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Responses of Leaf Processes in a Sensitive Birch (*Betula pendula* Roth) Clone to Ozone Combined with Drought

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Saplings of an ozone sensitive clone of birch (*Betula pendula* Roth, KL-5-M) were well-watered or exposed to mild drought-stress combined with ambient or elevated (1·5 × the ambient) ozone for 11 weeks in open-field conditions in central Finland. Stomatal response, visible injury, chlorophyll and nutrient content, and changes in cellular anatomy and plant growth were studied. Drought stress alone, in ambient ozone, reduced stomatal density and stomatal conductance. Drought stress and ozone effects were additive, reducing total leaf number, foliage area and starch formation in mesophyll cells. Drought stress and ozone effects were additive, increasing the N concentration in the leaves, the thickness of the upper epidermal cell wall, the number of pectinaceous projections of mesophyll cell walls, and the vacuolar tannin-like depositions and phenolic droplets, regarded as signs of activated stress defence mechanisms. The increase in specific foliage mass, cytoplasmic lipids (younger leaves), and a condensed appearance of the upper epidermal mucilaginous layer were caused by both drought and ozone, but were not additive. The results show that combined drought stress contributed to birch responses to 1·5 × current ambient ozone concentrations, corresponding to critical-level ozone exposure. The only beneficial effect of drought stress was the slight reduction of visible leaf symptoms induced by ozone in autumnal leaves.

Key words: Birch, *Betula pendula*, sensitive clone, ozone, drought, microscopy.

INTRODUCTION

Model evaluations predict large increases in the concentration of tropospheric ozone, especially in the northern hemisphere, if NO_x emissions are not drastically lowered (Hough and Derwent, 1990; Finlayson-Pitts and Pitts, 1997). Periods of elevated ozone concentrations are concomitant with sunny dry weather conditions (leading to drought stress), which are predicted to increase as a consequence of increasing CO_2 emissions (Schnug, 1998).

Typical metabolic ozone responses in forest trees are lower net photosynthesis and reduced foliar Rubisco, carbohydrate and chlorophyll contents, changes in carbon allocation to roots, increased dark respiration, reduced stomatal conductance and water-use efficiency, and accelerated leaf senescence leading to impaired biomass production (e.g. Pye 1988; Lefohn, 1992). Drought stress generally results in lower leaf water potential, stomatal closure, reduced stomatal conductance and net photosynthesis, disruption of membrane integrity, denaturation and reduction of proteins, increased ABA concentration, polyamine and solute (e.g. sucrose, pinitol) accumulation, and reduced growth (although root growth may be favoured) (Popp and Smirnoff, 1995; Wellburn *et al.*, 1996). The results from interactive effects of ozone and drought have been contradictory: a short summary of the interaction studies was presented in Pääkkönen *et al.* (1998*a*).

The concept of AOT (accumulated exposure over a threshold) has been demonstrated to be the most appropriate definition for critical ozone concentrations, above which adverse effects on vegetation may occur (Fuhrer, Skärby and Ashmore, 1997). The critical concentrations provide the best available scientific basis for the protection of plants from significant ozone effects in Europe. However, the data used to derive critical concentrations are mostly from open-top chamber experiments, using plants well supplied with water and nutrients. From the evaluation of biomass and visible leaf injury responses, critical ozone exposure AOT40 (AOT concentration of 40 nl l^{-1}) of 10 μl l^{-1} h was defined for forest trees (e.g. Ashmore and Wilson, 1992; Kärenlampi and Skärby, 1996). Further exposure-response data is currently needed, particularly on the different response parameters, effects of environmental factors on ozone responses and interactions, to develop more accurate and realistic critical concentrations (Fuhrer *et al.*, 1997).

Birch is a widespread tree, particularly in northern latitudes, and the effects of ozone on it have been intensively studied. Earlier experiments in birch have shown deleterious cellular ozone responses to different low (from ambient to double ambient) ozone concentrations: structural collapse and disintegration of mesophyll cells, discoloration, decreased photosynthetic capacity, and ultrastructural chloroplast and membrane injuries (Matyssek *et al.*, 1991, 1992; Günthardt-Goerg *et al.*, 1993; Pääkkönen *et al.*, 1995*a*, *b*) and also in hybrid poplar (Landolt *et al.*, 1994; Günthardt-Goerg, 1996). The ozone responses were dependent on genetical predisposition (Pääkkönen, Holopainen and Kärenlampi, 1995*a*, 1997*a*, 1997*b*), nutrition (Pääkkönen

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TABLE 1. *Ozone data measured from the beginning of the experiment on 11 Jun. to the harvest on 26 Aug. 1996. Mean values for the two ambient-ozone or elevated-ozone treatment areas are shown*

	Ambient ozone	Elevated ozone
AOT 00 (μl l^{-1} h)	43·8	67·6
AOT 40 (μl l^{-1} h)	0·5	10·7
24 h mean (nl l^{-1})	24·3 ± 5·7	37·0 ± 8·2
7 h mean (nl l^{-1})	31·7 ± 7·2	48·7 ± 9·7
Maximum 1 h concentration (nl l^{-1})	48·1	75·2

and Holopainen, 1995; Frey *et al*., 1996; Günthardt-Goerg *et al*., 1997, 1998), or on climatic factors (Pääkkönen *et al*., 1997*b*; Günthardt-Goerg *et al*., 1998). Certain ozone responses (increased scale and hair density, thickened cell walls) resembled drought-induced anatomical changes in leaf cells, and the hypothesis was postulated that the expression of xeromorphic leaf structures, within the genetical possibilities, is controlled not only by light and temperature, but also modified by ozone (Günthardt-Goerg *et al*., 1993). In addition to these responses to ozone stress in the leaf mesophyll cells, low chronic ozone concentrations increased the amount of sucrose and inositol at the expense of starch in leaves and stem bark, a process which may be seen as a biochemical adaptation to drought stress (Landolt *et al*., 1994; Popp and Smirnoff, 1995).

