Environmental Pollution 158 (2010) 1029–1035

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Leaf size and surface characteristics of *Betula papyrifera* exposed to elevated CO_2 and O_3

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The combined effects of CO_2 and O_3 on birch leaf surface characteristics cannot be predicted on the basis of studies examining each of these gases separately.

ARTICLE INFO

Article history:
Received 20 July 2009
Accepted 26 July 2009

Keywords:
Betula papyrifera
Carbon dioxide
Epicuticular wax
Epidermal cell
FACE
Leaf size
Ozone
Stomata
Trichome

ABSTRACT

Betula papyrifera trees were exposed to elevated concentrations of CO_2 (1.4 × ambient), O_3 (1.2 × ambient) or $CO_2 + O_3$ at the Aspen Free-air CO_2 Enrichment Experiment. The treatment effects on leaf surface characteristics were studied after nine years of tree exposure. CO_2 and O_3 increased epidermal cell size and reduced epidermal cell density but leaf size was not altered. Stomatal density remained unaffected, but stomatal index increased under elevated CO_2. Cuticular ridges and epicuticular wax crystallites were less evident under CO_2 and $CO_2 + O_3$. The increase in amorphous deposits, particularly under $CO_2 + O_3$, was associated with the appearance of elongated plate crystallites in stomatal chambers. Increased proportions of alkyl esters resulted from increased esterification of fatty acids and alcohols under elevated $CO_2 + O_3$. The combination of elevated CO_2 and O_3 resulted in different responses than expected under exposure to CO_2 or O_3 alone.

1. Introduction

Concentrations of atmospheric carbon dioxide (CO_2) and ozone (O_3) are currently increasing by 0.5–2% per year (Intergovernmental Panel on Climate Change (IPCC), 2001; Vingarzan, 2004). The leaf surface is the first point of contact between air pollutants and the plant. Gaseous pollutants enter the leaf primarily through the stomata, and have the potential to change the plant metabolic processes. Leaf size is determined by cell production and expansion, which are controlled in a coordinated manner during leaf organogenesis (Tsukaya, 2006). Leaf growth is often enhanced following exposure to elevated CO_2 (van Volkenburgh and Taylor, 1996; Taylor et al., 2003; Riikonen et al., 2004). Elevated CO_2 may influence both cell production and expansion (Ferris et al., 2001; Taylor et al., 2003; Tricker et al., 2004). In *Populus* spp., the enhanced cell expansion was associated with increased cell-wall extensibility and increased activity of the cell-wall loosening enzyme, xyloglucan endotransglycosylase (Ferris et al., 2001). It has been suggested that elevated CO_2 may affect the cell cycle by enhancing the carbohydrate status of the leaves (Kinsman et al., 1997). In several experiments with forest tree species, leaf size was reduced by elevated O_3 (Günthardt-Goerg et al., 1993; van Volkenburgh and Taylor, 1996; Riikonen et al., 2004). The O_3-induced decrease in photosynthesis and acceleration of leaf senescence decrease the supply and translocation of assimilates for formation of new leaves (Dizengremel, 2001; Oksanen, 2003; Riikonen et al., 2004). In *P. trichocarpa*, leaf expansion was initially reduced by elevated O_3 and then increased at the end of the growing season (Gardner et al., 2005). Increased leaf expansion was associated with stimulation of cell-wall elasticity and plasticity, and the authors concluded that O_3 may act directly on the cell-wall polysaccharides, resulting in altered cell-wall properties and leaf growth.

According to studies with herbarium leaves collected over the past 200 years, stomatal density (SD) and index (SI) have been decreasing with increasing CO_2 concentration (Woodward and Bazzaz, 1988). In field studies with broadleaved trees, the CO_2 response has been variable, showing no alteration (Vanhatalo et al., 2001;

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doi:10.1016/j.envpol.2009.07.034

Herrick et al., 2004; Maňkovská et al., 2005; Mcelrone et al., 2005; Riikonen et al., 2008b) or a decrease (Rey and Jarvis, 1997; Bettarini et al., 1998; Paoletti et al., 1998; Ferris et al., 2002) in SD. Stomatal density is determined by stomatal initiation during ontogenesis and by epidermal cell expansion (Woodward, 1987; Radoglou and Jarvis, 1990). In silver birch (*Betula pendula*) and *Fraxinus ornus*, SD decreased under elevated CO_2 as a consequence of an increase in leaf expansion, as SI was not altered (Rey and Jarvis, 1997; Bettarini et al., 1998). In *Populus* clones, the reduction in SD was accompanied by decreased SI in young leaves in the upper canopy, suggesting a change in stomatal initiation (Ceulemans et al., 1995; Ferris et al., 2002). In broadleaved trees, SD has either increased (Günthardt-Goerg et al., 1993; Pääkkönen et al., 1995; Frey et al., 1996), slightly decreased (Oksanen, 2003) or not been affected (Maňkovská et al., 2005; Riikonen et al., 2008b) by elevated O_3. In the case of O_3 exposure studies, SI has been seldom calculated, and thus it is difficult to assess whether the change in SD is caused by an altered stomatal initiation or by change in leaf growth.

