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Environmental Pollution 115 (2001) 437–446

ENVIRONMENTAL POLLUTION

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Impacts of elevated CO_2 and/or O_3 on leaf ultrastructure of aspen (*Populus tremuloides*) and birch (*Betula papyrifera*) in the Aspen FACE experiment

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Received 1 October 2000; accepted 17 July 2001

"Capsule": *Elevated ozone and CO_2 concentrations had varying effects on leaf ultrastructure of aspen and birch.*

Abstract

Impacts of elevated atmospheric O_3 and/or CO_2 on three clones of aspen (*Populus tremuloides* Michx.) and birch (*Betula papyrifera* Marsh.) were studied to determine, whether or not elevated CO_2 ameliorates O_3-induced damage to leaf cells. The plants were exposed for 3 years at the Aspen FACE exposure site in Wisconsin (USA) prior to sampling for ultrastructural investigations on 19 June 1999. In the aspen clones, elevated CO_2 increased chloroplast cover index, leaf and spongy mesophyll layer thickness, intercellular air space volume in mesophyll, amount of starch in chloroplasts and cytoplasmic lipids but decreased the number of plastoglobuli in chloroplasts. In contrast, elevated O_3 decreased chloroplast cover index, starch content, and the proportion of cytoplasm and intercellular space in mesophyll, and increased the proportion of vacuoles, the amount of condensed vacuolar tannins and the number of plastoglobuli. Ozone also caused structural thylakoid injuries (dilation, distortion) and stromal condensation in chloroplasts, which was ameliorated by elevated CO_2 by 5–66% in aspen clones and by 2–10% in birch. Birch ultrastructure was less affected by elevated CO_2 or O_3 stress compared to aspen. In the most O_3-sensitive aspen clone, thinner leaves and cell walls, lower proportion of cell wall volume, and higher volume for vacuoles was found compared to more-tolerant clones.

Keywords: Ozone; Carbon dioxide; Interaction; Aspen; Birch; FACE

1. Introduction

Several studies have indicated that projected climate change will impact strongly on forest growth and composition (e.g. Wuebbles et al., 1999; Kirschbaum, 2000; Lindner, 2000). In particular, there are major uncertainties regarding the responses of forest trees to the expected rise in greenhouse gases (O_3, CO_2, CH_4, NO_2, NO_x, CO, CFC-compounds). Tropospheric concentrations of O_3 and CO_2 are predicted to continue rising at 1–2% per year, with considerable consequences for forest tree productivity, and the stability, structure and functioning of forest ecosystems (e.g. IPCC, 1992; Keeling et al., 1995; Matyssek and Innes, 1999; Reilly et al., 1999). Whilst the effects of elevated O_3 and elevated CO_2 on forest trees are well documented for these gases individually, our understanding of the combined effects of elevated O_3 and elevated CO_2 is limited, and largely results from a number of short-term studies measuring growth, photosynthesis and antioxidant responses of seedlings raised in growth chambers (see review by Polle and Pell, 1999).

The existing database relating to the interactive effects of CO_2 and O_3 on conifers and deciduous trees provides a confused picture (Polle and Pell, 1999). Elevated CO_2 contributed negative impact of O_3 on growth, pigment concentrations, antioxidant activities and gas exchange in Norway spruce (*Picea abies*) after one growing season (Polle et al., 1993; Lippert et al., 1997), whereas O_3-induced photosynthetic responses (Barnes et al., 1995), needle loss and chromosomal aberrations in root tips

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0269-7491/01/$ - see front matter
PII: S0269-7491(01)00233-0

(Pfirrmann, 1992; Müller et al., 1994) were unaffected by the combined exposure. In contrast, elevated CO_2 increased the activities of superoxide dismutase (SOD), glutathione reductase (GR) and ascorbate peroxidase (AscPOD) in Norway spruce consistent with increased protection against O_3 injuries (Sehmer et al., 1998). In Scots pine (*Pinus sylvestris*), no significant interactions of CO_2 and O_3 on biomass, photosynthesis, pigments, nitrogen metabolism, needle structure or mycorrhizal infection were found in a short-term chamber experiment by Perez-Soba et al. (1995) or in a two-season OTC-experiment by Utriainen et al. (2000). In contrast, O_3-mediated reductions in gas exchange were ameliorated in 28–30-year-old trees by elevated CO_2 in an OTC field experiment that extended over two growing seasons (Kellomäki and Wang, 1997).

In trembling aspen (*Populus tremuloides* Michx.), elevated CO_2 (150 ppm above ambient) has not been found to alleviate the negative impacts of O_3 on leaf photosynthetic capacity in two soil-grown clones exposed in OTC-chambers between one and three growing seasons (Kull et al., 1996; Karnosky et al., 1998), whereas Volin and Reich (1996) and Volin et al. (1998) found that CO_2 counteracted the adverse effects of O_3 on photosynthesis and biomass in pot-planted seedlings after a short-time chamber experiment. An amelioration of phytotoxic effects of O_3 at elevated CO_2 appeared also in downy birch (*Betula pubescens*) as increased leaf growth (Mortensen, 1995), in beech (*Fagus sylvatica*) as stimulated photosynthesis (Grams et al., 1999) and in oak (*Quercus petraea*) as compensation growth and as reduced stomatal conductance leading to limited O_3 flux (Broadmeadow et al., 1999). In contrast, in sugar maple (*Acer saccharum*), O_3-induced increases in antioxidant activities were decreased by short-term exposure to 700 ppm CO_2 (Niewiadomska et al., 1999). In green ash (*Fraxinus pennsylvanica*) and yellow-poplar (*Liriodendron tulipifera*), significant $O_3 \times CO_2$ interactions were not evident, whereas in black cherry (*Prunus serotina*) a possible ameliorative effect of CO_2 on photosynthetic processes and growth was suggested after a 10-week chamber exposure (Loats and Rebbeck, 1999). The recent results from the present Aspen FACE experiment have indicated that the interaction of elevated CO_2 and O_3 in aspen is complex: elevated CO_2 afforded protection from ozone-induction of visible foliar injuries, whereas the opposite was true for epicuticular wax degradation, and activities of antioxidants and defensive phenylpropanoid pathway (Sheng et al., 1997; Noormets et al., 2000; Wustman et al., 2001). In addition, growth, Rubisco and pigment concentrations have been mostly unaffected by interaction (e.g. Karnosky et al., 1999; Isebrands et al., 2001).

