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RESEARCH PAPER

Demonstration of a diel trend in sensitivity of *Gossypium* to ozone: a step toward relating O_3 injury to exposure or flux

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Abstract

Plant injury by ozone (O_3) occurs in three stages, O_3 entrance through stomata, overcoming defences, and attack on bioreceptors. Concentration, deposition, and uptake of O_3 are accessible by observation and modelling, while injury can be assessed visually or through remote sensing. However, the relationship between O_3 metrics and injury is confounded by variation in sensitivity to O_3. Sensitivity weighting parameters have previously been assigned to different plant functional types and growth stages, or by differentially weighting O_3 concentrations, but diel and seasonal variability have not been addressed. Here a plant sensitivity parameter (S) is introduced, relating injury to O_3 dose (uptake) using three independent injury endpoints in the crop species, Pima cotton (*Gossypium barbadense*). The diel variability of S was determined by assessment at 2 h intervals. Pulses of O_3 (15 min) were used to assess passive (constitutive) defence mechanisms and dose was used rather than concentration to avoid genetic or environmental effects on stomatal regulation. A clear diel trend in S was apparent, with maximal sensitivity in mid-afternoon, not closely related to gas exchange, whole leaf ascorbate, or total antioxidant capacity. This physiologically based sensitivity parameter provides a novel weighting factor to improve modelled relationships between either flux or exposure to O_3, and O_3 impacts. This represents a substantial improvement over concentration- or phenology-based weighting factors currently in use. Future research will be required to characterize the variability and metabolic drivers of diel changes in S, and the performance of this parameter in prediction of O_3 injury.

Key words: Air pollution impact, antioxidant metabolism, chlorophyll content, cotton, diurnal sensitivity, gas exchange, O_3, ozone flux modelling, ozone injury, SPAD.

Introduction

Concentrations of the secondary oxidant air pollutant ozone (O_3) have increased during the industrial era and are projected to continue to do so under current emissions trends (Vingarzan, 2004; Royal Society, 2008). There is speculation that enlightened global regulatory action may limit or even reverse this trend in ozone concentration [O_3], with up to a 5% decrease (Stevenson *et al.*, 2006), although other scenarios project a 6–15% increase in [O_3] (Stevenson *et al.*, 2006). In many areas of the globe, current levels of ambient O_3 are already injurious to managed and native vegetation (Booker *et al.*, 2009; Avnery *et al.*, 2011*a*; Wilkinson *et al.*, 2012). Plausible scenarios suggest increased impact on vegetation under future climatic conditions (Avnery *et al.*, 2011*b*).

These ozone impacts on vegetation may be considered to occur in discrete steps, as:

(i) Ozone creation in the environment leading to ambient ozone concentration [O_3].

(ii) Ozone entry into the leaf, namely ozone flux, F, and its accumulation over time, dose, D.
(iii) Ozone exceeding constitutive plant defence capacity characterized as sensitivity, S, and leading to effective flux, F_{eff}, and effective dose, D_{eff}.
(iv) Ozone or secondary oxidants interacting with target biomolecules causing injury, I.
(vi) Ozone-induced synthesis of active defence capacity thereby reducing S.

Ozone flux (F) is an instantaneous parameter, and effective flux (F_{eff}) is that parameter corrected by S for the largely unknown suite of passive defensive factors. Dose (D) and effective dose (D_{eff}) are the integrated values of the corresponding fluxes (after Massman *et al.*, 2000; Musselman *et al.*, 2006).

The asynchronicity of diurnal peaks of stomatal conductance (g_s) and O_3 and the potential impact of diurnally varying sensitivity to O_3 were captured conceptually in the framework of Musselman *et al.* (2006) and Massman *et al.* (2000), as:

$$I \propto \Sigma [F–R] \quad (1)$$

where R describes removal of ozone molecules from the stomatal flux stream prior to interaction with bioreceptors. Here, a similar expression is used:

$$I \propto \Sigma [F \times S] = \Sigma Feff = Deff \quad (2)$$

which was presented (Massman *et al.*, 2000; Musselman *et al.*, 2006) as a simplified surrogate for Equation 1. However, the conceptualization of Equation 2 is both more tractable, allowing solution directly for S under a broad range of conditions, and more physiologically appropriate. This approach does not require the unwarranted assumption that injury is proportional to the difference [F–R], with its implication of a threshold. To date, no such threshold has been demonstrated, and a threshold is not consistent with the known complexity of O_3 defences and targets in plant tissues. A variety of metabolic and structural characteristics are likely to determine S within the leaf, and their relative importance may vary diurnally.