Ozone has been identified as affecting inward K^+ channels in guard cells (Torestaugen et al., 1999). However, there is not much information on the effects of CO_2 or O_3 on guard-cell length. According to Aasamaa et al. (2001), the small size of stomata enables a rapid variation in stomatal conductance. In *B. pubescens*, stomatal size was increased, whereas SD was not affected by elevated CO_2 after 4 years of open-top chamber (OTC) exposure (Vanhatalo et al., 2001). Stomatal density of *Quercus ilex* growing by a naturally CO_2-enriched spring was reduced but stomatal size was not (Paoletti et al., 1998). In experiments with silver birch, elevated O_3 either reduced (Prozherina et al., 2003), did not alter (Frey et al., 1996) or increased the length of guard cells (Riikonen et al., 2008b).

Glandular trichomes are thought to be connected to production of pest- or pollinator-interactive chemicals (*e.g.*, terpenes and essential oils) (Wagner, 1991), reduction of heat load (Ehleringer et al., 1976), prevention of water loss (Körner, 1999) and UV radiation filtration (Kostina et al., 2001). In silver birch, the glandular trichomes have been found to be induced by defoliation (Valkama et al., 2005), O_3 and spring-time frost (Prozherina et al., 2003). Higher trichome density was recorded in *Q. ilex* growing around a CO_2 spring (Paoletti et al., 2007), but otherwise no information is available on the possible effects of CO_2 on trichome formation.

The production, chemical composition and structure of epicuticular waxes have been found to be sensitive to both elevated CO_2 and O_3. Elevated CO_2 and O_3 have increased wax production, modified wax structure, and altered wax chemical composition, leading to stomatal occlusion by wax deposits in several forest tree species (Barnes et al., 1988; Paoletti et al., 1998; Percy et al., 1994, 2002; Turunen et al., 1997; Vanhatalo et al., 2001; Karnosky et al., 2002; Maňkovská et al., 2005). However, the independent effects of CO_2 and O_3 on wax production and composition were attenuated in the combination treatment in the aspen (*Populus tremuloides* Michx.) clones growing at the Aspen Free-air CO_2 Enrichment (FACE) Experiment (Percy et al., 2002). These changes have been connected to changes in plant quality and to phylloplane resistance to insect and disease populations (Karnosky et al., 2002; Percy et al., 2002).

In the present study, we examined paper birch (*Betula papyrifera* Marsh.) growing at Aspen FACE (www.aspenface.mtu.edu) and exposed to elevated CO_2, O_3 or elevated $CO_2 + O_3$. The hypotheses tested were: 1. Elevated CO_2 increases and O_3 reduces the mean leaf size, which is connected to alterations in the density and size of epidermal cells; 2. elevated CO_2 decreases and O_3 increases SD by affecting leaf growth or stomatal initiation during ontogenesis; 3. elevated CO_2 has no effect and O_3 increases trichome density either by decreasing cell growth or by increasing trichome initiation during ontogenesis; 4. elevated CO_2 and O_3 alter epicuticular wax biosynthesis, causing changes in wax chemical composition that result in modified leaf surface wax; 5. The interaction of elevated CO_2 and O_3 is offsetting for leaf surface properties.

2. Materials and methods

2.1. Aspen FACE site and plant material

The Aspen FACE facility is located near Rhinelander, WI, USA (45.6° N, 89.5° W). In 1997, rooted cuttings of aspen (five clones), and seedlings of paper birch and sugar maple (*Acer saccharum* Marsh.) were planted in 12 30-m diameter treatment rings, each representing either control (ambient CO_2 and O_3), elevated CO_2, elevated O_3 or a combination of elevated CO_2 and O_3. The present study used maturing birch trees originating from seeds collected in an open-pollinated natural stand in Houghton County, Michigan (Dickson et al., 2000). The experiment is a full-factorial design with three replicates of each treatment, blocked across northern, central, and southern regions of the site. Fumigation began in the spring of 1998 and has continued during daylight hours for nine growing seasons through 2006, from bud break until leaf fall.

