Ozone-induced H_2O_2 accumulation in field-grown aspen and birch is linked to foliar ultrastructure and peroxisomal activity

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Summary

• Saplings of three aspen (*Populus tremuloides*) genotypes and seedlings of paper birch (*Betula papyrifera*) were exposed to elevated ozone (1.5× ambient) and 560 p.p.m. CO_2, singly and in combination, from 1998 at the Aspen-FACE (free-air CO_2 enrichment) site (Rhinelander, USA).

• The plants were studied for H_2O_2 accumulation within the leaf mesophyll, number of peroxisomes, level of gene expression for catalase (*Cat*), and changes in ultrastructure.

• In tolerant clones, ozone-elicited excess H_2O_2 production was restricted to the apoplast, without any ultrastructural injuries. This was associated with ozone-induced proliferation of peroxisomes and increased transcript levels of *Cat*. In sensitive plants, ozone-induced H_2O_2 accumulation continued from the cell wall to the plasma membrane, cytosol and chloroplasts, particularly in older leaves. However, chloroplastic precipitation was absent in the presence of elevated CO_2. In the most sensitive aspen clone, H_2O_2 accumulation was found in conjunction with chloroplast injuries, low number of peroxisomes and low cell wall volume, whereas in birch a simultaneous increase in cell wall thickness indicated defence activation.

• Our results indicate that oxidative stress manifests as H_2O_2 effects on leaf ultrastructure in sensitive trees exposed to elevated ozone. However, CO_2 enrichment appears to alleviate chloroplastic oxidative stress.

Key words: ozone, carbon dioxide, aspen (*Populus tremuloides*), paper birch (*Betula papyrifera*), oxidative stress, H_2O_2, peroxisomes, catalase.

Introduction

Global atmospheric ozone concentrations have risen about 36% since the preindustrial times (IPCC, 2001), together with nearly 30% increase in CO_2 concentrations over the same period (Prather *et al.*, 2001). A continuous 1–2% annual increase in ozone level has been predicted in scenarios of global change by Intergovernmental Panel on Climate Change (IPCC, 2001). About 25% of the global forests are currently at risk from damaging ozone concentrations (> 60 ppb) during the growing seasons, and this is predicted to further expand to 50% by year 2100 (Fowler *et al.*, 1999).

Ozone is one of the most powerful oxidants known. In plants, primary damage is largely confined to the leaf mesophyll, where ozone dissolves into the wet surface of the exposed cell walls (Kangasjärvi *et al.*, 1994). Reactions of ozone with water and solutes in the apoplasm lead to the formation of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroperoxide ($\cdot O_2H$), superoxide ($\cdot O_2^-$), hydroxyl radicals (OH^-) and singlet oxygen (Foyer *et al.*, 1994; Kangasjärvi *et al.*, 1994; Wohlgemuth *et al.*, 2002). It is generally assumed that damage by ozone is mainly the result of ROS formation, promoting oxygen toxicity in the plant cell (Podila *et al.*, 2001). Unsaturated lipids and proteins in the

apoplasm and plasmalemma are the obvious targets for ozone and ROS (Podila *et al.*, 2001). Both H_2O_2 and $\cdot O_2H$ can cause lipid peroxidation, whereas OH^- has the potential to attack all components of plasmalemma (Foyer *et al.*, 1994).

Reactive oxygen species are continuously produced during normal physiological processes, such as light reactions of photosynthesis (Foyer *et al.*, 1994; Foyer, 1996). Under nonperturbed conditions, a steady-state equilibrium prevails between the formation of ROS and their scavenging through nonenzymatic antioxidants (mainly ascorbic acid, glutathione, carotenoids, flavonoids and phenolic compounds) and enzymatic antioxidants, such as superoxide dismutase, catalase and peroxidases (Noctor & Foyer, 1998; Podila *et al.*, 2001). In principle, each cellular organelle has mechanisms to prevent or minimize damage from ROS (Podila *et al.*, 2001). Under environmental stress (such as ozone, UV light or extreme temperatures), the production of excess H_2O_2 has been observed in chloroplasts, mitochondria, peroxisomes, plasma membrane and in the apoplast (Vanacker *et al.*, 1998; Pellinen *et al.*, 1999). Thereafter, excess H_2O_2 can be converted to water and molecular oxygen spontaneously or by catalase, or detoxified through action of various peroxidases or ascorbate-glutathione pathway (Podila *et al.*, 2001). Catalase is found mainly in peroxisomes (and in glyoxysomes), which therefore play a significant role in defence against ozone stress (del Rio *et al.*, 2002). Although the protective action of catalase is limited to peroxisomes, very high turnover rates allow fast responses to excess H_2O_2 (Willekens *et al.*, 1997).

In addition to ROS formation as degradation products, plant cells respond quickly to ozone stress by induced ROS production (Schraudner *et al.*, 1998). It is assumed that this ROS induction serves as a general alarm signal leading to modifications in gene expression and metabolism under stress (López-Huertas *et al.*, 2000; Podila *et al.*, 2001). In particular, H_2O_2 seems to function as a primary signal molecule in a transduction pathway that includes protein phosphorylation under oxidative stress (Kovtun *et al.*, 2000) leading to cell death and expression of defence genes (Pellinen *et al.*, 2002; Wohlgemuth *et al.*, 2002).