Recent research (reviewed by Kangasjärvi *et al*., 1994) has added new findings to our understanding of the molecular basis of plant response to elevated ozone concentrations, leading to rapid cell death. Ozone enters the leaf through the stomata and reacts very rapidly with components of the cell walls, forming reduced oxygen species. Detoxification mechanisms in the apoplast (Luwe, 1996; Ranieri *et al*., 1996) may hinder further action of activated oxygen molecules which might otherwise change the permeability of the plasma membrane, probably by lipid peroxidation (Wellburn and Wellburn, 1996). By a controversial signal transduction process, metabolites of cell wall formation and degradation are induced: phenylalanine ammonialyase (PAL) leading to the formation of wall-bound phenolics and flavonoids, and pathogenesis-related (PR) proteins with hydrolytic cell wall degrading properties (Schraudner, Langebartels and Sandermann, 1996). Recently, induction of both protein types (PAL and PR10) has been detected in the birch clone we have used here after ozone fumigation and drought stress (Tuomainen *et al*., 1996; Pääkkönen *et al*., 1998*b*).

Since different anthropogenic, climatic and edaphic stress factors influence the plants at the same time, but in different combinations, and since their interactions induce complex plant defence and acclimation reactions, our experiments consider combined stress factors, ozone and drought, and are carried out under near-natural conditions. Our recent study with the current birch clone showed that, under high ozone- and drought-stress conditions in a chamber experiment, water deficit protected the plants from ozone injuries (Pääkkönen *et al*., 1998*a*). However, mild drought stress under open-field conditions enhanced ozone damage (reduced root growth, increased visible and chloroplast injuries, and yellowing of leaves) when the plants were exposed for one growing season to elevated ozone (1·8 × ambient). Therefore, the aim of this experiment was to test the hypothesis that low realistic drought stress in field conditions increases ozone sensitivity of birch. In the present study we focus on the cellular responses in leaves of different age. The relative influence of drought and ozone was analysed to distinguish their effects at the cellular level, relative to the response of the whole leaf and plant (growth). The ozone responses were related to critical ozone exposure.

MATERIALS AND METHODS

Plant material

Birch (*Betula pendula* Roth) saplings were propagated by tissue culture from clone 5 (KL-5-M) selected for its ozone sensitivity under similar environmental conditions to the current experiment (Pääkkönen *et al*., 1993). The saplings were grown outdoors during summer 1995 and overwintered with the pots covered with snow. Sixty saplings were transferred to a glasshouse on 23 Feb. 1996. After thawing, on 1 March the dormant saplings were transplanted into 5 l pots, filled with quartz sand and then grown in charcoal-filtered air in a climate controlled growth chamber, where temperature was between 12 °C (night) and 19 °C (day) and relative humidity approx. 60 %. The potted saplings were transferred to the field 2 weeks before the elevated ozone/drought treatments began. All the first flush leaves, which had developed before the start of the treatment, were tagged, and excluded from the analyses. On 11 Jun. 1996, the saplings were randomly divided into four treatment areas, each with well-watered and drought-stressed regime and ambient ozone (control) or elevated ozone concentrations. The pots were covered with plastic caps to exclude rain.

Watering and fertilization

The water status of each pot was checked each day by measuring the electrical conductivity of the sand using golden electrode-containing resistance blocks, embedded close to the roots of each plant. The electrical conductivity was related to the relative moisture content of the pot, tested separately. Every 2 or 3 d each well-watered sapling was given 0·4–0·8 dm^3 of water (depending on weather conditions) to reach the field capacity, while the droughted saplings received half that amount of water (0·2–0·4 dm^3 per sapling). To determine the actual drought stress in the leaves, the pre-dawn leaf water potentials were measured with a thermocouple psychrometer (Wescor, model L-51, Logan, Utah, USA). In addition to the watering treatment, each sapling was fertilized once a week (until 29 July) with 0·2 % Superex solution (19 : 5 : 20 N : P : K), applied in 0·2 dm^3 water, resulting in an optimum nitrogen supply of 78 kg N ha^{-1}.

Ozone fumigation

The saplings in pots were exposed to ambient and 1·5 times elevated ozone using the open-field fumigation system, described in detail by Wulff *et al.* (1992). The saplings were grown under natural microclimate, and added ozone gas was released from perforated tubes surrounding the elevated-ozone plants. Ozone was generated from pure oxygen with a Fisher OZ500 instrument and monitored continuously with a model 1003-AH ozone analyser (Dasibi Environmental Corp.). The computer-controlled system maintained the natural daily and seasonal ozone fluctuation. The cumulative ozone exposures AOT00 and AOT40, the 24-h and 7-h means (±s.d.), and the maximum 1-h concentrations are given in Table 1. The AOT40 value 10·7 μl l^{-1} h for elevated-ozone treatment (Table 1), indicates that the critical ozone exposure (AOT40 of 10 μl l^{-1} h) was slightly exceeded under fumigation.

Stomatal conductance

Stomatal conductance was measured at five occasions between 27 June to 21 August to determine ozone uptake. Three fully-sized young to middle-aged (6–8 week old) leaves, attached to the main stem, from 10 saplings per treatment were measured between 1000 h to 1500 h using a LI-COR Steady State Porometer (LI-1600, LI-COR Inc., NE, USA).

Nutrient analysis

Nutrients were analysed from dried and ground bulk samples, collected on 26 August separately from young to middle-aged (6–8 week old) and old (9–10 week old) leaves (these ages correspond to those used for light and fluorescence microscopy). Nitrogen concentration was determined by Kjehldahl digestion, and atomic absorption spectrophotometry (AAS) was used for Ca and K analysis.

Chlorophyll analysis

For chlorophyll *a* and *b* analysis, part of the leaves sampled for light and fluorescence microscopy (described later) were frozen in liquid nitrogen. Chlorophyll concentrations were determined individually for each leaf sample using the DMSO method (Barnes *et al.*, 1992).

External symptoms, light and fluorescence microscopy

The number of leaves with ozone-induced brown dots and yellowing (> 50% of leaf area) was calculated as a proportion of the total number of leaves for ten fumigated saplings per water treatment 3 d before sampling. The number of dots per leaf was counted for 40 leaves per treatment.