The purpose of this study is to (1) examine structural leaf characteristics of aspen clones and birch, and (2) investigate whether or not rising CO_2 concentrations alleviate the negative effects of tropospheric O_3 on trembling aspen at the leaf ultrastructural level. The combined effects of CO_2 and O_3 were examined using a novel Free Air CO_2 and Ozone Enrichment facility (FACTS-II, Aspen FACE) in Northern Wisconsin, where soil-grown clones showing different O_3 sensitivity have been exposed to elevated concentrations of O_3 and/or CO_2 since 1997 (Dickson et al., 2000).

2. Materials and methods

2.1. The Aspen FACE experiment

The Forest Atmospheric Carbon Transfer and Storage (FACTS II) Free-Air CO_2 and O_3 Enrichment (Aspen FACE) facility is located at the USDA Forest Service Harshaw Experimental Farm in Oneida County near Rhinelander, Wisconsin (Dickson et al., 2000). The large-scale experiment consists of twelve 30 m-diameter exposure fields (with three replications), where randomized mixtures of trees, consisting of a combination of sugar maple (*A. saccharum* Marsh.), paper birch (*Betula papyrifera* Marsh.) and five genotypes of O_3-sensitive and O_3-tolerant trembling aspen clones (*Populus tremuloides* Michx.) are being exposed to (1) ambient air (serving as control), (2) elevated CO_2 during daylight hours (target = 560 ppm, which was about 200 ppm above the ambient), (3) elevated O_3 during daylight hours (target = 1.5×ambient, and (4) a combination of elevated CO_2 and elevated O_3 during daylight hours. The system is described in detail by Karnosky et al. (1999). Ozone data is given in Table 1. Detailed information about aspen clones and planting in the FACE site are described in Karnosky et al. (1998, 1999) and Dickson et al. (2000).

2.2. Transmission electron and light microscopy

For anatomical and ultrastructural investigations, samples from fully expanded sun leaves were collected from five replicate plants per clone per exposure field (240 leaves in total) on 19 June 1999 (between 10:00 and 12:00) from aspen clones 259, 216 and 271 and birch. Strips (5 mm) were cut 2 cm from the leaf apex, and placed immediately in a 2.5% (v/v) glutaraldehyde fixative (in 0.1M phosphate buffer, pH 7.0). In the laboratory, 1.5 mm^2 pieces were cut from the strips under fixative solution using a razor blade. Leaf samples were post-fixed in 1% buffered OsO_4 solution, dehydrated with an ethanol series followed by a propylene oxide treatment, and then embedded in LX 122 Epon. Thin sections for electron microscopy were stained with lead citrate and uranyl acetate and were examined using an electron microscope (JEOL 1200 EX) operating at 80

Table 1
Mean O_3 concentrations (ppb), and total cumulative O_3 exposures over a threshold of 0 ppb (AOT00, ppm-h) and over a threshold of 40 ppb (AOT40, ppm-h) calculated for daylight hours (May–July 07:00–15:00, August 09:00–15:00) for three replicate exposure fields separately (North, Center and South)

	Ambient	Elevated O_3			Elevated O_3+CO_2		
		North	Center	South	North	Center	South
May–September 1998							
Mean	36	56	57	56	57	57	55
AOT00	47.3	77.2	78.7	76.9	77.4	78.9	76.2
AOT40	0.3	24.3	30.6	27.2	29.8	30.5	25.9
May 1999							
Mean	44	55	56	55	56	56	55
AOT00	11.7	14.6	15.1	14.6	14.8	14.8	14.6
AOT40	1.1	5.2	5.3	5.1	5.1	5.3	5.2
June 1999							
Mean	41	60	61	59	61	61	61
AOT00	14.9	21.7	21.9	21.1	21.9	21.8	21.9
AOT40	2.3	9.5	9.7	9.2	9.7	9.8	9.7

kV. Sections for light microscopy were stained with aqueous Toluidine blue and studied with a Nikon MicroPhot-FXA microscope.

Under the light microscopy, measurements of total leaf thickness, spongy and palisade layer thickness, compartment volumes (%) for vacuoles, chloroplasts, cytoplasm and cell walls in mesophyll sections, leaf internal-exposed cell surface area (cm^2/leaf area cm^2) and chloroplast cover index (i.e. proportion of cell area occupied by chloroplasts) were made on digital micrographs at ×453 magnification using Adobe Photoshop (Version 5.0) program for 10 samples per clone per exposure field (480 samples in total). Section areas were determined on the basis of pixels in selected image histograms using standard tools of the program. Thin-sections (five sections per clone per exposure field, 240 in total) were photographed with a digital camera (connected to the electron microscope), followed by image analyses using the Digital Micrograph program plus Adobe Photoshop as described above. For each thin section, 10 mesophyll cells (five palisade and five spongy cells, 2400 cells in total) were examined to obtain average values for chloroplast and starch grain size (section area), cell wall thickness in mesophyll, number of plastoglobuli in chloroplast and amount (section area) of cytoplasmic lipids and vacuolar tannin deposition. To quantify the extent of ultrastructural oxidative chloroplast injuries, dilation (swelling) of thylakoid interspaces, distortion (undulated shape) of thylakoids leading to unstacking of grana, and condensed appearance of chloroplast stroma (precipitation) were determined for each cell (2400 chloroplasts in total). Replicate data was averaged for each exposure field. Data for palisade and spongy cells was also pooled to obtain average values for the whole mesophyll.

2.3. Statistical methods

There were no statistically significant effects of block (exposure field) within any treatment according to ANOVA. Block means ($n=3$) were used to reveal statistical differences between the treatments using Tukey's multiple range test or Kruskall-Wallis H-test (non-parametric relative proportions; SPSS v. 9.0). The main effects of O_3 and CO_2 and $O_3 \times CO_2$ interactions were determined using Multivariate General Linear Model procedures. To reveal the main O_3 effects, all O_3-exposed plants were compared with control plants. Similarly, the main CO_2 effects were found by comparing all CO_2-exposed plants with controls. In addition, clonal differences were tested for control plants using ANOVA. Differences were considered significant at $P \leqslant 0.05$.