Injury and O_3 exposure

The conditions of high temperature and radiation that are most conducive to rapid plant growth are also conducive to tropospheric production of O_3 from natural and anthropogenic precursors. Deposition of O_3 in vegetated landscapes (Massman and Grantz, 1995; Zhang *et al.*, 2006) and O_3-induced plant injury (I) are both typically dominated by stomatal uptake (flux) of O_3 (F). While additional pathways are associated with reactive surfaces and gases (Massman and Grantz, 1995; Fares *et al.*, 2010), these do not lead to injury. F is a function of $[O_3]$ near the leaf, and of stomatal opening (g_s). g_s also follows diel and seasonal trends in temperature and radiation (Grantz, 1989).

There is site-specific asynchronicity between the highest $[O_3]$ and the highest g_s (Grunhage *et al.*, 1997; Grunhage and Jager, 2003; Fares *et al.*, 2010). This reduces potential F and I, rendering projection of I from $[O_3]$ less robust than from F (Matyssek *et al.*, 2004; Zhang *et al.*, 2006). Recent models of ozone impacts on vegetation incorporate g_s and thereby F (e.g. Emberson *et al.*, 2000), though, in most cases, I is still parameterized as a function of $[O_3]$ (Lefohn, 1992) rather than F or D.

At regional scales, both g_s and $[O_3]$ near the leaf may be modelled using the species composition of regional land cover, calculated vertical gradients in $[O_3]$, and partitioning between stomatal and non-stomatal pathways, to determine stomatal uptake (F; Massman and Grantz, 1995; Grantz *et al.*, 1997; Fares *et al.*, 2010). These concepts are well understood. While modelling of F remains rudimentary and cumbersome to implement, the flux-based approach has the potential to be more physiologically sound than the concentration-based approach. Injury can be assessed experimentally, using a variety of techniques, and regionally through approaches such as remote sensing. However, the relationship between I and D remains poorly characterized. This reflects uncertainties in modelled $[O_3]$ and g_s, above, and in the rates, capacities, and mechanisms of O_3 detoxification.

Weighting factor

Reliance on either $[O_3]$ or F in current modelling paradigms implicitly assumes that defence capacity is non-varying. This assumption is known to be an oversimplification (Dizengremel *et al.*, 2008; Heath *et al.*, 2009), and its limitations have not been adequately tested across diverse vegetation types and environments. In particular, the temporal (diel and seasonal) variability of these defences, and the resulting sensitivity, S, remain to be characterized (Massman *et al.*, 2000; Heath *et al.*, 2009). Defence mechanisms may be passive (constitutive; not responsive to current oxidant challenge), or active (inductive; responsive to current challenge), and both will require consideration to model I from $[O_3]$ or F adequately (Musselman and Massman, 1999; Matyssek *et al.*, 2004; Musselman *et al.*, 2006). Whether constant or varying diurnally, seasonally, and developmentally (Massman and Grantz, 1995; Musselman and Massman, 1999; Massman *et al.*, 2000; Panek and Goldstein, 2001; Danielsson *et al.*, 2003; Grunhage *et al.*, 2004; Massman, 2004; Musselman *et al.* 2006; Fares *et al.*, 2010), defence capacity is a significant determinant of plant sensitivity to O_3 (S), and may be the principal determinant of injury (I) in cases such as genetic variation in O_3 sensitivity. This requires that $[O_3]$ or F be assigned differential weights to reflect S.

Weighting factors incorporated into the models MOSES-TRIFFID (Sitch *et al.*, 2007) and DLEM (Zhang *et al.*, 2007), while based on ozone flux, are time invariant and assigned according to plant functional type. The weighting factor, W126 (Lefohn and Runeckles, 1987; Lefohn *et al.*, 1988), is cumulative and based on $[O_3]$, emphasizing higher concentrations without ignoring lower concentrations. The concept of a physiologically based and seasonally varying weighting factor for $[O_3]$ was suggested by Krupa and Teng (1982) and realized in the phenological gamma weighting functions of Lee

(1988). Significantly, these functions performed best in combination with a sigmoidal weighting of $[O_3]$ such as W126.