Stomatal density on the abaxial surface of the leaves was determined for 50 fully-grown 6–8 week old leaves, attached to the main stem, from ten saplings in each treatment. Stomatal density was counted under a light microscope from 3 × 3 mm square pieces (four pieces per leaf) using a systematic uniform random sampling according to Kubínová (1994), and averaged for each leaf.

For light and fluorescence microscopy, 8 mm-diameter leaf discs were excised on 26 August (0700–0900 h) from each half of the central lamina (between second-order veins) from three saplings per treatment and from five leaves per sapling (leaf age 6–10 weeks). These leaves had therefore been formed under different treatment conditions. The 6–8 week old young to middle-aged leaves were still green and defined as 'young to middle-aged leaves', but the 9–10 week old leaves with incipient autumnal yellowing were defined as 'old leaves'. For the determination of the starch pattern, the leaf discs were excised in the field into methanol and stained with I_2-KI. For semi-thin sections the discs were excised into 2·5% buffered (pH 7·4) glutaraldehyde, and embedded in Technovit 7100. 2·5 μm-thick sections were stained with 1% methylene blue as a metachromatic stain, with 0·25% Coomassie brilliant blue for proteins, with 0·005% aniline blue for callose, with 0·01% calcofluor white Mr2 for 1,4-β-glucans in cellulose, or with 0·03% coriphosphine for pectins (for detailed description and discussion of the methods used see Günthardt-Goerg *et al.*, 1997).

Transmission electron microscopy (TEM)

Fifteen 7–8 week old middle-aged leaves were sampled per treatment (bulk sampling on 26 August) and their ultrastructure examined as described earlier (see Methods, and Holopainen *et al.*, 1992; Pääkkönen *et al.*, 1995*a*). The mean thickness of the leaf upper side transverse epidermal cell wall (middle point of the cell) was measured from photographs (60 measurements from 15 leaves per treatment).

Growth

At the final harvest on 26 August, ten saplings per treatment were measured for height, number of leaves, individual leaf area, total foliage area, and dry weights of leaves, stem and roots. For relative growth rates [RGR $(t,t_o) = \ln (W_t) - \ln (W_{to})$; with initial biomass weight W_{to} and a harvest biomass weight W_t at the end of the experiment] the initial dry weights were measured for additional saplings at a destructive harvest on 10 June, before the start of treatment.

Statistical analysis

Analysis of variance (ANOVA) was calculated with a SPSS program with Tukey's multiple range test for the significance of the means ($P < 0·05$). Significance of ozone, drought and leaf age interactions, and the main effects (Table 4) were determined using MANOVA. To reveal the main ozone effects, all elevated-ozone plants were compared with all ambient-ozone plants (watering levels combined). Similarly, the main drought effects were determined by comparing all droughted plants with all well-watered plants, regardless of ozone treatment. Data were presented as means ± s.e.

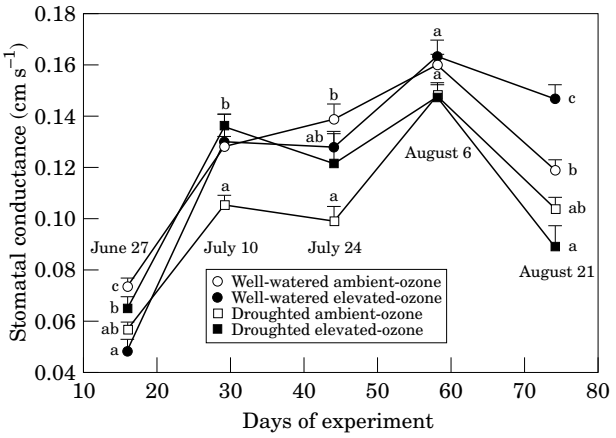

FIG. 1. Stomatal conductance of 6–8-week-old leaves on the main stem of *Betula pendula* saplings, sensitive clone 5, measured from 27 Jun. to 21 Aug. 1996. Symbols represent mean values (±s.e.) of 30 leaves from ten different saplings per treatment. ANOVA; Tukey's multiple range test, $P < 0{\cdot}05$. Means indexed by the same characters are not significantly different.

RESULTS

Water potentials

The mean pre-dawn water potentials, measured on three occasions before the sampling date from five saplings per treatment, were $-0{\cdot}32 \pm 0{\cdot}04$ (s.e.) MPa for well-watered ambient-ozone saplings, $-1{\cdot}32 \pm 0{\cdot}1$ MPa for droughted ambient-ozone saplings, $-0{\cdot}57 \pm 0{\cdot}08$ MPa for well-watered elevated-ozone saplings, and $-1{\cdot}56 \pm 0{\cdot}2$ MPa for droughted elevated-ozone saplings. The water potentials for droughted saplings were significantly lower than in well-watered treatments, however, the saplings did not reach wilting point.

Stomata

The stomatal density (number of stomata mm^{-2} leaf area) was 101 ± 8 (mean ± s.e.) in well-watered ambient-ozone saplings, 84 ± 4 mm^{-2} in droughted ambient-ozone saplings, 116 ± 6 mm^{-2} in well-watered elevated-ozone saplings and 94 ± 5 mm^{-2} in droughted elevated-ozone saplings. The stomatal density was significantly higher in well-watered elevated-ozone saplings ($P < 0{\cdot}021$), but significantly lower in droughted ambient-ozone saplings ($P < 0{\cdot}036$), than in the well-watered ambient-ozone treatment.

Stomatal conductance and the significance of the difference between the treatments were variable due to different weather conditions on the measuring dates (cloudy and cool weather on 27 June, Fig. 1). Low values were measured particularly in the droughted ambient-ozone saplings throughout the experiment, and on 21 August in the droughted elevated-ozone treatment (Fig. 1).

Nutrient concentrations

Nitrogen concentration of the younger leaves was significantly increased in droughted elevated-ozone saplings, compared to well-watered ambient-ozone treatment (Table 2), whereas in the older leaves differences were not significant. Compared to the well-watered ambient-ozone treatment, Ca concentration was significantly decreased in the younger droughted elevated-ozone leaves, but increased in the older droughted ambient-ozone leaves (Table 2). K concentration was not significantly affected by ozone or drought treatments.

