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Interacting elevated CO_2 and tropospheric O_3 predisposes aspen (*Populus tremuloides* Michx.) to infection by rust (*Melampsora medusae* f. sp. *tremuloidae*)

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Abstract

We investigated the interaction of elevated CO_2 and/or (Ozone) O_3 on the occurrence and severity of aspen leaf rust (*Melampsora medusae* Thuem. f. sp. *tremuloidae*) on trembling aspen (*Populus tremuloides* Michx.). Furthermore, we examined the role of changes in leaf surface properties induced by elevated CO_2 and/or O_3 in this host–pathogen interaction. Three- to five-fold increases in levels of rust infection index were found in 2 consecutive years following growing-season-long exposures with either O_3 alone or $CO_2 + O_3$ depending on aspen clone. Examination of leaf surface properties (wax appearance, wax amount, wax chemical composition, leaf surface and wettability) suggested significant effects by O_3 and $CO_2 + O_3$. We conclude that elevated O_3 is altering aspen leaf surfaces in such a way that it is likely predisposing the plants to increased infection by aspen leaf rust.

Keywords: epicuticular wax, FACE experiment, leaf rust, leaf surface properties

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Introduction

Global atmospheric CO_2 concentration has risen by nearly 30% since preindustrial times (Barnola *et al*. 1995), largely because of the industrial emissions (Keeling *et al*. 1995). Similarly, background concentrations of tropospheric ozone (O_3) related to emissions of nitrogen oxides (NO_x) and volatile organic compounds (VOC) from fossil fuel, such as thermal generation and transportation, have increased from *circa*. 10 ppb to over 40 ppb (Finlayson-Pitts & Pitts 1997; Fowler *et al*. 1998; Stevenson *et al*. 1998). Fowler *et al*. (1999) suggest that nearly one-quarter of the earth's forests are currently at risk from tropospheric O_3 when July peak concentrations exceed 60 ppb.

Elevated CO_2 and O_3 affect trees through different mechanisms. With trembling aspen (*Populus tremuloides* Michx.) elevated CO_2 stimulates photosynthesis (Tjoelker *et al*. 1998; Noormets *et al*. 2001), delays foliar senescence in autumn (Isebrands, personal communication), and stimulates above-ground (Isebrands *et al*. 2001) and below-ground (King *et al*. 2001) growth. Trees grown with elevated CO_2 generally have lower nitrogen concentrations in their foliage, lower Rubisco concentrations, altered defense compounds (Lindroth *et al*. 1993, 1997) and decreased concentration of antioxidants (Wustman *et al*. 2001).

In contrast to the largely beneficial effects of CO_2 on aspen, O_3 is generally detrimental to aspen growth and productivity. Ozone has been shown to induce foliar injury (Karnosky 1976), decrease foliar chlorophyll content (Gagnon *et al*. 1992), accelerate leaf senescence (Karnosky *et al*. 1996), decrease photosynthesis (Coleman *et al*. 1995a), alter carbon allocation (Coleman *et al*. 1995b), alter epicuticular wax structure and composition

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(Mankovska *et al*. 1998; Karnosky *et al*. 1999), and decrease growth (Wang *et al*. 1986; Karnosky *et al*. 1992, 1996, 1998; Coleman *et al*. 1996). Extrapolation of data on the impacts of O_3 on aspen in open-top chambers to aspen in nature suggests that 14–33% biomass loss may be occurring over 50% of aspen's natural range in the eastern US (Hogsett *et al*. 1997).

Current climate change scenarios predict further increases in global CO_2 concentrations (Stott *et al*. 2000) and area of global forests exposed to damaging levels of O_3 (increases to 49% or 17×10^6 km^2) (Stevenson *et al*. 1998; Fowler *et al*. 1999). Predicting the potential impacts of plant diseases in this background of climate change is difficult as little is known about the occurrence and severity of disease under interacting CO_2 and O_3 (Chakraborty *et al*. 2000; Sandermann 2000). Thus, it is not possible at present to predict whether increased levels of these gases will lead to a lower or higher disease likelihood in particular plant-pathogen systems (Sandermann 2000). For example, very little is known about how foliar pathogens of forest trees will respond to elevated CO_2 and/or O_3 (Kickert & Krupa 1990).

Ozone predisposes some plants to infection by facultative leaf pathogens (Wukasch & Hofstra 1977; Rist & Lorbeer 1984). However, there is uncertainty about the role of O_3 in affecting infection by obligate pathogens such as rusts. Previous studies with poplar response to O_3 and foliar pathogens have shown both decreased (Coleman *et al*. 1987, 1988) and increased (Beare *et al*. 1999) disease levels with elevated O_3. In a short-term (5 h) greenhouse chamber study, Coleman *et al*. (1987) showed that an acute O_3 dose decreased the susceptibility of two eastern cottonwood (*Populus deltoides* Bartr.) clones to *Melampsora medusae*. However, Beare *et al*. (1999) found opposite response when a longer O_3 exposure period (17 d) increased severity of *Melampsora* rust on 'Balsam spire' hybrid poplar (*Populus trichocarpa* Torr. & Gray $\times$ *Populus balsamifera* L). To the best of our knowledge, no one has examined the impact of O_3 on *Melampsora* rust occurrence on trembling aspen and furthermore, no one has examined the role of interacting elevated CO_2 on the host $\times$ O_3 $\times$ rust interaction in trees.

