and 10% glycerol were added to the dissolved sample, boiled for 4 min and 12% SDS-PAGE was electrophorized (Laemmli 1970) with purified spinach Rubisco standard (Sigma Ltd) in each gel. Gels were scanned with a Pharmacia ImageMaster program (Version 1.0). An aliquot for soluble protein was quantified by centrifuging the sample at $12\,000 \text{ g}$ for 5 min, resuspending the pellet in 0.1 mmol m^{-3} NaOH and measuring the concentration according to Bradford (1976). The chlorophyll concentration was determined by Arnon's (1949) method taking a 100 mm^3 aliquot of the crude extract.

Electron microscopy

For ultrastructural studies, leaf samples were collected at harvest date 19 June in the chamber experiment and on

5 September in the field experiment from the main stem leaves (three leaves from five plants per clone per treatment). Strips about 5 mm wide, 2 cm from the leaf apex were cut in the field. The strips were immediately placed in a fixative solution of 2.5% (v/v) glutaraldehyde in phosphate buffer (0.1 mmol m⁻³, pH 7.0). In the laboratory, 1.5 × 1.5 mm square pieces were cut from the strips under a drop of fixative using a razor blade. The samples were postfixed in 1% buffered OsO₄ solution, dehydrated using an ethanol series and embedded in LX 122 Epon. The thin sections were stained with uranyl acetate and lead citrate and were studied with an electron microscope (JEOL 1200 EX) operating at 80 kV.

Palisade and spongy mesophyll cells were studied separately. Forty randomly selected cross sections of chloroplasts per clone per treatment from both mesophyll tissues were photographed. The size of chloroplasts, and the number and size of plastoglobuli, were measured from the photographs. The proportion of starch grain and thylakoid membranes in relation to total chloroplast cross-sectional area was determined with a point analysis method, where a cross-hatched grid with random points was positioned over the photograph and the points within starch grain or thylakoid membranes were counted in relation to total chloroplast area. The chloroplasts were classified as normal lens-shaped, abnormal (protrusions), or spherical. The appearance of thylakoids was classified according to degree of swelling and curling (0 = no injuries; 1 = slight swelling or curling; 2 = severe swelling or curling), and the density of chloroplast stroma was recorded (0 = light stroma; 1 = electron-dense stroma).

Statistical analyses

The factorial design of the experiments consisted of two clones × two levels of ozone × two (open-field) or three (chamber) levels of water treatments. Because of the rotation and randomized location of plants during the experiments, no significant chamber or block effects were revealed by ANOVA. Therefore, data for the two replicate chambers or blocks were allowed to pool for further analysis. Tukey's multiple-range test was used to detect the differences between all treatments, as shown in Tables 2–5 and Figs. 1 & 2. Comparisons between elevated-ozone plants and control plants (0 or ambient ozone) within each watering group (WW, MW and DS) are described in the text of the Results section. Significance of ozone × drought, clone × ozone, and clone × drought interactions, and the main effects of ozone and drought, shown in Tables 2–6, were determined using two-way ANOVA. To reveal the main ozone effects, all ozone-treated plants were compared with all control plants (watering levels combined). Similarly, the main drought effects were determined by comparing all the drought-stressed plants with plants receiving higher water supply, regardless of ozone treatment. Differences and interactions were considered significant at $P < 0.05$.

RESULTS

Leaf water potentials and soil moisture

In the chamber experiment, the mean predawn leaf water potentials measured three times before and at the harvest date in control chambers were –0.15 MPa for WW plants, –0.24 MPa for MW plants, and –2.64 MPa for DS plants. The corresponding water potentials under ozone fumigation were lower: –0.42 MPa, –0.64 MPa and –2.97 MPa, respectively. In the field exposure, the mean predawn leaf water potentials under ambient ozone treatment were –0.97 MPa for WW plants and –1.9 MPa for DS plants. Under elevated-ozone treatment, the corresponding water potentials were –0.96 MPa and –1.47 MPa, respectively. According to leaf water potentials, the water stress of DS plants was higher in the chamber experiment than in the field experiment.

Growth and physiological responses to drought

Chamber experiment

In the chamber experiment, water deficiency significantly reduced the foliage area and the dry mass of leaves, stem and roots in clone 2 in MW and DS plants, and in clone 5 in DS plants, compared with WW controls (Table 2). In clone 2, these allocation changes resulted in significantly higher shoot:root ratio. The number of stomata was significantly lower in both clones in MW and DS plants, accompanied by significantly lower stomatal conductance in DS plants of clone 5 (Table 2). Although there were no significant differences between the single treatments in net photosynthesis, transpiration, chlorophyll and Rubisco content, or Rubisco activity, the significant main effects of drought were found in both clones as higher contents of chlorophyll and Rubisco and lower transpiration rates (Table 3). In addition, in clone 5, increased Rubisco activity but decreased net photosynthesis were also observed as significant main effects of drought (Table 3).

Field exposure

In the open-field exposure, drought treatment reduced significantly the height growth in clone 2, whereas both clones showed significant reduction in foliage area, and the relative growth rates of leaves, stem and roots (Table 4). Stomatal conductance was reduced in DS plants throughout the measurement period in both clones (Fig. 1). Significantly higher contents of chlorophyll and Rubisco were observed in DS plants of both clones on 19 September (Fig. 2). In both clones, the significant main effects of drought ($P < 0.05$) were also found as increased net photosynthesis, Rubisco activity and water-use efficiency on 19 September (Fig. 2).

Growth and physiological responses to ozone

Chamber experiment

Significant ozone responses were found in WW and MS plants but not in DS plants. In the MW plants, ozone

Table 2. Significant growth and stomatal responses and interactions of *Betula pendula* clones to 130 nmol mol⁻¹ ozone and drought in the chamber experiment. Values are means ± SE. Two-way ANOVA; Tukey's multiple range test; n = 10. WW, well watered; MW, moderately watered; DS, drought stressed. Means followed by different letters are significantly different within the clone