Visible leaf injury, yellowing and chlorophyll concentration

Early ozone injury symptoms (small brown dots) appeared on 12 August in the oldest, 9-week-old leaves from well-watered elevated-ozone saplings, during the episode of ambient high ozone concentrations. Three days before sampling, on 23 August, $18{\cdot}2 \pm 2{\cdot}9$ % (s.e.) of leaves in well-watered elevated-ozone saplings and $14{\cdot}1 \pm 2{\cdot}2$ % of leaves in droughted elevated-ozone saplings showed small brown dots on the upper leaf side (total number of leaves per sapling = 100 %, ten saplings per treatment). No visible injuries were seen in well-watered or droughted ambient-ozone saplings. The range (minimum-maximum) of elevated-ozone-induced brown dots (diameter below 0·80 mm) per leaf in well-watered saplings was 2–22 for 6-week-old leaves, 4–25 for 7–8-week-old leaves (used for transmission electron microscopy), and 6–30 for 9–10-week-old leaves. The corresponding ranges for droughted leaves were 0–1, 0–15, and 0–17, indicating that drought-stress protected the leaves from ozone-induced visible leaf dotting.

On 23 August, there were no significant differences among the treatments in yellowed leaves as a proportion of

TABLE 2. *N, Ca and K concentrations (mg g^{-1} d.wt) in leaves of well-watered or droughted, and ambient- or elevated-ozone grown* Betula pendula

Nutrient	Leaf age (weeks)	Ambient well-watered	Ambient droughted	Elevated O_3 well-watered	Elevated O_3 droughted
N	6–8	$19{\cdot}3 \pm 0{\cdot}5^{a}$	$22{\cdot}6 \pm 0{\cdot}5^{ab}$	$21{\cdot}8 \pm 1{\cdot}0^{ab}$	$23{\cdot}2 \pm 0{\cdot}5^{b}$
	9–10	$17{\cdot}5 \pm 0{\cdot}9^{a}$	$18{\cdot}3 \pm 0{\cdot}9^{a}$	$17{\cdot}7 \pm 0{\cdot}3^{a}$	$19{\cdot}5 \pm 0{\cdot}22^{a}$
Ca	6–8	$3{\cdot}4 \pm 0{\cdot}3^{bc}$	$3{\cdot}3 \pm 0{\cdot}01^{b}$	$4{\cdot}1 \pm 0{\cdot}2^{c}$	$2{\cdot}5 \pm 0{\cdot}01^{a}$
	9–10	$3{\cdot}7 \pm 0{\cdot}3^{a}$	$7{\cdot}2 \pm 1{\cdot}3^{b}$	$3{\cdot}7 \pm 0{\cdot}4^{a}$	$4{\cdot}5 \pm 1{\cdot}0^{a}$
K	6–8	$16{\cdot}2 \pm 0{\cdot}4^{a}$	$13{\cdot}2 \pm 2{\cdot}5^{a}$	$14{\cdot}0 \pm 0{\cdot}6^{a}$	$13{\cdot}6 \pm 1{\cdot}7^{a}$
	9–10	$17{\cdot}5 \pm 0{\cdot}9^{a}$	$15{\cdot}1 \pm 0{\cdot}3^{a}$	$17{\cdot}8 \pm 0{\cdot}9^{a}$	$13{\cdot}9 \pm 1{\cdot}2^{a}$

ANOVA; Tukey's multiple range test, $P < 0{\cdot}05$, $n = 3$ (ten leaves per plant). Means indexed by the same superscript are not significantly different.

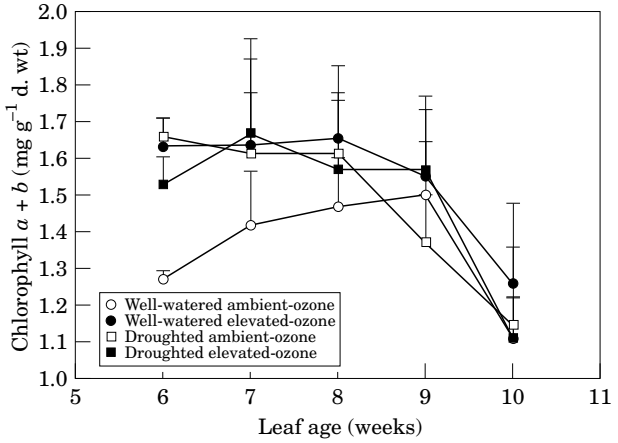

FIG. 2. Total chlorophyll ($a+b$) concentration (mg g^{-1} d.wt) in relation to leaf ageing in *Betula pendula*, sensitive clone 5. Symbols indicate mean values from three leaves (one leaf per leaf age per sapling per treatment) with standard error bars. ANOVA; Tukey's multiple range test, $P < 0{\cdot}05$, $n = 3$, with no significant difference where the pair-wise comparison of the means is concerned. For main effects see Table 5.

the total number of leaves per sapling: ten saplings per treatment). This was $23{\cdot}3 \pm 2{\cdot}0$(s.e.)% for well-watered ambient-ozone saplings, $26{\cdot}1 \pm 2{\cdot}9$% for droughted ambient-ozone saplings, $34{\cdot}3 \pm 2{\cdot}6$% for well-watered elevated-ozone saplings, and $48{\cdot}6 \pm 1{\cdot}6$% for droughted elevated-ozone saplings.

The changes in contents of chlorophyll *a* and *b* during leaf ageing were similar in all treatments, and contents of total chlorophyll ($a+b$) are shown in Fig. 2. In well-watered ambient-ozone plants, contents of chlorophyll increased continuously until the leaves were 9 weeks old, then decreased rapidly. Although significant differences were not found between the treatments, both elevated-ozone and drought stress tended to increase chlorophyll content in the young and middle-aged leaves. The chlorophyll content was significantly increased by drought treatment as a main effect over all leaf ages (Table 5). In the older leaves of all treatments, chlorophyll contents decreased and they appeared yellow.

