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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?

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"Capsule": *Elevated CO_2 did not ameliorate all of the adverse effects of O_3.*

Abstract

To determine whether elevated CO_2 reduces or exacerbates the detrimental effects of O_3 on aspen (*Populus tremuloides* Michx.), aspen clones 216 and 271 (O_3 tolerant), and 259 (O_3 sensitive) were exposed to ambient levels of CO_2 and O_3 or elevated levels of CO_2, O_3, or CO_2+O_3 in the FACTS II (Aspen FACE) experiment, and physiological and molecular responses were measured and compared. Clone 259, the most O_3-sensitive clone, showed the greatest amount of visible foliar symptoms as well as significant decreases in chlorophyll, carotenoid, starch, and ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) concentrations and transcription levels for the Rubisco small subunit. Generally, the constitutive (basic) transcript levels for phenylalanine ammonia-lyase (*PAL*) and chalcone synthase (*CHS*) and the average antioxidant activities were lower for the ozone sensitive clone 259 as compared to the more tolerant 216 and 271 clones. A significant decrease in chlorophyll *a*, *b* and total ($a+b$) concentrations in CO_2, O_3, and CO_2+O_3 plants was observed for all clones. Carotenoid concentrations were also significantly lower in all clones; however, *CHS* transcript levels were not significantly affected, suggesting a possible degradation of carotenoid pigments in O_3-stressed plants. Antioxidant activities and *PAL* and 1-aminocyclopropane-1-carboxylic acid (ACC)-oxidase transcript levels showed a general increase in all O_3 treated clones, while remaining low in CO_2 and CO_2+O_3 plants (although not all differences were significant). Our results suggest that the ascorbate-glutathione and phenylpropanoid pathways were activated under ozone stress and suppressed during exposure to elevated CO_2. Although CO_2+O_3 treatment resulted in a slight reduction of O_3-induced leaf injury, it did not appear to ameliorate all of the harmful affects of O_3 and, in fact, may have contributed to an increase in chloroplast damage in all three aspen clones.

Keywords: Aantioxidants; Aspen; Carbon dioxide; FACE; Oxidative stress; Ozone; Phenylalanine ammonia-lyase; Pigments; Rubisco

1. Introduction

Atmospheric CO_2 is increasing rapidly and is expected to double by the end of the next century (Barnola et al., 1995). Tropospheric ozone (O_3) is also increasing globally at a rate of 1–2% per year (Chameides et al., 1995) and it is likely that these two gases will have significant impacts in the future on forest tree species and ecosystems (Matyssek and Innes, 1999; Reilly et al., 1999). However, it is not clear at this point whether or not elevated CO_2 reduces or exacerbates the detrimental effects of O_3. Moreover, leaf age can greatly affect the direction of the CO_2+O_3 response. McKee et al. (1995) showed no protective effect of elevated CO_2 against O_3 on emerging wheat flag leaves, but strong effects in mature leaves. Experimental results provide evidence for both hypotheses, but neither has been tested under open field conditions, and both could be strongly influenced by the lower boundary layer conductances that characterize enclosures (Polle and Pell, 1999). There is clearly a need to test this in an open-air facility such as

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Nomenclature

AccOx	1-aminocyclopropane-1-carboxylic acid-oxidase
AOS	active oxygen species
APX	ascorbate peroxidase
CAT	catalase
Chs	chalcone synthase
DEPC	diethylpyrocarbonate
FACE	free-air CO_2 and/or O_3 exposure
GR	glutathione reductase
IAA	isoamyl alcohol
PAL	phenylalanine ammonia-lyase
RbcS	small subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase
Rubisco	ribulose-1, 5-bisphosphate carboxylase/oxygenase
SOD	super oxide dismutase
SSC	sodium saline citrate buffer
TE	tris–EDTA buffer

FACE where artifacts are minimized. The view that elevated CO_2 should reduce the detrimental effects of O_3 is based primarily on the expected decrease in stomatal conductance in elevated CO_2, which reduces O_3 flux received by the plant. The increase in substrate availability for repair and detoxification at elevated CO_2 may also reduce the effect of elevated O_3 (Rao et al., 1995).