3. Results

3.1. Aspen

Clonal differences were significant in total leaf thickness ($P \leqslant 0.04$), compartment volume for vacuoles ($P \leqslant 0.01$) and exposed cell surface area ($P \leqslant 0.001$). In the most O_3-sensitive clone (259) leaf thickness and exposed mesophyll cell surface area were smaller and mesophyll vacuolation higher as found in more-tolerant aspen clones (Tables 2 and 3). Elevated CO_2 tended to increase the thickness of leaf and spongy mesophyll layer (significant main effects in clones 259 and 216), whereas O_3 tended to decrease leaf thickness (Table 2). Exposure to the combination of elevated CO_2+O_3 resulted in significantly increased thickness of leaf

Table 2
Effects of elevated O_3 and CO_2 alone and in combination on total leaf thickness, palisade and spongy mesophyll thickness, chloroplast cover index (% of cell section area), number of plastoglobuli and size of starch grain in chloroplasts in aspen (*Populus tremuloides*) clones 259, 216 and 271, and birch (*Betula papyrifera*)[a]

Response	Clone	Control	CO_2	Ozone	CO_2 + ozone	Main effects		Interaction
						Ozone	CO_2	
Leaf thickness (μm)	259	138±4ab	154±5b	135±9a	154±9b	ns	**	ns
	216	139±3a	153±11ab	136±5a	177±5b	ns	*	ns
	271	160±13a	166±7a	145±7a	156±6a	ns	ns	ns
	Birch	140±6a	155±11a	131±3a	144±4a	ns	ns	ns
Palisade layer thickness (μm)	259	69±1a (1.3)	73±4a (1.1)	68±4a (1.2)	67±5a (1.0)	ns	ns	ns
	216	68±1a (1.4)	74±8ab (1.4)	71±2a (1.3)	91±3b (1.3)	ns	ns	ns
	271	80±7a (1.2)	80±2a (1.1)	66±2a (1.3)	65±4a (1.0)	ns	ns	ns
	Birch	48±5a (0.8)	55±6a (0.7)	53±4a (0.9)	53±4a (0.8)	ns	ns	ns
Spongy layer thickness (μm)	259	54±3a	66±5a	55±2a	67±4a	ns	**	ns
	216	50±2a	54±4ab	53±2a	70±6b	ns	ns	ns
	271	67±2ab	74±3b	52±4a	64±5ab	ns	ns	ns
	Birch	61±6a	75±8a	61±3a	65±3a	ns	ns	ns
Chloroplast cover index	259	20.8±1.8a	21.8±8.0a	21.6±3.5a	33.2±4.0a	ns	ns	ns
	216	26.4±10.2ab	28.2±4.1b	20.5±0.5a	20.0±3.4a	ns	ns	ns
	271	23.3±7.1b	32.0±2.8b	10.8±4.2a	25.1±3.3b	*	*	ns
	Birch	31.3±5.8 b	26.9±2.4ab	28.8±9.5ab	21.04±6.2a	ns	ns	ns
Number of plastoglobuli/chloroplast	259	3.3±0.5a	4.0±0.2a	10.2±1.0b	3.4±0.4a	**	ns	**
	216	4.9±0.7a	4.4±0.9a	9.0±2.0a	8.4±0.9a	ns	ns	ns
	271	5.9±0.8ab	3.7±0.5a	10.3±1.9b	4.1±0.8a	ns	*	ns
	Birch	4.3±0.8a	3.9±0.7a	6.6±0.7a	4.2±0.6a	ns	ns	ns
Starch grain size (μm^2)	259	3.6±0.6b	3.4±0.5b	0.7±0.2a	3.8±0.5b	*	ns	ns
	216	3.9±0.8b	2.7±0.5a	1.3±0.3a	3.9±0.4b	ns	ns	ns
	271	2.8±0.5a	4.8±0.9ab	1.3±0.7a	8.3±2.3b	ns	ns	ns
	Birch	6.6±1.1ab	10.0±1.1c	2.7±0.8a	8.6±1.4bc	ns	***	*

[a] Palisade mesophyll thickness relative to spongy mesophyll thickness is given in parentheses. Samples were collected on 19 June 1999 (between 10:00 and 12:00). ANOVA followed by Tukey's multiple range test; Multivariate GLM-test for main effects; Kruskall-Wallis H test for relative proportions, $P<0.05$. $n=3$. Values are means±S.E. Significant differences between the treatments are indicated by different letters.

(total), palisade and spongy layers in clone 216 (Table 2). Palisade mesophyll thickness relative to spongy thickness was significantly decreased in elevated CO_2+O_3 exposure in clone 259 compared to controls. Chloroplast cover index was significantly increased in CO_2-exposed plants and reduced in ozone-exposed plants of aspen clone 271 appearing also as significant main effects (Table 2). There were no significant differences between treatments in average cell wall thickness for palisade and spongy cells, but in the sensitive aspen clone 259 thinner average mesophyll cell walls (248±16 S.E. μm) were measured compared to clone 216 (311±23 μm) and clone 271 (287±27 μm).

Compartment volume for vacuoles was significantly increased in O_3-exposed plants of clone 271, which was found also as a significant main effect (Table 3). Compartment volumes for chloroplasts and cell walls were unaffected by O_3 and CO_2, whereas proportion of cytoplasm in mesophyll tissue tended to decrease due to O_3 (significant main effect in clone 271; Table 3). Proportion of intercellular space was significantly increased in CO_2 and CO_2+O_3 plants in clone 259, while O_3-induced decrease was found in clone 271 (Table 3). Significant $CO_2\times O_3$ interactions were observed in proportion of cytoplasm and intercellular space in clone 271 (Table 3).

Elevated O_3 treatment generally increased the number of plastoglobuli (significantly in aspen clones 259, 271, Fig. 1b–d) both in palisade and spongy tissue, whilst CO_2 tended to decrease that number (significant main effect in clone 271; Table 2). At the same time, amount of starch decreased in ozone plants (significantly in clones 259 and 216), whereas combined CO_2+O_3 resulted in significant increase in starch content in clone 271 compared with controls. Significant $CO_2\times$O3 interactions were found in the number of plastoglobuli in clone 259 (Table 2).

