Environmental Pollution 158 (2010) 2664–2671

Contents lists available at ScienceDirect

Environmental Pollution

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

Ozone exposure and stomatal sluggishness in different plant physiognomic classes

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Sluggish stomatal responses are suggested to be both an effect of O_3 exposure and a reason of increased O_3 sensitivity in plants.

ARTICLE INFO

Article history:
Received 30 November 2009
Received in revised form
27 April 2010
Accepted 28 April 2010

Keywords:
Snap bean
Oak
Stomatal conductance
Stomatal sluggishness
Tropospheric ozone

ABSTRACT

Gas exchange responses to static and variable light were tested in three species: snap bean (*Phaseolus vulgaris*, two cultivars), California black oak (*Quercus kelloggii*), and blue oak (*Q. douglasii*). The effects of 1-month (snap beans) and 2-month (oaks) O_3 (ozone) exposure (70 ppb over 8 h per day in open-top chambers) were investigated. A delay in stomatal responses (i.e., 'sluggish' responses) to variable light was found to be both an effect of O_3 exposure and a reason for increased O_3 sensitivity in snap bean cultivars, as it implied higher O_3 uptake during times of disequilibrium. Sluggishness increased the time to open (thus limiting CO_2 uptake) and close stomata (thus increasing transpirational water loss) after abrupt changes in light level. Similar responses were shown by snap beans and oaks, suggesting that O_3-induced stomatal sluggishness is a common trait among different plant physiognomic classes.

1. Introduction

Tropospheric ozone (O_3) pollution is an under-considered component of global climate change (Bytnerowicz et al., 2007). Background O_3 levels over the mid-latitudes of the Northern Hemisphere have continued to rise over the past three decades, at 0.5–2% rate per year (Vingarzan, 2004). Ozone is the most phytotoxic air pollutant. To explain loss of plant yield and productivity, attention has primarily focused on O_3 effects on net photosynthesis (Reich, 1987). Photosynthetic impairment has been attributed to a decline in the efficiency of carboxylation, the electron transport system, and indirect effects on stomata (Paoletti and Grulke, 2005). The research presented here extends knowledge of elevated O_3 effects on stomatal behavior.

Stomatal closure is a secondary response to O_3 (stomatal conductance (gs) decreases following a decrease in intercellular CO_2 concentration), but an understanding of direct stomatal responses to O_3 is still imperfect (Paoletti and Grulke, 2005). Incomplete stomatal closure under unfavorable conditions during the day (Wieser and Havranek, 1995; Matyssek et al., 1995; Grulke et al., 2007b; Mills et al., 2009) or at night (Grulke et al., 2004) can occur with moderate and above O_3 exposures. Sluggish stomatal response is defined here as a delay in stomatal response to changing environmental factors relative to controls. Sluggish stomatal response to light with O_3 exposure was first postulated in Norway spruce under transpirational assay, i.e. by measuring water losses over time in detached needles (Keller and Häsler, 1984). Sluggish stomatal responses with O_3 exposure suggests an uncoupling of the normally tight relationship between carbon assimilation and gs as measured under steady-state conditions (Paoletti and Grulke, 2005). Stomatal aperture increases directly due to photosynthetic photon flux density (PPFD) or indirectly by PPFD-induced changes in photosynthesis that affect intercellular CO_2 concentrations (Aphalo and Jarvis, 1993). In this paper, we focused on stomatal responses to abrupt changes in light. Steady-state light conditions are not common in the field: variable light may represent two thirds of the incident light in forest canopies (Pearcy, 1990). Sluggish stomatal response following O_3 exposure has also been reported with changes in leaf-to-air vapor pressure deficits (e.g. *Acer saccharum*, Tjoelker et al., 1995; *Pinus sylvestris*, Kellomäki and Wang, 1997) and water stress (e.g. *Populus deltoides* × *trichocarpa*, Reich and Lassoie, 1984; *Arbutus unedo*, Paoletti, 2005; *Dactylis glomerata* and *Leontodon hispidus*, Mills et al., 2009; *Rudbeckia laciniata* var. *digitata*, Grulke et al., 2007a).

We conducted measurements for three species in different physiognomic classes: an annual crop (an O_3-sensitive (S156) and

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doi:10.1016/j.envpol.2010.04.024

an O_3-tolerant (R331) cultivar of snap bean, *Phaseolus vulgaris* L.), a broadleaf deciduous tree (California black oak, *Quercus kelloggii* Newb.), and a broadleaf drought-deciduous tree (blue oak, *Q. douglasii* Hook. and Arn.). *Q. douglasii* retains multiple years of leaves; when it experiences severe drought, it completely loses its leaves (Pavlik et al., 2002). We addressed the following questions: 1) Are O_3-induced sluggish stomatal responses a common trait across different plant physiognomic classes? 2) Does stomatal sluggishness affect O_3 sensitivity of plants? 3) Is stomatal sluggishness related to photosynthetic responses?