Response	WW				MW				DS				Main effects						
	Control		Ozone		Control		Ozone		Control		Ozone		Drought		Ozone		Interaction		
Foliage area (cm ²)																			
Clone 2	1383 ± 153c	1268 ± 151c	668 ± 106ab	981 ± 156bc	445 ± 76a	377 ± 45a							***	ns	ns			ns	
Clone 5	1516 ± 129c	1352 ± 127c	1168 ± 154c	930 ± 129b	392 ± 37a	467 ± 67ab							***	ns	ns			ns	
Dry wt leaves (g)																			
Clone 2	4.1 ± 0.2c	3.5 ± 0.3c	2.5 ± 0.2bc	3.2 ± 0.2b	1.9 ± 0.1a	1.9 ± 0.2a							***	ns	ns			*	
Clone 5	4.6 ± 0.2c	4.4 ± 0.4c	3.4 ± 0.2bc	3.0 ± 0.1b	1.7 ± 0.2a	1.9 ± 0.2a							***	ns	ns			ns	
Dry wt stem (g)																			
Clone 2	3.9 ± 0.3c	3.7 ± 0.2bc	2.7 ± 0.1a	3.0 ± 0.2b	2.6 ± 0.1a	2.6 ± 0.1a							***	ns	ns			ns	
Clone 5	4.0 ± 0.2b	4.1 ± 0.2b	3.0 ± 0.2ab	3.3 ± 0.2b	2.2 ± 0.2a	2.9 ± 0.2a							***	*	ns			ns	
Dry wt roots (g)																			
Clone 2	10.9 ± 1.7b	9.6 ± 1.1b	3.9 ± 0.3a	4.7 ± 0.3a	3.4 ± 0.2a	3.6 ± 0.4a							***	ns	ns			ns	
Clone 5	9.6 ± 2.0b	8.9 ± 1.5b	7.4 ± 1.1ab	5.0 ± 0.6ab	2.9 ± 0.2a	3.8 ± 0.4a							***	ns	ns			ns	
Shoot:root ratio (g g ⁻¹ dry wt)																			
Clone 2	0.84 ± 0.10a	0.82 ± 0.092a	1.37 ± 0.09b	1.35 ± 0.06b	1.39 ± 0.08b	1.38 ± 0.15b							***	ns	ns			ns	
Clone 5	1.12 ± 0.14a	1.11 ± 0.13a	1.02 ± 0.14a	1.37 ± 0.13a	1.37 ± 0.07a	1.28 ± 0.10a							ns	ns	ns			ns	
O ₃ -injured leaves (% of total no.)																			
Clone 2	0.0a	4.0 ± 0.9b	0.0a	7.5 ± 1.8b	0.0a	3.5 ± 3.0ab							ns	***	***			***	
Clone 5	0.0a	4.0 ± 0.8b	0.0a	5.7 ± 1.2b	0.0a	1.1 ± 0.6a							ns	***	***			***	
Stomata mm ⁻²																			
Clone 2	79.3 ± 8.7b	72.1 ± 3.4b	39.3 ± 5.6a	42.4 ± 3.1a	35.2 ± 4.3a	33.5 ± 1.2a							***	ns	ns			ns	
Clone 5	62.0 ± 5.2b	64.6 ± 3.2b	53.9 ± 5.7a	90.9 ± 18.6b	35.3 ± 1.2a	59.9 ± 6.1ab							*	**	ns			ns	
Stomatal conductance (cm s ⁻¹)																			
Clone 2	0.325 ± 0.034a	0.251 ± 0.028a	0.218 ± 0.027a	0.295 ± 0.040a	0.338 ± 0.051a	0.298 ± 0.055a							ns	ns	ns			ns	
Clone 5	0.409 ± 0.053b	0.225 ± 0.022a	0.299 ± 0.050ab	0.183 ± 0.014a	0.203 ± 0.034a	0.112 ± 0.013a							***	***	***			ns	

Table 3. Effects of ozone and drought treatments of *Betula pendula* clones on chlorophyll and Rubisco quantity, Rubisco activities, net photosynthesis and transpiration rate in the chamber experiment on June 15. Values are means \pm SE. Two-way ANOVA; Tukey's multiple range test, $n = 5$. WW, well watered; MW, moderately watered; DS, drought stressed. Values followed by different letters are significantly different within the clone

Response	WW			MW			DS			Main effects		
	Control	Ozone		Control	Ozone		Control	Ozone		Drought	Ozone	Interaction
Chlorophyll quantity (g m^{-2})												
Clone 2	0.18 \pm 0.02ab	0.14 \pm 0.02a		0.20 \pm 0.03ab	0.19 \pm 0.01ab		0.24 \pm 0.02b	0.23 \pm 0.02b		**	0.23 \pm 0.02b	ns
Clone 5	0.17 \pm 0.02a	0.17 \pm 0.02a		0.16 \pm 0.01a	0.23 \pm 0.04a		0.25 \pm 0.02a	0.26 \pm 0.03a		**	0.26 \pm 0.03a	ns
Rubisco quantity (g m^{-2})												
Clone 2	2.01 \pm 0.07a	2.06 \pm 0.27a		2.67 \pm 0.35a	2.42 \pm 0.29a		3.06 \pm 0.22a	2.43 \pm 0.20a		*	2.43 \pm 0.20a	ns
Clone 5	2.36 \pm 0.33ab	1.59 \pm 0.23a		2.01 \pm 0.11ab	2.42 \pm 0.44ab		3.25 \pm 0.39b	3.05 \pm 0.40ab		**	3.05 \pm 0.40ab	ns
Rubisco initial activity ($\mu\text{mol CO}_2 \text{ mg}^{-1} \text{ soluble protein min}^{-1}$)												
Clone 2	0.093 \pm 0.010a	0.073 \pm 0.009a		0.093 \pm 0.010a	0.092 \pm 0.010a		0.106 \pm 0.007a	0.097 \pm 0.007a		ns	0.097 \pm 0.007a	ns
Clone 5	0.095 \pm 0.013a	0.078 \pm 0.010a		0.075 \pm 0.010a	0.100 \pm 0.002a		0.115 \pm 0.011a	0.114 \pm 0.011a		*	0.114 \pm 0.011a	ns
Rubisco total activity ($\mu\text{mol CO}_2 \text{ mg}^{-1} \text{ soluble protein min}^{-1}$)												
Clone 2	0.120 \pm 0.013a	0.097 \pm 0.015a		0.117 \pm 0.010a	0.116 \pm 0.012a		0.142 \pm 0.011a	0.134 \pm 0.012a		ns	0.134 \pm 0.012a	ns
Clone 5	0.115 \pm 0.015a	0.094 \pm 0.012a		0.091 \pm 0.010a	0.133 \pm 0.007a		0.148 \pm 0.018a	0.140 \pm 0.013a		*	0.140 \pm 0.013a	ns
Net photosynthesis ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)												
Clone 2	8.23 \pm 0.81a	6.11 \pm 0.96a		8.91 \pm 1.09a	7.10 \pm 1.23a		7.08 \pm 1.92a	3.61 \pm 1.36a		ns	3.61 \pm 1.36a	ns
Clone 5	8.75 \pm 1.25a	8.51 \pm 1.27a		4.51 \pm 0.54a	6.87 \pm 1.52a		5.60 \pm 0.74a	5.41 \pm 1.19a		*	5.41 \pm 1.19a	ns
Transpiration rate ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)												
Clone 2	18.13 \pm 1.07b	15.53 \pm 0.84ab		18.29 \pm 1.10b	16.75 \pm 0.99ab		15.37 \pm 0.77ab	13.50 \pm 0.84a		**	13.50 \pm 0.84a	ns
Clone 5	14.89 \pm 1.20ab	17.88 \pm 0.46b		15.00 \pm 0.79b	16.79 \pm 1.45b		13.23 \pm 0.31a	13.72 \pm 0.12a		**	13.72 \pm 0.12a	ns