There were no structural chloroplast injuries in any control plants (Fig. 1a). Ozone- and CO_2-induced changes in structural condition of thylakoids and stroma and contents of cytoplasmic lipids and tannin-like vacuolar deposition are given in Table 4. Ozone exposure resulted in a 7–52% increase in thylakoid injuries (dilation+distortion; Fig. 1b) and a 4–67%

Table 3
Compartment volumes (%) for vacuoles, chloroplast, cytoplasm, cell walls and intercellular space, for aspen (*Populus tremuloides*) clones 259, 216 and 271, and birch (*Betula papyrifera*)[a]

Response	Clone	Control	CO_2	Ozone	CO_2 + ozone	Main effects		Interaction
Compartment volumes (%)						Ozone	CO_2	
Vacuoles	259	32.8±3.1a	27.5±1.9a	9.7±7.1a	19.0±5.4a	ns	ns	ns
	216	18.8±4.9a	24.0±5.5a	29.8±2.7a	27.5±5.3a	ns	ns	ns
	271	16.5±3.3a	18.1±3.0a	38.4±9.6b	19.5±4.4a	*	ns	ns
	Birch	19.8±5.6a	18.3±4.4a	18.0±4.2a	14.9±2.1a	ns	ns	ns
Chloroplasts	259	12.2±0.9a	12.2±5.4a	12.2±1.0a	16.4±2.8a	ns	ns	ns
	216	11.4±4.7a	12.4±0.5a	10.8±0.7a	9.9±2.9a	ns	ns	ns
	271	9.8±4.9	14.5±2.4a	5.3±1.3a	8.7±0.8a	ns	ns	ns
	Birch	14.8±4.2a	14.4±4.9a	13.1±2.4a	19.9±4.5a	ns	ns	ns
Cytoplasm	259	14.2±4.1a	11.6±1.4a	17.2±2.6a	13.4±4.5a	ns	ns	ns
	216	14.3±2.9a	9.3±1.2a	12.2±4.1a	11.5±2.1a	ns	ns	ns
	271	10.8±1.1a	12.5±2.3a	8.4±0.4a	7.1±1.2a	*	ns	*
	Birch	10.6±3.3a	12.2±2.8a	11.6±1.7a	13.7±0.9a	ns	ns	ns
Cell walls	259	19.8±2.6a	21.8±0.2a	19.0±4.1a	21.6±5.9a	ns	ns	ns
	216	28.7±1.7a	21.1±3.9a	25.7±1.4a	21.5±5.2a	ns	ns	ns
	271	26.2±3.4a	20.3±4.7a	25.3±1.3a	24.5±1.1a	ns	ns	ns
	Birch	20.2±3.0a	17.0±3.5a	16.6±5.0a	12.6±2.8a	ns	ns	ns
Intercellular space	259	21.0±7.2a	26.9±6.5b	21.8±6.3a	29.6±6.1b	ns	ns	ns
	216	26.8±7.8ab	33.3±3.6b	21.6±3.1a	29.6±9.6b	ns	ns	ns
	271	36.7±10.1b	34.6±2.3ab	22.4±6.4a	40.2±2.2b	ns	ns	*
	Birch	34.6±4.0a	38.3±9.1a	40.6±2.5a	38.9±4.7a	ns	ns	ns

[a] Samples were collected on 19 June 1999 (at 10:00–12:00). Kruskall-Wallis H-test; Multivariate GLM-test for main effects, $P<0.05$. $n=3$. Values are means±S.E. Significant differences between the treatments are indicated by different letters.

increase in precipitation of stroma (Fig. 1c), whereas combined CO_2 ameliorated these injuries. Greatest O_3 injuries were found in sensitive clone 259, while clone 216 was least affected. In all clones, elevated CO_2 alone and with ozone increased the amount of cytoplasmic lipids by 1–33%, possibly reflecting high storage lipid production due to increased C availability. Tannin-like depositions were, on the other hand, increased by 11–34% in O_3 ($+CO_2$)-exposed plants, suggesting an activation of the phenylpropanoid pathway leading to accumulation of condensed tannins (Fig. 1d; Table 4).

3.2. Birch

In birch, combined CO_2+O_3 resulted in significantly lower chloroplast cover index (Table 2) and exposed cell surface area (data not shown) as compared to controls. There were no significant differences in compartment volumes for vacuoles, chloroplasts, cytoplasm, cell walls or intercellular space between the treatments (Table 3). Number of plastoglobuli was significantly increased in O_3-exposed plants, whereas elevated CO_2 led to significantly increased starch grain size (Table 2). Significant $CO_2\times O_3$ interaction was found also in starch grain size (Table 2). Ozone-induced increase in thylakoid injuries was ameliorated by 10% due to elevated CO_2 (Table 4). Precipitation of stroma was not conspicuous in any treatments, whereas cytoplasmic lipids tended to decrease and tannin depositions increase in O_3 ($+CO_2$)-exposed plants (Table 4).

4. Discussion

4.1. Advantages of FACE exposure

Free-air fumigation methodology allows quantitative risk assessment for the long-term reactions of forest trees to increasing greenhouse gases. The present Aspen FACE exposure system, capable of accurate CO_2 and O_3 administration, offers reliable opportunities for examining the influence of these pollutants on forest trees and ecosystems, since artificial disturbance effects have been minimized. Therefore, the present results provide realistic information about morphological responses of aspen and birch to concomitantly increasing CO_2 and O_3.

4.2. Antagonistic responses to CO_2 and O_3

In general, impacts of elevated CO_2 and O_3 on leaf structure were contradictory in aspen. CO_2 tended to increase leaf thickness, chloroplast cover index, amount of intercellular space in mesophyll, amount of starch in