Carbon dioxide was fumigated daily according to sun angle (*ca.* 30 min post-sunrise to pre-sunset) during the growing season. In 2006, CO_2 concentration averaged 377 ppm in the three control rings, and 533 ppm (1.4 × ambient) in the six elevated CO_2 rings (3 CO_2, 3 $CO_2 + O_3$). Ozone was fumigated using the same protocol, except when leaf surfaces were wet or ambient maximum temperature was below 15 °C (Karnosky et al., 2007). In 2006, ambient growing season mean O_3 concentration was 37.1 ppb and averaged 44.7 ppb (1.2 × ambient) in the six elevated O_3 rings (3 O_3, 3 $CO_2 + O_3$). More details on the field site, experimental design and performance are available elsewhere (Dickson et al., 2000; Karnosky et al., 2003, 2005, 2007).

2.2. Light microscopy

For microscopy, four light-exposed short-shoot leaves per tree (four trees per ring) were collected from south-facing branches in the middle third of the living canopy, during 16–25 August 2006. For light microscopy (Zeiss, Axioplan, Oberkochen, Germany; camera attachment, Kodak DCS 460), a rectangular section (5 × 10 mm) was dissected between the second and third lateral veins from the leaf base of each leaf. In birch the stomata are located on the abaxial surfaces of the leaves, and thus fifteen randomly chosen microscopic fields of the abaxial surface of each leaf section were sampled (4.7 mm^2 per tree, 12 trees per treatment) under 400× magnification. Stomatal density (number of stomata per leaf area) and length of the guard cells (on average of 180 guard cells per tree) were determined using ImageJ 1.37v. The rest of the leaves were air dried for scanning electron microscopy (SEM).

2.3. Scanning electron microscopy

Of the four leaves from each tree used for light microscopy, two were randomly selected, and a rectangular section was cut from them on the opposite side of the leaf blade than that used for light microscopy. These leaf segments were sputter-coated (Polaron E 5100, Polaron Equipment Ltd., Watford, UK) with 48 nm of gold and examined under a scanning electron microscope (XL30 ESEM TMP, FEI Company, Netherlands). Three randomly chosen fields on the abaxial leaf surface were viewed under magnifications of 50× (trichome density, 14 mm^2 per tree), 200× (epidermal cell size; percent coverage of trichomes, veins, stomata and epidermal cells; occurrence of elongated plate crystallites in stomatal chambers) [number of stomata with stomatal plate crystallite deposits, % of total number of stomata]; and 500× (structural condition of epicuticular wax in inter-stomatal areas, 0.14 mm^2 per tree). The micrographs were analyzed using ImageJ 1.37v.

Approximately 65 cells from each tree were measured for epidermal cell size. The point-counting method (Romppanen and Collan, 1984) was used to calculate the percent coverage of the trichomes, veins, stomata, and epidermal cells in the microscopic fields. The percent coverage was then used to determine the epidermal cell size in each leaf. The leaf area, and the mean size and coverage area of the epidermal cells in each leaf were used to calculate the epidermal cell density per leaf. To study the relationship between co-occurring changes in SD and epidermal cells, SI was calculated: $[SD/(e + SD)] \times 100$, where e is epidermal cell density. The trichome index was calculated as follows: $[t/(e + t)] \times 100$, where t is trichome density.

Seven classes based on wax morphology were defined for determining structural condition of epicuticular wax in inter-stomatal areas. The classes were: 1) intact wax crystallites; 2) combination of classes 1 and 3; 3) crystallites less prominent, rounded and structure less defined; 4) combination of classes 3 and 5; 5) crystallites covered by amorphous wax film; 6) combination of classes 5 and 7; 7) wax crystalline structure absent. After the analysis, the silhouettes of all leaves were drawn and scanned, and the leaf area was calculated using Adobe Photoshop Elements 2.0. The pixels in the measured areas were transformed to μm^2 based on the calibration line of each picture. The specific leaf area (SLA) was calculated as leaf area per dry weight ($cm^2\ g^{-1}$).

2.4. Epicuticular wax chemical composition

Recently fully expanded and physiologically similar leaves were collected from full-sun, main lateral shoots. Five leaves from each of five trees were pooled for analysis. Epicuticular waxes (EW) were recovered on site by $CHCl_3$ rinsing, using a glass syringe. Solvent/wax solution was filtered, solvent evaporated, and EW weighed to 10 μg and expressed as μg cm^{-2} leaf area. Quantitative wax chemical composition was determined using a Varian 3410 GC (Varian Associates Inc., Sunnyvale, CA) equipped with a flame ionization detector (FID) (Percy et al., 1994). Retention times were determined on a DB1-HT capillary column (15 m; 0.32 mm i.d.) with methyl silicone liquid phase (0.1 μm film thickness). Carrier gas (He) flow was 4.5 mL min^{-1}. Column programming was: 70–120 °C at 20 °C min^{-1}; 121 °C–390 °C at 6 °C min^{-1}. The septum programmable injector was programmed at: 75 °C–125 °C at 18 °C min^{-1}; 126–395 °C at 12 °C min^{-1}; hold 15 min. The FID was operated at 400 °C.