The alternate hypothesis, that elevated CO_2 will exacerbate the detrimental effects of O_3, is based primarily on the prediction that reduced photorespiration, decreased Rubisco content, increased availability of CO_2 for photosynthesis, and a decrease in non-photochemical energy dissipation will reduce the need for cellular detoxification of reactive oxygen species (Polle et al., 1993). Thus, the lower antioxidant levels result in reduced tolerance to O_3. In aspen, there is some evidence of antioxidants playing a key role in O_3 tolerance (Sheng et al., 1997; Noormets et al., 2000) and an apparent increase in O_3 sensitivity with elevated CO_2 (Kull et al., 1996). In aspen softwood cuttings grown in open-top chambers, CO_2 did not compensate for the deleterious effects of elevated O_3 and, in some cases, photosynthetic capacity decreased more than with O_3 alone (Kull et al., 1996). This was particularly true for the O_3 sensitive clone. Moreover, the tolerant clones sometimes became more sensitive to O_3 with CO_2 enrichment (Kull et al., 1996; Karnsoky et al., 1998). Data from various tree species is conflicting on the interacting effects of O_3 and CO_2. These findings fall into two categories, (1) the elevated CO_2 has some positive effects in ameliorating the negative effects of O_3 (Mortensen et al., 1996; Kellomaki and Wang, 1997; Dickson et al., 1998; Sehmer et al., 1998; Grams et al., 1999; Broadmedow et al., 2000; Utriainen et al., 2000) or it increases the negative effects of O_3 (Kull et al, 1996; Karnosky et al., 1998; Niewiadomska et al., 1999).

To better understand the interactive affects of increased CO_2 and O_3 levels, we have measured various leaf antioxidant activities and gene expression patterns of several stress-response pathways for aspen trees growing in a Free Air CO_2 and/or O_3 Enrichment (FACE) experiment. Chlorophyll, carotenoid, and Rubisco contents have also been measured to help determine how the photosynthetic capacities are affected for clones of differing O_3 sensitivity. Results from this open air experiment indicate that elevated CO_2 levels increase O_3 sensitivity in all three clones by suppressing ascorbate-glutathione and phenylpropanoid pathways. An increase in AOS, consequently, may be responsible for the observed decreases in chlorophyll and carotenoid pigments.

2. Materials and methods

2.1. Plant material and fumigation protocols

The research facility is located in the USDA Forest Service Harshaw farm site in Oneida County, Wisconsin, USA (Karnosky et al., 1999; Dickson et al., 2000). Three replicate FACE rings were established for each treatment [(control (=ambient), elevated O_3, elevated CO_2, and elevated O_3+CO_2)]. In elevated-O_3 rings, O_3 concentrations were maintained approximately 1.5 times higher than the ambient concentrations. The mean O_3 concentrations during the 1998 growing season were 36 ppb for ambient air and 56 ppb for elevated O_3 exposures. The monthly AOT40 values ranged from 5.2 to 9.2 ppm h (mean 7.0 ppm h) for elevated O_3 treatments and ranged from 25.9 to 30.6 ppm h for the seasonal dose (Table 1). The mean concentrations and total cumulative O_3 exposures over a threshold of 0 ppb (AOT00) and of 40 ppb (AOT40) calculated for daylight hours for each exposure ring are given in Table 1. Carbon dioxide was dispersed during daytime hours at 560 ppm, approximately 200 ppm above our ambient. The plants were established at 1×1 m spacing across each ring to simulate an aggrading aspen forest. Details regarding the aspen clones are presented in Karnosky et al. (1998) and Sheng et al. (1997) and the planting and handling of the aspen clones is described in Karnosky et al. (1999).

The same plants were used for biomaterial measurements, determinations of visible leaf injury, antioxidant activities, chlorophyll, carotenoid, rubisco and starch concentrations, and gene expression levels (Rubisco small subunit and defense-related proteins), and therefore the data from different analyses are comparable. In addition, chlorophyll, carotenoid and rubisco

Table 1
Mean O_3 concentrations (ppb), 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 during the 1998 growing season[a]

Month	O_3	Ambient			Elevated O_3			Elevated $O_3 + CO_2$		
		North	East	South	1.3	2.3	3.3	1.4	2.4	3.4
May	Mean ppb	44	40	40	52	54	53	52	53	50
	AOT00 ppm h	12.6	11.5	11.5	17.9	18.6	18.1	17.2	18.3	17.5
	AOT40 ppm h	0.3	0.3	0.3	5.7	6.6	6.2	6.1	6.3	5.2
June	Mean ppb	35	31	32	55	57	55	55	57	55
	AOT00 ppm h	12.5	11.3	11.7	19.7	20.5	19.7	19.9	20.3	19.7
	AOT40 ppm h	0	0	0	6.7	7.9	6.9	7.3	7.9	6.7
July	Mean ppb	32	27	31	57	58	56	55	57	57
	AOT00 ppm h	11.9	10	11.5	21.2	21.6	20.8	20.5	21.2	21.2
	AOT40 ppm h	0	0	0	7.6	8.0	7.1	7.2	7.9	7.5
August	Mean ppb	44	37	41	59	58	59	64	62	57
	AOT00 ppm h	13.6	11.4	12.6	18.4	18	18.3	19.8	19.1	17.8
	AOT40 ppm h	0	0	0	7.0	8.1	7.0	9.2	8.4	6.5
May–August	Mean ppb	39	34	36	56	57	56	57	57	55
	AOT00 ppm h	50.6	44.2	47.3	77.2	78.7	76.9	77.4	78.9	76.2
	AOT40 ppm h	0.3	0.3	0.3	27.0	30.6	27.2	29.8	30.5	25.9