Microscopy

In well-watered ambient-ozone saplings, palisade and spongy mesophyll cells of the younger leaves were homogeneously filled with starch. Starch content decreased considerably with drought, but also with age (Table 3). In elevated-ozone saplings, patches of mesophyll cells without starch were found increasingly as leaves aged (concomitant with visible brown dots). The droughted elevated-ozone samples therefore contained least starch.

In well-watered leaves the number of cytoplasmic lipid droplets in the mesophyll bundle sheath cells, spongy cells and lower epidermal cells was cumulatively increased with leaf age and elevated ozone (Fig. 3A and B), whereas in droughted leaves it decreased with leaf age independent of ozone (Table 3). Tannin-like depositions appeared generally in vacuoles as granulated (Fig. 3B), or net-like and precipitated along tonoplast membranes (Fig. 3A and C). Like the cytoplasmic lipids, the tannin-like depositions were most abundant in bundle sheath cells (Figs 3B, 4D) and upper and lower epidermal cells. Tannin-like depositions increased with leaf age except in droughted elevated-ozone leaves. More tannin-like depositions were found in leaves from the elevated-ozone and droughted treatments than in the well-watered ambient-ozone leaves (Table 3). Phenolic droplets were observed by fluorescent microscopy under ultraviolet light in vacuoles of mesophyll, upper and lower epidermal cells, and bundle sheath cells, increasing with leaf age and elevated ozone (Fig. 4B and D; Table 3).

With cellulose (β-D-glucan) staining, a thickening of the transversal walls of upper epidermal cells was observed after both stress treatments in 7–8-week-old middle-aged leaves. This thickening was significant in the droughted elevated-ozone treatment. The mean thickness of the outer cell wall of the upper epidermis (excluding the cuticle proper) was $1{\cdot}8 \pm 0{\cdot}3$ (s.e.) μm for well-watered ambient-ozone plants, $2{\cdot}3 \pm 0{\cdot}4$ μm for droughted ambient-ozone plants, $2{\cdot}2 \pm 0{\cdot}4$ μm for well-watered elevated-ozone plants, and $2{\cdot}5 \pm 0{\cdot}5$ μm for droughted elevated-ozone plants. In addition to cell wall thickening, a few phenolic-containing peltate scales on the upper leaf side (Fig. 4C) were found in droughted ambient-ozone and elevated-ozone leaves, but not in well-watered leaves (due to rare occurrence, statistical testing did not reveal a significant difference).

In sections stained with methylene blue, darkening of the polysaccharide-containing mucilaginous layer of the upper epidermal cells was observed after drought and elevated-ozone treatments, and during leaf ageing (Fig. 4E and F).

Droplet-like projections of cell walls, facing the intercellular space, were observed after the droughted and elevated-ozone treatments in spongy mesophyll cells (Table 3; Fig. 3D). These projections, with a diameter of 1·0–4·5 μm were continuous with the outermost pectinaceous layer of the cell wall (middle lamella), (Fig. 3D). Cytochemically, these wall projections stained positive for pectin, protein (weak reaction) and carbohydrates (weak reaction). Callose formation was not observed in any sample, and no influence of the treatments on the occurrence of Ca-oxalate crystals was detected, despite differences in Ca-concentrations.

Growth

Height increase, mean area of single leaves and relative growth rates of leaves, stem and roots were unaffected by droughted and elevated-ozone treatments. Since the number of leaves per sapling was smaller in the stress treatments (significant in droughted elevated-ozone plants *vs.* well-watered ambient-ozone), smaller foliage area and increased specific foliage mass resulted (Table 4).

Interactions and main effects of ozone, drought and leaf age

In addition to the main effects of ozone, drought and leaf ageing presented in Table 5, certain significant interactions occurred, namely in stomatal density (increased by ozone, but decreased by drought, ozone × drought interaction $P < 0{\cdot}041$), in stomatal conductance (decreased by ozone, but

TABLE 3. *Cell properties in* Betula pendula *leaves exposed to drought and ozone*

Response	Leaf age (weeks)	Ambient well-watered	Ambient droughted	Elevated O_3 well-watered	Elevated O_3 droughted
Starch	6–8	+++	+	++	+
	9–10	++	+	+	+
Lipids	6–8	−	++	++	++
	9–10	+	+	+++	+
Tannin-like deposition	6–8	+	++	++	+++
	9–10	++	+++	+++	+++
Phenolic droplets	6–8	−	−	++	++
	9–10	+	++	+++	+++
Droplet-like wall projections	6–8	−	+	−	+
	9–10	−	+	+	++

Estimated abundance: +, little; ++, considerable; +++, abundant; −, not observed.

increased by drought on 24 July, ozone × drought interaction $P < 0{\cdot}006$), but were reversed in autumnal leaves on 6 August (ozone × drought interaction $P < 0{\cdot}023$), and in Ca concentration (increased by leaf age except in well-watered elevated-ozone plants, and decreased in droughted elevated-ozone saplings, ozone × drought × leaf age interaction $P < 0{\cdot}008$). Significant age × ozone interactions were not found.

DISCUSSION

The applied drought stress, which significantly decreased pre-dawn water potentials, was reflected by lower stomatal conductance (modified by the weather conditions) and reduced foliage area growth. N, K, and Ca concentrations indicated a non-limited nutritional status and a small retranslocation of N from older leaves in all treatments. In the younger leaves of the droughted and elevated-ozone treatments, increased N corresponded with a tendency of increased chlorophyll content, agreeing with our recent drought-ozone interaction experiment (Pääkkönen *et al.*, 1998*a*). Increased chlorophyll and N contents, in leaves of droughted and ozone-stressed birch with indeterminate growth, may be related to the photosynthetic ability of young leaves to compensate for a reduced photosynthetic active foliage area, as reported in Beyers, Riechers and Temple (1992), Hom (1990) and Pääkkönen *et al.* (1998*a*).

Drought stress reduced the visible leaf injury caused by elevated ozone which was related to lower stomatal conductance during the period of high ozone concentrations. Previously, stomatal closure and reduced ozone uptake under drought stress were reported to result in reduced mottling and flecking of foliage in *Pinus taeda*, in *Picea abies* and also in the current birch clone under high stress concentrations in the chamber experiment (Pääkkönen *et al.*, 1998*a*).