Table 4. Significant effects of elevated ozone and drought on *Betula pendula* clones in the open-field exposure. Two-way ANOVA; Tukey's multiple range test; $n = 10$. WW, well watered; MW, moderately watered; DS, drought stressed; RGR, relative growth rate. Values followed by different letters are significantly different within the clone

Response	WW		DS		Main effects		
	Control	Ozone	Control	Ozone	Drought	Ozone	Interaction
Height (cm)							
Clone 2	47.5 ± 3.9b	56.6 ± 2.7b	28.4 ± 2.9a	25.1 ± 2.3a	**	ns	*
Clone 5	26.6 ± 1.5a	35.0 ± 2.2b	21.0 ± 2.1a	26.1 ± 3.6a	ns	**	*
Foliage area (cm ²)							
Clone 2	2676 ± 295b	3388 ± 254b	1030 ± 169a	1004 ± 120a	**	ns	**
Clone 5	2127 ± 224b	2320 ± 333b	945 ± 129a	1215 ± 127a	**	*	ns
RGR leaf							
Clone 2	0.13 ± 0.01b	0.13 ± 0.01b	0.06 ± 0.01a	0.05 ± 0.0a	**	ns	ns
Clone 5	0.12 ± 0.01b	0.10 ± 0.02b	0.06 ± 0.01a	0.07 ± 0.01ab	**	ns	ns
RGR stem							
Clone 2	0.13 ± 0.01b	0.14 ± 0.01b	0.07 ± 0.01a	0.04 ± 0.01a	**	ns	ns
Clone 5	0.12 ± 0.01b	0.12 ± 0.02b	0.06 ± 0.01a	0.07 ± 0.01a	**	ns	ns
RGR root							
Clone 2	0.21 ± 0.01c	0.22 ± 0.01c	0.13 ± 0.01b	0.07 ± 0.01a	**	ns	*
Clone 5	0.19 ± 0.01b	0.21 ± 0.01b	0.10 ± 0.01a	0.10 ± 0.01a	**	ns	ns
Visible injuries (%) on 4 September							
Clone 2	0.5 ± 0.1a	3.4 ± 1.0a	1.5 ± 0.2a	12.1 ± 0.3b	ns	*	ns
Clone 5	1.4 ± 1.4a	7.8 ± 2.1b	2.5 ± 0.4a	16.9 ± 1.7b	ns	*	ns
Stomata mm ⁻² (10 August)							
Clone 2	93.9 ± 5.0a	115.2 ± 6.9a	108.4 ± 7.4a	89.8 ± 5.6a	ns	*	*
Clone 5	81.4 ± 4.1b	113.5 ± 3.9c	102.7 ± 6.5bc	78.1 ± 4.4a	*	*	**
Yellowed leaves (%) on 25 September							
Clone 2	9.6 ± 1.4a	13.7 ± 1.4a	13.0 ± 1.5a	24.1 ± 8.5a	ns	*	ns
Clone 5	13.8 ± 2.7a	24.8 ± 3.5a	26.5 ± 1.8a	45.0 ± 7.8b	ns	*	**

fumigation increased significantly the number of stomata and reduced the foliage area in clone 5, whereas a significantly higher stem dry mass was observed in clone 2, compared with correspondingly watered controls. Visible ozone injuries were significantly increased in WW and MW ozone plants of both clones, despite lower stomatal conductance rates (significant difference in clone 5) (Table 2). Photosynthesis, chlorophyll and Rubisco were unaffected by ozone exposure in both clones in all watering treatments. However, significant main effects of ozone were found as decreased net photosynthesis in clone 2 and as decreased transpiration rate in both clones (Table 3).

Field experiment

Elevated ozone stimulated the height growth of WW plants of clone 5, compared with the corresponding ambient-ozone treatment, whereas relative growth rate of roots was significantly lower in DS elevated-ozone plants of clone 2. Ozone-induced visible injuries were significantly increased in DS elevated-ozone plants of both clones, and also in WW elevated-ozone plants of clone 5. Significantly accelerated autumn yellowing of leaves was also found in DS elevated-ozone plants of clone 5 (Table 4).

Elevated ozone increased significantly the stomatal density in WW plants of clone 5; however, the DS elevated-ozone plants showed a significant reduction in stomatal density. Stomatal conductance and the significance of difference between the treatments were variable because of different weather conditions (Fig. 1). Ozone-induced reduction in stomatal conductance was measured particularly on 3 July and 2 August in WW and DS plants of clone 2, and on 18 July in WW plants of clone 5. In DS plants of clone 2, chlorophyll contents were significantly increased under elevated-ozone on 5 September (Fig. 2). The significant main effects of ozone ($P < 0.05$) also appeared as lower Rubisco content in clone 5 on 19 September, lower net photosynthesis in clone 2 on 10 August and in clone 5 on 5 and 19 September, and lower water-use efficiency in clone 2 on 10 August (Fig. 2).

Ultrastructural study

No major qualitative differences in ultrastructural responses to ozone and drought were found between spongy and palisade mesophyll cells in either experiment. The results of the ultrastructural responses of palisade cells are shown, as the visible ozone injuries appear in the

Table 5. Significant ultrastructural changes under 130 nmol mol⁻¹ ozone fumigation and drought stress in leaf palisade tissue of *Betula pendula* clones in the chamber experiment. Values are means ± SE. Two-way ANOVA; Tukey's multiple range test; n = 5. WW, well watered; MW, moderately watered; DS, drought stressed. Values followed by different letters are significantly different within the clone

Response	WW		MW		DS		Main effects		Interaction
	Control	Ozone	Control	Ozone	Control	Ozone	Drought	Ozone	
Size of plastoglobuli (nm)									
Clone 2	100.1 ± 7.8a	171.6 ± 9.9b	120.7 ± 5.0a	152.0 ± 13.0ab	177.3 ± 26.2b	241.8 ± 14.2b	*	**	ns
Clone 5	177.3 ± 26.9a	134.7 ± 7.1b	176.4 ± 9.2a	256.7 ± 12.8b	168.7 ± 10.6a	186.4 ± 17.7a	ns	ns	ns
Number of plastoglobuli									
Clone 2	2.4 ± 0.4a	4.5 ± 1.1ab	4.1 ± 0.6a	6.5 ± 0.7b	8.0 ± 1.3b	4.7 ± 0.7ab	*	ns	ns
Clone 5	1.6 ± 0.7a	8.8 ± 0.6b	2.0 ± 0.7ab	4.5 ± 0.7a	3.9 ± 0.5a	2.3 ± 0.7a	ns	*	ns
Starch (% of total chloroplast area)									
Clone 2	34.8 ± 5.3b	39.8 ± 4.2b	27.6 ± 4.5b	13.1 ± 5.6a	14.5 ± 4.0a	23.9 ± 4.7ab	**	ns	*
Clone 5	38.8 ± 8.8bc	54.4 ± 4.1c	41.8 ± 3.8bc	0.8 ± 0.1a	36.9 ± 3.3b	37.7 ± 5.1b	ns	*	*
Thylakoids (% of total chloroplast area)									
Clone 2	73.3 ± 3.3c	46.9 ± 2.6ab	54.7 ± 5.9b	38.8 ± 4.2a	52.3 ± 4.4ab	49.2 ± 2.0ab	**	***	ns
Clone 5	38.3 ± 3.6ab	22.9 ± 2.0a	27.5 ± 3.7b	42.3 ± 2.0b	30.0 ± 3.0ab	35.2 ± 5.1ab	ns	ns	ns

upper side of leaves, and the palisade mesophyll layer is also the most important photosynthetic tissue.