[a] Ambient O_3 was calculated for three points (North, East and South) within the exposure area.

concentrations were determined for the same leaf extraction samples.

2.2. Samples

Foliar samples from aspen were collected in late July of 1998 from each treatment ring as close as possible to mid-day (noon). Samples were collected from 6–10 trees in each treatment from each of the three replicates. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until use. Leaf samples were collected from the developing leaf zone [LPI 3–5 (Larson and Isebrands, 1971)] and care was taken to collect same age leaf samples from each tree.

2.3. Sample extraction and analysis

Frozen leaves (0.25 g) were ground with liquid nitrogen and extracted briefly with 6 ml of cold 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) insoluble polyvinylpyrrolidone. The homogenate was centrifuged at 3000 ***g*** (4 °C) for 2 min, 1 ml of supernatant was added to a 1 ml column of P2 Biogel (Bio Rad) and centrifuged at 600× ***g*** for 1 min. Biogel purified samples were immediately assayed for SOD, APX and CAT activity. Samples assayed for GR activity were extracted as described above, using 40 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 3% (w/v) insoluble polyvinylpyrrolidone, and 40 mM 3-amino-1, 2,4-triazole. Homogenate was centrifuged at 6000 ***g*** for 10 min (4 °C) and the supernatant was assayed for GR activity.

2.3.1. Antioxidant enzymes

Aspen clones (from samples collected in July) were analyzed to determine the levels of antioxidant enzymes. Biochemical analyses included enzyme assays for SOD (Dhindsa et al., 1981), ascorbate peroxidase (Chen and Asada, 1989), GR activity (Price et al., 1990), and catalase (Kato and Shimizu, 1987). All the biochemical analyses were normalized for age of the plants, weight of the sampled foliar material and for amount of total proteins or cell lysates used for each analysis. Assays were repeated on each extracted sample at least three times.

2.3.2. Rubisco, soluble protein, starch and pigments

For Rubisco and soluble protein assays, frozen leaf samples were weighed, and a crude extract was prepared using 2 ml of extraction buffer (Gezelius and Hallen, 1980). The amount of total Rubisco protein was determined by PAGE as described by Rintamäki et al. (1988), using purified Rubisco protein (Sigma Chemical Co.) as a standard. The areas and intensities of Rubisco bands were determined by scanning the gels with the Adobe PhotoShop Program (Version 5.0), and the Rubisco concentrations were calculated on a dry weight basis. An aliquot for soluble proteins was quantified as described by Pääkkönen et al. (1998). Chlorophyll analysis was done as per the protocol of Porra et al. (1989) and carotenoid measurements were done as per the protocol of Wellburn and Lichtenthaler (1984). In order to avoid variability between samples for rubisco and protein analysis and chlorophyll and associated pigments, samples for pigments were taken from the

crude extract before centrifugation and subjected to proper extraction protocol for either chlorophyll or carotenoid pigments. Leaf samples for starch determination were freeze-dried, milled, and analyzed by standard enzymatic techniques (Boehringer Kit for Food analysis).