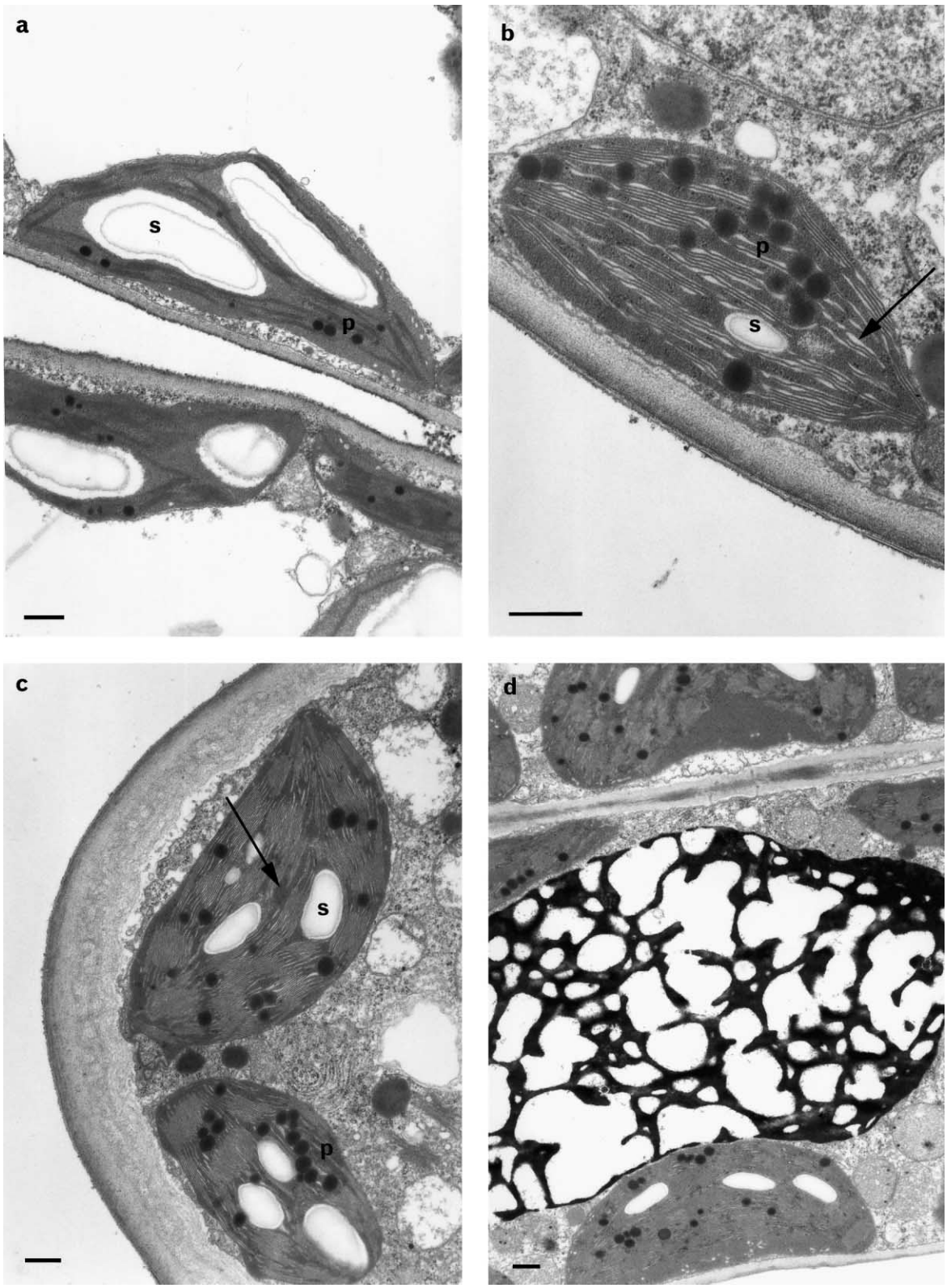

Fig. 1. Transmission electron microscopy of aspen (*Populus tremuloides*), O_3 sensitive clone 259, illustrating O_3-induced chloroplast injuries and changes in leaf mesophyll cells in leaf samples collected on 19 June 1999. Scale bar = 500 nm. p, plastoglobuli; s, starch grain; t, tannin. (a) Intact chloroplast of control plant, showing large starch grains and low number of plastoglobuli. (b) Ozone-induced dilation of chloroplast thylakoids (arrow), accumulation of plastoglobuli, and small starch grain (compared to control chloroplast). (c) Precipitation (darkening) of stroma (arrow). (d) Accumulation of condensed tannin deposition in vacuole of palisade cell.

Table 4
The mean percentage increase/decrease (−) in ultrastructural symptoms, accumulation of cytoplasmic lipids and vacuolar tannins in palisade and spongy mesophyll chloroplasts (combined data for whole mesophyll) in response to elevated CO_2 and O_3 alone and in combination in aspen (*Populus tremuloides*) clones 259, 216 and 271, and birch (*Betula papyrifera*), when compared with control plants[a]

Response	Clone	CO_2	Ozone	CO_2 + ozone
Thylakoid dilation/ distortion	259	nf	51.6	8.0
	216	nf	6.7	1.1
	271	nf	48.7	3.3
	Birch	0.2	43.5	33.3
Precipitation of stroma	259	nf	66.8	0.9
	216	nf	4.3	0.5
	271	nf	49.4	3.1
	Birch	nf	6.9	5.1
Amount of cytoplasmic lipids	259	25.0	0.8	16.7
	216	15.9	0.7	11.6
	271	0.9	0.6	33.3
	Birch	−7.1	−8.2	−9.7
Amount of tannin-like deposition	259	0.1	34.2	32.9
	216	−7.6	19.2	10.5
	271	−18.9	21.1	25.6
	Birch	2.2	31.9	35.4

[a] Samples collected on 19 June 1999 (between 10:00 and 12:00), $n = 3$; nf, symptoms were not found.

chloroplasts but decrease the number of plastoglobuli, whereas O_3 tended to decrease leaf thickness, chloroplast cover index, starch accumulation, and intercellular space but increase the number of plastoglobuli. In birch, however, this opposite action of CO_2 and O_3 was not evident except in leaf thickness. Previously, CO_2-induced increase in leaf thickness and density was suggsted by Luo et al. (1994) and O_3-induced decrease in leaf thickness was reported in a sensitive birch clone by Pääkkönen et al. (1995a). Ozone-increased number of plastoglobuli has been well documented in several tree species, e.g. European birch (Oksanen and Saleem, 1999), beech (*Fagus sylvatica*; Mikkelsen and Heide-Jørgensen, 1996) and conifers (Holopainen et al., 1992), indicating accelerated foliar/needle senescence.

Ozone-induced oxidative structural injuries in chloroplast thylakoids and stroma were reduced by the presence of elevated CO_2 in all plants, and most clearly in aspen clones 259 and 271 (injury reduction up to 66%). In several species, protective action of CO_2 has been explained by a closure of stomata thus limiting uptake of O_3 into the leaves, by increased availability of carbohydrate for repair and defence, by increased availability of detoxifying compounds (especially antioxidants) due to lower photorespiration, and by anatomical changes in the leaf such as increasing thickness and density of mesophyll (e.g. Luo et al., 1994). In chloroplasts, stimulation of chloroplastic FeSOD by elevated CO_2 was suggested to give some protection against oxidative ozone effects in sugar maple, when antioxidant enzymes were considered (Niewiadomska et al., 1999).