2. Materials and methods

The snap bean O_3-sensitivity cultivars were developed at the Raleigh USDA-ARS. Cultivar R331 is considered O_3 tolerant, while cultivar S156 is known to be highly sensitive (Burkey and Eason, 2002; Flowers et al., 2007; Booker et al., 2009). Although R331 and S156 were similar in size and productivity in low-O_3 air under field conditions (Flowers et al., 2007), yield of S156 was reduced by up to 60% by ambient O_3 (45 ppb as 12-h mean) compared with R331 (Booker et al., 2009). *Q. kelloggii* is a drought-adapted species common throughout California in the transition between chaparral and the montane, mixed conifer forest (McDonald, 1990a). *Quercus kelloggii* was symptomatic in southern California during the high O_3 exposures of the mid 1970s, and continues to exhibit a low level of visible foliar injury under ambient field conditions (Grulke et al., 2005). Earlier onset of senescence and sluggish stomatal responses with high background O_3 exposure and nitrogen deposition has been reported in this species in the field (Grulke et al., 2005). *Q. douglasii* is found in the interior foothills throughout California, and is the most drought tolerant of California's deciduous oaks (McDonald, 1990b). Little physiological data are available for this species. In the greenhouse, these seedlings retained 3–5 leaf age classes, whereas mature trees retain 0–30% of foliage through the winter depending on water availability.

Oaks were germinated from acorns collected locally, and grown in greenhouses (Riverside, CA; 8 ± 2 ppb O_3 on average) until 3 and 5 yr old for *Q. kelloggii* and *Q. douglasii*, respectively. Snap beans were germinated in the same greenhouses and moved to open-top exposure chambers (OTCs; Heagle et al., 1973) when plants were 6-weeks-old (from time of emergence). Plants were measured as described below and placed in three charcoal-filtered or three elevated O_3 OTCs. Three plants of each oak species and six of each snap bean O_3-sensitivity were placed in each OTC. Every week, plants were moved to an OTC with same O_3 treatment level. The O_3 levels were 22 ± 4 ppb and 70 ± 7 ppb for 8 h per day (10 a.m. – 6 p.m.) in the control and ozone chambers, respectively. Snap bean responses were tested after one month of exposure, and oaks were tested after two months of exposure. During O_3 exposure, average temperature and relative humidity were 23 °C and 24% over one month and 24 °C and 24% over two months based on hourly values as measured in each chamber ("Hobos," Model S-THA-M017, Onset Computer Corp.). Plants were watered daily.

Gas exchange responses were measured with two intercalibrated open gas exchange systems (Model 6400, LiCor Instruments Lincoln, NE) at controlled leaf temperature (25 °C), leaf-to-air vapor pressure differences (VPD_L, 1.5 kPa), saturating photosynthetic photon flux density (PPFD, 1800 μmol m^{-2} s^{-1}, unless varied for dynamic light responses), and CO_2 concentration (385 ppm, unless varied for response curves). Measurements were carried out at the beginning of the experiment (pre-exposure) and 1–2 months later (post-exposure) between 8:30 and 15:30 on 1 plant per OTC, 1–2 healthy leaves per plant, at set positions from the terminal shoot in oaks (3rd–5th) and snap bean (2nd).

To simulate dynamic "flecky" light responses, the methodology developed and described in Paoletti (2005) was applied (Fig. 1). After gs achieved equilibrium at saturating light (gs_{high} at 1800 μmol m^{-2} s^{-1}), the light intensity was sharply decreased to 200 μmol m^{-2} s^{-1}. After achieving a new equilibrium (gs_{low}), the light level was sharply increased back to 1800 μmol m^{-2} s^{-1}. The light cycle was conducted twice on each leaf measured. The system continuously logged the data at 15-s intervals. The average of 5–6 data points at equilibrium (low (A_{low}) and high light (A_{high})) was used to calculate: range of gs variation, $\Delta gs = gs_{high} - gs_{low}$; time to close stomata, $T_{close} = Tgs_{low} - Tgs_{high}$; time to open stomata, $T_{open} = Tgs_{high} - Tgs_{low}$; rate of change of gs for both opening ($\Delta gs/T_{open}$) and closing ($\Delta gs/T_{close}$), where Tgs_{low} and Tgs_{high} were the time to reach minimum and maximum equilibrated gs in each cycle.

For deriving CO_2 response curves (A/Ci), A was measured over a range of CO_2 concentrations (375, 275, 125, 375, 675, 975, 1275, and 1675 ppm) under saturating light. Gas exchange was recorded for 900 s at each new CO_2 concentration before the next concentration was imposed. Maximum RuBP-saturated rate of carboxylation (V_{cmax}) and maximum rate of electron transport (J_{max}) were estimated with an iterative procedure according to Farquhar et al. (1980) and Long and Bernacchi (2003). Stomatal limitation (L, i.e. the proportionate decrease in A that may be attributed to stomata at ambient CO_2 concentration) was calculated by comparing the observed and expected values of A at 370 ppm CO_2 concentration (Farquhar and Sharkey, 1982). A/Ci responses for *Q. douglasii* after O_3 exposure were not measured.

The statistical unit was the single plant. Data were checked for normal distribution by the Kolmogorov–Smirnov d test and eventually ln-transformed before ANOVA. Original data were used for the tables. Data were evaluated by a one-way ANOVA to test for the effects of single factors, or a two-way ANOVA to test for the effects of O_3 exposure and either snap bean cultivar or oak species. Result were considered significant at $p \leq 0.05$, while a tendency towards significance was considered when $0.05 \leq p < 0.1$. This choice is justified by the fact that this kind of measurements is time consuming (up to 40 min per each) and variable with environmental fluctuations, while there was a need to maximize replication and concentrate the measurements during the daytime environmental variables were relatively constant. Linear correlations between parameters and pre- vs. post-exposure t tests were tested at $p \leq 0.05$. Statistical analysis was performed with STATISTICA software (6.0, StatSoft Inc., Tulsa, OK).