Concerning cell differentiation to stomata, the effect of drought and ozone were of equal importance, but opposite. Both stresses combined were therefore similar to the well-watered ambient-ozone treatment. Changes in stomatal density were consistent with findings in previous experiments (Matyssek *et al.*, 1991; Günthardt-Goerg *et al.*, 1993; Pääkkönen *et al.*, 1993, 1995*b*, 1997*a*). The lower stomatal density was related to reduced stomatal conductance in droughted ambient-ozone leaves *vs.* well-watered ambient-ozone leaves, from 27 June to 24 July. These results support the previous experiments indicating high responsiveness of stomatal differentiation to different stress-related factors. For example, stomatal density was greatly increased by low nutrient supply in parallel with a reduced leaf size in birch (Frey *et al.*, 1996).

Starch formation and carbon allocation is affected by most internal and external changes in plants. Earlier ozone studies have reported: (1) that starch formation is reduced in birch leaves with increasing ozone injury and leaf age; (2) that starch accumulates along small leaf veins; and (3) that an impaired phloem transport is related to an increased investment of structural carbon in the leaves (increased specific leaf weight) (Matyssek *et al.*, 1992; Günthardt-Goerg *et al.*, 1993, 1996). These previous starch results were confirmed in the present study in the well-watered elevated-ozone treatment in young to middle-aged leaves, with drought being more efficient in reducing mesophyll starch formation than ozone alone. The calculated shoot/root ratios indicated that the regulation of carbon allocation is dissimilar during drought and ozone stress: carbon allocation to the shoot was favoured by drought, as reported in *Pinus halepensis* by Gerant *et al.*, 1996, but reduced by ozone. Thereby, high phenotypic plasticity seems to be characteristic for *Betula pendula*.

Microscopical studies were able to give a more detailed answer to the different allocation of structural carbon, which was responsible for increased specific leaf mass due to drought and ozone, but not cumulative. Increased thickness of upper epidermal cell walls has been detected previously in connection with ageing or drought stress (Mikkelsen and Heide-Jørgensen, 1996; Bussotti *et al.*, 1995) or low fertilisation (Günthardt-Goerg *et al.*, 1997). Because ozone can also lead to thickened cell walls (not only in epidermal cells, Günthardt-Goerg *et al.*, 1997) the additive effects of drought and ozone significantly increased the thickness of the upper epidermal cell wall in the present study. Increased epidermal cell wall thickness as well as decreased stomatal density were interpreted as slowing down water loss through stomatal and cuticular transpiration in droughted saplings, as shown by stomatal conductance measurements. Droplet-like pectinaceous cell wall projections (for detailed analysis