In this paper, we present observations on the occurrence and severity of *Melampsora medusae* Thuem. f. sp. *tremuloidae* Shain infection on three trembling aspen genotypes differing in O_3 tolerance and grown for multiple years under interacting elevated CO_2 and/or O_3 in a free-air enrichment (FACE) system. The objectives of the study were to determine (a) if O_3 increases or decreases the occurrence and severity of *M. medusae* infection and if this response varies by genotype and its O_3 susceptibility (b) if CO_2 ameliorates, aggravates or has no effect on O_3-induced responses, and (c) if O_3-induced changes to leaf surface physiochemical characteristics and properties might predispose the trees to rust attack.

Materials and methods

The impacts of rapidly changing gaseous (CO_2, O_3) composition of the atmosphere on forest trees is being investigated at the Aspen FACE site located on the USDA Forest Service Harshaw Research Farm near Rhinelander, Wisconsin (Karnosky *et al*. 1999; Dickson *et al*. 2000). The experiment comprises a full factorial with twelve 30 m diameter treatment rings composed of 3 control rings, 3 rings with elevated O_3, 3 rings with elevated CO_2, and 3 rings with elevated O_3 + elevated CO_2. All rings are a minimum of 100 m apart. The rings were planted in mid summer 1997 and treatments ran from aspen budbreak to budset in 1998, 1999 and 2000. The eastern one-half of each ring was randomly planted with 3- to 6-month-old potted trees (20–40 cm in height) at 1 m $\times$ 1 m spacing in two tree plots of five aspen clones differing in O_3 tolerance (8 L, 216 and 271 = relatively tolerant and 42E and 259 = relatively sensitive). For this study of rust occurrence and foliar surface properties, we focused on the three clones (216, 259, and 271) for which we had extensive previous information on O_3 tolerance and pest susceptibility (Karnosky *et al*. 1992, 1996, 1998, 1999).

CO_2 and O_3 were released during the daylight hours. The targeted elevated CO_2 treatment concentration was 560 ppm, 200 ppm above ambient and O_3 exposure was at 1.5 $\times$ ambient. O_3 was not administered during periods of cold weather, or when leaf surfaces were wetted from fog, dew, or rain events. Complete details on the experimental design and pollutant generation and monitoring can be found in Karnosky *et al*. (1999) and Dickson *et al*. (2000).

Rust assessment

Melampsora medusae Thuem. f. sp. *tremuloidae* was identified by observations of urediniospores with scanning electron microscopy and/or light microscopy. The inoculum was natural with the primary source likely being adjacent stands of larch (*Larix* spp. and hybrids). Voucher specimens of rusted leaves, representing each of the five severity classes, were placed in the Pacific Forestry Centre's Forest Pathology Herbarium (DAVFP 25858) in Victoria, BC, Canada. *Melampsora* rust incidence was assessed on September 27–28, 1999 and on August 29–30, 2000. The percentage of infected leaves per tree for every tree in the core of each ring (n = 1839 trees total) was estimated and the severity of rust occurrence was scored on an average of 20 leaves per tree from 1 to 5 with 1 = 1–20% of the leaf area covered with urediniospores, 2 = 21–40%, 3 = 41–60%, 4 = 61–80%, and 5 = 81–100%. An index of mean

severity of infection was then calculated as percent leaves injured × severity of rust occurrence.

Leaf surface properties

Epicuticular waxes provide a physical barrier to protect leaves from pathogen infection (Mendgen 1996). It is well known that the appearance of these waxes can be seriously altered by air pollutants (Kerstiens 1996) and that these changes are dramatically manifested in leaves of aspen growing under elevated O_3 (Mankovska *et al*. 1998; Karnosky *et al*. 1999).

To characterize the impacts of O_3 on wax structure, we collected leaves (recently mature, LPI = 10–15 (Larson & Isebrands 1971)) in mid August of 1999 and 2000. Leaf segments (5 mm × 10 mm) were air-dried, gold-coated on a cold stage, and examined under a JSM 6400 (JEOL Ltd., Peabody, Massachusetts) scanning electron microscope (SEM) . Five leaves per clone were examined for each treatment.

Epicuticular waxes were recovered by a chloroform rinse of five fully expanded (LPI 8–12) leaves pooled per wax sample. Five samples were collected randomly across the two-tree plantings per clone, per ring ($n = 15$ samples per treatment). The solvent/wax solution was filtered, solvent evaporated, and epicuticular wax weighed to ± 10 μg and expressed as μg cm^{-2} leaf area. Quantitative wax chemical composition (± 0.001%) was determined with a high-temperature capillary Varian 3410 (Varian Inc., Walnut Creek, California) gas chromatograph (Percy *et al*. 1994). Homologue assignments and peak integration were determined with Varian version 6.0 WorkStar software and injection of reference homologueues and wax mixtures. Final confirmation of homologueue assignments was determined with GC–MS.