3. Results

3.1. Pre-exposure gas exchange attributes

Prior to O_3 exposure, O_3-sensitive and tolerant snap beans under static light conditions had similar gas exchange parameters

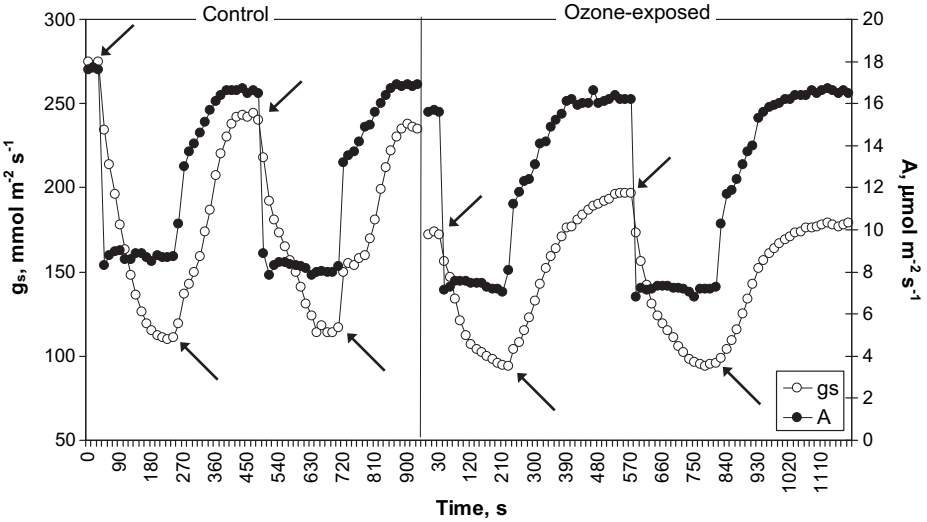

Fig. 1. Example of "flecky" light curves, i.e. stomatal conductance (gs) and photosynthesis (A) responses to light variation in ozone-sensitive snap beans exposed to charcoal-filtered air (control) or to O_3 (70 ppb over 8 h per day in open-top chambers) for one month. Arrows show the time of light down to 200 μmol m^{-2} s^{-1} and up to 1800 μmol m^{-2} s^{-1}.

Table 1
Gas exchange attributes under static light conditions (±SE) before O_3 exposure. gs_{high}, maximum stomatal conductance at saturating light; gs_{low}, minimum stomatal conductance at low light; A_{high}, light-saturated maximum photosynthesis; A_{low}, minimum photosynthesis at low light; V_{cmax}, maximum RuBP-saturated rate of carboxylation; J_{max}, maximum rate of electron transport; L., stomatal limitation; PV, *Phaseolus vulgaris* (an O_3-sensitive and an O_3-tolerant cultivar); QK, *Quercus kelloggii*; QD, *Q. douglasii*. Levels of significance of a one-way ANOVA are shown (N = 6 plants in PV, N = 3 plants in QD and QK). A t shows a significant t test ($p \leq 0.05$) in the comparison between pre- and post-exposure control values. Post-exposure values are in Table 3.

Species	gs_{high} [mmol m^{-2} s^{-1}]		gs_{low} [mmol m^{-2} s^{-1}]		A_{high} [μmol m^{-2} s^{-1}]		A_{low} [μmol m^{-2} s^{-1}]	V_{cmax} [μmol m^{-2} s^{-1}]		J_{max} [μmol m^{-2} s^{-1}]		L [%]	
PV sensitive	327 ± 36	t	64 ± 13		22.2 ± 2.2	t	5.4 ± 1.4	121 ± 18	t	199 ± 19	t	30 ± 5.6	t
PV tolerant	364 ± 13	t	88 ± 16		20.8 ± 0.9	t	6.2 ± 0.5	128 ± 15	t	199 ± 22	t	25 ± 1.8	t
ANOVA results													
Cultivar	*0.175 ns*		*0.096+*		*0.396 ns*		*0.464 ns*	*0.662 ns*		*0.972 ns*		*0.248 ns*	
QD	123 ± 31		37 ± 2		8.3 ± 1.7		3.6 ± 0.8	41 ± 5		66 ± 2.4		24 ± 3.4	
QK	64 ± 14		45 ± 16		7.1 ± 0.9		4.5 ± 0.5	40 ± 10		71 ± 18		31 ± 4.1	
ANOVA results													
Species	*0.153 ns*		*0.624 ns*		*0.587 ns*		*0.417 ns*	*0.925 ns*		*0.806 ns*		*0.242 ns*	

ns, $p > 0.1$; +, $p < 0.1$; *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

(Table 1). Maximum and minimum gs and A, and parameters from A/Ci curves were statistically indistinguishable. However, gs_{low} showed a tendency to be higher in the tolerant cultivar (ca 40%), implying higher endogenous O_3 uptake at low light levels. Dynamic responses to light variation were similar in the two snap bean cultivars, although the sensitive cultivar had a tendency to lower rate of change in gs (-17%) during closure than that of the tolerant cultivar (Table 2). No other differences were statistically significant. The magnitude of change in gs from high to low light showed a tendency to be greater, and the response time of stomatal opening and closure of *Q. douglasii* was significantly longer, than that of *Q. kelloggii* (+358%, +170% and +267%, respectively).