The concept of weighting F or D according to time of day or metabolic state has been suggested previously (Massman *et al*., 2000; Fares *et al*., 2010). These weighting parameters have been conceptual surrogates for the unknown sensitivity to O_3 (Massman *et al*., 2000). Metabolic reasoning to predict the time course of such a parameter has also been presented (Dizengremel *et al*., 2008). However, no direct measurements of plant sensitivity have been made available that could provide a quantitative basis for an inherent sensitivity weighting parameter such as S.

Present study

Defence capacity may vary diurnally, such that a specific D will cause different I at different times of the day, even within the same species and growth environment. Two hypotheses are tested: (H1) that leaf sensitivity to O_3 does not vary diurnally; and (H2) that whole leaf content of antioxidants is inversely related to S. A study of Pima cotton is presented, with one 15 min pulse exposure to O_3 per plant. Exposures were to a broad range of D at 2 h intervals throughout the photoperiod. I was determined using three independent assays: (i) a measure of chlorophyll content; (ii) stomatal conductance; and (iii) non-injured leaf area. I was assayed following a 1 week lag to allow symptom development. S was determined as the slope of the linear regression of each I versus D; that is, the dose–response (D–R) relationship, determined separately at each time of day.

The results will determine if assumption of constant sensitivity, S, is warranted. If H1 is disproven, then it will be necessary to incorporate S into process models of O_3 impacts on vegetation. This will suggest that S may also vary over seasonal and developmental time. If H2 is disproven it will support increasing evidence that these simple measures of defence against O_3 are not adequate to predict S.

Materials and methods

Plant growth

Seed of Pima cotton (*Gossypium barbadense* L.) cv. S-6 (J.G. Boswell Company, Corcoran, CA, USA) was obtained from foundation seed stock. Several seeds were planted in moist commercial potting mix (Earthgro Potting Soil; Scotts Company, Marysville, OH, USA) in Conetainers (3.8 cm depth×21 cm height; Ray Leach Model SC10; Stuewe & Sons, Inc., Tangent, OR, USA), and thinned to one uniform plant per pot.

Plants were grown in a research greenhouse at Kearney Research and Extension Center (103 m asl; 36.598°N, 119.503°W) for 26 d. Automated drip emitters irrigated all pots to excess daily and provided a complete fertilizer solution (1.3 g l^{-1}, Miracle Gro, Scotts Miracle-Gro Products Inc., Port Washington, NY, USA) to excess twice weekly. Temperature in the growth bay was 15–30 °C, with photosynthetic photon flux density (PPFD) near solar noon of ~1100 µmol m^{-2} s^{-1} at bench level.

The study material, Pima cotton, was chosen because of its economic importance and the wealth of previous information available on responses to ozone in this system grown under these conditions (e.g. Grantz *et al*., 2010; Grantz and Vu, 2012). Additionally, leaf size and shape were convenient for the required manipulations.

Ozone exposure

Each plant was exposed to a single, 15 min square wave pulse of O_3. Pulses were administered over a wide range of nominal O_3 concentrations (0.0, 0.5, 1.0, 1.5, and 2.0 µmol mol^{-1} O_3) and at 2 h intervals throughout the daylight period (7:00–19:00 h Pacific daylight time). After a single pulse, plants were returned to the greenhouse bench in the growth bay and left undisturbed for 6 d for symptoms to develop prior to assessment of injury.

Plants were transported from the growth bay of the greenhouse to the exposure chamber immediately prior to pulse exposure. g_s was determined with a steady-state porometer ((LI-1600, LI-COR Inc., Lincoln, NE, USA), immediately prior to exposure and again immediately afterwards, on the youngest fully expanded leaf (YFEL). Measurements were made on the adaxial and abaxial surfaces, which were summed. Adaxial conductance contributed <10% of total conductance.