Leaf surface wettability was assessed by measuring leaf surface-droplet contact angle (DCA) (Percy & Baker 1988) on five leaves per clone per treatment. Small volume (0.2 μL; *circa*. 700 μm dia.) distilled water droplets were placed on leaf surfaces as described by Percy & Baker (1988). Equilibrium angles were measured (± 1 °) using an NRL Contact Angle Goniometer (Ramé-Hart Inc., Mountain Lakes, New Jersey).

Data analysis

The experiment was designed as a randomized complete block design with four treatment rings in three replicate blocks. An experimental ring was regarded as an experimental unit for all statistical analyses and the mean parameter values for individual rings were used as input values.

The main and interactive effects of clone, year, and CO_2 and O_3 levels on the percent of infected leaves, severity of infection and infection index were calculated with analysis of variance (ANOVA) using general linear models procedure (SAS, SAS Institute, Cary, NC, USA). The significance of clonal differences within a given treatment was calculated with Duncan's Multiple Range Test.

Epicuticular wax amount and alkane ratios [ΣC 29–C35(long)/ΣC23–C28(short) homologues; homologue value expressed per unit leaf area (μg cm^{-2})] were analysed using ANOVA (SAS 1993). Main effects tested were treatment, block, clone, and interactions. Differences between means were analysed using Duncan's multiple range test. The DCA data were analysed using ANOVA with an additional main effect leaf surface included.

Results and discussion

Treatment summary

Treatment rings with elevated CO_2 had an average concentration of 543 ± 75 ppm CO_2 in 1999 and 531 ± 86 ppm CO_2 in 2000 during daylight hours. Background daylight ambient CO_2 averaged 350 ± 10 ppm in 1999 and 347 ± 13 ppm in 2000. Daytime ambient O_3, O_3 treatment rings, and O_3 + CO_2 treatment rings averaged 36.9, 51.1 and 52.3 ppb O_3, in 1999, and 36.0, 47.4, and 48.9 ppb in 2000, respectively (Table 1, Fig. 1). Actual hourly average O_3 data for the 1999 and 2000 growing season are presented in Fig. 2.

The CO_2 concentrations we used (ambient plus 200 ppm) are predicted to occur globally within the next 40–60 years (Stott *et al*. 2000). The O_3 concentrations are similar to those characterized for several forested areas of the eastern United States (Pinkerton & Lefohn 1987). The O_3 values are similar in terms of the 1 × O_3 seasonal dose for the 1 × treatments (SUM 0 of 50–70 ppmh) described by Karnosky *et al*. (1996) which decreased stem biomass for the O_3 sensitive clone 259 but not for the more O_3 tolerant clones 216 and 271.

Table 1 Daytime (7 a.m.–7 p.m) ozone (O_3) average concentration (ppb) and SUM 0 (ppmh) and standard deviation for ambient air plots and for O_3 and O_3 + CO_2 treatments in the Aspen FACE project in 1999 and 2000

Year	Treatment	Daytime average (ppb)	SUM 0 (ppmh)
1999	Ambient	36.9 ± 6.5	62.8 ± 11.1
	O_3 treatments	51.1 ± 17.1	87.9 ± 29.4
	CO_2 + O_3 treatments	52.3 ± 18.0	90.0 ± 31.9
2000	Ambient	36.0 ± 11.0	58.2 ± 17.7
	O_3 treatments	47.4 ± 21.6	78.8 ± 35.9
	CO_2 + O_3 treatments	48.9 ± 22.2	80.9 ± 36.7

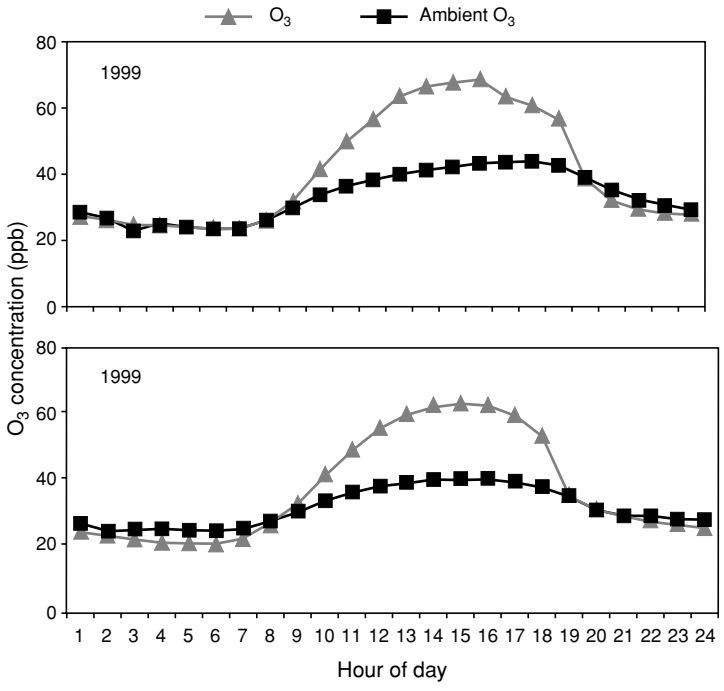

Fig. 1 The hourly profiles (mean hourly values over the growing season) for O_3 (grey) concentrations in O_3 (top) and $CO_2 + O_3$ treatments (bottom) and ambient rings (black) are shown from 1999 data. The 2000 profiles were similar.