The attributes measured under static light, i.e. gs_{high}, gs_{low}, A_{high}, A_{low}, V_{cmax}, J_{max} and L, were not correlated to each other in the two oaks, except for J_{max} vs. V_{cmax} (Table 5). In snap bean, A_{low} was significantly correlated with V_{cmax}, J_{max} and A_{high}, and A_{high} was significantly correlated with gs_{high}, and gs_{low}. Several of the gas exchange attributes measured under dynamic light, i.e. ΔA, Δgs, T_{open} and T_{close}, were correlated to each other in both beans and oaks: ΔA vs. Δgs; ΔA vs. T_{open}; and T_{open} vs. T_{close}.

3.2. Phenological and non-O_3 environmental effects over the course of the OTC experiment

Due to phenological and non-O_3 environmental effects after 1 month in charcoal-filtered OTCs, snap bean exhibited significant reductions in most gas exchange attributes relative to pre-experiment values (see t test in Tables 1 and 2): gs_{high} (−47% and −55%, in the sensitive and tolerant cultivar, respectively), A_{high} (−39% and −37%), V_{cmax} (−70% and −62%), J_{max} (−70% and −61%), ΔA (−57% and −59%), Δgs (−70% and −79%), T_{open} (−79% and −82%), and T_{close} (−82% and −86%). Stomatal limitation to assimilation, L, increased in control OTC plants (+46% and +87%, for sensitive and tolerant cultivars, respectively). After 1 month in control OTCs, stomata were more sluggish in response to changes in light in the sensitive cultivar (+70%, $\Delta gs/T_{open}$; +72%, $\Delta gs/T_{close}$).

Quercus kelloggii grown in charcoal-filtered air showed a significant reduction in T_{open} (−62%) and T_{close} (−61%), relative to pre-experiment values (see t test in Tables 1 and 2), consequently inducing the statistical significance of the differences between $\Delta gs/T_{open}$ and $\Delta gs/T_{close}$ of the two oaks (Table 4). Stomatal opening and closing in post-exposure *Q. kelloggii* controls were faster than those of *Q. douglasii* controls (−85% and −90%, respectively), so that the rate of change of gs in both opening and closing stomata was higher in the former species (+120% and +400%, respectively). Similar figures were recorded for O_3-treated oaks, with faster opening (-81%) and closing of stomata (−86%) and higher rates of gs change in opening (+155%) and closing (+350%) in *Q. kelloggii* than in *Q. douglasii*.

3.3. Gas exchange responses after O_3 exposure

Sensitive snap beans showed a tendency to longer times for stomatal opening (+29% and +36% in control vs. O_3-exposed plants, respectively) and closing (+37% and +10%) than tolerant cultivars (Table 4). *Q. kelloggii* control and O_3-treated plants showed a tendency to higher gs_{low} (+110% and +60%, respectively; Table 3) and lower Δgs in response to dynamic light (−53% and −35%; Table 4) than *Q. douglasii*. *Quercus douglasii* also showed higher ΔA than *Q. kelloggii* (+68% and +35% in control and O_3-exposed plants, respectively).

Relative to plants grown in charcoal-filtered air, O_3 exposure increased the time for stomatal closure in both snap bean cultivars (+13% and +42% in the sensitive and tolerant cultivar, respectively)

Table 2
Dynamic gas exchange responses (±SE) to light variation (from high to low light and vice versa) before ozone exposure. ΔA, span of photosynthesis variation; Δgs, span of stomatal conductance variation; T_{open}, time to open stomata; T_{close}, time to close stomata; $\Delta gs/T_{open}$ and $\Delta gs/T_{close}$, rate of change of gs in opening and closing stomata. For other acronyms, see Table 1. Levels of significance of a one-way ANOVA are shown (N = 6 plants in PV, N = 3 plants in QD and QK). A t shows a significant t test ($p \leq 0.05$) in the comparison between pre- and post-exposure control values. Post-exposure values are in Table 4.

Species	ΔA [μmol m^{-2} s^{-1}]		Δgs [mmol m^{-2} s^{-1}]		T_{open} [s]		T_{close} [s]		$\Delta gs/T_{open}$		$\Delta gs/T_{close}$	
PV sensitive	15.7 ± 1.2	t	264 ± 31	t	984 ± 85	t	893 ± 53	t	0.27 ± 0.03	t	0.29 ± 0.02	t
PV tolerant	15.9 ± 1.2	t	276 ± 21	t	912 ± 83	t	805 ± 61	t	0.32 ± 0.03		0.35 ± 0.03	
ANOVA results												
Cultivar	*0.874 ns*		*0.626 ns*		*0.376 ns*		*0.121 ns*		*0.294 ns*		*0.097+*	
QD	4.6 ± 1.0		87 ± 29		989 ± 173		1168 ± 181		0.08 ± 0.02		0.08 ± 0.03	
QK	2.6 ± 0.5		19 ± 3		366 ± 75	t	318 ± 111	t	0.05 ± 0.01		0.07 ± 0.02	
ANOVA results												
Species	*0.138 ns*		*0.080+*		*0.030**		*0.016**		*0.108 ns*		*0.878 ns*	

ns, $p>0.1$; +, $p<0.1$; *, $p<0.05$; **, $p<0.001$; ***, $p<0.0001$.