4.3. Anatomical leaf characteristics providing O_3 tolerance

Anatomical measurements revealed that in the most O_3-sensitive aspen clone (259) the leaves were 0.6–13% thinner as compared to more tolerant clones in control plants, which was accompanied by 6–9% smaller proportion of cell walls, 15–17% smaller cell surface area in mesophyll tissue, 14–20% thinner cell walls, 6–16% smaller proportion of intercellular air space, but 43–50% higher proportion of vacuoles. This is in accordance with previous results in green ash (*Fraxinus pennsylvanica*), black cherry (*Prunus serotina*; Bennett et al., 1992) and European birch (*Betula pendula*), where O_3 tolerance of different genotypes was related to thicker leaves compared to sensitive trees (Pääkkönen et al., 1997) and a better ability to structural acclimation (Pääkkönen et al., 1995b, 1998). In another study with black cherry, however, an O_3-sensitive genotype had a significantly thinner palisade layer, thicker spongy layer, lower ratio of palisade to spongy tissue, and slightly greater total leaf thickness (Ferdinand et al., 2000).

Lower internal cell surface area and thinner average cell wall thickness in mesophyll was determined for the sensitive aspen clone in the present experiment, suggesting a decreased protection of the plasmalemma against O_3 by the cell apoplast. Although in two differently sensitive poplar clones (*Populus deltoids*×*maximowiczii* and *Populus deltoids*×*euramericana*) cell wall ascorbate and related leaf internal cell wall exposed area and cell wall thickness were not sufficient to explain the differences in O_3 sensitivity (Ranieri et al., 1999), the dominant role of cell wall thickness in the quenching reactive oxygen species has been confirmed by Moldau (1998) and Chameides (1989). It has been estimated that typical cell wall thickness of 0.1–0.5 μm (as found in the present samples) with average (0.6–1.0 mM) ascorbate concentration is able to detoxify only about half of the O_3 entering cell surface, whereas in thicker cell walls direct reaction may result in more complete decomposition of O_3 (Moldau et al., 1997). Thereby, further investigations are needed for these aspen genotypes to determine whether or not the cell wall thickness and related effective reaction pathway with cell wall ascorbate is important factor in O_3 detoxification and sensitivity.

4.4. Vacuolar responses eliciting new questions

The finding of a higher vacuolation state of mesophyll cells in the most sensitive aspen clone (control plants) has not been reported elsewhere. Vacuoles are multifunctional organelles, acting as storage sites for

inorganic ions, sugars, organic acids, amides and amino acids, tannins, lipids, pigments, storage proteins and hydrolytic enzymes creating turgor pressure. Because they are crucial to the processes of detoxification, alleviation of oxidative stress (function of transporters like multidrug resistance-associated proteins in tonoplast) and maintaining homeostasis within the cytoplasm under environmental stress conditions (Marty, 1999), O_3-induced increase in the volume of vacuoles in tolerant aspen clones (216 and 271) may be regarded as a sign of functional and structural acclimation to stress. However, the importance of this leaf characteristic with respect to O_3 sensitivity of aspen remains unclear.

Accumulation of condensed tannins in vacuoles of all O_3-exposed plants was in accordance with the stimulated phenylpropanoid pathway indicated by increased transcription level of PAL, determined during the previous season for these aspen clones (Wustman et al., 2001). Previously, O_3-induced vacuolar tannin-like depositions and phenolic droplets, indicating activated stress defence mechanisms were reported in European birch (*B. pendula*) by Pääkkönen et al. (1998) and Oksanen and Saleem (1999).

4.5. Starch content reflecting down-regulation of photosynthesis?

Decreased starch content in O_3-exposed plants was in accordance with photosynthetic responses, showing greatest decline in carboxylation efficiency and stomatal conductance in O_3 ($+CO_2$)-exposed trees of clone 259 (Sober et al., 2001). CO_2-induced accumulation of starch, as found in aspen clone 271 and birch, may indicate down-regulation of photosynthesis. In several species it as been reported that exposure to high CO_2 leads to accumulation of carbohydrates in leaves (Ray and Jarvis, 1998). Because export of C-compounds is rate-limiting, negative feedback on Rubisco activity and content, photochemical processes, and amount of chlorophyll has been found (Saxe et al., 1998). However, down-regulation seems to be mostly associated with stressed plants, and field-grown trees under realistic conditions rarely have shown any feedback-regulation of photosynthesis (Scarascia-Mugnozza et al., 1996; Curtis, 1996).

5. Conclusions

In this study, a contrasting action of CO_2 and O_3 on leaf structure was observed in leaf thickness, chloroplast cover index, proportion of intercellular space, and amount of starch and plastoglobuli. Significant interactions of CO_2 and O_3 in the above-mentioned parameters were generally antagonistic to O_3 responses. Impacts of O_3 and CO_2 on leaf structure were more pronounced in aspen clones compared to birch. Ultrastructural study also provided evidence that plants grown under elevated CO_2 are better able to limit O_3-caused injuries in chloroplasts. The results suggest that several morphological characteristics may have a role in conferring sensitivity to O_3 for aspen clones, because thinner leaves and cell walls, lower proportion of cell wall volume, and increased vacuolar volume were found for the most sensitive clone.

Acknowledgements

This research was supported by the Academy of Finland (project no. 42702), the USDA Forest Service Global Change Program, the US Department of Energy (DE-FG02-95ER62125 and DE-FG02-93ER6166) and the Brookhaven National Labs/US Department of Energy (725079), the National Science Foundation (DBI-9601942, DBI-9601942, and IBN-9652675), the National Council of Air and Stream Improvement of the Pulp and Paper Industry (NCASI).

References

Barnes, J.D., Pfirrmann, T., Steiner, K., Lütz, C., Busch, U., Küchenhoff, H., Payer, H-D., 1995. Effects of elevated CO_2, elevated O_3 and potassium deficiency on Norway spruce (*Picea abies* (L.) Karst): seasonal changes in photosynthesis and non-structural carbohydrate content. Plant, Cell and Environment 18, 1345–1357.