2.3.3. Gene expression

Leaf material (1–2 g) was ground in liquid nitrogen, and added to the prewarmed extraction buffer (100 mM Tris–HCl pH 8.0, 20 mM EDTA, 0.5 M NaCl, 2% PVP, 0.5% SDS, 0.5% β-mercaptoethanol). After three extractions with chloroform:IAA (24:1), 1/5×volume of 10 M LiCl was added and the RNA was precipitated one hour on ice. The pellet was dissolved into DEPC-TE, extracted once more with chloroform:IAA, and reprecipitated in ethanol at −80 °C overnight. The RNA pellet was purified with 70% ethanol, dried and resuspended in DEPC-treated water. RNA quality was checked by gel electrophoresis, and concentration determined by GeneQuant II RNA/DNA Calculator (Pharmacia Biotech). Single-stranded digoxigenin (DIG)-labeled DNA-probes were generated by PCR using DynaZyme DNA-polymerase (Finnzymes, Finland) and DIG-DNA labeling mixture (Boehringer Mannheim, Germany). Birch RbcS, Pal, Chs, and AccOx probes (Pääkkönen et al., 1998) were used after careful optimization of hybridization conditions for aspen samples. RNA hybridizations were performed according to The DIG System User's Guide for Filter Hybridization (Boehringer Mannheim, Germany). Total RNA, 10 μg each per lane, was separated in formaldehyde containing 1% agarose gel. Equivalent loading of RNA was verified by SYBR-Green staining (Molecular Probes Inc. OR). After gel electrophoresis, RNA was transferred to nylon membranes overnight at room temperature with 10×SSC (1.5 M NaCl, 150 mM sodium citrate pH 7.0). RNA was fixed to the filter by UV-crosslinking, followed by washing in 2×SSC for 10 min. The filters were prehybridized for 1 1/2 h at 48 °C in DIG Easy Hyb (Boehringer Mannheim, Germany) and hybridized overnight at 48 °C with denatured DIG-labeled purified DNA-probes. Filters were washed in 2×SSC, 0.1% SDS at room temperature (2×5 min) and in 0.1×SSC, 0.1% SDS at 68 °C (3×15 min) Hybridization signals were visualized using the DIG Luminescent Detection Kit (Boehringer Mannheim, Germany). Membranes were incubated in blocking reagent and treated with anti-DIG-alkaline phosphatase-conjugate. The chemiluminescent substrate was pipetted onto the filter and the light signal was recorded on X-ray films (Fig. 1). The relative intensities of stress protein inductions were determined by scanning the X-ray films and measuring the band densities with Adobe PhotoShop program (Version 5.0). The relative band densities were compared only within the film and the band intensities were indicated as percentage of the maximal intensity on the film.

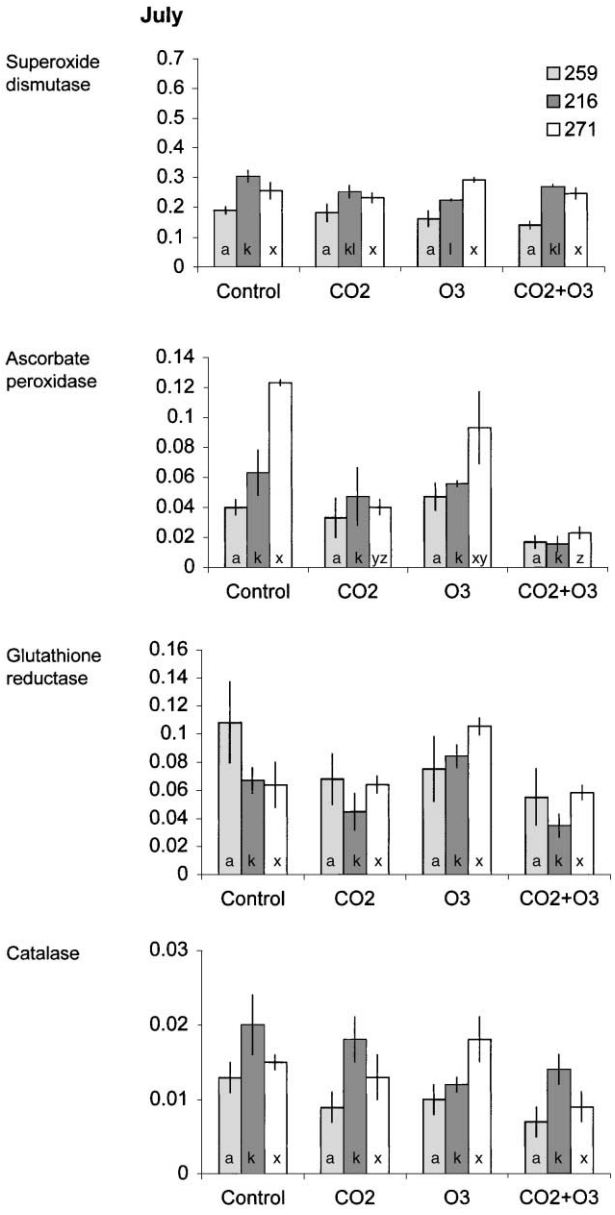

Fig. 1. Effects of elevated O_3 and CO_2 alone or in combination on SOD, APX, GR and CAT activities in aspen clones 259, 216 and 271. Data for three replicate FACE rings/treatment were pooled and expressed as standard deviations.

2.3.4. Growth analysis

Fifteen plants per clone per treatment were measured between 27 and 31 July 1998 for base diameter (mm), number of leaves per branch (3–6th branch from the top), mean leaf size (cm^2; 9–11th leaf from the top) and proportion of visibly injured leaves (%; determined for the 3–6th branch from the top) as described (Karnosky et al., 1980, 1996). Visible injuries were necrotic

or dark-brown pigmented dots and flecks appearing on the upper leaf surface.