Table 3
Gas exchange responses under static light conditions (±1 SE) after O_3 exposure. Plants were exposed to charcoal-filtered air (C) or ozone (O_3) in open-top chambers for one month (PV) or two months (QK and QD). Acronyms like in Table 1. A/Ci curves were not carried out on QD. Levels of significance of one- or two-way ANOVA are shown (N = 6 plants in PV, N = 3 plants in QD and QK).

Species	Exposure	gs_{high} [mmol $m^{-2} s^{-1}$]	gs_{low} [mmol $m^{-2} s^{-1}$]	A_{high} [μmol $m^{-2} s^{-1}$]	A_{low} [μmol $m^{-2} s^{-1}$]	V_{cmax} [μmol $m^{-2} s^{-1}$]	J_{max} [μmol $m^{-2} s^{-1}$]	L [%]
PV sensitive	C	172.1 ± 21.4	93.0 ± 9.9	13.5 ± 1.1	6.8 ± 0.4	36.1 ± 2.5	58.9 ± 4.6	43.8 ± 2.5
PV sensitive	O_3	180.2 ± 19.4	97.7 ± 12.0	13.7 ± 1.4	6.7 ± 0.6	45.5 ± 4.7	72.7 ± 7.5	45.8 ± 2.1
PV tolerant	C	162.8 ± 14.8	107.4 ± 15.6	13.1 ± 0.7	6.6 ± 0.3	48.5 ± 4.3	78.5 ± 7.1	46.7 ± 1.4
PV tolerant	O_3	147.4 ± 21.3	85.5 ± 11.8	12.4 ± 0.9	6.1 ± 0.2	43.6 ± 1.9	68.6 ± 1.9	49.0 ± 1.5
ANOVA results								
Cultivar		*0.291 ns*	*0.928 ns*	*0.434 ns*	*0.378 ns*	*0.207 ns*	*0.255 ns*	*0.135 ns*
Ozone		*0.855 ns*	*0.498 ns*	*0.847 ns*	*0.474 ns*	*0.583 ns*	*0.773 ns*	*0.290 ns*
Cultivar × Ozone		*0.552 ns*	*0.301 ns*	*0.693 ns*	*0.680 ns*	*0.090+*	*0.087+*	*0.941 ns*
QD	C	79.5 ± 19.7	28.4 ± 5.7	6.34 ± 0.3	2.29 ± 0.29			
QD	O_3	140.4 ± 14.5	61.0 ± 10.6	8.87 ± 0.55	3.82 ± 0.47			
QK	C	84.0 ± 6.1	59.7 ± 10.2	5.84 ± 0.78	3.43 ± 0.71	35.2 ± 1.8	58.8 ± 3.8	28.7 ± 2.8
QK	O_3	152.7 ± 26.0	97.6 ± 21.1	7.15 ± 1.40	3.41 ± 0.65	32.5 ± 3.6	52.0 ± 4.4	25.0 ± 1.2
ANOVA results								
Species		*0.698 ns*	*0.075+*	*0.364 ns*	*0.624 ns*			
Ozone		*0.008***	*0.066+*	*0.127 ns*	*0.314 ns*	*0.441 ns*	*0.197 ns*	*0.211 ns*
Species × Ozone		*0.855 ns*	*0.883 ns*	*0.612 ns*	*0.307 ns*			

(Table 4, Fig. 1). Because of O_3 exposure, both V_{cmax} and J_{max} showed a tendency to be higher in the O_3-sensitive cultivar only (+26% and +23%, respectively) (Table 3). Relative to control plants, O_3-exposed oaks showed higher gs_{high} (+77% and +82% in *Q. douglasii* and *Q. kelloggii,* respectively), gs_{low} (+115% and +63%) and Δgs (+60% and +124%). Also relative to controls, stomata of both oaks were slightly slower to open and close after O_3 exposure: +20% and +5% in *Q. douglasii* and +44% and +37% in *Q. kelloggii*, respectively.

Correlations among static light parameters were similar to those at pre-exposure time in both control and O_3-treated snap beans with a higher correlation coefficient between maximum and minimum A and gs (Table 6). The strength of the correlations among static light parameters increased slightly in oaks compared to the pre-exposure ones, e.g. gs_{high} vs. V_{cmax} and J_{max} (Table 7). V_{cmax} and J_{max} were always correlated to each other. Relative to pre-exposure, ΔA vs. Δgs were still correlated in both beans and oaks, independent on O_3, and T_{open} and T_{close} were still correlated to each other in both control and O_3-exposed oaks (Tables 6 and 7). Pre-exposure and post-exposure control and O_3-treated beans had consistent correlations among gas exchange attributes, while oaks did not show consistent trends over the course of the experiment (Tables 5, 6 and 7). T_{close} was higher with higher gs_{high} in pre-exposure and post-exposure O_3-treated snap beans (Tables 5 and 6), while T_{open} increased with increasing gs_{high} only in oaks at pre-exposure time (Table 5).