Bennett, J.P., Rassat, P., Berrang, P., Karnosky, D.F., 1992. Relationships between leaf anatomy and ozone sensitivity of *Fraxinus pennsylvanica* Marsh. and *Prunus serotina* Ehrh. Environmental and Experimental Botany 32, 33–41.

Broadmeadow, M.S.J., Heath, J., Randle, T.J., 1999. Environment limitations to O_3 uptake—some key results from young trees growing at elevated CO_2 concentrations. In: Sheppard, L.J., Cape, J.N. (Eds.), Forest Growth Responses to the Pollution Climate of the 21st Century. Water, Air and Soil Pollution. Kluwer Academic Publishers, pp. 299–310.

Chameides, W.L., 1989. The chemistry of ozone deposition to plant leaves: role of ascorbic acid. Environmental Science Technology 23, 595–600.

Curtis, P.S., 1996. A meta-analysis of leaf gas exchange and nitrogen in trees grown under elevated carbon dioxide. Plant, Cell and Environment 19, 127–137.

Dickson, R.E., Lewin, K.F., Isebrands, J.G., Coleman, M.D., Heilman, W.E., Riemenschneider, D.E., Sober, J., Host, G.E., Zak, D.F., Hendrey, G.R., Pregitzer, K.S., Karnosky, D.F., 2000. Forest Atmosphere Carbon Transfer Storage-II (FACTS II)–The Aspen Free-air CO_2 and O_3 Enrichment (FACE) Project in an Overview (General Tech. Rep. NC-214). USDA Forest Service North Central Research Station.

Ferdinand, J.A., Fredericksen, T.S., Kouterick, K.B., Skelly, J.M., 2000. Leaf morphology and ozone sensitivity of two open pollinated genotypes of black cherry (*Prunus serotina*) seedlings. Environmental Pollution 108, 297–302.

Grams, T.E.E., Anegg, S., Häberle, K.-H., Langebartels, C., Matyssek, R., 1999. Interactions of chronic exposure to elevated CO_2 and O_3 levels in the photosynthetic light and dark reactions of European beech (*Fagus sylvatica*). New Phytologist 144, 95–107.

Holopainen, T., Anttonen, S., Wulff, A., Palomäki, V., Kärenlampi, L., 1992. Comparative evaluation of the effects of gaseous pollutants, acidic deposition and mineral deficiencies: structural changes in the cells of forest plants. Agriculture, Ecosystems and Environment 42, 365–398.

IPCC, 1992. Climate change 1992. In: Houghton, J.T., Callander, B.A., Varney, S.K. (Eds.), The Supplementary Report to the IPCC Scientific Assessment. IPCC, Cambridge University Press, Cambridge.

Isebrands, J.G., McDonald, E.P., Kruger, E., Hendrey, G., Pregitzer, K., Percy, K., Sober, J., Karnosky, D.F., 2001. Growth responses of *Populus tremuloides* clones to interacting carbon dioxide and tropospheric ozone. Environmental Pollution (in press).

Karnosky, D.F., Podila, G.K., Gagnon, Z., Pechter, P., Akkapeddi, A., Sheng, Y., Riemenschneider, D.E., Coleman, M., Dickson, R.E., Isebrands, J.G., 1998. Genetic control of responses to interacting tropospheric ozone and CO_2 in *Populus tremuloides*. Chemosphere 36, 807–812.

Karnosky, D.F., Mankovska, B., Percy, K., Dickson, R.E., Podila, G.K., Sober, J., Noormets, A., Hendrey, G., Coleman, M.D., Kubiske, M., Pregitzer, K.S., Isebrands, J.G., 1999. Effects of tropospheric O_3 on trembling aspen and interaction with CO_2: results from an O_3-gradient and a FACE experiment. In: Sheppard, L.J., Cape, J.N. (Eds.), Forest Growth Responses to the Pollution Climate of the 21st Century. Water, Air and Soil Pollution. Kluwer Academic Publishers, The Netherlands, pp. 311–322.

Keeling, C.D., Whort, T.P., Wahlen, M., van der Plicht, J., 1995. Interannual extremes in the rate of rise of atmospheric carbon dioxide since 1980. Nature 375, 666–670.

Kellomäki, S., Wang, K.-Y., 1997. Effects of elevated O_3 and CO_2 concentrations on photosynthesis and stomatal conductance in Scots pine. Plant, Cell and Environment 20, 995–1006.

Kirschbaum, M.U., 2000. Forest growth and species distribution in a changing climate. Tree Physiology 20, 309–322.

Kull, O., Sober, A., Coleman, M.D., Dickson, R.E., Isebrands, J.G., Gagnon, Z., Karnosky, D.F., 1996. Photosynthetic responses of aspen clones to simultaneous exposures of ozone and CO_2. Canadian Journal of Forest Research 26, 639–648.

Lindner, M., 2000. Developing adaptive forest management strategies to cope with climate change. Tree Physiology 20, 299–307.

Lippert, M., Steiner, K., Pfirrmann, T., Payer, H.-D., 1997. Assessing the impact of elevated CO_2 and O_3 on gas exchange characteristics of differently K supplied clonal Norway spruce trees during exposure and the following season. Trees 11, 306–315.

Loats, K.V., Rebbeck, J., 1999. Interactive effects of ozone and elevated carbon dioxide on the growth and physiology of black cherry, green ash and yellow-poplar seedlings. Environmental Pollution 106, 237–248.

Luo, Y., Field, C.B., Mooney, H.A., 1994. Predicting responses of photosynthesis and root fractions to elevated CO_2: interactions among carbon, nitrogen and growth, theoretical paper. Plant, Cell and Environment 17, 1195–1204.

Matyssek, R., Innes, J.L., 1999. Ozone—a risk factor for trees and forests in Europe? Water, Air and Soil Pollution 116, 199–226.

Marty, F., 1999. Plant vacuoles. Plant Cell 11, 587–599.

Mikkelsen, T.N., Heide-Jørgensen, H.S., 1996. Acceleration of leaf senescence in *Fagus sylvatica* L. by low levels of tropospheric ozone demonstrated by leaf color, chlorophyll fluorescence and chloroplast ultrastructure. Trees 10, 145–156.

Moldau, H., Padu, E., Bichele, I., 1997. Quantification of ozone decay and requirement for ascorbate in *Phaseolus villgaris* L. mesophyll cell walls. Phyton 37, 175–180.