Exposures were administered using one of two protocols. In Run 1, the intact, attached, YFEL of each plant was placed within a clear plexiglass [poly(methyl methacrylate)] cuvette, illuminated with a tungsten–halogen projection bulb (ELD; 150 W, 21 V; Sylvania, Danvers, MA, USA), through an infrared reflective mirror (Optical Coating Laboratory, Inc., Santa Rosa, CA, USA), providing ~200 µmol m^{-2} s^{-1} at leaf level. In Run 2, the entire plant was exposed to the O_3 pulse in a continuously stirred tank reactor (CSTR) (Heck *et al*., 1978; Grantz *et al*., 2010) illuminated with natural sunlight of ~300 µmol m^{-2} s^{-1} at leaf level near solar noon. The $[O_3]$ in both protocols was brought to steady state, and each pulse was initiated/terminated by placing/removing the plant material, allowing precise control of pulse duration (15.0 ± 0.1 min) with minimal disruption of $[O_3]$. Plants were immediately returned to the greenhouse bench after the pulse.

O_3 was provided to the cuvette by ultraviolet radiation of room air using an integrated ozone source/monitor (Dasibi 1003C; Dasibi Corp., Glendale, CA, USA). O_3 was provided to the CSTRs by corona discharge (Model SGC-11, Pacific Ozone Technology, Brentwood, CA, USA) from purified oxygen (Series ATF-15, Model 1242, SeQual Technologies Inc., San Diego, CA, USA). Feedback for the O_3 generators in each case was provided by the exit stream of the exposure chamber (Grantz *et al*., 2010).

Each protocol was repeated three times (6 d total), with five $[O_3]$ per hour. Each plant was exposed to a single pulse. The single cuvette approach required sequential pulse exposures at each $[O_3]$, so that one plant was exposed to each $[O_3]$ during each 2 h window. In contrast, each of five CSTRs was held at constant but different $[O_3]$. This allowed all $[O_3]$ to be administered simultaneously so that two plants were exposed to each $[O_3]$ at each 2 h time point on 2 d, and one plant per $[O_3]$ on the third day, due to a shortage of uniform plants. The data were pooled for analysis, so that each D–R relationship and estimate of sensitivity was obtained from 40 independent measurements of I and D.

Calculation of ozone dose–response relationships

To calculate O_3 flux, g_s was measured immediately prior to enclosure in the exposure chamber, and immediately after removal from the chamber. Ozone flux at each minute [F(t)] during the pulse was calculated as the product of O_3 concentration $[O_3]$×(t) directly measured at 60 s intervals during the pulse and minute by minute g_s(t) obtained by interpolation at 60 s intervals between pre- and post-pulse values of g_s. Stomatal conductance generally declined during the pulses, independent of $[O_3]$. Instantaneous flux [F(t)] was calculated as:

$$F(t)=gs(t)\,[O3]\,(t) \quad (3)$$

and ozone dose, D, was calculated as total cumulative O_3 flux during the 15 min pulse, as:

$$D=\Sigma F(t) \quad (4)$$

Three independent endpoints were used to assess ozone injury (I) on the YFEL. At 6 d following exposure, near midday, a relative measure of chlorophyll content was determined as SPAD (Minolta SPAD-502, Spectrum Technologies, Plainfield, IL, USA) and a direct measure of g_s was obtained using the steady-state porometer (LI-1600) in a region of leaf away from major veins. At 7 d following exposure, the third endpoint, non-injured leaf area, was assessed by analysis of digital photographs of entire leaf laminae. SPAD and g_s were normalized by the mean value obtained from control leaves (exposed to control pulses of charcoal-filtered air, and non-injured leaf area was expressed relative to total leaf area, on a pixel basis.

Digital photographs were obtained (Nikon D90 with 18–105 mm; f 3.5–5.6, Nikkor lens; Nikon USA Inc., Melville, NY, USA) while attached to the plant. Photographs were taken on the growth greenhouse bench against a white bond paper background (Run 1) or in a white reflective box with constant illumination provided by a 27 W Daylight Compact Fluorescent bulb (Run 2). Photographs were recorded in.jpg format (480 × 321 pixels; 18–28 kb). Images were digitally excised and pasted onto a white digital background to provide a colour standard, then deconstructed into colour classes (Digital Image Pro 9; Microsoft Inc., Redmond, WA, USA). These were linked to injury type (Image J; National Institutes of Health; http://rsbweb.nih.gov/ij/download.html) discriminating dark green (non-injured) leaf area from both light green (chlorotic) and beige-brown (necrotic) area.