4. Discussion

4.1. Responses without O_3 exposure

Ozone-sensitive snap beans were slower in stomatal closing and opening than tolerant snap beans. Sluggish stomatal response to changes in environmental conditions would be expected to increase water loss under unfavorable conditions and decrease carbon acquisition under favourable conditions, respectively. All other gas exchange parameters were similar between the two cultivars. In contrast, Flowers et al. (2007) reported higher photosynthesis in the sensitive vs. the tolerant cultivar, but similar gs. In their paper, Rubisco activity (V_{cmax}) was also higher in the sensitive cultivar, while stomatal limitation (L) did not differ between cultivars. In their O_3 exposure (60 ppb as a 12-h average), the decline in photosynthesis following flowering was significantly accelerated in the sensitive cultivar (Flowers et al., 2007). As a result, photosynthesis of the sensitive cultivar under O_3 was similar to that of the tolerant one across the entire growing season. The results by Flowers et al. (2007) thus suggest a strong effect of phenology on snap bean gas exchange. Their result was confirmed by our experiments presented here. The strong variation in both static and dynamic light parameters in control snap beans from pre-exposure (6-week-old plants) to post-exposure conditions in

Table 4
Dynamic gas exchange responses (±1 SE) to light variation after O_3 exposure. Acronyms like in Table 3. Levels of significance of a two-way ANOVA are shown (N = 6 plants in PV, N = 3 plants in QD and QK).

Species	Exposure	ΔA [μmol $m^{-2} s^{-1}$]	Δgs [mmol $m^{-2} s^{-1}$]	T_{open} [s]	T_{close} [s]	$Δgs/T_{open}$	$Δgs/T_{close}$
PV sensitive	C	6.7 ± 0.8	79.8 ± 13.2	211 ± 22	158 ± 13	0.46 ± 0.11	0.50 ± 0.05
PV sensitive	O_3	7.0 ± 0.9	83.2 ± 10.6	223 ± 24	179 ± 18	0.38 ± 0.04	0.47 ± 0.04
PV tolerant	C	6.5 ± 0.4	57.9 ± 7.9	163 ± 22	115 ± 14	0.38 ± 0.05	0.55 ± 0.11
PV tolerant	O_3	6.3 ± 0.7	67.1 ± 13.1	164 ± 10	163 ± 14	0.41 ± 0.07	0.40 ± 0.06
ANOVA results							
Cultivar		*0.507 ns*	*0.112 ns*	*0.065+*	*0.061+*	*0.720 ns*	*0.899 ns*
Ozone		*0.907 ns*	*0.589 ns*	*0.806 ns*	*0.029**	*0.736 ns*	*0.229 ns*
Cultivar × Ozone		*0.724 ns*	*0.802 ns*	*0.859 ns*	*0.371 ns*	*0.428 ns*	*0.408 ns*
QD	C	4.05 ± 0.04	51.0 ± 14.8	913 ± 146	1190 ± 95	0.05 ± 0.01	0.04 ± 0.01
QD	O_3	5.06 ± 0.35	81.5 ± 6.1	1095 ± 37	1243 ± 131	0.09 ± 0.02	0.06 ± 0.01
QK	C	2.41 ± 0.34	23.8 ± 5.5	140 ± 16	124 ± 6	0.16 ± 0.04	0.20 ± 0.04
QK	O_3	3.74 ± 0.84	53.3 ± 15.9	201 ± 46	170 ± 20	0.23 ± 0.06	0.27 ± 0.04
ANOVA results							
Species		*0.046**	*0.058+*	*<0.001****	*<0.001****	*0.067+*	*0.001***
Ozone		*0.105 ns*	*0.042**	*0.096+*	*0.061+*	*0.403 ns*	*0.336 ns*
Species × Ozone		*0.821 ns*	*0.972 ns*	*0.389 ns*	*0.974 ns*	*0.752 ns*	*0.757 ns*

Table 5
Pearson r coefficient and level of significance in the correlation matrix among parameters measured under static or dynamic light conditions before O_3 exposure. Top half: snap beans (N = 12); Bottom half: oaks (N = 6).

Pre-exposure – Snap beans											
	gs_{high}	gs_{low}	A_{high}	A_{low}	V_{cmax}	J_{max}	L	ΔA	Δgs	T_{open}	T_{close}
gs_{high}		ns	r=0.777 p<0.001	ns	ns	ns	ns	r=0.621 p=0.003	r=0.845 p<0.001	ns	r=0.459 p=0.042
gs_{low}	ns		r=0.517 p=0.020	ns	ns	ns	ns	ns	ns	ns	ns
A_{high}	ns	ns		r=0.692 p=0.001	ns	ns	ns	r=0.663 p=0.001	r=0.535 p=0.015	ns	ns
A_{low}	ns	ns	ns		r=0.602 p=0.038	r=0.738 p=0.006	ns	ns	ns	r=-0.813 p<0.001	ns
V_{cmax}	ns	ns	ns	ns		r=0.929 p<0.001	ns	ns	ns	r=-0.653 p=0.021	r=-0.614 p=0.034
J_{max}	ns	ns	ns	ns	r=0.914 p=0.011		ns	r=-0.667 p=0.018	ns	r=-0.869 p<0.001	r=-0.782 p=0.003
L	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns
ΔA	r=0.899 p=0.015	ns	r=0.868 p=0.025	ns	ns	ns	ns		r=0.507 p=0.023	r=0.459 p=0.042	r=0.705 p=0.001
Δgs	r=0.926 p=0.008	ns	ns	ns	ns	ns	r=-0.811 p=0.050	r=0.934 p=0.006		ns	ns
T_{open}	r=0.841 p=0.036	ns	ns	ns	ns	ns	r=-0.861 p=0.028	r=0.873 p=0.023	r=0.960 p=0.002		r=0.454 p=0.044
T_{close}	ns	ns	ns	ns	ns	ns	ns	ns	ns	r=0.871 p=0.024	
Pre-exposure - Oaks											

ns, Not significant.