Free-air fumigations are novel methods for approaching a quantitative risk assessment of the chronic ozone and CO_2 exposure of adult trees and forest stands (Karnosky *et al.*, 2001). In the Aspen free-air CO_2 enrichment (FACE) site (Rhinelander, WI, USA), elevated ozone and CO_2, singly and in combination, have been found to affect growth and productivity, gas exchange, foliar gene expression and biochemistry, ultrastructure of leaves, quality of leaves and wood, leaf surface properties and pest performance in aspen, leading to disturbances in ecosystem balance (Oksanen *et al.*, 2001; Percy *et al.*, 2002; Karnosky *et al.*, 2003). Elevated CO_2 concentrations increased the growth of aspen, while ozone completely offset the growth enhancement by CO_2 (Karnosky *et al.*, 2003). In the sensitive aspen clone 259, lower average antioxidant activities, lower constitutive levels of defence-related genes (phenylalanine ammonia-lyase (PAL), chalcone synthase), thinner leaves and cell walls, a lower proportion of cell wall volume and higher volume of vacuoles have been reported, compared with more tolerant clones (Oksanen *et al.*, 2001; Wustman *et al.*, 2001). However, detailed studies on location and severity of oxidative stress within leaf mesophyll cells have not been performed on these trees.

In this paper we examine (1) the extent that aspen and birch leaves exposed to elevated ozone and/or CO_2 in the field show signs of oxidative stress (H_2O_2 accumulation) within the mesophyll cells, and (2) whether or not H_2O_2 accumulation is connected to structural damage and increase in detoxifying peroxisomes. (3) Furthermore, we examine the link between ozone-induced proliferation of peroxisomes and transcript levels of catalase which converts H_2O_2 to water and molecular oxygen.

Materials and Methods

Aspen FACE site

The Aspen FACE site is located within a fenced 32-ha USDA Forest Service Harshaw Research Farm near Rhinelander, WI, USA (Karnosky *et al.*, 1999; Dickson *et al.*, 2000). The experimental site contains 12 individual treatment rings (30 m in diameter), which are spaced 100 m apart to minimize between-ring drift of ozone and CO_2. The study is a full-factorial design with three control rings (ambient ozone and CO_2), three elevated CO_2 rings (target 560 p.p.m., 200 ppm above ambient), three elevated ozone rings (1.5× ambient), and three CO_2 + ozone rings. Trees were not exposed to ozone during cold weather (when the maximum temperatures were projected to be less than 15°C), rain or when the leaves were wet with dew. Mean ozone concentrations and AOT40 exposures (accumulated over the threshold of 40 p.p.b.) for the growing season 2001 from budbreak to sampling are given in Table 1. Detailed information on the experimental design, gas generation, and monitoring can be found in Dickson *et al.* (2000) and at http://aspenface.mtu.edu.

Each exposure ring is divided into east and west halves, and the latter is further subdivided into north and south quadrants (see http://aspenface.mtu.edu/ring_maps.htm). Aspen (*Populus tremuloides* Michx.) clones (numbered 8L, 42E, 216, 259, and 271) were planted at 1 × 1 m spacing as randomized pairs within the eastern half of each ring so that the clonal position within a ring is unique. The north-west quadrant was planted with alternating sugar maple (*Acer saccharum* Marsh.) and aspen clone 216, while the south-west quadrant was planted with paper birch (*Betula papyrifera* Marsh.) and aspen clone 216. The aspen clones were known to differ in ozone sensitivity: 8L, 216 and 271 were relatively tolerant genotypes, while 42E and 259 were relatively sensitive (Karnosky *et al.*, 2003). The rings were planted during the summer of 1997, and the plants were exposed over each growing season from May 1998 to different ozone and/or CO_2 treatments from aspen budbreak to leaf fall.

Table 1 Average 24-h ozone concentrations and AOT40 exposures for control (ambient air) trees and elevated ozone trees from 11 May until the end of August 2001

Treatment	Replicate	May (21 d) Mean (p.p.b.)	AOT40 (p.p.m.h)	June Mean (p.p.b.)	AOT40 (p.p.m.h)	July Mean (p.p.b.)	AOT40 (p.p.m.h)	August Mean (p.p.b.)	AOT40 (p.p.m.h)
Ambient		41 ±11	1.67	41 ±12	0.80	37 ±11	1.85	34 ±10	0.89
O_3	1	55 ±22	4.76	59 ±24	6.85	60 ±25	8.63	49 ±21	5.19
$O_3 + CO_2$	1	56 ±21	4.97	61 ±24	7.33	61 ±22	8.81	51 ±20	5.41
O_3	2	54 ±20	4.27	62 ±23	7.34	61 ±24	8.66	50 ±20	5.20
$O_3 + CO_2$	2	56 ±21	4.85	60 ±23	6.86	63 ±31	9.31	48 ±26	5.00
O_3	3	47 ±31	3.06	63 ±32	7.54	62 ±29	9.53	53 ±38	0.04
$O_3 + CO_2$	3	57 ±24	5.24	61 ±23	7.16	60 ±23	8.36	51 ±25	5.77

Replicate rings: 1, northern; 2, central; 3, southern. AOT40, accumulated over the threshold of 40 ppb.