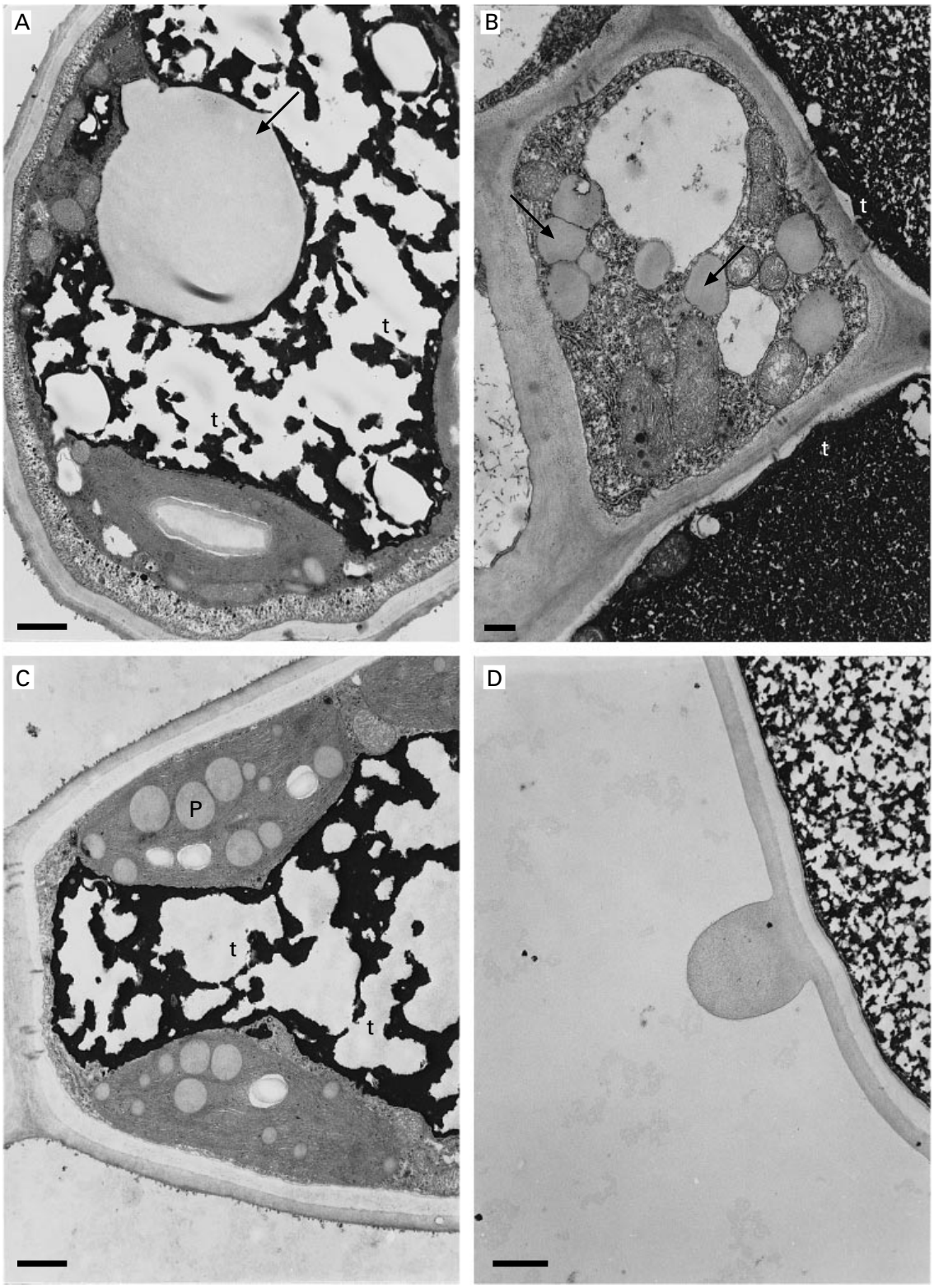

FIG. 3. Transmission electron microscopy of *Betula pendula*, illustrating the stress-induced changes in the cells of droughted elevated-ozone middle-aged 7–8-week-old leaves, presented in Table 3. Scale bar = 1 μm. A, Large cytoplasmic lipid body (arrow) and net-like tannin depositions (t) in the vacuole of a spongy parenchyma cell; B, increased cytoplasmic lipid droplets (arrows) in a bundle sheath cell. The neighbouring cells have large vacuoles with tannin-like deposits (t); C, accumulation of net-like tannin deposits (t) in a vacuole of a spongy parenchyma cell. (p) indicates plastoglobuli in the chloroplast; D, cell wall projection facing the intercellular space, continuous with the outermost pectinaceous layer of the spongy parenchyma cell wall.

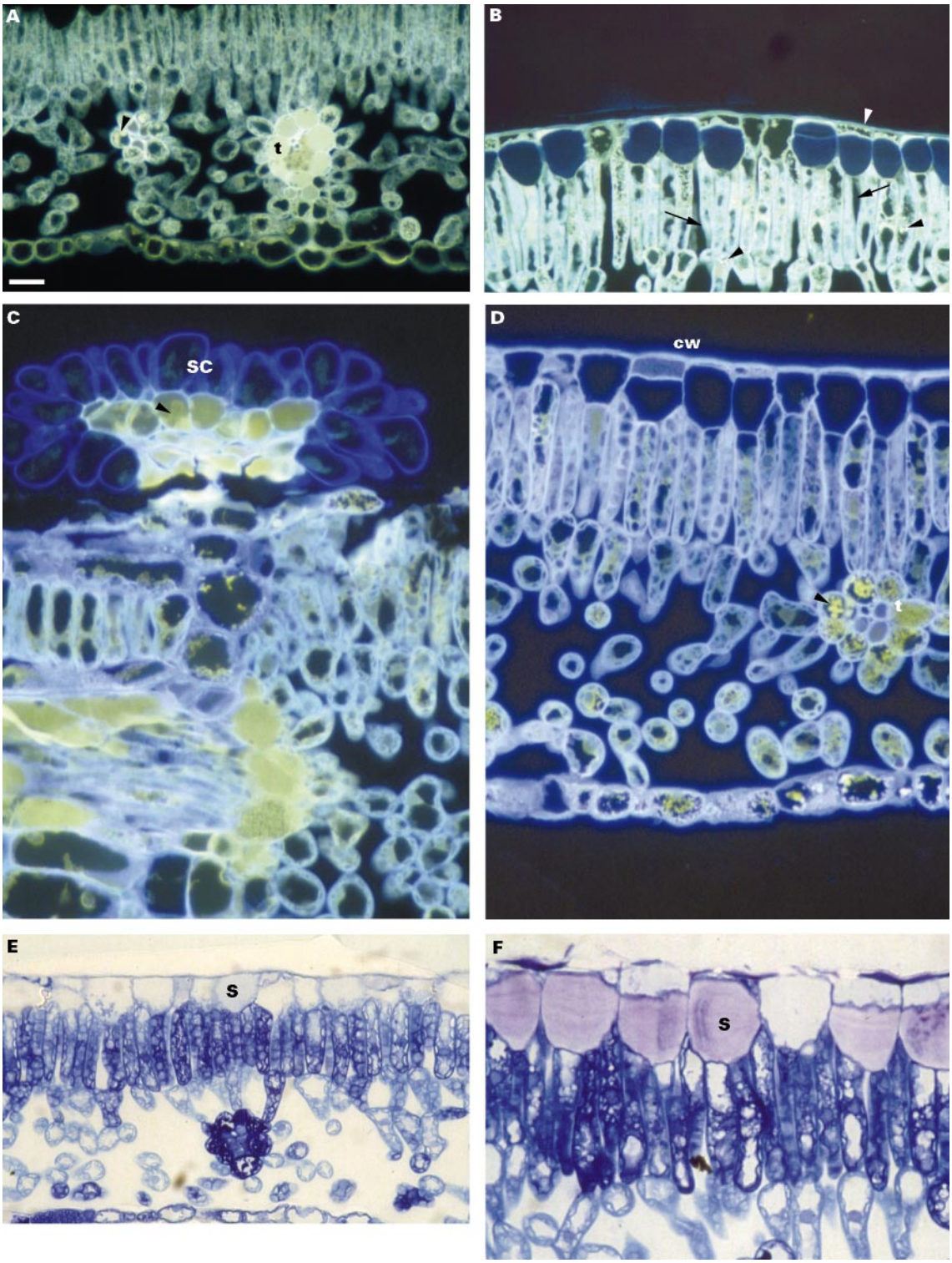

FIG. 4. *Betula pendula*, leaf cross sections in intercostal fields. Bar = 22 μm. A–E, 6–8 week-old; F, 9 week-old leaves. A–D, Fluorescence microscopy 340–380 nm excitation radiation, black or white arrow heads indicate phenolic droplets, (t) tannin cells, long arrows collapsed cells (4B). A and B, Unstained sections; C and D, stained with Calcofluor White. A, Well-watered ambient-ozone leaf with few phenolic droplets in lower epidermal cells, vacuoles of mesophyll cells and bundle sheat cells; B, droughted elevated-ozone leaf with abundant phenolic droplets in epidermal and mesophyll cells; C, droughted ambient-ozone with a peltate scale (sc) on the upper leaf side, containing yellow fluorescent phenolics; D, droughted elevated-ozone leaf showing the thickened epidermal cell wall (cw) at the upper leaf side, and accumulation of tannin-like depositions (yellowish-green fluorescence) and phenolic droplets (bright-yellow fluorescence) in the bundle sheath cells. E and F, light microscopy, stained with methylene blue. E, Young well-watered ambient-ozone leaf with light slime (s) in the epidermal cell wall; F, old well-watered ambient-ozone leaf with darkened, condensated slime.