3. Results

3.1. Leaf growth and visible injury

Ozone treated plants of all three clones had significantly smaller base diameters, average number of leaves per branch, and mean leaf sizes when compared to their respective controls, and similar results were obtained for average leaf sizes and leaves per branch of $CO_2 + O_3$ treated plants (Table 2). Average leaf sizes for clones 216 and 271 were most significantly affected by $CO_2 + O_3$, indicating an increased sensitivity to O_3 in the presence of elevated CO_2 (Table 2). However, visible leaf injury (average number of injured leaves per branch as well as injury index per leaf measured as described by Karnosky et al., 1980, 1996) was significantly higher for O_3 treated plants when compared to $O_3 + CO_2$ treated plants, and clone 259 showed the greatest amount of leaf injury (Table 2).

3.2. Antioxidant activities

With the exception of samples from clone 259, SOD activities varied little, while APX, GR and CAT activities were generally highest for O_3 treated plants and lowest for CO_2 and $O_3 + CO_2$ treated plants for all three clones when compared to controls (Fig. 1).

3.3. Pigment, Rubisco, and starch contents

Ozone, CO_2, and $O_3 + CO_2$ treatments had significant effects on Chl *a* and Chl *b* content, whereas Rubisco content was not significantly affected (Fig. 2). Chl *a*, chl *b*, and total chl ($a + b$) contents significantly decreased for all CO_2, O_3, and $CO_2 + O_3$ treated clones (Fig. 2). Chl a/b ratios only varied significantly for clone 259, with an increase (low chl *b* content; Fig. 2). Total carotenoid and Rubisco concentrations generally decreased in CO_2, O_3, and $CO_2 + O_3$ treated plants. Starch concentrations for clones exposed to all three treatments, exhibited some trends, with CO_2, O_3, and $CO_2 + O_3$ plants generally having higher starch concentrations in clone 216 and lower concentrations in clones 259 and 271 when compared to controls (Fig. 2).

3.4. Transcription levels of RbcS, Chs, Pal, AccOx

Expression levels of RbcS, for all clones and all treatments, generally decreased compared to controls with the exception of O_3 treated clone 271 plants, which showed a significant increase (Figs. 3 and 4). These results correlate, most significantly for clone 259, with the Rubisco contents shown in Fig. 2, however, transcription levels do not appear to correlate with starch contents (Figs. 2 and 3). *CHS* expression levels did not vary significantly, except for O_3 treated clone 271 plants, which showed a significant increase compared to controls. Both clones 259 and 271 showed a significant decrease in total carotenoid contents under CO_2 and O_3 treatments (Fig. 2). The combination of $CO_2 + O_3$ had a rather dramatic effect in the decrease of carotenoid component on 259, but not on gene expression of *CHS*. *PAL* expression levels of all three clones were significantly increased by O_3 treatments and significantly decreased by CO_2 and $CO_2 + O_3$ treatments. Although the differences are not significant, changes in *AccOx* expression levels correlate with changes in *PAL* mRNA levels.

Table 2
Effects of elevated O_3 and CO_2 alone and in combination on growth parameters in aspen (*Populus tremuloides*) clones[a]

Response	Clone	Control	CO_2	O_3	$CO_{2+}O_3$
Base diameter, mm	259	18.8±0.4 bc	20.3±0.4 c	16.9±0.5 a	20.1±0.7 c
	216	22.2±0.4 b	24.4±0.4 c	18.9±0.3 a	22.2±0.4 b
	271	22.4±0.5 b	22.6±0.4 b	19.5±0.5 a	21.9±0.4 b
Number of leaves/branch	259	37.5±1.0 b	36.5±0.9 b	23.6±0.6 a	23.2±0.7 a
	216	34.8±0.6 b	41.2±1.2 c	23.9±0.7 a	24.4±0.7 a
	271	39.8±0.9 b	39.9±1.5 b	26.4±0.7 a	25.5±0.7 a
Mean leaf size, cm^2	259	80.9±4.7 a	67.8±2.8 a	70.8±5.3 a	70.1±4.4 a
	216	67.4±2.2 b	61.7±3.4 ab	57.6±2.1 ab	56.4±2.9 a
	271	66.6±3.0 b	63.1±3.6 ab	59.8±1.9 ab	54.3±2.1 a
Injured leaves/branch,%	259	0.0±0.0 a	0.0±0.0 a	10.76±0.65 b	8.11±0.65 b
	216	0.0±0.0 a	0.0±0.0 a	8.42±0.72 b	4.26±0.54 b
	271	0.0±0.0 a	0.0±0.0 a	7.51±0.71 b	3.30±0.46 b

[a] Significant block effects were not found, and therefore the data for three replicate FACE rings/treatment were pooled for further analyses. Anova, Tukey's test. $P < 0.05$, $n = 15$. Values are means±S.E. Significant differences between the treatments are indicated by different letters.