Rust assessment

Significant differences in rust occurrence and severity occurred owing to clone and treatment (Table 2, Fig. 3). Seasonal exposures to O_3 and $CO_2 + O_3$ both resulted in significantly increased rust occurrence and severity (three- to five-fold) for all the three clones. Clonal differences in rust susceptibility were greater in 1999 than 2000, although some of these differences may be in part because of the earlier sampling time of 2000. Some of the more severely rusted leaves might have already fallen from the trees by late September 1999. Weather conditions varied considerably for the 2 years as 2000 was on average cooler and wetter than 1999 (Heilman, unpublished). The O_3 sensitive clone (259) was consistently the most susceptible to rust occurrence though it was not always significantly different from the other two clones.

Our results support the findings of Beare *et al*. (1999) that O_3 exacerbates the extent of *Melampsora* rust infection in *Populus* and is counter to the results of Coleman *et al*. (1987) who found that *Melampsora* rust infection was decreased in the presence of O_3. The desperate findings could be because of the different *Populus* species used in the three experiments or to the differences in O_3 exposure durations and techniques. Ozone is known to predispose some host plants to pathogens (Manning & Tiedemann 1995; Sandermann 1996). Coleman *et al*. (1987) noted the impact of environment on genotypic reactions and the potential impact on disease resistance. Our data suggest that the more rust-resistant aspen clones show increased levels of infection after extended exposure to O_3, suggesting suppression of host resistance after prolonged exposure to O_3. Coleman *et al*. (1987) only tested very short-term O_3 exposures (5 h) whereas the Beare *et al*. (1999) O_3

Table 2 Analysis of variance for the effects of clone, year, CO_2, O_3 and $CO_2 + O_3$ on the occurrence and severity of *Melampsora medusae* rust on *Populus tremuloides*

Source of variation	df	%L[1]	Severity[2]	Index[3]
Clone	2	0.0001	0.0026	0.0003
Year	1	0.0056	n.s.	n.s.
Clone × Year	2	0.0002	0.0049	0.0008
CO_2	1	0.0035	n.s.	0.0153
Clone × CO_2	2	0.0131	n.s.	0.0453
Year × CO_2	1	n.s.	n.s.	n.s.
Clone × Year × CO_2	2	n.s.	n.s.	n.s.
O_3	1	0.0001	0.0001	0.0001
Clone × O_3	2	0.0094	n.s.	0.0001
Year × O_3	1	0.0194	n.s.	n.s.
Clone × Year × O_3	2	0.0037	n.s.	0.0055
CO_2 × O_3	1	0.0007	0.0500	0.0022
Clone × CO_2 × O_3	2	n.s.	n.s.	n.s.
Year × CO_2 × O_3	1	0.0032	n.s.	0.0334
Clone × Year × CO_2 × O_3	2	n.s.	n.s.	n.s.

[1]Percent leaves with *Melampsora medusae* uridiniospores
[2]Severity of *Melampsora medusae* infection (see Fig. 3 for details)
[3]Index comprised of average percent leaves infected × average severity