Light and transmission electron microscopy

Leaf samples were collected on 3 August 2001 between 11.00 hours and 14.00 hours from aspen clones 216, 259 and 271, and paper birch from all experiment rings (three rings per treatment, $n = 3$). The youngest fully expanded leaf from branch tip (from upper third of canopy) and one old short-shoot leaf (from branch in the middle third of the canopy) from southern side of each selected tree (two trees per ring per clone per treatment) were sampled. All the leaves sampled were partly shaded. Thereafter, leaf sections for transmission electron microscopy were cut in the laboratory between the second and third leaf vein, followed by cutting into 1.5 mm^2 square pieces. To localize subcellular accumulation of H_2O_2, half of the cut leaf pieces were incubated under vacuum with 5 mM $CeCl_3$ solution (in 0.1 M phosphate buffer, pH 7.0) for 1 h before prefixation. During incubation Ce^{3+} ions react with H_2O_2 forming electron dense cerium perhydroxide precipitates in reaction: $H_2O_2 + CeCl_3 \rightarrow Ce(OH_2)OOH$ (Bestwick *et al.*, 1997). The $CeCl_3$ treated samples and control samples (without $CeCl_3$ incubation) were prefixed with 2.5% (v : v) glutaraldehyde solution (in 0.1 M phosphate buffer, pH 7.0) at +4°C overnight. Thereafter, the samples were postfixed in 1% buffered OsO_4 solution, dehydrated with an ethanol series followed by a propylene-oxide treatment, and embedded in LX 122 Epon (Ladd Research Industries, Burlington, NC, USA). The thin sections for electron microscopy were stained with lead citrate and uranyl acetate, and were studied with an electron microscope JEOL 1200 EX (JEOL, Tokyo, Japan) operating at 80 kV. The sections for light microscopy were stained with aqueous Toluidine blue, and studied with a Nikon MicroPhot-FXA microscope (Nikon, Tokyo, Japan). The light microscopy samples were measured for total leaf thickness and palisade and spongy layer thickness from digital micrographs using Adobe Photoshop version 5.0 (Adobe Systems, San Jose, CA, USA) program. Thin sections were photographed with Bioscan camera (Gatan, Pleasanton, CA, USA) connected to electron microscope using Digital Micrograph program (Gatan, Pleasanton, CA, USA) for further image analyses. The samples were analysed for sectional area of chloroplasts and starch grains, cell wall thickness in mesophyll cells, number of peroxisomes per cell section area, and localization of H_2O_2 accumulation from 10 cells per tissue per sample (1920 cells in total; 3 rings × 4 treatments × 4 clones × 2 leaves × 2 tissues × 10 cell) with Adobe Photoshop program. To confirm that the precipitation was derived from H_2O_2, ozone-exposed samples from all aspen clones and birch were also infiltrated with H_2O_2-destroying catalase (100 U ml^{-1}) before fixation, as described in Pellinen *et al.* (1999).

Gene expression for catalase

The remaining young leaves (from two replicate fields) were frozen in liquid nitrogen and used for RNA gel blot analysis to reveal expression of catalase (CAT). RNA was extracted from 1–2 g of frozen leaves with the procedure described in detail in Wustman *et al.* (2001). Total RNA (10 μg per lane) was fractionated and transferred onto positively charged nylon membrane (Boehringer Mannheim, Germany). Equal loading of RNA was verified by methylene blue staining. Membranes were fixed by UV-crosslinking, and prehybridized in DIG EasyHyb hybridization buffer (Boehringer Mannheim) for 3 h at 48°C and hybridized overnight with DIG-labelled double stranded DNA probes for *Cat* [AJ302710], which was generated by polymerase chain reaction (PCR) (95°C, 1 min; 55–59°C, 1 min; 72°C, 1 min; for 30 cycles) using 1 unit of DNA-polymerase (DynaZyme, Finnzymes, Finland), 5 μl dNTP labelling Mix (0.1 mM digoxigenin-labelled dUTP; Boehringer Mannheim), 2.5 mM $MgCl_2$, 30 pmol degenerate *Cat* primers and 0.1 μg of corresponding cDNA template. DIG Luminescent Detection Kit (Boehringer Mannheim) was used for detection. Chemiluminescent substrate (CSPD, Boehringer Mannheim, Germany) was pipetted onto the membrane, which was autoradiographed at room temperature. The relative band densities were measured with the Adobe Photoshop program and the band densities were indicated as percentage of the maximal intensity on the film.

Statistics

The experimental design was a randomized complete block design with four treatments in three replicate rings. The exposure ring was the experimental unit for all statistical testing ($n = 3$). Analysis of variance (ANOVA) using general linear models procedure (SPSS 10.0; SPSS Chicago, IL, USA) was used to test the main and interactive effects of ozone, CO_2, clone and leaf age. Thereafter, the significance of treatments within each clone/species was calculated with Tukey's multiple range test. Before analyses, the data were checked for normality and homogeneity of variance, and when necessary the values were transformed to satisfy the assumptions of ANOVA. For nonparametric relative proportions (H_2O_2 accumulation and transcript level of *Cat*), Kruskall–Wallis *H*-test was used. Differences were considered significant at $P \leq 0.05$.