Table 6
Pearson r coefficient and level of significance in the correlation matrix among parameters measured under static or dynamic light conditions in control and O_3-exposed snap bean plants after exposure (N = 12).

Post-exposure - Control snap beans											
	gs_{high}	gs_{low}	A_{high}	A_{low}	V_{cmax}	J_{max}	L	ΔA	Δgs	T_{open}	T_{close}
gs_{high}		r=0.734 p=0.007	r=0.813 p=0.004	r=0.754 p=0.012	ns	ns	ns	r=0.775 p=0.008	r=0.715 p=0.009	ns	ns
gs_{low}	r=0.907 p<0.001		ns	ns	ns	ns	ns	ns	ns	ns	ns
A_{high}	r=0.871 p<0.001	r=0.838 p=0.001		r=0.897 p<0.001	ns	ns	ns	r=0.970 p<0.001	r=0.840 p=0.002	ns	r=0.646 p=0.044
A_{low}	r=0.688 p=0.019	r=0.638 p=0.035	r=0.911 p<0.001		ns	ns	ns	r=0.763 p=0.010	r=0.695 p=0.026	ns	ns
V_{cmax}	ns	ns	ns	ns		r=0.956 p<0.001	ns	ns	ns	ns	ns
J_{max}	ns	ns	ns	ns	r=0.981 p<0.001		r=0.243 p<0.001	ns	ns	ns	ns
L	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns
ΔA	r=0.907 p<0.001	r=0.886 p<0.001	r=0.972 p<0.001	r=0.789 p=0.004	ns	ns	ns		r=0.846 p<0.001	ns	ns
Δgs	r=0.948 p<0.001	r=0.785 p=0.002	r=0.846 p<0.001	r=0.668 p=0.025	ns	ns	ns	r=0.881 p<0.001		ns	r=0.715 p=0.009
T_{open}	ns	ns	r=0.657 p=0.028	r=0.830 p=0.002	ns	ns	ns	ns	ns		ns
T_{close}	r=0.761 p=0.004	r=0.656 p=0.020	r=0.773 p=0.005	r=0.740 p=0.009	r=-0.650 p=0.022	r=-0.609 p=0.035	ns	r=0.731 p=0.011	r=0.692 p=0.013	ns	
Post-exposure – Ozone-treated snap beans											

ns, Not significant.

Table 7
Pearson r coefficient and level of significance in the correlation matrix among parameters measured under static or dynamic light conditions in control and O_3-exposed oak seedlings after exposure (N = 3–6).

Post-exposure - Control oaks											
	gs_{high}	gs_{low}	A_{high}	A_{low}	V_{cmax}	J_{max}	L	ΔA	Δgs	T_{open}	T_{close}
gs_{high}		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
gs_{low}	r=0.774 p=0.014		ns	r=0.696 p=0.037	ns	ns	ns	ns	ns	ns	ns
A_{high}	ns	ns		r=0.767 p=0.016	ns	ns	ns	ns	ns	ns	ns
A_{low}	ns	ns	r=0.892 p=0.001		ns	ns	ns	ns	ns	ns	ns
V_{cmax}	r=-0.995 p=0.005	ns	ns	ns		r=0.941 p=0.005	r=0.878 p=0.021	r=0.840 p=0.037	ns	ns	ns
J_{max}	r=-0.992 p=0.008	ns	ns	ns	r=0.998 p=0.002		r=0.988 p<0.001	ns	ns	ns	ns
L	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns
ΔA	ns	ns	r=0.941 p<0.001	r=0.686 p=0.041	ns	ns	ns		r=0.854 p=0.022	r=0.731 p=0.025	r=0.721 p=0.028
Δgs	ns	ns	r=0.859 p=0.003	r=0.670 p=0.048	ns	ns	ns	r=0.881 p=0.002		r=0.729 p=0.026	r=0.773 p=0.015
T_{open}	ns	ns	ns	ns	ns	ns	ns	ns	ns		r=0.987 p<0.001
T_{close}	ns	ns	ns	ns	ns	ns	ns	ns	ns	r=0.921 p=0.001	
Post-exposure ? Ozone-treated oaks											

ns, Not significant.

chambers with charcoal-filtered air (10-week-old plants, flowering in progress) was due to a different developmental stages and associated resource sinks. Another factor that may contribute to differences between these results and those of Flowers et al. (2007) may be relative humidity (RH) during plant growth prior to and during exposure. In this study, RH during exposure was 24% which is much lower than in the Flowers et al. (2007) study where reported vapor pressure deficits of ~1.4 kPa translate into an RH of ~58%. This may affect plant phenology so that gas exchange characteristics over time may be different as leaf development and senescence patterns acclimate to environments that differ in RH. This may be reflected in the fact that flowering was in progress in 10-week-old plants whereas flowering occurs at 4–5 weeks in plants grown at higher RH. Leaf development appears to be another factor regulating stomatal response. For example, pre-exposure stomatal opening and closing times for snap bean were 4-fold slower than post-exposure controls so that leaf age effects are much larger than the ozone effects for this species. The 3–5 yr old oaks did not show significant changes in photosynthetic and stomatal parameters over the 2-month exposure period, except reduced time for stomatal opening and closing in *Q. kelloggii*. Once oak leaves expanded, as they had prior to gas exchange measurements in this study, there were only small changes in physiology over time relative to annuals.