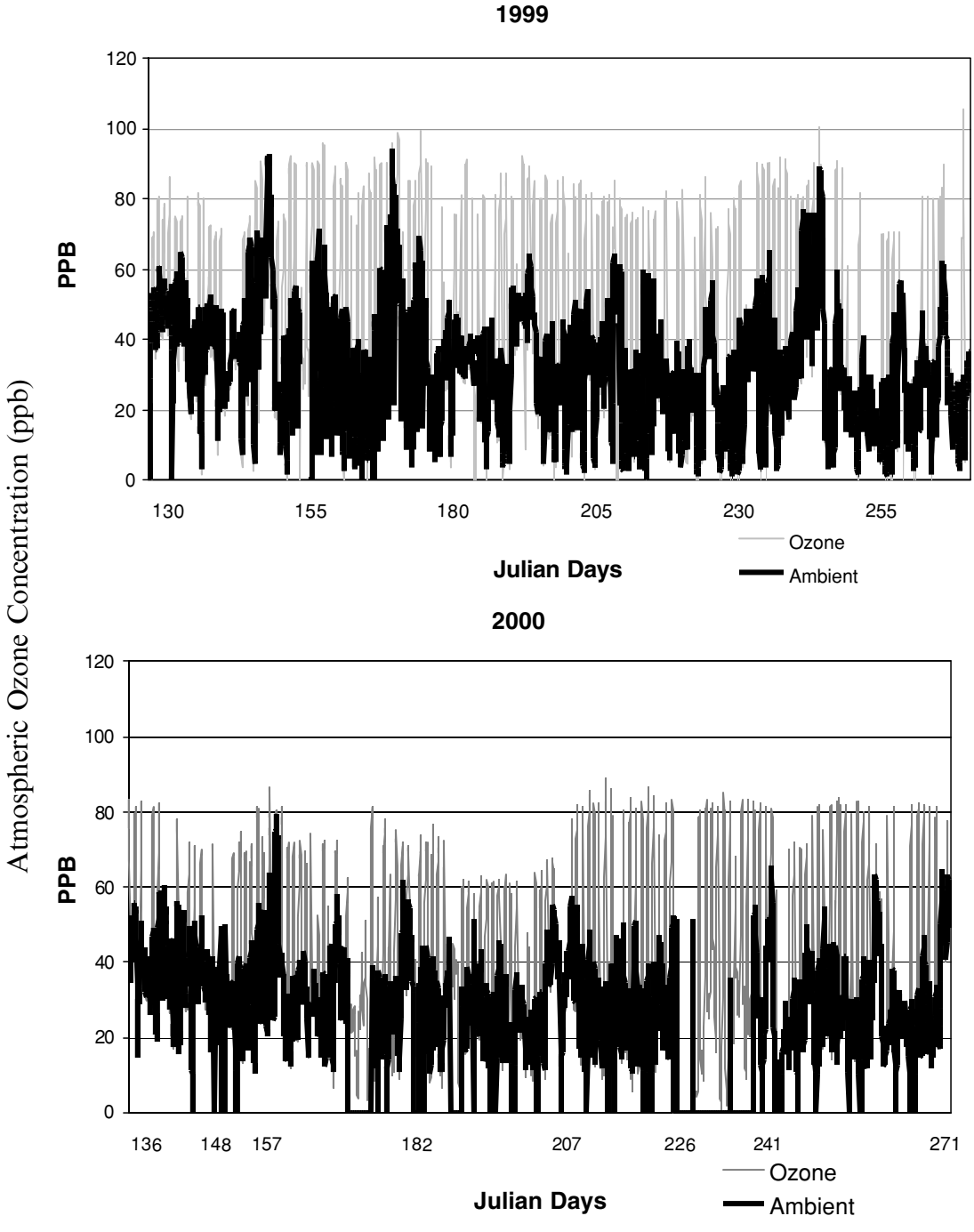

Fig. 2 The hourly average O_3 concentrations in the O_3 treatments (grey) and ambient rings (dark) are shown for 1999 and 2000.

exposures were over multiple weeks and our study involved seasonal exposures. In two of the few CO_2/O_3/pathogen interaction studies reported, Tiedemann & Firsching (1998, 2000) found that O_3 decreased the occurrence of *Puccinia recondita* rust on spring wheat while elevated CO_2 had no effect on rust occurrence. Because of the conflicting results, predicting interactions among trees and foliar pathogens under rapidly increasing atmospheric CO_2 and/or O_3 concentrations will be difficult at best (Kickert & Krupa 1990; Chakraborty *et al*. 2000; Seem *et al*. 2000).

Leaf surface physicochemical characteristics

Ozone had a large effect on the structure of wax deposits formed on aspen leaves in our study. Leaves from trees