TABLE 4. *Significant ozone and drought induced growth responses*

Response	Ambient well-watered	Ambient droughted	Elevated O_3 well-watered	Elevated O_3 droughted
Number of leaves per sapling	135 ± 16^{b}	94 ± 17^{ab}	101 ± 13^{ab}	87 ± 6^{a}
Foliage area (cm^2)	2707 ± 206^{b}	1981 ± 311^{ab}	1881 ± 256^{ab}	1807 ± 86^{a}
Shoot: root ratio (g g^{-1} d.wt)	$1{\cdot}65 \pm 0{\cdot}14^{ab}$	$1{\cdot}79 \pm 0{\cdot}15^{b}$	$1{\cdot}31 \pm 0{\cdot}09^{a}$	$1{\cdot}54 \pm 0{\cdot}08^{ab}$
Specific foliage mass (mg cm^{-2})	$1{\cdot}67 \pm 0{\cdot}17^{a}$	$2{\cdot}64 \pm 0{\cdot}24^{b}$	$2{\cdot}52 \pm 0{\cdot}21^{b}$	$2{\cdot}22 \pm 0{\cdot}19^{b}$

Tukey's multiple range test, $P < 0{\cdot}05$, $n = 10$. Means indexed by the same superscript are not significantly different.

TABLE 5. *Main effects of ozone, drought and leaf aging*

Response	Ozone	Drought	Leaf age
Stomatal density	↑ ($P < 0{\cdot}029$)	↓ ($P < 0{\cdot}031$)	–
Stomatal conductance	ns	↓*	–
Ca	ns	ns	↑ ($P < 0{\cdot}022$)
N	↑ ($P < 0{\cdot}046$)	↑ ($P < 0{\cdot}001$)	↓ ($P < 0{\cdot}001$)
Chlorophyll	ns	↑ ($P < 0{\cdot}044$)	↓ ($P < 0{\cdot}05$)
Visible leaf injury	↑ ($P < 0{\cdot}019$)	ns	↑ ($P < 0{\cdot}040$)
Leaf number	↓ ($P < 0{\cdot}032$)	↓ ($P < 0{\cdot}025$)	–
Single leaf area	↓ ($P < 0{\cdot}034$)	ns	–
Foliage area	↓ ($P < 0{\cdot}020$)	↓ ($P < 0{\cdot}033$)	–
RGR of total plant	ns	↓ ($P < 0{\cdot}026$)	–
Shoot: root ratio	↓ ($P < 0{\cdot}039$)	ns	–
Specific foliage mass	↑ ($P < 0{\cdot}022$)	↑ ($P < 0{\cdot}024$)	–

* $P < 0{\cdot}045$ on 27 June; $P < 0{\cdot}049$ on 21 August.
↑ = significant increase; ↓ = significant decrease; ns = not significant; – = not analysed. MANOVA.

and discussion see Günthardt-Goerg *et al*., 1997) are a further reaction of cell walls to the additive effect of ozone and drought stress. This cell wall reaction is thought to be induced by oxidative stress occurring in the intercellular space, when the cells are separated by a strong external force (cutting, infection), or when they collapse as a result of drought or ozone injury. A darkened mucilaginous layer, which was observed in an increasing number of epidermal cells with drought stress and leaf ageing, probably represents a 'condensation' of polysaccharides due to water loss. This effect may be reversible at rewatering.

Accumulation of cytoplasmic lipids is known to be an alternative storage form to starch in conifer needles and seeds. The present observations suggest a complex interaction of leaf age, drought and ozone, since lipid droplets increased with leaf age in well-watered saplings (and particularly in elevated-ozone treatment), as reported in Pääkkönen *et al*. (1995*a*), but decreased with leaf aging in droughted treatments.

In the present study, tannin-like depositions and phenolic droplets increased with leaf age, drought, and elevated ozone in the vacuoles of certain cells. These secondary metabolites originate from activation of the PAL pathway. Increased total phenolic, proanthocyanidin and cathecin concentrations were reported in conifer needles after chronic exposure to elevated ozone (Brooker, Anttonen and Heagle, 1996; Wulff *et al*., 1996). This accumulation of secondary metabolites may indicate activated defence reactions, as suggested by the increased ozone- and drought-induced stress-related proteins in our previous study (Pääkkönen *et al*., 1998*b*). Measurements of mitochondrial NAD malic enzyme activity showed directly that ozone and drought enhance the catabolic activity in needles of *Pinus halepensis* Mill. (Gerant *et al*., 1996). From present knowledge it is not possible to judge whether these tannin-like and phenolic metabolites are stored and reactivated later, whether they are involved in an osmotic adjustment in response to a water deficit, or whether they indicate an irreversible oxidative injury in the cytoplasm.

In summary, in this open-field experiment, slightly exceeding the critical ozone concentration, AOT40 exposure of $10{\cdot}7\ \mu l\ l^{-1}\ h$, induced visible leaf damage, reduced mesophyll starch content, and increased specific foliage mass and lipid, tannin, and phenolic depositions in well-watered elevated-ozone plants. When drought treatment was applied with elevated-ozone exposure, significantly reduced foliage area, stomatal conductance (21 August) and mesophyll starch were observed, indicating detrimental interaction of ozone and drought stress in this relatively low-stress field experiment. These results suggest that the critical ozone concentration for droughted plants may be lower than for well-watered plants. Concomitant with these negative responses, structural and metabolic stress defence-related reactions, such as thickening of the cell wall and accumulation of secondary metabolites, were more evident due to combined drought and ozone stress. In addition, birch leaves adapted to drought and ozone stress by changing leaf differentiation and structure (stomatal density,

leaf number and specific foliage mass), and carbon allocation, indicating high phenotypic plasticity.

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