Moldau, H., 1998. Hierarchy of ozone scavenging reactions in the plant cell wall. Physiologia Plantarum 104, 617–622.

Mortensen, L.M., 1995. Effect of carbon dioxide concentration on biomass production and partitioning in *Betula pubescens* Ehrh. seedlings at different ozone and temperature regimes. Environmental Pollution 87, 337–343.

Müller, M., Köhler, B., Grill, D., Guttenberger, H., Lütz, C., 1994. The effect of various soils, different provenances and air pollution on root tip chromosomes in Norway spruce. Trees 9, 73–79.

Niewiadomska, E., Gaucher-Veilleux, C., Chevrier, N., Mauffette, Y., Dizengremel, P., 1999. Elevated CO_2 does not provide protection against ozone considering the activity of several antioxidant enzymes in the leaves of sugar maple. Journal of Plant Physiology 155, 70–77.

Noormets, A., Krishna Podila, G., Karnosky, D.F., 2000. Rapid response of antioxidant enzymes to O_3-induced oxidative stress in *Populus tremuloides* clones varying in O_3 tolerance. Forest Genetics 7, 339–342.

Oksanen, E., Saleem, A., 1999. Ozone exposure results in various carry-over effects and prolonged reduction in biomass in birch (*Betula pendula* Roth). Plant, Cell and Environment 22, 1401–1411.

Pérez-Soba, M., Dueck, T., Puppi, G., Kuiper, J.C., 1995. Interactions of elevated CO_2, NH_3 and O_3 on mycorrhizal infection, gas exchange and N metabolism in saplings of Scots pine. Plant and Soil 176, 107–116.

Pfirrmann, T., 1992. Wechselwirkungen von Ozone, Kohlendioxid und Wassermangel bei zwei Klonen unterschiedlich mit Kalium ernährter Fichten. Giessner Bodenkundliche Abhanglungen, Giessen.

Polle, A., Pfirrmann, T., Chakrabarti, S., Rennenberg, H., 1993. The effects of enhanced ozone and enhanced carbon dioxide concentrations on biomass, pigments and antioxidative enzymes in spruce needles (*Picea abies* L.). Plant, Cell and Environment 16, 311–316.

Polle, A., Pell, E., 1999. Role of carbon dioxide in modifying the plant response to ozone. In: Luo, Y., Mooney, H.A. (Eds.), Carbon Dioxide and Environmental Stress. Academic Press, ISBN 0-12-460370-X, pp. 193–213.

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., 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., Holopainen, T., Kärenlampi, L., 1997. Variation in ozone sensitivity of *Betula pendula* and *Betula pubescens* clones from southern and central Finland. Environmental Pollution 95, 37–44.

Pääkkönen, E., Günthardt-Goerg, M., Holopainen, T., 1998. Responses of leaf processes in a sensitive birch (*Betula pendula* Roth) clone to ozone combined with drought. Annals of Botany 82, 49–59.

Ranieri, A., Castagna, A., Padu, E., Moldau, H., Rahi, M., Soldatini, G.F., 1999. The decay of O_3 through direct reaction with cell wall ascorbate is not sufficient to explain the different degrees of O_3-sensitivity in two poplar clones. Journal of Plant Physiology 154, 250–255.

Ray, A., Jarvis, P.G., 1998. Long-term photosynthetic acclimation to increased atmospheric CO_2 concentration in young birch (*Betula pendula*) trees. Tree Physiology 18, 441–450.

Reilly, J., Prinn, R., Harnisch, J., Fitzmaurice, J., Jacoby, H., Kicklighter, D., Mellilo, J., Stone, P., Sokolov, A., Wang, C., 1999. Multi-gas assessment of the Kyoto Protocol. Nature 401, 549–555.

Saxe, H., Ellsworth, D.S., Heath, J., 1998. Tansley review: tree and forest functioning in an enriched CO_2 atmosphere. New Phytologist 139, 395–436.

Scarascia-Mugnozza, G., De Angelis, P., Matteucci, G., Valentina, R., 1996. Long-term exposure to elevated CO_2 in a natural *Quercus ilex* L. community: net photosynthesis and photochemical efficiency of PS II at different levels of water stress. Plant, Cell and Environment 19, 643–654.

Sehmer, L., Fontaine, V., Antoni, F., Dizengremel, P., 1998. Effects of ozone and elevated atmospheric carbon dioxide on carbohydrate metabolism of spruce needles. Catabolic and detoxification pathways. Physiologia Plantarum 102, 605–611.

Sheng, Y., Podila, G.K., Karnosky, D.F., 1997. Differences in O_3-induced SOD and glutathione antioxidant expression in tolerant and sensitive aspen (*Populus tremuloides* Mixch.) clones. Forest Genetics 4, 25–33.

Sober, A., Noormets, A., Kull, O., Isebrands, J.G., Dickson, R. E., Sober, J., Karnosky, D.F., 2001. Photosynthetic parameters in aspen grown with interacting elevated CO_2 and tropospheric ozone concentrations as affected by leaf nitrogen. Tree Physiology (in press).

Utriainen, J., Janhunen, S., Helmisaari, H.-S., Holopainen, T., 2000. Biomass allocation, needle structural characteristics and nutrient composition in Scots pine seedlings exposed to elevated CO_2 and O_3 concentrations. Trees 14, 475–484.

Volin, J.C., Reich, P.B., 1996. Interaction of elevated CO_2 and O_3 on growth, photosynthesis and respiration of three perennial species grown in low and high nitrogen. Physiologia Plantarum 97, 674–684.

Volin, J.C., Reich, P.B., Givnish, T.J., 1998. Elevated carbon dioxide ameliorates the effects of ozone on photosynthesis and growth: species respond similarly regardless of pathway or plant functional group. New Phytologist 138, 315–325.

Wuebbles, D.J., Jain, A., Edmonds, J., Harvey, D., Hayhoe, K., 1999. Global change: state of the science. Environmental Pollution 100, 57–86.

Wustman, B.A., Oksanen, E., Karnosky, D.F., Sober, J., Isebrands, J.G., Hendrey, G.R., Pregitzer, K.S., Podila, G.K., 2001. Effects of elevated CO_2 and O_3 on aspen clones varying in O_3 sensitivity: can CO_2 ameliorate the harmful effects of O_3? Environmental Pollution (in press).