Results

Ozone exposure

Table 1 shows that the critical ozone level for trees, AOT40 of 10 ppm h, was exceeded in all ozone exposure rings by in July, and that the average AOT40 exposure for the sampled trees (3 August) was 20.6 p.p.m.h. The maximum hourly ozone concentrations in elevated ozone (+ CO_2) rings were 83 p.p.b. in May, 90 p.p.b. in June, 110 p.p.b. in July and 104 p.p.b. in August, suggesting that oxidative stress was very likely in exposed trees.

H_2O_2 accumulation

No visible accumulation of H_2O_2 was found in any leaves collected from control trees or elevated CO_2 trees, or in catalase-treated samples, verifying that all cerium perhydroxide precipitation in leaf tissue was derived from H_2O_2. Ozone-induced H_2O_2 accumulation was found in all aspen clones and birch regardless of CO_2 treatment (no significant difference between these treatments when 480 cells/clone or species/treatment were scored) (Table 2). In ozone and CO_2 + ozone plants, H_2O_2 accumulation was more evident in the older leaves: in aspen 15–39% of the young leaf cells showed H_2O_2 accumulation, whereas the number was 22–60% for older leaf cells (significant difference, $P = 0.031$, when the data were pooled for ozone treatments, tissues and clones; Table 2). The corresponding percentages for birch were 30% and 57%, respectively (no significant difference, $P = 0.057$, when the data were pooled for ozone treatments and tissues). For both leaf ages, palisade parenchyma displayed a 1–11% higher number of H_2O_2 accumulated cells in aspen compared with spongy parenchyma (significant difference, $P = 0.032$, when the data were pooled for ozone treatments), whereas in birch there were no significant differences between these tissues (Table 2).

Table 2 Variation in H_2O_2 accumulation between palisade and spongy mesophyll tissues, and between young and old leaves in ozone tolerant aspen (*Populus tremuloides*) clones 216 and 271, and in sensitive aspen clone 259 and birch

Clone	Tissue	Ozone		CO_2 + ozone	
		Young	Old	Young	Old
216	Palisade	21 ±5	32 ±8	20 ±3	33 ±5
	Spongy	15 ±3	30 ±4	17 ±4	29 ±4
259	Palisade	39 ±6	60 ±7	39 ±4	57 ±6
	Spongy	32 ±7	51 ±5	37 ±5	53 ±4
271	Palisade	20 ±3	25 ±6	19 ±4	33 ±4
	Spongy	16 ±5	22 ±4	17 ±4	22 ±4
Birch	Palisade	30 ±6	59 ±5	31 ±6	58 ±6
	Spongy	29 ±5	55 ±7	28 ±5	54 ±5

Values are percentage numbers (±SD) of cells showing H_2O_2 precipitation, when 120 cells/treatment/clone or species/leaf/tissue were examined.

In the tolerant aspen clones 216 and 271, H_2O_2 precipitation was located only on the outer hydrophobic surface of the cell wall facing the intercellular space (Fig. 1a), which may indicate efficient decomposition of ozone before reaching the plasma membrane. In the sensitive aspen clone 259, however, accumulation was found mainly in the inner side of cell wall adjacent to plasma membrane (Fig. 1b), whereas in birch, the precipitation extended throughout the cell wall. In aspen clone 259 and birch, H_2O_2 accumulation continued from the plasma membrane to the cytoplasm and adjacent chloroplasts (Fig. 1c), and damage in plasma membrane was apparent. In chloroplasts, however, accumulation was found only in older, ozone-exposed leaves, not in the presence of elevated CO_2. Peroxisomal H_2O_2 accumulation was found in all ozone-exposed leaves both in aspen and birch (Fig. 1d), indicating excess H_2O_2 production in addition to normal cellular processes (including photorespiration, β-oxidation of fatty acids, glyoxylate metabolism and detoxification of reactive oxygen species).

Gene expression for catalase

Transcript levels for *Cat* were significantly increased under ozone in ozone-tolerant clones 216 and 271, while more-sensitive clone 259 and birch were unaffected (Fig. 2). Elevated CO_2 concentrations completely offset the ozone-induced *Cat* transcript increase in aspen clones 216 and 271 (Fig. 2).

Ultrastructure

For the structural study of aspen clones, the effects of treatment were significant for total leaf thickness, size of chloroplast and starch grain, cell wall thickness and number of peroxisomes (Table 3). There were significant differences among aspen clones in total leaf thickness, palisade to spongy