Wax samples were silylated with N,O-bis (tri-methylsilyl) acetamide (TMS) at 50 °C for 30 min. Varian Workstar software programming was used to integrate peak areas and calculate homologue percentages. Relative retention times (RRT) were calculated against the retention time (RT) for the dominant octadecyl docosanoate (C_{40}) alkyl ester homologue. Assignments were based on series of injected pure compounds including: alkyl esters (ex P.J. Holloway, UK), alkanes (C_{10}–C_{60}; Supelco), free fatty acids (hexadecanoic, octadecanoic, hexacosanoic, octacosanoic and dotricontanoic acids; Sigma) and primary alcohols (hexacosanol, octacosanol; Sigma). The FID response factors were calculated as means for each EW class from repeated (5% total counts) injections ($n = 10$) of pure reference homologues.

Final confirmation of homologue assignments was completed using a Hewlett–Packard 5989 GC–MS (Palo Alto, CA, USA). A DB1-HT column (30 m; 0.25 mm ID; 0.1 μm film thickness) was temperature programmed from 70 °C to 120 °C at 12 °C^{-1}; 120 °C–390 °C at 6 °C^{-1} (hold 10 min). Helium carrier gas flow rate was 1.0 mL^{-1} (constant flow). Injection was on-column at 250 °C. Electron ionization (EI) mass spectra were searched against Wiley and/or NIST libraries. Ion source and quadruple temperatures were 275 °C and 100 °C, respectively.

2.5. Statistical analysis

The experimental design is a randomized complete block design with three replicates (FACE rings) per treatment arrayed across the site. Block was considered a random effect. Main effects and interaction of CO_2 and O_3 were tested using SPSS 14.0 (SPSS Chicago, IL, USA) for Windows linear mixed model analysis of variance (ANOVA). Statistical analyses were performed using ring means (mean of four birches). Pairwise comparisons were performed for interpreting the significant interaction between CO_2 and O_3. The normality of the data and homogeneity of variances were checked from residual plots. Data were ln-transformed to meet ANOVA requirements if necessary.

3. Results

3.1. Leaf size and epidermal cell characteristics

Mean epidermal cell size was 15, 8, and 7% higher in elevated CO_2, O_3 and $CO_2 + O_3$ treatments than in controls, respectively (Tables 1 and 2). The CO_2 effect on epidermal cell size was stronger under ambient O_3 concentration, as revealed by a significant interaction of elevated CO_2 and O_3 (Table 2). Significant interaction of elevated CO_2 and O_3 (Table 2) showed that elevated CO_2 and O_3 applied singly reduced epidermal cell density by 10 and 14%, respectively, whereas under $CO_2 + O_3$ this was not observed (Table 1). Mean leaf size was not significantly affected by the treatments, although the leaves tended to be smaller in elevated O_3 (Tables 1 and 2). Elevated CO_2 increased SI, but SD remained unaffected (Tables 1 and 2). SLA, length of the guard cells and trichome density and index were not significantly affected by the treatments (Tables 1 and 2).

Table 1
Mean values (±1 SE, $n = 3$) for epidermal characteristics and leaf area in birch short-shoot leaves exposed to elevated CO_2, O_3 or $CO_2 + O_3$.

	Control	CO_2	O_3	$CO_2 + O_3$
Stomatal density, mm^{-2}	124.5 ± 0.7	119.4 ± 1.9	115.2 ± 3.5	123.9 ± 5.2
Stomatal index	7.5 ± 0.07	8.2 ± 0.22	7.5 ± 0.23	7.9 ± 0.39
Guard-cell length, μm	43.0 ± 1.3	42.4 ± 1.2	43.7 ± 0.7	41.1 ± 1.2
Epidermal cell size, μm^2	647.4 ± 9.8	746.9 ± 9.7	701.2 ± 5.6	695.8 ± 8.1
Epidermal cell density per leaf, $\times 10^6$	2.8 ± 0.12	2.5 ± 0.14	2.4 ± 0.02	2.8 ± 0.18
Mean leaf size, cm^2	22.7 ± 1.0	22.3 ± 0.8	20.3 ± 0.2	21.9 ± 0.8
Trichome density, mm^{-2}	11.1 ± 1.2	9.2 ± 0.2	11.2 ± 0.8	10.8 ± 0.7
Trichome index	0.87 ± 0.10	0.83 ± 0.01	0.95 ± 0.07	0.93 ± 0.07
Specific leaf area, $cm^2\ g^{-1}$	115.7 ± 4.2	108.4 ± 9.6	110.1 ± 2.8	110.9 ± 4.9