Ultrastructural responses to drought

Chamber experiment

In the DS control plants of clone 2, the number and size of plastoglobuli was significantly increased (Table 5), whereas the relative amount of starch and thylakoids of chloroplast area was significantly lower than in WW and MS control plants (Table 5; Fig. 3b,c). In clone 5, no significant drought responses were observed (Table 4).

Field experiment

In the field conditions, exposure to drought resulted in significantly bigger plastoglobuli in both clones and in a reduced relative amount of starch in clone 2, supporting the chamber experiment (Table 6).

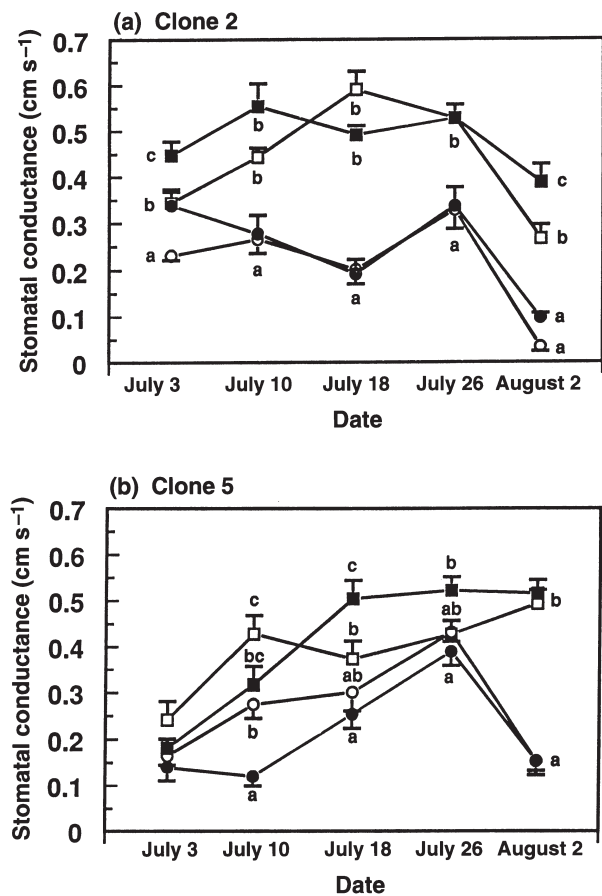


Figure 1. Effects of ozone and drought treatments on stomatal conductance of birch (*Betula pendula*) clones (a) 2 and (b) 5 in the field experiment. ANOVA; Tukey's multiple range test; n = 10. Symbols represent: WW ambient ozone (■); WW elevated ozone (□); DS ambient ozone (●); DS elevated ozone (○). WW, well watered; DS, drought stressed.

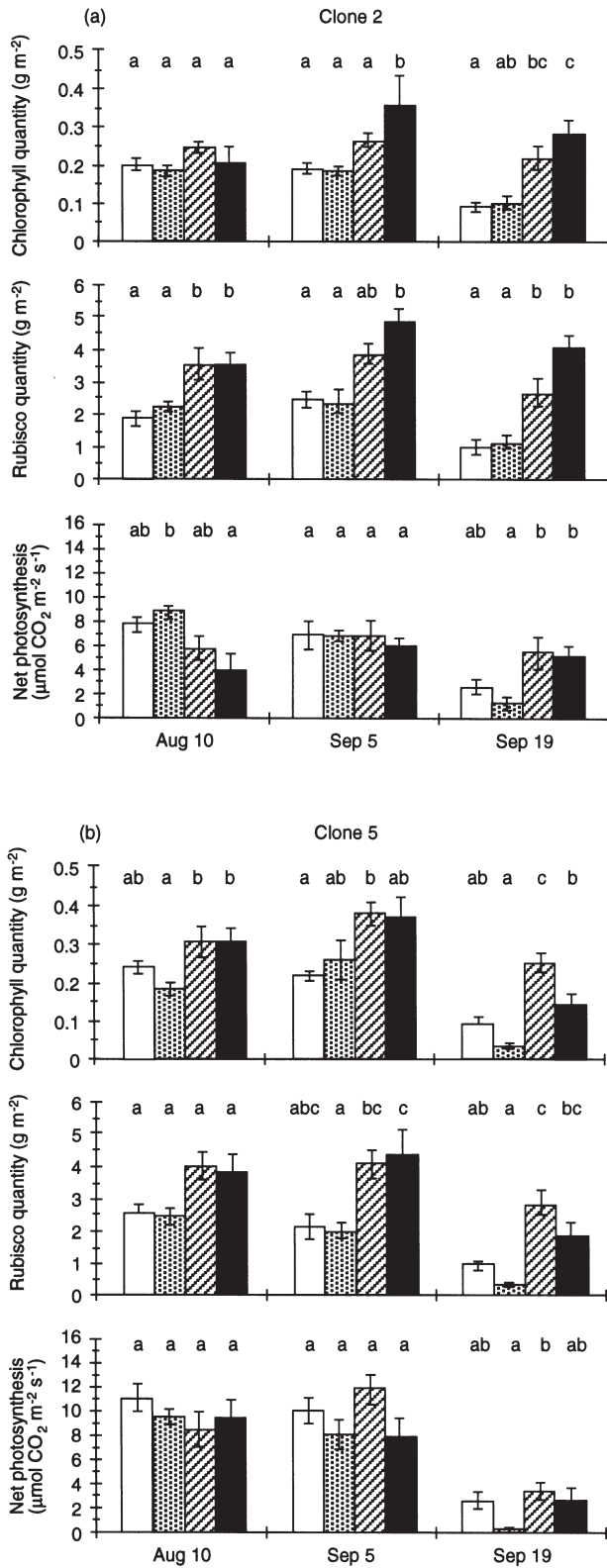


Figure 2. Effects of ozone and drought treatments on chlorophyll quantity, Rubisco quantity and net photosynthesis of birch (*Betula pendula*) clones (a) 2 and (b) 5 in the field experiment. ANOVA; Tukey's multiple range test; $n = 5-6$. Symbols represent: WW ambient ozone (□); WW elevated ozone (▨); DS ambient ozone (▧); DS elevated ozone (■). WW, well watered; DS, drought stressed.