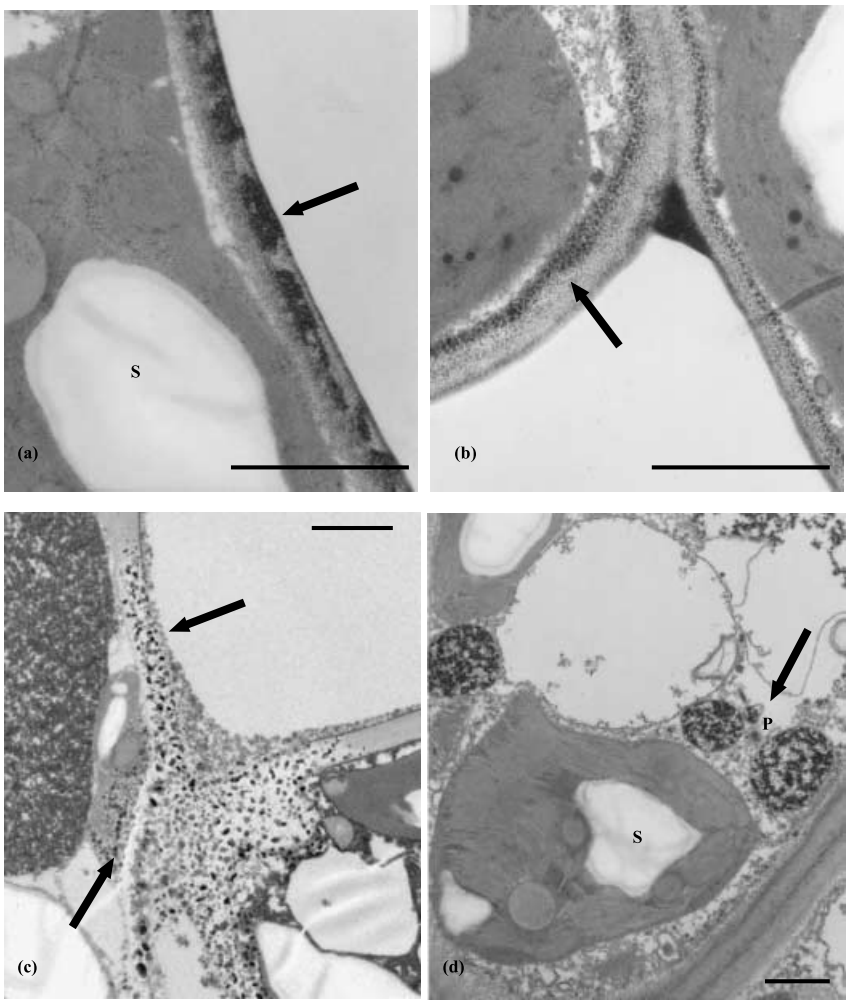

Fig. 1 Ozone-induced accumulation of H_2O_2 within the mesophyll cells of aspen (*Populus tremuloides*) clones is shown here as electron dense cerium perhydroxide precipitates (arrows). (a) H_2O_2 formation in external side of cell walls in ozone-tolerant clone 216 with intact chloroplast; (b) H_2O_2 precipitation in cytoplasmic side of apoplast in ozone-sensitive clone 259 extending to cytosol and adjacent chloroplasts through plasma membrane (c); (d) proliferation and increased staining of peroxisomes for clone 271. S, starch grain; P, peroxisome. Bars, 1 μm.

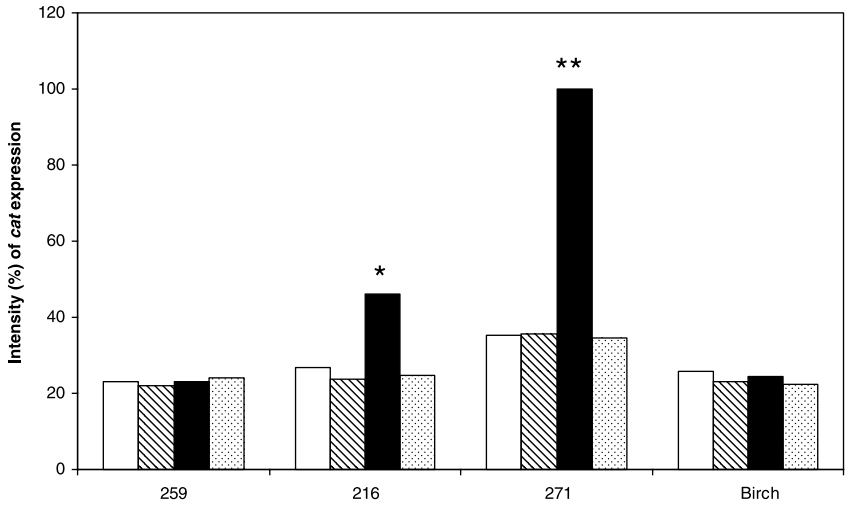

Fig. 2 The effect of ozone, CO_2 and CO_2 + ozone on relative transcript levels of catalase in aspen (*Populus tremuloides*) clones and birch (*Betula papyrifera*). Maximum density of the band = 100. Values are means + SD. Open bars, control plants; hatched bars, elevated CO_2; closed bars, elevated ozone; dotted bars, elevated CO_2 + ozone. Multivariate ANOVA (n = 2), Tukey's multiple range test, $P < 0.05$.

layer ratio, size of chloroplast and starch grain, and number of peroxisomes (Table 3). The most ozone-sensitive clone, 259, showed 8–16% thinner mesophyll cell walls and an 11–19% lower proportion of palisade to spongy mesophyll layer compared with more-tolerant clones, 216 and 271, suggesting lower cell wall volume and less efficient photosynthesis per leaf area (Table 4). Significant treatment–clone interaction was found in size of chloroplasts, cell wall thickness and

Table 3 ANOVA results for effects of treatment and clone in aspen (*Populus tremuloides*) ultrastructural parameters