Table 2
P-values for the main effects and interaction of CO_2 and O_3 on paper birch leaf surface structures. Data were analyzed using mixed model ANOVA. Replication (block) was considered as a random effect. Significant treatment effects and interactions ($P < 0.05$) are shown in bold.

	CO_2	O_3	$CO_2 \times O_3$
Stomatal density	0.559	0.447	0.070
Stomatal index	**0.048**	0.633	0.579
Guard-cell length	0.530	0.823	0.422
Epidermal cell density	0.709	0.690	**0.041**
Epidermal cell size	**0.001**	0.879	**0.000**
Mean leaf size	0.396	0.078	0.168
Trichome density	0.083	0.160	0.211
Trichome index	0.441	0.063	0.792
Specific leaf area	0.553	0.777	0.471
Wax structure	**0.000**	0.097	**0.017**
Stomatal crystalline deposits	**0.048**	0.258	0.184

3.2. Epicuticular wax structure

Under ambient air (control), epidermal wax structure on the abaxial leaf surface of paper birch comprised cuticular ridges most evident around stomata and irregular epicuticular crystalline structures surmounting the epidermal cells (Figs. 1A and 2). The crystalline structures were embedded in an amorphous wax deposit (Fig. 1A). Under CO_2 exposure, cuticular ridges adjacent to stomata were barely visible beneath a film of amorphous EW that conformed to epidermal cell shape. Crystalline wax structure was almost completely absent (Figs. 1C and 2, Table 2). Modifications to EW structure observed under CO_2 exposure were much more pronounced under elevated $CO_2 + O_3$ (Figs. 1D and 2, Table 2). Larger, thicker amorphous films obscured epidermal cell outlines and no crystalline structure was evident (Fig. 1D). Elevated CO_2 and $CO_2 + O_3$ caused elongated plate crystallites in some stomatal chambers (Figs. 1D and 2, Table 2).

Almost all (99%) EW constituents were found in only four classes. Alkyl esters (C_{32}–C_{50} homologues; even chains dominant; C_{42} major homologue) predominated, comprising 58.7% of the EW on control leaves (Fig. 3). Alkanes (C_{20}–C_{36}; odd chains dominant; hentriacontane major homologue) comprised 27.0% of the EW deposit on control leaves. Fatty acids (C_{16}–C_{32}; even chains dominant; triacontanoic acid major homologue) comprised 8.1% of the EW deposit on control leaves. Primary alcohols (C_{22}–C_{30}; even chains only; triacosanol major homologue) comprised 6.2% of the EW deposit on control leaves. Minor constituents, comprising about 1% of recovered EW, included the pentacyclic triterpenoid, ursolic acid.

Amounts of alkyl esters in leaf EW tended to increase relative to the control (58.7%) when *B. papyrifera* was exposed to CO_2 (66.0%), O_3 (63.2%) or $CO_2 + O_3$ (68.2%) (Fig. 3). However, the difference due to treatment was not statistically significant ($P = 0.237$). There was no difference ($P = 0.492$) due to treatment in proportion of alkanes recovered in EW. The proportion of fatty acids in *B. papyrifera* EW was affected by treatment ($P = 0.003$). Relative to the control (8.1%), fatty acids comprised 5.2% (CO_2), 9.9% (O_3) and 3.04% ($CO_2 + O_3$) of the EW deposit. The proportion of primary alcohols in *B. papyrifera* EW was also significantly affected by treatment ($P = 0.038$). Relative to the control (6.2%), primary alcohols comprised 4.8% (CO_2), 5.6% (O_3), and 3.0% ($CO_2 + O_3$) of the EW deposit (Fig. 3). Exposure to $CO_2 + O_3$ significantly reduced the proportion of both fatty acids and primary alcohols in *B. papyrifera* leaf epicuticular wax.