Ultrastructural responses to ozone

Chamber experiment

In the chamber experiment, significant ozone responses were found in the WW and MS plants, but not in the DS plants, in accordance with the results from the growth and physiology studies. Ozone fumigation induced similar kinds of responses to drought stress (significant differences being found in both clones): significant increase in the number of plastoglobuli and reduction in the relative amount of starch in both clones; decrease in the relative amount of thylakoids in clone 2; increase in the size of plastoglobuli in clone 2, but variable responses in clone 5 (Table 5). Ozone exposure increased considerably the proportion of chloroplasts showing abnormal shape (protrusions, spherical shape), injured thylakoids (swelling and curling) and high density of stroma in WW and MW trees (Fig. 3d), whereas DS trees were almost unaffected by ozone in both clones (Table 7).

Field experiment

In the field experiment, significant ozone responses were predominantly found in DS plants (Tables 6 & 7). Exposure to elevated ozone increased significantly the relative amount of starch in WW plants of clone 5 but, in contrast, a reduced amount of starch was observed in DS plants of clone 2. Thylakoids were significantly reduced in DS plants of clone 5 under elevated ozone exposure (Table 6). In contrast to the chamber experiment, the proportion of chloroplasts showing abnormal shape, high density of stroma and injured thylakoids was greater in ozone-fumigated DS plants compared to WW plants in both clones (Table 7).

Interactions

Chamber experiment

A two-way interaction between drought and ozone reduced significantly the number of visibly injured leaves in both clones, but also the dry mass of leaves in clone 2 (Table 2). In the ultrastructural study, significant interactions were found as a higher proportion of starch in both clones compared with DS controls (Table 5). Single drought stress and combined ozone and drought treatment caused similar reductions in growth (Table 2).

Significant clone \times ozone interactions in foliage area growth ($P < 0.05$) and stomatal conductance ($P < 0.01$) indicated the high ozone sensitivity of clone 5. Significant clone \times moisture interactions in number ($P < 0.001$) and size of plastoglobuli ($P < 0.016$), dry weight of leaves ($P < 0.045$), shoot:root ratio ($P < 0.017$) and number of stomata ($P < 0.001$), instead, revealed the higher drought sensitivity of clone 2 (Tables 2 & 5).

Field experiment

In both clones, significant interactions between drought and elevated ozone were found as reduced height growth

Table 6. Significant ultrastructural responses and interactions of elevated ozone and drought in leaf palisade tissue of *Betula pendula* clones in the open-field exposure. Two-way ANOVA; Tukey's multiple range test. n = 5. WW, well watered; DS, drought stressed. Values followed by different letters are significantly different within the clone

Response	WW		DS		Main effects		
	Control	Ozone	Control	Ozone	Drought	Ozone	Interaction
Size of plastoglobuli (nm)							
Clone 2	288 ± 17a	268 ± 13a	408 ± 15b	429 ± 23b	**	ns	*
Clone 5	261 ± 12a	337 ± 16a	409 ± 28b	336 ± 17ab	**	ns	ns
Starch (% of total chloroplast area)							
Clone 2	42.3 ± 3.8c	53.2 ± 4.0c	24.5 ± 3.5b	5.0 ± 3.0a	**	ns	*
Clone 5	29.1 ± 4.5a	43.9 ± 4.2b	20.4 ± 5.3a	18.3 ± 3.9a	*	ns	ns
Thylakoids (% of total chloroplast area)							
Clone 2	28.9 ± 2.4b	19.1 ± 1.7a	33.2 ± 2.1bc	37.8 ± 4.0c	ns	ns	ns
Clone 5	25.8 ± 2.0ab	25.3 ± 2.0ab	31.1 ± 4.1b	20.5 ± 1.7a	ns	*	ns

and lower stomatal density (Table 4). In clone 2, significant interactions appeared as reduced foliage area, relative growth rate of roots and amount of starch, but higher content of chlorophyll and bigger size of plastoglobuli (Tables 4 & 6; Fig. 2a). In clone 5, accelerated yellowing of leaves was observed as a significant interactive effect of ozone and drought (Table 4). The ozone–drought interaction resulted in a 66.2% (clone 2) and 44.1% (clone 5) decrease in relative growth rate of the total plant, whereas drought treatment alone caused 44.6% and 51.0% decreases, respectively. The negative effect of interaction in the field experiment was also indicated by 14.5% (clone 2) and 31.2% (clone 5) higher proportions of yellowed leaves compared with control plants, whereas drought stress alone increased the percentage number of yellowed leaves by 3.4% and 12.7%, respectively (Table 4).

Significant clone × ozone interactions in leaf yellowing ($P < 0.04$) (Table 4), chlorophyll quantity (19 September, $P < 0.048$), and Rubisco quantity (19 September, $P < 0.035$) (Fig. 2a,b) showed the high ozone sensitivity of clone 5, whereas significant clone × moisture interactions in stomatal conductance on 3 July, 18 July and 26 July supported the higher drought sensitivity of clone 2, as found in the chamber experiment (Fig. 1).

DISCUSSION

The results from these two experiments, using similar plant material but different exposure regimens, confirms the general understanding that ozone responses are mediated by drought, but the degree and sign of that mediation varies with experiment (especially severity of stress), clone and variable. In the present study, under high stress conditions in the chamber experiment, exposure to drought protected the plants from visible and ultrastructural ozone injuries. The results from the open-field experiment showed, however, that ozone concentrations that were on average 1.8 times ambient caused damage (reduced root growth, visible ozone injuries, yellowing of the leaves, and ultrastructural injuries to the chloroplasts) to birch trees that

were exposed to relatively mild water stress. In many previous experiments in the literature (summarized in the Introduction), conducted with open-top or covered chambers, it has been concluded from studies of conifers (e.g. Dobson *et al.* 1990; Beyers *et al.* 1992; Karlsson *et al.* 1995) and beech (e.g. Le Thiec *et al.* 1994) that drought protects plants from ozone injury. The present experiments clearly indicate that this conclusion may be incorrect in field conditions, because it is based on exposure of plants to severe (and perhaps unrealistic) water stress in chambers or open-top enclosures.

As described in earlier papers (e.g. Pääkkönen *et al.* 1996), the high ozone sensitivity of clone 5 appeared as reductions in foliage area growth, stomatal conductance and contents of chlorophyll and Rubisco, as well as higher proportions of yellowed leaves and chloroplast injuries when compared with the more ozone-tolerant clone 2. In the present study, however, significant ozone responses could also be found in DS plants of clone 2 as reduced relative growth rates of roots (the field experiment), visible leaf injuries and reduced proportions of starch and thylakoids, and ultrastructural chloroplast injuries (both experiments). This demonstrates that ozone sensitivity/tolerance of birch is not consistent, and depends on the variable. Ozone-tolerant clone 2 showed somewhat higher sensitivity to drought as well as ozone × drought interaction, which was manifested as greater growth reduction and more pronounced drought-related ultrastructural and stomatal changes (increased size and number of plastoglobuli and stomatal density, lowered stomatal conductance) compared with clone 5.