Source of variation	Dependent variable	Type III sum of squares	df	Mean square	*F*	*P*
Treatment	Leaf thickness	5543	3	1848	5.513	0.003**
	Palisade to spongy ratio	0.194	3	0.065	1.264	0.295
	Size of chloroplast	93	3	31	7.031	0.000***
	Size of starch grain	17	3	6	6.522	0.000***
	Cell wall thickness	210832	3	70277	3.839	0.010*
	Number of peroxisomes	2405	3	802	816.4	0.000***
Clone	Leaf thickness	7730	2	3865	11.534	0.000***
	Palisade to spongy ratio	0.988	2	0.494	9.640	0.000***
	Size of chloroplast	101	2	50	11.389	0.000***
	Size of starch grain	15	2	7	8.463	0.000***
	Cell wall thickness	38870	2	19435	1.062	0.347
	Number of peroxisomes	924.0	2	462	313.7	0.000***
Treatment × Clone	Leaf thickness	4189	6	689	2.083	0.073
	Palisade to spongy ratio	0.130	6	0.022	0.421	0.862
	Size of chloroplast	69	6	12	2.619	0.017*
	Size of starch grain	6	6	1	1.223	0.294
	Cell wall thickness	369493	6	61582	3.364	0.003**
	Number of peroxisomes	484	6	81	54.8	0.000***

*, **, ***, indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Table 4 Main effects of ozone and/or CO_2 on structure of ozone-tolerant aspen (*Populus tremuloides*) clones 216 and 271, sensitive aspen clone 259 and birch (*Betula papyrifera*)

Parameter	Clone	Control	CO_2	Ozone	CO_2 + ozone
Leaf thickness (μm)	216	136 ±16a	157 ±12ab	158 ±21b	169 ±13b
	259	164 ±25a	178 ±27a	149 ±149a	188 ±27a
	271	146 ±20a	152 ±17a	133 ±10a	146 ±11a
	Pooled	149 ±23ab	163 ±22ab	147 ±21a	168 ±25b
	Birch	166 ±18a	173 ±27a	150 ±22a	171 ±16a
Palisade to spongy ratio	216	1.38 ±0.17a	1.54 ±0.44a	1.41 ±0.23a	1.48 ±0.17a
	259	1.23 ±0.23a	1.23 ±0.25a	1.10 ±0.20a	1.20 ±0.22a
	271	1.51 ±0.19a	1.48 ±0.11a	1.30 ±0.06a	1.38 ±0.22a
	Pooled	1.37 ±0.22a	1.41 ±0.32a	1.27 ±0.22a	1.36 ±0.22a
	Birch	0.63 ±0.08a	0.71 ±0.07a	0.69 ±0.11a	0.71 ±0.09a
Size of chloroplast (μm^2)	216	5.6 ±1.8a	7.1 ±2.7b	5.6 ±1.3a	5.5 ±1.7a
	259	7.7 ±2.3b	7.4 ±2.2b	5.2 ±2.5a	2.3 ±2.1a
	271	7.9 ±1.4a	7.4 ±2.2a	6.9 ±2.3a	7.4 ±2.2a
	Pooled	7.1 ±2.1b	7.3 ±2.4b	5.9 ±2.2a	6.3 ±2.2ab
	Birch	8.4 ±3.1a	7.6 ±2.0a	8.5 ±2.1a	7.7 ±1.9a
Size of starch grain (μm^2)	216	0.3 ±0.1a	1.1 ±0.2b	0.4 ±0.2a	1.0 ±0.7b
	259	0.8 ±0.3a	1.4 ±0.7b	0.9 ±0.1a	1.4 ±0.9b
	271	0.6 ±0.2a	0.5 ±0.1a	0.5 ±0.1a	0.9 ±0.3a
	Pooled	0.5 ±0.3a	1.0 ±0.3b	0.6 ±0.3ab	1.1 ±0.8a
	Birch	3.1 ±2.2a	2.1 ±1.1a	2.6 ±2.0a	2.8 ±1.7a
Cell wall thickness (nm)	216	307 ±84ab	281 ±93a	399 ±207b	358 ±166ab
	259	283 ±113a	358 ±192a	291 ±106a	316 ±144a
	271	337 ±108b	248 ±64a	377 ±109b	391 ±156b
	Pooled	308 ±103ab	296 ±135a	355 ±154b	355 ±157b
	Birch	353 ±81a	363 ±74a	447 ±207b	357 ±88ab
Number of peroxisomes	216	9.0 ±1.2a	8.2 ±1.2a	13.9 ±1.3b	11.9 ±1.4a
	259	8.1 ±1.0a	9.0 ±1.2a	11.0 ±1.1a	11.7 ±1.3a
	271	10.0 ±1.5a	9.5 ±0.9a	19.0 ±1.3b	15.2 ±1.7a
	Pooled	9.0 ±1.4a	8.9 ±1.2a	14.6 ±3.5b	12.9 ±1.8a
	Birch	8.0 ±1.1a	8.8 ±1.1a	12.8 ±0.9a	11.8 ±1.2a

Values are means ±SD. Multivariate ANOVA ($n = 3$), Tukey's multiple range test. Means followed by different letters are significantly different, $P < 0.05$.