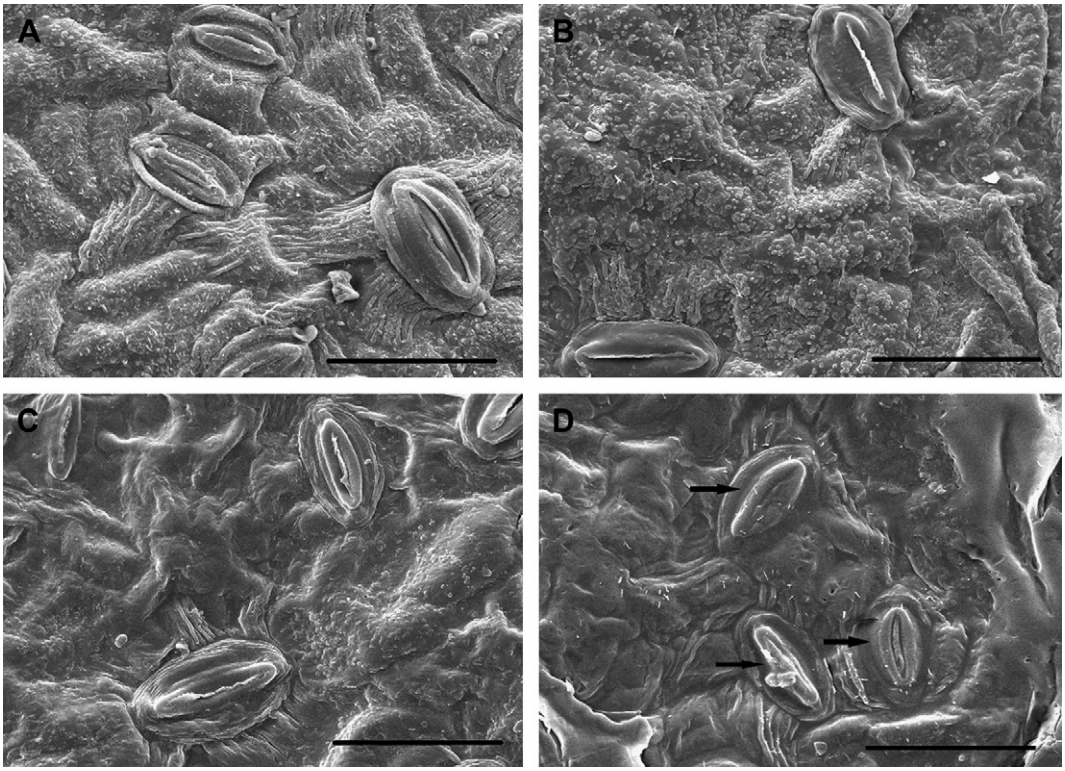

Fig. 1. Effect of treatment on epicuticular wax structure on the abaxial surfaces of *Betula papyrifera* leaves. (A) Class 1: Intact wax crystallites, control; (B) Class 3: Wax crystallites less prominent and structure less defined, elevated O_3; (C) Class 5: Wax crystallites covered by an amorphous wax film, elevated CO_2; (D) Class 7: Crystalline structure absent, elevated $CO_2 + O_3$. Arrows in the figure D indicate stomata with plate crystallite deposits. Bars, 50 μm. The classes based on wax morphology are described in the Materials and methods.

4. Discussion

At time of sampling for epidermal characteristics and epicuticular waxes, leaf area index of the closed canopy birch–aspen stands was still increased under elevated CO_2 compared with controls (Riikonen et al., 2008a). However, elevated CO_2 did not increase the mean leaf size in the present study, nor in the leaves collected the previous autumn (Riikonen et al., 2008a). Thus, in birch, elevated CO_2 must have increased leaf area index by enhancing leaf production. Cell division and expansion are controlled by a complex network of factors, including plant hormones, in response to environmental cues (Nishimura et al., 2004; Tsukaya, 2006; Savaldi-Goldstein et al., 2007). Taylor et al. (2003) suggested that the CO_2-induced effects on the cell cycle are likely to involve an interaction between plant hormones and cell carbon status. Tricker et al. (2004) found that increased leaf size was connected with increased epidermal cell expansion and production in poplar, but

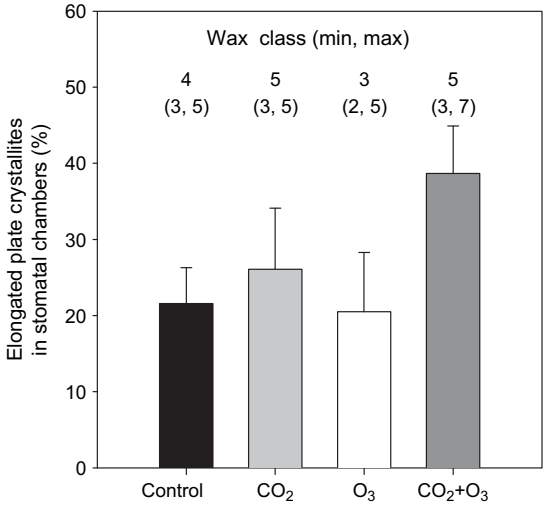

Fig. 2. Stomata with elongated plate crystallites in stomatal chambers (expressed as a percentage of total number of stomata) and structural condition of wax in the interstomatal areas on the abaxial surfaces of birch leaves exposed to elevated CO_2, O_3 or $CO_2 + O_3$ at the Aspen FACE site. Seven wax classes were scored for (see Fig. 1A–D). Data are means ± 1 SE. Median (min, max) values are also shown.