In addition to differential ozone and drought-stress sensitivity, the results of the present experiments indicate that high phenotypic plasticity is characteristic for young birch trees, supporting those of our earlier studies (Pääkkönen *et al.* 1993, 1996). This high phenotypic plasticity (acclimation) of birch is based on indeterminate growth pattern and adaptable leaf cell differentiation. As the phenotype of birch is greatly affected by environmental conditions as well as by stress factors, some of the observed response

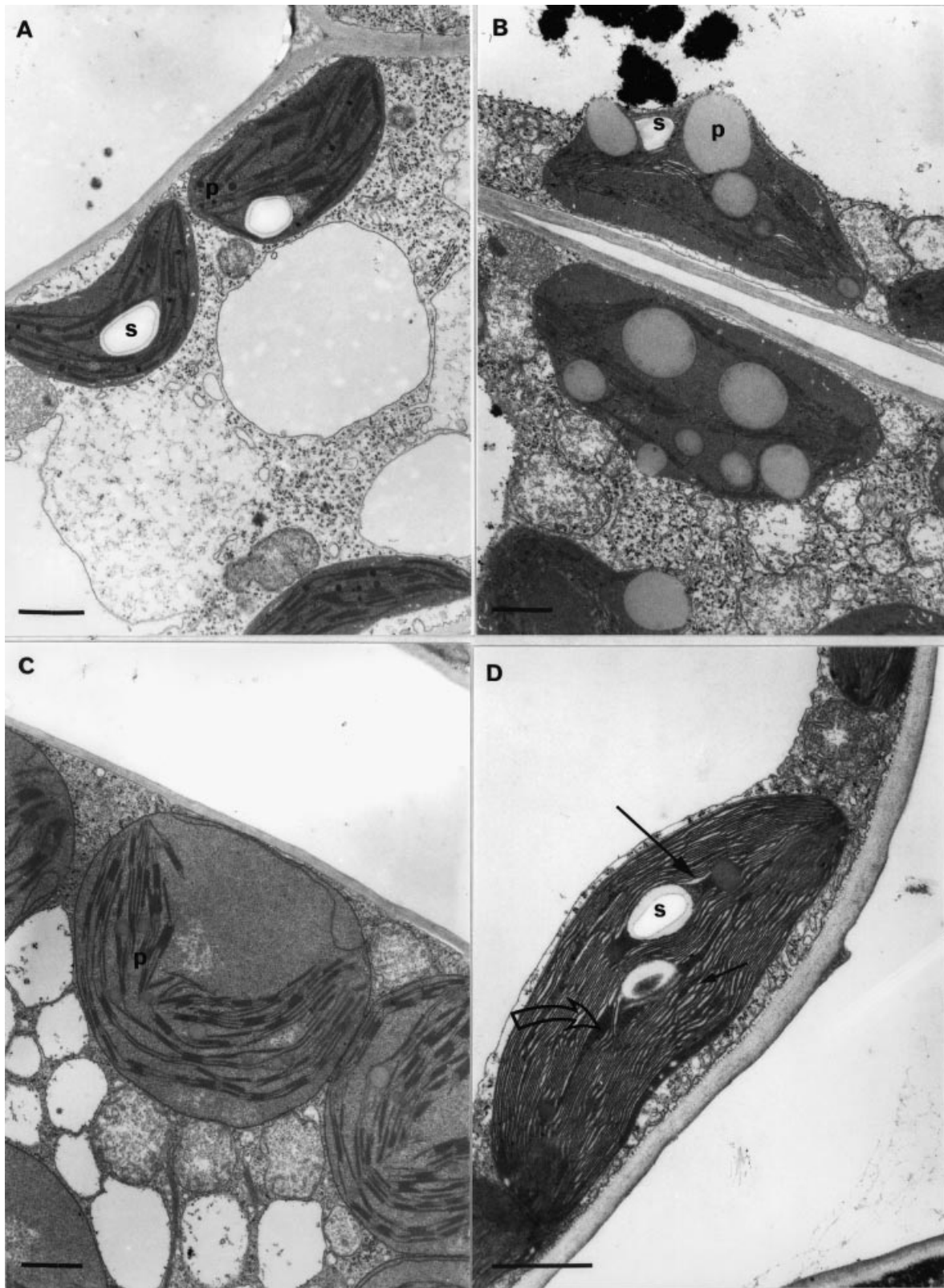


Figure 3. Drought- and ozone-induced ultrastructural changes in palisade mesophyll cells of *Betula pendula* after chamber experiment; p, plastoglobuli; s, starch. Bar = 1 μm . (A) Normal lens-shaped chloroplasts of a MW control plant, clone 2. (B) Drought-induced increased size of plastoglobuli and reduced proportion of thylakoids of chloroplast area in a DS control plant of clone 2. (C) Spherical shape of chloroplast and reduced proportion of thylakoids after combined ozone and drought stress in clone 2. (D) Swelling (long arrow) and curling (short arrow) of thylakoids and high density of stroma (curved open arrow) in WW plants of clone 5 exposed to $130 \text{ nmol mol}^{-1}$ ozone. MW, moderately watered; DS, drought stressed; WW, well watered.

Table 7. Percentage increase or decrease (–) in ultrastructural injuries in palisade mesophyll cells of *Betula pendula* clones in response to elevated ozone (chamber and open-field experiment) compared with controls in different watering groups. WW, well watered; MW, moderately watered; DS, drought stressed; $n = 5$

Response	Chamber experiment			Field experiment	
	WW	MW	DS	WW	DS
Abnormal chloroplast shape					
Clone 2	28.1	52.6	6.7	20.9	47.8
Clone 5	78.4	42.1	–2.8	15.7	45.2
High density of stroma					
Clone 2	14.2	17.4	8.5	9.1	23.0
Clone 5	63.5	53.9	1.7	17.4	40.7
Thylakoid injuries					
Clone 2	51.4	55.9	–2.4	–0.4	12.5
Clone 5	73.3	44.7	1.4	33.1	42.7

differences between the chamber and field experiments (for example, in height and foliage area growth) may have been affected by different exposure environments. For instance, light intensity in the chambers was lower than in the field conditions, not exceeding photosynthetically saturating levels of radiation.