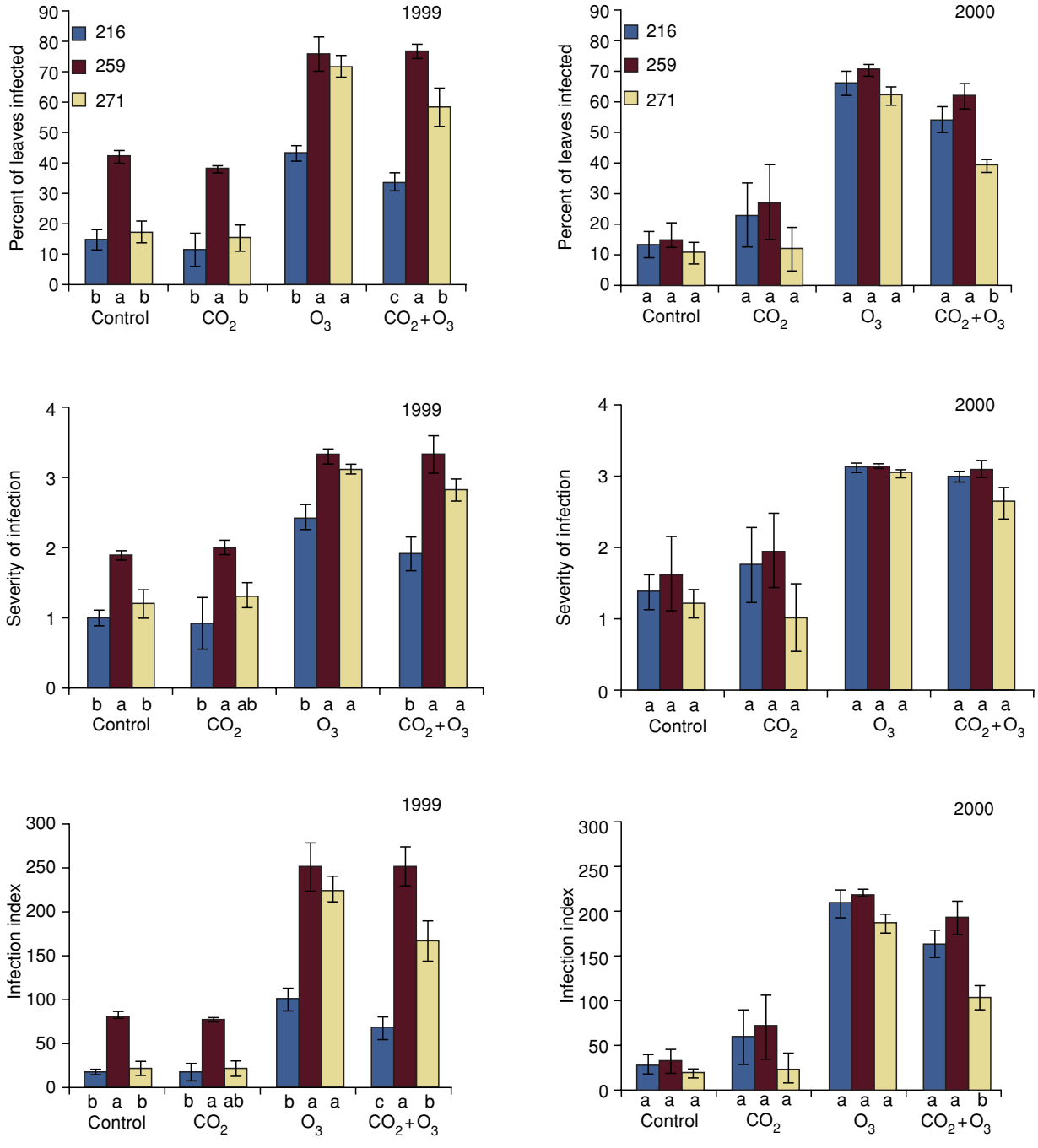

Fig. 3 *Melampsora medusae* occurrence (percent of leaves infected), severity of infection (1 = 1–20% of the leaf area covered with urediniospores, 2 = 21–40%, 3 = 41–60%, 4 = 61–80%, and 5 = 81–100%), and infection index (percent leaves infected × severity) for aspen clones 216, 259 and 271 exposed to growing season – long conditions of elevated CO_2 (ambient + 200 ppm), O_3 (1.5 × ambient), or CO_2 (ambient + 200 ppm) + O_3 (1.5 × ambient) at the Aspen FACE site in Rhinelander, Wisconsin during 1999 (top) and 2000 (bottom panels). All values are means ± SE.

growing with elevated O_3 had an increased proportion of amorphous; flat deposits (Fig. 4) crystallized over a dense array of plate crystallites (Fig. 4) normally observed distributed between pronounced cuticular ridges on control leaves.

Allen (1991) demonstrated the key role of topographical signals from the leaf surface in the development of appressoria from urediniospores of *M. medusae* (unspecified f. sp. but probably *deltoidae*) collected from *P. deltoides*. Optimal ridge heights preferred for

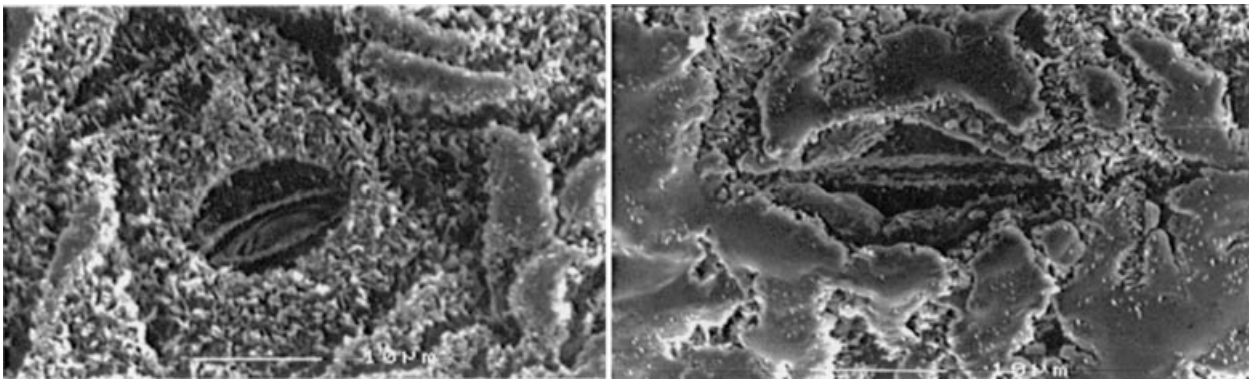

Fig. 4 Ozone impacts on the adaxial leaf surface waxes are shown here in a comparison of scanning electron micrographs of leaves from aspen clone 259 grown under control (left) and elevated O_3 (right) in the FACTS II (Aspen FACE) project. The visible changes of wax structure from crystallite (left) to amorphous forms (right) have been confirmed by extensive SEM characterization of hundreds of leaves (Mankovska *et al*. 1998; Karnosky *et al*. 1999) and by subsequent wax chemical composition analysis data (see Fig. 6).