Table 5 Significant differences in ozone and/or CO_2 caused changes in ultrastructure of aspen (*Populus tremuloides*) (tolerant clones 216 and 271) between young and old leaves and between palisade and spongy tissue (tissues 1 and 2, respectively)

Parameter	Leaf	Tissue	Control	CO_2	Ozone	CO_2 + ozone
Aspen clone 216						
Size of chloroplast (μm^2)	Young	Palisade	6.6 ±1.1a	8.1 ±1.7a	6.4 ±1.4a	6.1 ±2.7a
	Young	Spongy	6.3 ±2.0a	4.9 ±1.2a	5.6 ±0.8a	5.2 ±1.0a
	Old	Palisade	4.4 ±1.8a	10.4 ±2.1b	5.4 ±1.0a	6.0 ±1.4a
	Old	Spongy	4.9 ±1.4a	5.1 ±1.1a	5.1 ±1.9a	4.7 ±1.5a
Aspen clone 271						
Cell wall thickness (nm)	Young	Palisade	249 ±32a	242 ±71a	375 ±104a	340 ±162a
	Young	Spongy	435 ±55b	277 ±54a	362 ±70ab	417 ±151ab
	Old	Palisade	270 ±78ab	210 ±75a	416 ±151b	324 ±123ab
	Old	Spongy	381 ±124ab	262 ±48a	354 ±117ab	484 ±167b

Values are means ±SD. Multivariate ANOVA (n = 3), Tukey's multiple range test. Means followed by different letters are significantly different, $P < 0.05$.

number of peroxisomes (Table 3). In most cases, there were no significant differences in leaf age or mesophyll tissue in response to treatments, except for significant treatment–leaf age and treatment–tissue interaction in size of chloroplast in clone 216 (P = 0.011), and treatment–tissue interaction in cell wall thickness in clone 271 (P = 0.017). In these cases, data are given separately for young and old leaves, and for palisade and spongy mesophyll (Table 5). In birch, the effect of treatment was significant in size of starch grain (P = 0.043) and cell wall thickness (P = 0.012).

The main effects of treatments on aspen and birch are given in Table 4 (data pooled for young and old leaves, and both mesophyll tissues). Enrichment of CO_2 significantly increased the size of chloroplasts (clone 216) and starch grains (clones 216 and 259), and decreased the mesophyll cell wall thickness (clone 271) (Table 4). Elevated ozone concentrations significantly increased the total leaf thickness (clone 216), decreased the size of chloroplasts (clone 259), increased the cell wall thickness (birch), and increased the number of peroxisomes (clones 216 and 271), indicating proliferation of the peroxisomal population under ozone stress. Combined action of CO_2 + ozone led to significantly thicker leaves (clone 216), smaller chloroplasts (clone 259), and increased size of starch grains (clones 216 and 259) (Table 4). Ozone-induced structural injury in thylakoid membranes (dilation and distortion, as described previously in Oksanen *et al.*, 2001) was found only in older leaves of aspen clone 259 (data not shown). Table 5 indicates that, in aspen clone 216, the increasing effect of elevated CO_2 on chloroplast size was significantly stronger in older leaves and palisade tissue compared with young leaves and spongy tissue. In aspen clone 271, the decreases in cell wall thickness were greater in young leaves and spongy tissues under elevated CO_2 (Table 5).

Discussion

In this study, we were able to visualize and locate ozone-induced H_2O_2 accumulation within leaf mesophyll cells, and relate oxidative stress with structural injuries in aspen and birch. In addition, increased transcript levels for catalase were demonstrated to be related to ozone-induced proliferation of peroxisomes. In tolerant aspen clones 216 and 271, H_2O_2 precipitation was restricted to the apoplasm, and no structural injuries could be found within the cells. In ozone-sensitive aspen clone 259, cytoplasmic and chloroplastic H_2O_2 precipitation was accompanied by reduced chloroplast size and increased thylakoid injuries in ozone-exposed leaves, and by 23–27% thinner mesophyll cell walls, 15–22% lower palisade to spongy ratio and 21–42% fewer peroxisomes compared with more tolerant clones. In birch, however, ozone-elicited H_2O_2 accumulation within cells was observed in conjunction with reduced starch grain and increased thickness of cell walls, indicating increased defence against ozone stress. Previously, H_2O_2 accumulation was reported in cell walls, plasma membranes, mitochondria, peroxisomes and cytosol of ozone-exposed birch (*Betula pendula*) leaves by Pellinen *et al.* (1999), and in apoplast of drought-stressed leaves of *Cistus clusii* and *Cistus albidus* by Munné-Bosch *et al.* (2003).

We could not distinguish the H_2O_2 formation from ozone degradation (taking place within the apoplast) from the endogenous active H_2O_2 production by the cells, because the samples were collected from field-growing trees during the exposures, and no inhibitors for ROS generation were used. However, plasma membrane NADPH-dependent superoxide synthase and cell wall peroxidases have been reported to be the most likely sources for active apoplastic H_2O_2 generation under ozone stress in birch (Pellinen *et al.*, 1999). Although it is unlikely that ozone is able to cross the plasmalemma because of to its high reactivity with cell wall antioxidants, phenolic and olefinic compounds, proteins and lipids (Podila *et al.*, 2001), Moldau (1998) and Chameides (1989) have calculated that the direct reaction between ozone and cell wall ascorbate (average concentration of 0.5 mM) in cell walls 300–500 nm thick is able to detoxify only 50–70% of the ozone entering the exposed cell wall surface. Therefore, we

assume that in the mesophyll cells (cell wall thickness between 291 nm and 447 nm) ozone and the ROS that it formed had the potential to react with the plasmalemma at high ozone attack sites in ozone exposed leaves of sensitive plants. Considering that H_2O_2 is rather stable, not very reactive and electrically neutral, we suggest that H_2O_2 had the potential to pass through cell membranes at oxidative burst sites and reach the neighbouring chloroplasts. Oxidative damage in plasmalemma by ozone and ROS is further supported by Jakob & Heber (1998) who reported the inability of even high apoplastic ascorbate to detoxify oxidative radical in ozone exposed leaves.