The two oak species showed similar gas exchange responses under static and dynamic light. An exception was that the evergreen (drought-deciduous) *Q. douglasii* had more sluggish stomatal responses than the deciduous *Q. kelloggii*. Although both species are drought tolerant, stomata of the more xerophilic *Q. douglasii* were expected to open and close more rapidly than stomata of *Q. kelloggii* as stomatal regulation improves with increasing drought tolerance of a species (Zeifel et al., 2007). However, stomatal regulation is usually assessed in a static state, rather than a dynamic response to changing environmental conditions (an equally important attribute of drought tolerance), and in response to water stress (rather than to light variation). Similar time for stomatal equilibration in snap bean and *Q. douglasii* suggest that more investigations across a wider range of plant species and stress conditions are warranted.

In general, stomatal equilibrium to changes in environmental conditions required longer time periods with higher endogenous gas exchange rates. The length of time for stomatal opening was generally correlated to that of closure. The higher gs and photosynthesis at saturating light, the higher the range of gs and A in beans, but not in oaks.

4.2. Responses to O_3 exposure

Previous studies demonstrated that exposure to 40 ppb in OTC and 41 ppb in ambient air (12-h O_3 mean) from emergence to mature pod harvest decreased pod yield by 41% and 58%, respectively, in the sensitive S156 cultivar compared with the tolerant R331 snap bean (Booker et al., 2009). When grown in the field under ambient O_3 conditions (44 ppb as 12-h mean), fresh market pod yield and mature bean yield were 32% and 44% lower, on average, in S156 than R331 (Booker et al., 2009). Both cultivars, however, showed similar responses of steady-state gs with and without O_3 exposure (over the range of 0–60 ppb as a 12-h mean) (Flowers et al., 2007). Our experiment confirmed that gs did not vary with either the cultivar or the O_3 exposure. Steady-state endogenous gs, therefore, cannot explain the different response in yield of the two snap bean cultivars to O_3. Flowers et al. (2007) suggested that the difference in O_3-sensitivity between snap

bean cultivars may be due to different anti-oxidant capacity and/or ability to regulate Rubisco activity. They found that V_{cmax}, an indicator of Rubisco activity, decreased with increasing O_3 exposure in the sensitive cultivar and did not vary in the tolerant R331 type (Flowers et al., 2007). In contrast, we found that O_3 exposure increased Rubisco activity (V_{cmax}) and RuBP availability (J_{max}) in the sensitive snap beans, suggesting up-regulation as a response to O_3 injury. We found sluggish stomatal responsiveness in the sensitive cultivar to environmental change in light intensity relative to the tolerant cultivar. Thus, O_3 sensitivity in snap bean may be due to O_3-induced sluggish stomatal responses to changes in environmental conditions, despite up-regulation in Rubisco activity and RuBP availability in response to O_3 exposure. Ozone-induced stomatal sluggishness has been already demonstrated in forest (e.g. Reich and Lassoie, 1984; Tjoelker et al., 1995; Kellomäki and Wang, 1997; Paoletti, 2005; Handley and Grulke, 2008) and grassland species (Mills et al., 2009).

We postulate that stomatal sluggishness is a cause of increased O_3-sensitivity, as it implies higher O_3 uptake before reaching equilibrium. Stomata of the sensitive cultivar in charcoal-filtered controls were slower to open and close than that of the tolerant cultivar, although differences in stomatal response time to changes in environmental conditions were not marked in pre-exposure controls. Sluggish stomatal responses are thus suggested to be both an effect of O_3 exposure and a reason of increased O_3 sensitivity in plants.

Ozone-impaired stomatal responses were shown by both snap beans and oaks, suggesting similar stomatal responses to dynamic light conditions with O_3 exposure. In O_3-exposed oaks, however, both maximum and minimum gs were affected, in that they were higher than in the controls. Aberrant stomatal responses may last long after O_3 exposure (Paoletti, 2005), resulting in incomplete closure even at nighttime (Wieser and Havranek, 1995; Matyssek et al., 1995; Grulke et al., 2004, 2007b). Surprisingly, these impaired stomatal responses did not translate into significant effects on the photosynthetic responses of *Q. kelloggii*, suggesting effective detoxification mechanisms.

In conclusion, we presented evidence that O_3 itself can modify stomatal function resulting in slowed responses to normal environmental cues. Aperture modulation of the trillions of stomata per hectare of actively growing vegetation warrants special attention (Cochrane and Cochrane, 2009). Stomata exert major controls on both the water and carbon cycles of the world (Hetherington and Woodward, 2003), but little is still known about the dynamics of stomatal responses to O_3. Aberrant stomatal response to O_3 exposure has been largely ignored in current modeling efforts of O_3 effects on plants (Emberson et al., 2000; Grunhage et al., 2001). The consequence is to minimize the importance of O_3 impact on plant water balance and susceptibility to drought stress and, in the case of forests, of fire. As climate change is increasing the risk of flooding, drought and forest fire (Bytnerowicz et al., 2007), whole ecosystem consequences of the O_3-induced stomatal sluggishness should be evaluated.

Acknowledgments

We thank Kent Burkey for providing the seeds of the sensitive and tolerant varieties of snap bean, and providing information on their cultivation. Many thanks to Pam Padgett for allowing use her open-top chamber system and providing the oak seedlings. The use of trade names is for information only and does not imply commercial condonement by the US government. A travel grant was awarded to EP by the short-term mobility program of the CNR in order to conduct this research.

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