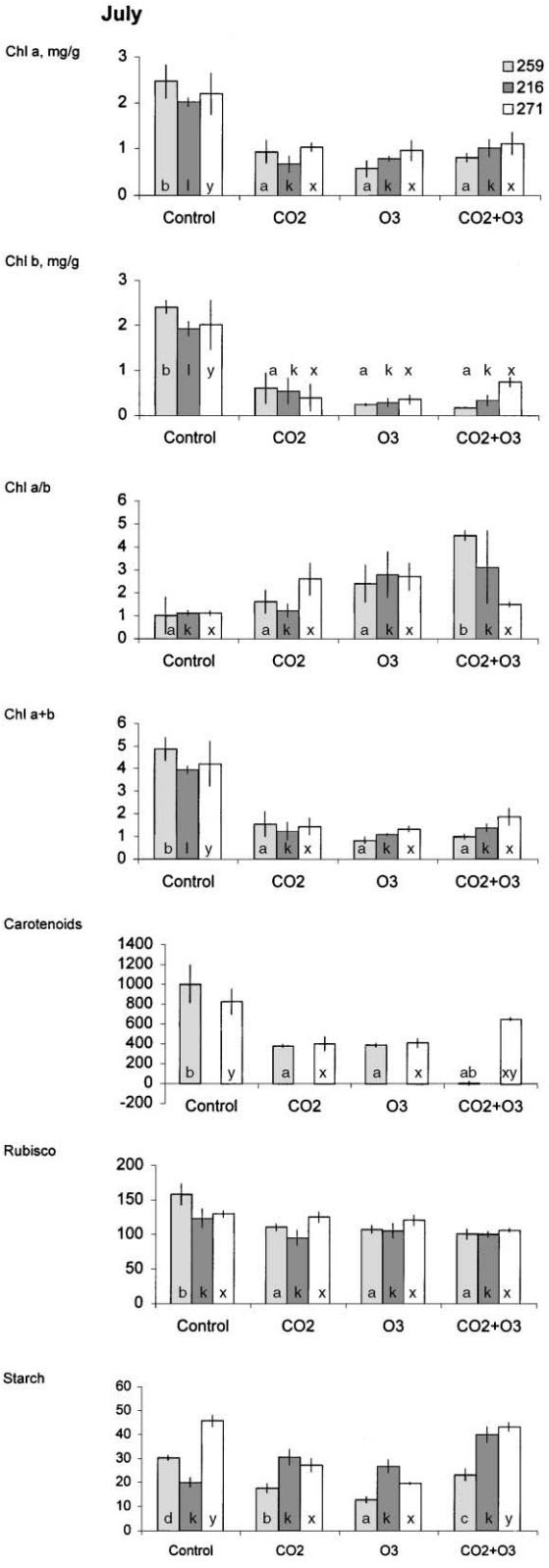

Fig. 2. Effects of elevated O_3 and CO_2 alone or in combination on chl *a* and *b*, total chl (mg/g dry wt.), carotenoid (μg/g dry wt.), total Rubisco (mg/g dry wt.), and starch (mg/g dry wt.) concentrations, and chl a/b ratio in aspen clones 259, 216 and 271. Anova, Tukey's test. $P<0.05$, $n=15$. Values are means±S.E. Significant differences between the treatments are indicated by different letters.

4. Discussion

Polle et al. (1997) provided evidence that oxidative stress in beech leaves (*Fagus sylvatica* L.) increases when N and/or C assimilation was limited. Podila et al. (1998) found that clones grown in soils with the lowest average NH_4^+ and C:N ratios demonstrated a slight decrease in antioxidant activity. However, trends in the effects of elevated CO_2 on antioxidant activities could still be seen in these aspen clones. Furthermore, Coleman et al. (1998) and Sober et al. (2001), found that N levels were linearly related to photosynthesis.