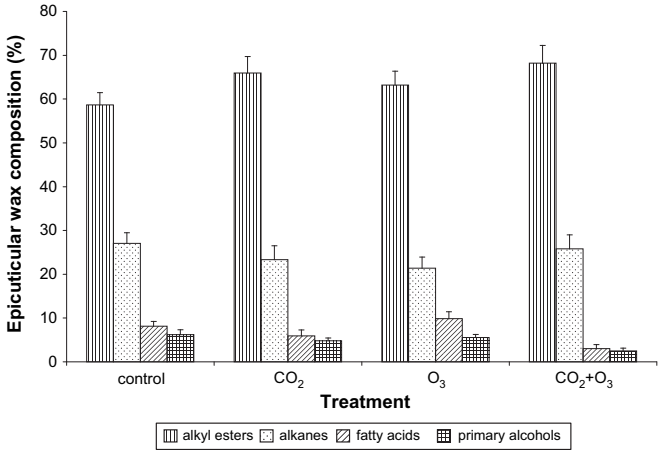

Fig. 3. Effect of elevated CO_2, O_3 and $CO_2 + O_3$ on *Betula papyrifera* epicuticular wax chemical composition. Data are means ± 1 SE of major (99%) epicuticular wax classes.

the CO_2 effect diminished over three growing seasons as the canopy closed. In contrast, in our study, both elevated CO_2 and O_3 reduced epidermal cell density and increased the epidermal cell size. The separate effects from single exposure on epidermal cell density and size were diminished in the combination treatment; i.e., elevated CO_2 reduced epidermal cell density and increased the epidermal cell size more under ambient O_3 concentration. It is known that the morphogenetic controls of cell number and cell size interact during leaf organogenesis, and that in some cases, decreased cell proliferation triggers compensation in cell enlargement (Tsukaya, 2006). Under elevated CO_2, reduced epidermal cell density was apparently compensated for by enhanced epidermal cell size. The current study suggests that epidermal cell density and size were not related to carbohydrate production, as elevated CO_2 significantly increased (49%) and O_3 decreased (27%) the photosynthetic C gain (Riikonen et al., 2008a) but epidermal cell responses were similar in both treatments.

In some O_3 studies with birch, SD was increased in association with reduced leaf growth under elevated O_3 (Günthardt-Goerg et al., 1993; Pääkkönen et al., 1995; Frey et al., 1996), which may result in a more even O_3 distribution within the leaf tissue that facilitates more efficient detoxification processes (Pääkkönen et al., 1995; Hetherington and Woodward, 2003). In most long-term experiments, such an increase in SD has not been detected (Maňkovská et al., 2005; Oksanen, 2003; Riikonen et al., 2008b), which is in accordance with the current study.

A general view is that there is a trend toward lower SD and SI in elevated CO_2 (Hetherington and Woodward, 2003; Paoletti and Grulke, 2005). In the current experiment, elevated CO_2 did not significantly reduce SD. In fact, SD remained fairly stable despite fewer and larger epidermal cells, and consequently, SI was increased under elevated CO_2. Similar CO_2-responses were found in an experiment with *Zea mays* (Driscoll et al., 2006). According to a recent review by Casson and Gray (2008), elevated CO_2 could affect SD and SI by affecting cell cycle machinery, including the number of initial divisions of meristemoid mother cell (stomatal entry), or spacing and amplifying divisions. However, the signal transduction pathway is still largely unknown (Casson and Gray, 2008). There is evidence suggesting that alterations in wax composition and accumulation can affect stomatal development, possibly by modulating the perception of diverse environmental signals (Holroyd et al., 2002; Casson and Gray, 2008).