Wendler & Millard (1996) have reported that birch (*Betula pendula*) avoids rather than tolerates drought by closing stomata to reduce transpiration and, eventually, by leaf abscission. Reduced stomatal conductance was also a general response to drought stress in our experiment. Lower stomatal conductance has also been observed in most ozone-treated trees, consistent with several previous studies (Dobson *et al.* 1990; Pell *et al.* 1993; Le Thiec *et al.* 1994). In the chamber experiment, stomatal closure and the smaller number of stomata of DS plants under fumigation probably resulted in reduced gas exchange and ozone flux inside the leaf tissue, thus providing protection from visible and ultrastructural ozone damage. On the other hand, lowered gas exchange rates under water deficit may have been partially responsible for growth reductions, as suggested previously in saplings of ash by Reiner *et al.* (1996). Contrary to the results from the chamber experiment, the reduced stomatal conductance and number of stomata of DS trees under elevated ozone in the field experiment did not offer protection from ozone injuries; instead, greater ozone damage was observed when the DS trees were compared with WW trees. As illustrated in Fig. 1b, ozone fumigation in field conditions initially increased, but later decreased the stomatal conductance in WW plants of clone 5, whereas among DS plants a general increase of conductance was observed under ozone stress. Those variable data suggest disturbance of the stomatal water regulation capacity of ozone- and drought-exposed plants of ozone-sensitive clone 5. Direct deleterious influence of air pollutants and drought stress on stomatal function was described previously in spruce (*Picea abies*) by Maier-Maercker &

Koch (1992). They also regarded the loss of stomatal control and disturbed water budget as a main reason for foliage damage in a declining spruce stand exposed to ozone (combined air pollutants) and dry soil conditions (Maier-Maercker & Koch 1995). Impaired capacity for stomatal water balance regulation under combined water and ozone stress was previously observed also in beech (Pearson & Mansfield 1993), bush bean (Bender *et al.* 1991) and yellow poplar (Roberts 1990).

Activated photosynthetic processes appearing as increased chlorophyll and Rubisco contents, and higher Rubisco activity and net photosynthesis (except in clone 5 in the chamber experiment) was generally found as the main effect of drought. Both clones showed increased chlorophyll contents in drought-stressed trees even under ozone fumigation. Similarly, Le Thiec *et al.* (1994) reported increased photosynthesis and chlorophyll contents in beech under interactive ozone and drought stress compared with well-watered equivalents. In wheat (*Triticum aestivum*), ozone-induced impairment in photochemical capacity was smallest if severe drought stress coincided with ozone fumigation (Soja, Khan & Bolhar-Nordenkamp 1996). In the present study, the drought-induced stimulation of photosynthesis-related processes can be regarded as a compensation mechanism for foliage loss resulting from water deficit. On the other hand, smaller foliage area also had a compensatory function, resulting in reduced area of the transpiration surface, agreeing with studies on aspen (*Populus tremuloides*) by Greintner *et al.* (1994) and radish (*Raphanus sativus*) by Pell *et al.* (1993). Increased contents of chlorophyll and Rubisco and higher net photosynthesis in drought-stressed plants of clone 2 were accompanied by increased thylakoids. However, the proportion of starch was reduced, which may indicate active transport and metabolism of starch in drought-stressed plants.

Drought stress impaired plant growth considerably in both experiments, whereas growth responses to ozone varied from reduction to stimulation of shoot growth, in accordance with our previous ozone experiments with the same birch clones (Pääkkönen *et al.* 1993, 1996). Lowered stomatal conductance was a general response to both stress factors. In this study, typical drought-induced responses were activated photosynthetic apparatus and decreased stomatal density. In contrast, ozone treatment increased the stomatal density and impaired photosynthesis. At the ultrastructural level, increased size and number of plastoglobuli, and reduced proportions of starch and thylakoids in chloroplasts, were found after both stress treatments. Instead, swelling and curling of thylakoids, abnormal shape of chloroplasts and increased density of chloroplast stroma were regarded as specific ozone responses, as described previously in Pääkkönen *et al.* (1995a, 1995b, 1996). A decreased amount of starch in drought-stressed leaves has been reported elsewhere, for example in willow (*Salix* sp.) (Vapaavuori & Nurmi 1982), Scots pine (*Pinus sylvestris*), Norway spruce (*Picea abies*) (Palomäki, Holopainen & Holopainen 1994) and jack pine (*Pinus banksiana*) (Zwiazek & Shay 1987).

In the field experiment, the ozone exposure value of AOT40 for fumigated plants was 11.6 $\mu\text{mol mol}^{-1} \text{ h}$, thereby exceeding the critical ozone level defined for forest trees of 10 $\mu\text{mol mol}^{-1} \text{ h}$ (e.g. Ashmore & Wilson 1993). In this exposure, AOT40 of 11.6 $\mu\text{mol mol}^{-1} \text{ h}$ resulted in significantly impaired relative growth rate of roots (66%, clone 2), increased visible injuries (11.6%, clone 2; 15.5%, clone 5) and accelerated leaf yellowing (31.2%, clone 5) in drought-exposed plants, compared with WW controls. Therefore, as a practical implication, our results suggest that damage to birch that would normally be attributed to drought (for example, accelerated leaf senescence and reduced growth) may actually be the result of an ozone–drought interaction. These findings, which were obtained on immature pot-grown saplings, should be applied with caution to mature, field-grown trees.