It has been reported that elevated CO_2 may result in decreased production of antioxidant metabolites (e.g. glutathione and ascorbate) and in antioxidant enzymes (Badiani et al., 1993; Polle et al., 1993; Schwanz et al., 1996; Polle et al., 1997; Polle and Pell, 1999) including aspen (Karnosky et al., 1998), while O_3 generally increases antioxidant activities. Furthermore, several reports have demonstrated that overexpression of SOD increases resistance to oxidative stress (Jansen et al., 1989; Sen Gupta et al., 1993a, 1993b), and that APX activities also increased in the leaves of transformed plants. In aspen (*Populus tremula*×*P. alba*), Tyystjarvi et al. (1999) found that an 5–8-fold increase in FeSOD expression was unable to protect the plants from photoinhibition, while tobacco plants, with a similar increase in GR expression levels, had a lower level of photoinhibition. Thus, the ascorbate-glutathione pathway may not be limited by the SOD catalyzed reduction of superoxide to H_2O_2, but instead by the conversion of H_2O_2 to H_2O by APX (and catalases) and the reduction of oxidized dehydroascorbate and glutathione by dehydroascorbate reductase and glutathione reductase. In our experiments, we found that APX, CAT and GR activities for all three clones generally increased as a result of exposure to elevated O_3, and decreased due to elevated CO_2 and CO_2+O_3 levels, suggesting a suppression of the ascorbate-glutathione pathway by CO_2.

A decrease in antioxidant activities would decrease the plants primary defense against AOS, and thus an increase of oxyradicals within the chloroplast may account for the significant decreases observed for chl *a* and *b* and total carotenoid contents under elevated O_3, especially since transcription levels of *CHS* were not significantly affected. This may also account for the decrease in Rubisco content. However, RcbS expression levels were also significantly lower in plants treated with CO_2, O_3, and CO_2+O_3. While it appears that more damage within the chloroplast occurs due to elevated CO_2 and O_3 levels, it was found in our work and in previous reports (Karnosky et al., 1999; Tjoelker et al., 1993; Volin et al., 1993) that CO_2 can reduce the amount of visible leaf injury. It has also been shown that CO_2 increases the light saturated photosynthetic rate for aspen (Karnosky et al., 1999; Volin and Reich,

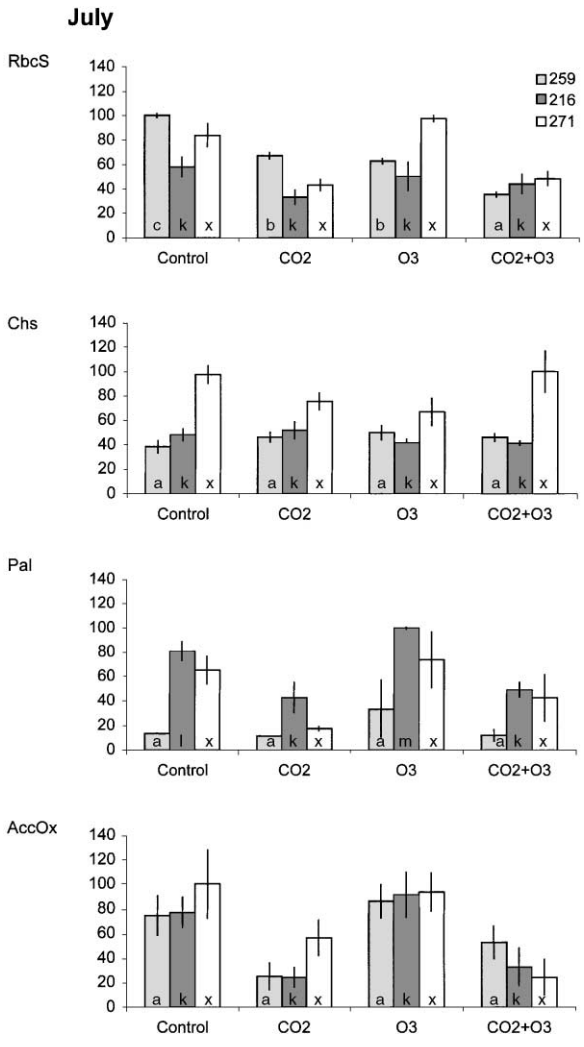

Fig. 3. Transcript levels of Rubisco small subunit (RbcS), chalcone synthase (Chs), phenylalanine ammonia-lyase (Pal) and AccOx (means ± S.E.) in leaves of aspen clones 259, 216 and 271. Maximum density of the band in each gel = 100. Kruskall–Wallis test. Significant differences between the treatments are indicated by different letters.