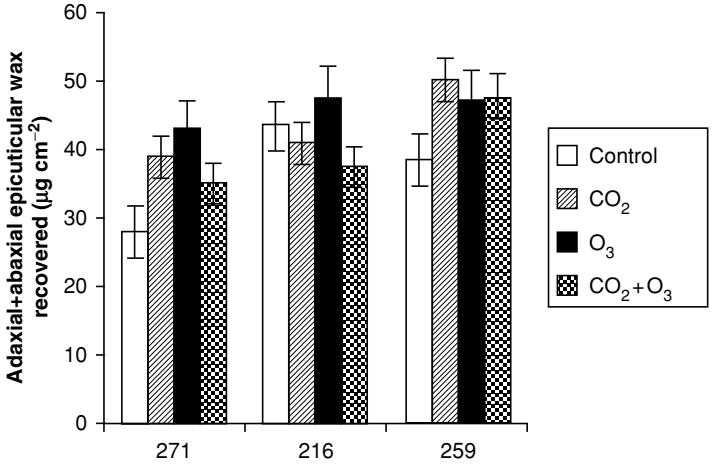

Fig. 5 Epicuticular wax amounts recovered from three aspen clones exposed to control, elevated CO_2, elevated O_3 or elevated $CO_2 + O_3$ over the 1999 growing season in the Aspen FACE project near Rhinelander, Wisconsin. Data are means $\pm$ SE.

appressorium formation (a prerequisite for leaf penetration by the pathogen) are quite low (0.4–0.8 μm). This morphology would likely be similar to the flattened amorphous epicuticular wax deposits we see on aspen leaves exposed to O_3 (Fig. 4b) and as described by Mankovska *et al*. (1998) and Karnosky *et al*. (1999). The O_3-induced topography is in marked contrast to the large ridges seen in waxes of control aspen leaves (Fig. 4a).

To be sure that the structural changes we saw were not induced during specimen preparation or SEM examination, we examined the production and chemical composition of aspen leaf surface waxes following seasonal O_3 and/or CO_2 exposures. Averaged across all the four treatments in our experiment, the O_3 sensitive clone (259) had significantly greater ($P < 0.0001$) adaxial/abaxial wax deposits (45.7 μg cm^{-2}) compared to the O_3 tolerant clones 216 (42.8 μg cm^{-2}) and 271 (36.6 μg cm^{-2}). Ozone and $CO_2 + O_3$ treatments resulted in changes in wax production in two of the three clones (Fig. 5). Wax amount on O_3 treated (42.8 μg cm^{-2}) and $CO_2 + O_3$-treated (34.9 μg cm^{-2}) leaves of clone 271 were significantly greater ($P < 0.001$) than on control (29.1 μg cm^{-2}) leaves. In the O_3 sensitive clone 259, wax amount on O_3-treated (47.1 μg cm^{-2}) and $CO_2 + O_3$-treated (47.6 μg cm^{-2}) leaves was significantly greater ($P < 0.001$) than on control (38.4 μg cm^{-2}) leaves (Fig. 5). Increased wax amount implies an O_3-mediated stimulation of *de novo* synthesis, which may be a protective adaptation similar to the generalized wounding mechanism of plant response to O_3 (Heath 1999).

Significant ($P < 0.01$) treatment effects on epicuticular wax chemical composition were detected for two (216 and 259) of the three clones. Along with increases in