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REFERENCES

- Aron D.I. (1949) Copper enzymes in isolated chloroplast. Polyphenoloxidases in *Beta vulgaris*. *Plant Physiology* **24**, 1–15.
- Ashmore M.R. & Wilson R.B. (eds) (1993) *Critical Levels of Air Pollutants for Europe*. UK Department of the Environment, London.
- Bender J., Tingey D.T., Jäger H.J., Rodecap K.D. & Clark C.S. (1991) Physiological and biochemical responses of bush bean (*Phaseolus vulgaris*) to ozone and drought stress. *Journal of Plant Physiology* **137**, 565–570.
- Beyers J.I., Riechers G.H. & Temple P.J. (1992) Effects of long-term ozone exposure and drought on the photosynthetic capacity of ponderosa pine (*Pinus ponderosa* Laws.). *New Phytologist* **122**, 81–90.
- Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- Darrall N.M. (1989) The effects of air pollutants on physiological processes in plants. *Plant, Cell and Environment* **12**, 1–30.
- Dobson M.C., Taylor G. & Freer-Smith H. (1990) The control of ozone uptake by *Picea abies* (L.) Karst and *P. sitchensis* (Bong.) Carr. during drought and interacting effects on shoot relations. *New Phytologist* **116**, 465–474.
- Greitner C.S., Pell E.J. & Winner W.E. (1994) Analysis of aspen foliage exposed to multiple stresses: ozone, nitrogen deficiency and drought. *New Phytologist* **127**, 579–589.
- Heggstad H.E., Gish T.J., Lee E.H., Bennett J.H. & Douglass L.W. (1985) Interaction of soil moisture stress and ambient ozone on growth and yields of soybeans. *Phytopathology* **75**, 472–477.
- Hunt R. (1990) *Basic growth analysis*. Unwin Hyman, London.
- Karlsson P.E., Medin E.-L., Wickström H., Seldén G., Wallin G., Ottosson S. & Skärby L. (1995) Ozone and drought stress — interactive effects on the growth and physiology of Norway spruce (*Picea abies* (L.) Karst.). *Water, Air and Soil Pollution* **85**, 1325–1330.
- Kickert R.N. & Krupa S.V. (1990) Forest responses to tropospheric ozone and global climate change: an analysis. *Environmental Pollution* **68**, 29–65.
- Krupa S.V. & Manning W.J. (1988) Atmospheric ozone: formation and effects on vegetation. *Environmental Pollution* **50**, 101–137.
- Kúbinová L. (1994) Recent stereological methods for measuring leaf anatomical characteristics: estimation of the number and sizes of stomata and mesophyll cells. *Journal of Experimental Botany* **45**, 119–127.
- Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Le Thiec D., Dixon M. & Garrec J.P. (1994) The effects of slightly elevated ozone concentrations and mild drought stress on the physiology and growth of Norway spruce, *Picea abies* (L.) Karst. and beech, *Fagus sylvatica* L., in open-top chambers. *New Phytologist* **128**, 671–678.
- Lee W.S., Chevone B.I. & Seiler J.R. (1990) Growth and gas exchange of loblolly pine seedlings as influenced by drought and air pollutants. *Water, Air and Soil Pollution* **51**, 105–116.
- Lilley R., McC. & Walker D.A. (1974) An improved spectrophotometric assay for ribulose biphosphate carboxylase. *Biochimica et Biophysica Acta* **358**, 226–229.
- Maier-Maercker U. & Koch W. (1992) The effect of air pollution on the mechanism of stomatal control. *Trees* **7**, 12–25.
- Maier-Maercker U. & Koch W. (1995) Poor stomatal control of water balance and the abscission of green needles from declining stand of spruce trees (*Picea abies* (L.) Karst.) from the northern Alps. *Trees* **10**, 63–73.
- Matyssek R., Günthardt-Goerg M., Saurer M. & Keller T. (1991) Seasonal growth $\delta^{13}\text{C}$ in leaves and stem, and phloem structure of birch (*Betula pendula*) under low ozone concentration. *Trees* **6**, 69–76.
- Pääkkönen E. (1996) *Ozone — complex risk for birch*. Doctoral dissertation, Kuopio University.
- Pääkkönen E., Holopainen T. & Kärenlampi L. (1995a) Ageing-related anatomical and ultrastructural changes in leaves of birch (*Betula pendula* Roth.) clones as affected by low ozone exposure. *Annals of Botany* **75**, 285–294.
- Pääkkönen E., Holopainen T. & Kärenlampi L. (1997) Variation in ozone sensitivity among clones of *Betula pendula* and *Betula pubescens*. *Environmental Pollution* **95**, 37–44.
- Pääkkönen E., Metsärinne S., Holopainen T. & Kärenlampi L. (1995b) The ozone sensitivity of birch (*Betula pendula* Roth.) in relation to the developmental stage of leaves. *New Phytologist* **132**, 145–154.
- Pääkkönen E., Paasisalo S., Holopainen T. & Kärenlampi L. (1993) Growth and stomatal responses of birch (*Betula pendula* Roth.) clones to ozone in open-air and chamber fumigations. *New Phytologist* **125**, 615–623.
- Pääkkönen E., Vahala J., Holopainen T., Karjalainen R. & Kärenlampi L. (1996) Growth responses and related biochemical and ultrastructural changes of the photosynthetic machinery in birch (*Betula pendula* Roth.) exposed to low level ozone fumigation. *Tree Physiology* **16**, 597–605.
- Palomäki V., Holopainen J. & Holopainen T. (1994) Effects of drought and waterlogging on ultrastructure of Scots pine and Norway spruce needles. *Trees* **9**, 98–105.
- Pearcy R.W., Ehleringer J., Mooney H.A. & Rundel P.W. (1989) *Plant Physiological Ecology: Field Methods and Instrumentation*, pp. 29–56. Chapman and Hall, London.
- Pearson M. & Mansfield T.A. (1993) Interacting effects of ozone and water stress on the stomatal resistance of beech (*Fagus sylvatica* L.). *New Phytologist* **123**, 351–358.

- Pell E.J., Sinn J.P., Eckardt N., Johansen C.V., Winner W.E. & Mooney H.A. (1993) Responses of radish to multiple stresses II. Influence of season and genotype on plant response to ozone and soil moisture deficit. *New Phytologist* **123**, 153–163.
- Reiner S., Wiltshire J.J.J., Wright C.J. & Colls J.J. (1996) The impact of ozone and drought on the water relations of ash trees (*Fraxinus excelsior* L.). *Journal of Plant Physiology* **148**, 166–171.
- Roberts B.R. (1990) Physiological responses of yellow-poplar seedlings to simulated acid rain, ozone fumigation, and drought. *Forest Ecology and Management* **31**, 215–224.
- Soja G., Khan S. & Bolhar-Nordenkamp H.R. (1996) Drought stress as a modifier of ozone responses in wheat. In: *Critical Levels for Ozone in Europe: Testing and Finalizing the Concepts*. (eds L. Kärenlampi & L. Skärby), pp. 308–313. 1996 UN-ECE Workshop Report. Department of Ecology and Environmental Science, University of Kuopio, Finland.
- Tingey D.T. & Hogsett W.E. (1985) Water stress reduces ozone injury via a stomatal mechanism. *Plant Physiology* **77**, 944–947.
- Tingey D.T. & Taylor G.E. Jr (1982) Variation in plant response to ozone: a conceptual model of physiological events. In: *Effects of Gaseous Air Pollution in Agriculture and Horticulture* (eds M. H. Unsworth & D. P. Ormrod), pp. 113–138. Butterworths, Cambridge.
- Van den Driessche R. & Langebartels C. (1994) Foliar symptoms, ethylene biosynthesis and water use of young norway spruce (*Picea abies* (L.) Karst.) exposed to drought and ozone. *Water, Air and Soil Pollution* **78**, 153–168.
- Vapaavuori E. & Nurmi A. (1982) Chlorophyll-protein complexes in *Salix* sp. 'Aquatica gigantea' under strong and weak light. II. Effects of water stress on the chlorophyll-protein complexes and chloroplast ultrastructure. *Plant and Cell Physiology* **23**, 791–801.
- Wendler R. & Millard P. (1996) Impacts of water and nitrogen supplies on the physiology, leaf demography and nitrogen dynamics of *Betula pendula*. *Tree Physiology* **16**, 153–159.
- Wulff A., Hänninen O., Tuomainen A. & Kärenlampi L. (1992) Open-air exposure of plants to ozone in Kuopio; analysis of method. *Annales Botanici Fennici* **29**, 263–267.
- Zwiazek J.J. & Shay J.M. (1987) Fluoride- and drought-induced structural alterations of mesophyll and guard cells in cotyledons of jack pine (*Pinus banksiana*). *Canadian Journal of Botany* **65**, 2310–2317.

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