The number of peroxisomes was increased by 26–47% in aspen clones under elevated ozone and by 38% in birch, suggesting an ozone-induced proliferation of peroxisomes. This may have been induced by peroxisome biogenesis genes (*PEX*) and signalled through the H_2O_2 molecule (López-Huertas *et al.*, 2000), as reported in plants exposed to other stress factors, such as cadmium (Romero-Puertas *et al.*, 1999; del Rio *et al.*, 2002). Proliferation was most evident in the tolerant aspen clones 216 and 271, suggesting improved detoxifying capacity under ozone stress. The increased number of peroxisomes (containing mainly catalase enzyme) under ozone exposure is also in accordance with increased *Cat* gene expression and with our previous study, where ozone-induced increase was found in enzymatic activity of catalase, especially in aspen clone 271 (Wustman *et al.*, 2001). Ozone-induced increases in catalase gene expression were reported in birch by Pellinen *et al.* (2002). Peroxisomes, and particularly catalase contained therein, have been shown to be important sinks for the bulk of the H_2O_2 from other subcellular compartments by Willekens *et al.* (1997), who also reported that susceptibility to ozone increased in catalase-deficient tobacco plants. In addition to catalase, several other antioxidative systems were found to be located in plant peroxisomes, including different superoxide dismutases and the ascorbate–glutathione cycle (del Rio *et al.*, 2002). Moreover, peroxisomes have been proposed to have signal-producing functions through generating messenger molecules such as H_2O_2 and $\cdot O_2^-$, and releasing them into the cytosol (del Rio *et al.*, 2002). Recently, pathogen-induced accumulation of H_2O_2 in mesophyll cell walls of barley (*Hordeum vulgare*) was reported to be connected to increases in glutathione and catalase activities (Vanacker *et al.*, 2000).

In chloroplasts of trees in our study, H_2O_2 accumulation was found only in ozone-exposed leaves and not in the presence of elevated CO_2. This may be partly explained by increased rate of photosynthesis in the presence of elevated CO_2 (Noormets *et al.*, 2001), leading to higher NADPH concentrations and increased activity of enzymatic detoxification (e.g. ascorbate–glutathione cycle through NADPH-dependent glutathione reductase; Podila *et al.*, 2001). However, counteracting effects of elevated CO_2 on chloroplastic H_2O_2 accumulation, as well as on peroxisomal proliferation and *Cat* transcription may also be related to CO_2-induced decrease in photorespiration (as a result of an increase in the CO_2 to O_2 ratio) (Eamus & Ceulemans, 2001), leading to lower production of toxic H_2O_2 therein. Therefore, CO_2 enrichment might increase scavenging capacity by releasing the resources for peroxisomal antioxidant defence against overproduction of H_2O_2 during oxidative stress. Similarly, Willekens *et al.* (1997) reported that photorespiration was required for photooxidation-induced lesion formation in catalase-deficient tobacco, and damage was prevented under elevated CO_2 concentrations. Beneficial effects of elevated CO_2 concentrations on chloroplast and starch grain size and on leaf and palisade layer thickness in the present experiment are in accordance with our previous study (Oksanen *et al.*, 2001) and are consistent with the increased assimilation rate (Noormets *et al.*, 2001).

In ozone-exposed trees, decreased photosynthesis (Noormets *et al.*, 2001; Karnosky *et al.*, 2003) might have resulted in excess light excitation energy (over-reduction of photosystem II) and accumulation of ROS in chloroplasts, leading to thylakoid membrane deterioration (Foyer, 1996). Furthermore, ozone-induced decreases in carotenoid pigments, as reported for these aspen clones by Wustman *et al.* (2001), may have predisposed the chloroplasts to excess ROS and peroxidation of membrane lipids, because carotenoids have an important role as membrane stabilizers and free radical scavengers by intercepting the chlorophyll triplet state (Demming-Adams & Adams, 1996; Havaux, 1998). Increased accumulation of excess H_2O_2 in chloroplasts of older leaves exposed to ozone is consistent with the study by Munné-Bosch and Alegre (2002), which reported that oxidative stress in chloroplasts of *Cistus clusii* increases progressively during leaf ageing. Therefore, it is likely that the detoxification capacity of chloroplasts was exceeded in the aged leaves of our trees.

In conclusion, this study demonstrated (1) that sensitive trees in the field are impacted by O_3 exposure, (2) that photosynthesizing palisade cells and older leaves had the most excess H_2O_2 formation, (3) that peroxisomal activity, mesophyll cell wall thickness and palisade to spongy layer ratio are closely related to ozone tolerance of aspen, and (4) that CO_2 enrichment seems to ameliorate the impact of oxidative stress within the chloroplasts.

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