It is uncommon in northern tree species for the alkyl ester class to be so dominant, and the early successional northern hardwood species *B. papyrifera* (58% alkyl ester; 27% alkanes; 8% fatty acids; 6% primary alcohols) along with its cohort *P. tremuloides* (Percy et al., 2002) can be distinguished in this way. In *Fagus sylvatica*, primary alcohols are dominant (50%), with alkyl esters comprising only 25%, fatty acids 10%, and hydrocarbons 9% (Gülz et al., 1992). In *Quercus robur*, primary alcohols also dominate (38%) with alkyl esters comprising only 3.2% along with other constituents fatty acids (16%), aldehydes (13%), hydrocarbons (5%), and triperpenols and their esters (8%) (Gülz and Müller, 1992).

Exposure of *B. papyrifera* to CO_2 or O_3 singly did not have as significant an impact on epicuticular wax biosynthesis as has been previously reported for *P. tremuloides* clones exposed to the same treatments (Percy et al., 2002; Karnosky et al., 2002). In particular, with respect to O_3, changes in the EW deposit of *B. papyrifera* were in the range reported for the most tolerant aspen clones. The fact that only the combined $CO_2 + O_3$ exposure resulted in statistically significant changes to fatty acid and primary alcohol proportions, and not O_3 singly, is very interesting and indicates that this species is responding differently, because it is generally more O_3 tolerant than aspen (Karnosky et al., 2003).

It is apparent in *B. papyrifera* that the reductive pathway (fatty acids → aldehydes → primary alcohols) of epicuticular wax synthesis predominates over the competing decarbonylative/decarboxylative pathway (alkanes → secondary alcohols → ketones) for available C. The overall efficiency of the acyl transferase enzyme system in *B. papyrifera*is clearly evident by the low proportions of free fatty acids (8.1%) and primary alcohols (6.2%) present, indicating a relatively high level of consumption of free fatty acids and primary alcohols during esterification of these acyl chains into alkyl esters (von Wettstein-Knowles, 1995). It is interesting that, although not statistically significant, alkyl esters tended to increase with treatment relative to control (Fig. 3). The fact that alkyl esters comprised 16% more of the EW deposit under $CO_2 + O_3$ than under the control treatment, and fatty acids and primary alcohols were significantly reduced in EW under $CO_2 + O_3$ compared with the control, could imply that the acyl transferase enzyme system in *B. papyrifera* was stimulated following co-exposure to CO_2 and O_3.

The aerial surfaces of all higher plants carry a partial or continuous coverage of amorphous epicuticular wax. These thin films are often surmounted by embedded crystalline wax structures, the shape (plates, tubes, ribbons, filaments, etc.) of which is predetermined by the chemical composition of the wax precursors (Jeffree et al., 1975). Alkyl esters and fatty acids crystallize as amorphous films, whereas alkanes and primary alcohols crystallize as plates (Baker, 1982). At the time of sampling in mid-August, modification to epicuticular wax structure was apparent on abaxial surfaces of leaves exposed to CO_2 and $CO_2 + O_3$. The alteration from a largely crystalline wax structure in the control to a progressively more amorphous structure with treatment for the wax deposit is not only logical (Jeffree et al., 1975) but entirely consistent with the reported significant increase in alkyl ester proportions and a trend in decreasing proportions of alkanes and primary alcohols. Taken together, these results imply a direct action of at least $CO_2 + O_3$ together on EW biosynthesis (von Wettstein-Knowles, 1995) as reported elsewhere (Percy et al., 1994; Maňkovská et al., 2005), especially as there is no known mechanism for oxidative transformation of *in situ* waxes at even supra-realistic O_3 concentrations (Jetter et al., 1996).

According to this study, the combined effects of CO_2 and O_3 on EW structure and chemical composition cannot be predicted on the basis of studies examining each of these factors separately. Different acclimatization responses may be required for growth under combination of elevated CO_2 and O_3, as also growth and leaf metabolism responses to separate exposures differ from those of combination of elevated CO_2 and O_3 (Karnosky et al., 2005; Paoletti and Grulke, 2005; Valkama et al., 2007; Riikonen et al., 2008a). The alterations in EW structure and chemical composition are likely to affect the plant responses to abiotic and biotic stresses such as light, temperature, and drought stress, and may have important consequences on plant water-use efficiency and C gain, as well as on plant quality and resistance to insect and disease populations in the future.

Acknowledgments

Aspen FACE is principally supported by the Office of Science (BER), U.S. Department of Energy, Grant No. DE-FG02-95ER62125 to Michigan Technological University, and Contract No. DE-AC02-98CH10886 to Brookhaven National Laboratory, the U.S. Forest Service Northern Global Change Program and Northern Research Station, Michigan Technological University, and Natural Resources Canada – Canadian Forest Service. This research was supported by the Emil Aaltonen Foundation and the Kone Foundation. We thank the support personnel of the Aspen FACE facility.

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