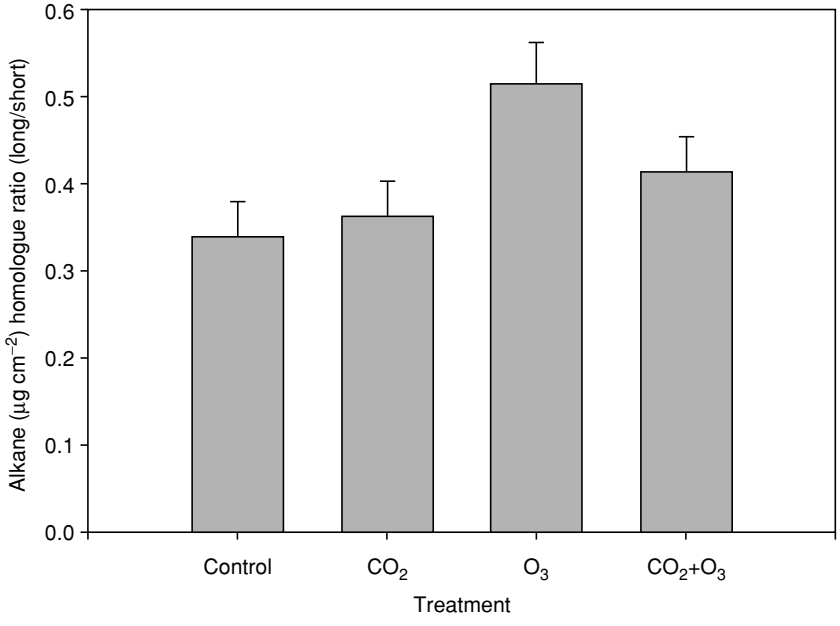

Fig. 6 Alkane carbon chain ratio [(ΣC29–C35/ΣC23–C28) homologues; expressed per unit leaf area (μg cm^{-2})] averaged across three aspen clones exposed to control, elevated CO_2, elevated O_3, elevated $CO_2 + O_3$ during the 1999 growing season in the Aspen FACE project near Rhinelander, Wisconsin. Data are means $\pm$ 1 SE.

proportions of wax classes crystallizing largely into amorphous forms (alkyl esters, fatty acids), there were proportional decreases in classes crystallizing as plates (alkanols, alkanes). Early work by Holloway (1969) showed that alkanes were most hydrophobic, marginally more so than esters and considerably more so than fatty acids. Changes in wax class composition therefore would be expected to result in changes to leaf wettability, possibly leading to a biological effect.

Ozone additionally induced significant changes in wax biosynthesis in the homologue carbon chains length. For instance, the ratio of long to short alkane carbon chains was significantly increased ($P < 0.05$) because of the O_3 in clone 259 (Fig. 6). More of the carbon allocated to the elongase-decarboxylase pathway was metabolized into the longer (> C28) chains, possibly contributing to the observed subtle alteration in leaf surface properties.

Leaf surface wettability

The key role of cuticle wettability in fungal pathogen spore survival has been pointed out by Kerstiens (1996). It is also known that wettability of tree foliar cuticles can be altered by O_3 (Percy *et al*. 1994). Contact angles on both adaxial and abaxial surfaces were affected by O_3. With particular reference to *Melampsora medusae* urediniospores which germinate on aspen abaxial leaf surfaces, exposure to O_3 decreased ($P < 0.0001$) the contact angle (leaves were more wettable) in the two O_3 tolerant clones but not in the O_3 sensitive clone 259 (Fig. 7). Interestingly, cofumigation with CO_2 ameliorated the negative effects of O_3 to different degrees on contact angle in the two tolerant clones.

Conclusions

Our results clearly show that exposure to O_3 alters leaf surface integrity. Leaf surface topography, microroughness and physicochemical characteristics such as chemical composition and epicuticular wax structure (conferred by its chemical composition) all combine to determine leaf surface properties such as wettability, retention of solutes, and foliar uptake among others. The microenvironment thus produced at the phylloplane is a critical determinant in the process of fungal attachment and infection (Kerstiens 1996; Mendgen 1996).

We believe that the O_3-induced changes to leaf surface physicochemical characteristics and wettability will significantly increase incidence of *Melampsora medusae* as documented here and these changes may have deleterious short-term impacts on carbon fixation in aspen and influence long-term competitive ability of the different clones. Further experiments, such as the artificial inoculation of aspen leaf surfaces having altered physicochemical characteristics produced under controlled O_3 exposure are suggested to test this hypothesis.

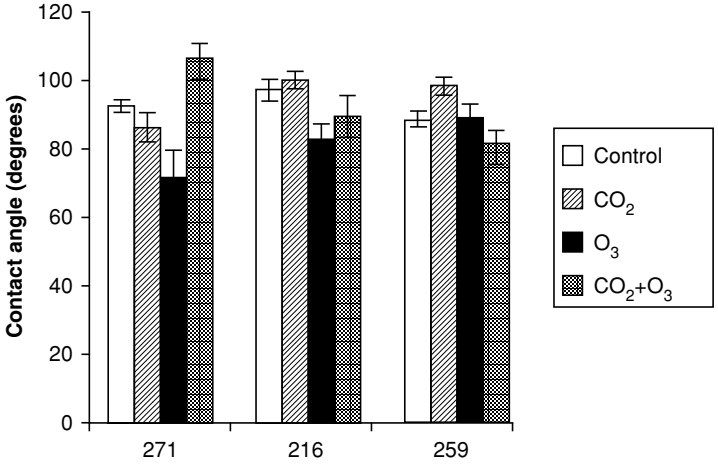

Fig. 7 Droplet contact angle (DCA) of the abaxial leaves of three aspen clones exposed to control, elevated CO_2, elevated O_3, elevated $CO_2 + O_3$ over the 1999 growing season in the Aspen FACE project near Rhinelander, Wisconsin. Data are means $\pm$ SE.

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