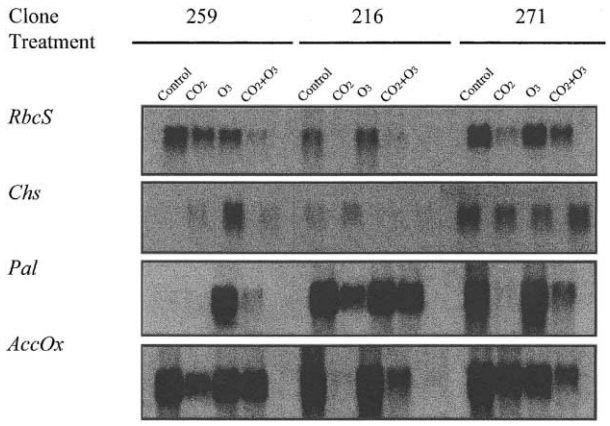

Fig. 4. Northern analysis of *RbcS, PAL, CHS*, and *AccOx* in aspen clones. Total RNA (10 μg each) from aspen clones 259, 216, and 271 was probed with single-stranded digoxigenin (DIG)-labeled Birch *RbcS, PAL, CHS*, and *AccOx* DNA probes.

1996; Volin et al., 1998), thus it is possible that, in the presence of CO_2, an increased rate of photosynthesis increases the concentration of available NADPH in the chloroplast, which can then be used by GR to reduce the glutathione pool and increase the flux through the ascorbate-glutathione cycle. However, leaf size was most significantly reduced for $CO_2 + O_3$ treated plants, suggesting that photosynthesis may be inhibited. Thus, it is also possible that an increase in AOS, due to suppression of antioxidants as discussed above, may be offset by a decrease in rate of photosynthesis which may only occur in $CO_2 + O_3$ treated plants, resulting in a decrease in both visible leaf injury and leaf growth.

Ozone sensitivity in *Populus* may be related to a lack of defense gene activation (Koch et al., 1998, 2000). Salicylic acid (SA) and jasmonic-acid-mediated (JA) signaling pathways, known mediators of pathogen and wound responses, may also be involved in responses to oxidative stress (Örvar et al., 1997; Koch et al., 1998, 2000; Rao et al., 1997, 2000). Our results indicate that *PAL* expression may be suppressed by CO_2 since *PAL* mRNA levels were lowest for CO_2 and $CO_2 + O_3$ treated plants (all clones). Suppression of the phenylpropanoid pathway, and a resulting decrease in various pathogen defense compounds, could increase the plants sensitivity to secondary pathogens especially when exposed to O_3 in combination with elevated CO_2. This may explain the increased rate of infection by a sooty mold fungus, (*Alternaria* spp.) observed for all three clones grown at field sites with intermediate and high O_3 levels (Karnosky et al., 1999) and a 3- to 5-fold increase in *Melampsora* leaf rust in aspen exposed to O_3 or $O_3 + CO_2$ (Karnosky et al., 2001). Just as Karnosky et al. (1999) found clone 259 to be most dramatically affected by the secondary infection, we found that *PAL* expression was most significantly suppressed in clone 259. However, increased infection levels for clone 259 correlated well with increased visible leaf damage (Karnosky et al., 1999), suggesting that O_3-induced foliar injury may result in a high susceptibility to secondary infections. It is also probable, that clone 259 may be inherently less sensitive to SA-mediated signaling which may explain its susceptibility to both O_3 and pathogens (Koch et al., 2000; Overmyer et al., 2000). On the other hand, clones 216 and 271 may be more sensitive to SA-mediated signaling resulting in launching a more effective defense against O_3-mediated damage and/or pathogens.

The observation that *AccOx* levels were reduced under elevated CO_2 conditions for all three clones suggests that under elevated CO_2 conditions the production of ethylene may be reduced, leading to less visible injury due to oxidative damage as well as damage by pathogens. Recently, it has been shown that in *Arabidopsis* ethylene promotes SA-mediated (ozone-induced) lesion formation and the ozone-induced AccOx transcript levels match with ozone-induced ethylene production

(Overmyer et al., 2000). Similarly, the reduction in AccOx levels under $CO_2 + O_3$ may have a role in reducing ethylene induced SA-mediated or O_3-induced damage in clones 216 and 271. However, the low level of reduction for AccOx in clone 259 may yet lead to increased visible damage under $CO_2 + O_3$ conditions.

5. Conclusions

Although responses to elevated CO_2, O_3, and $CO_2 + O_3$ were similar in all clones, clone 259 showed the highest sensitivity to all treatments. Our results suggest that elevated CO_2 levels may exaserbate the harmful effects of O_3 by suppressing the ascorbate-glutathione and *PAL* pathways, resulting in increased cellular damage as a result of increasing AOS levels and a reduced resistance to secondary infections. Degradation due to an increase in AOS, resulting from a decrease in antioxidant activities, was most likely responsible for the observed decrease in chlorophyll and carotenoid contents, while decreased expression levels of rcbS correlated well with decreased Rubisco contents.

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