Plant sensitivity to O_3 (S) was obtained separately for each time of day as the slope of the linear dose–response (D–R) relationship between I and D. Data from both runs were pooled, with D–R relationships determined independently for each endpoint, yielding I_{SPAD}, I_{COND}, and $I_{NON\text{-}INJ}$. Independent slopes were determined at each time of day. The slope of each D–R curve was taken as a sensitivity parameter, S_{SPAD}, S_{COND}, or $S_{NON\text{-}INJ}$, respectively, as:

$$I = I_0 + S\,D \quad (5)$$

The significance of each D–R relationship was expressed as r^2 and as a one-tailed *t*-test.

S was plotted as mean ±SE against time of day to evaluate diel trends in O_3 sensitivity.

Antioxidant activity

Non-exposed leaves were sampled directly from the growth greenhouse, simultaneously with pulse exposures. Leaves were flash-frozen in liquid nitrogen and stored in an ultra-low freezer until processing. Frozen leaf tissue was ground in liquid nitrogen using a mortar and pestle, and used as starting material for extraction of antioxidants.

Ascorbic acid (AA) and dehydroascorbic acid (DHA) were extracted from frozen leaf tissue powder in cold 6% (w/v) metaphosphoric acid, 0.2 mM diethylenetriaminpentaacteic acid, and assayed as described previously (Cheng *et al*., 2007). Total ascorbate was calculated as AA+DHA and the ascorbate redox ratio as AA/(AA+DHA). Total non-specific antioxidant capacity of leaf tissue was measured using an adaptation of the approach described by Neill *et al*. (2002), based on antioxidant scavenging of the stable free radical α,α-diphenyl-β-picrylhydrazyl (DPPH). Fresh-frozen tissue replaced the freeze-dried material recommended in the original procedure because AA in cotton leaves was found to be unstable during lyophilization. Frozen leaf tissue powder (50 mg) was extracted in 2.5 ml of acetic acid:water:methanol [7:23:70; v/v/v] for 18 h in the dark. Following centrifugation to remove cellular debris, an aliquot of the supernatant (50 µl) was combined with 1.4 ml of fresh 180 µM DPPH in methanol and incubated for 27 min in the dark. Absorbance was read at 517 nm. The reduction in absorbance of DPPH due to antioxidants in the unknown was compared with a standard curve established using known quantities (0–120 nmol) of AA so that total antioxidant capacity was reported in AA equivalents.

Results

Plants were exposed to a broad range of ozone concentrations (0–3.0 µmol mol^{-1}) during the 15 min pulse. This resulted in a range of O_3 doses D (0–25 mmol m^{-2}) dependent upon prevailing g_s and [O_3]. Most of the range in D was due to [O_3] because g_s did not vary substantially between O_3 treatments.

Leaves exposed to a pulse of low [O_3] (e.g. 5 mmol m^{-2}; Fig. 1A) in the cuvette did not display visible injury symptoms, demonstrating that enclosure in the chamber did not induce artefactual injury or accelerate senescence. Leaves exposed to a pulse of high [O_3] (e.g. 23.9 mol m^{-2}; Fig. 1B) in the cuvette, in contrast, exhibited considerable visible injury. The same result was observed with the CSTR protocol.

The dose dependence of visible leaf injury was quantified by an indirect measure of chlorophyll content (Fig. 2), by stomatal response (Fig. 3), and by digital photography (Fig. 5). All three measures declined with increasing D as indications of O_3-induced injury. Data were expressed relative to controls, with both endpoints clustering near 1.0 at low D.

Dose–response relationships for chlorophyll, conductance, and injury

Early in the photoperiod (07:00 h; Fig. 2A), the slope of the D–R relationship between SPAD and D for chlorophyll content was shallow (m= – 0.22 m^2 mmol^{-1}) and non-significant (*P*=0.15). This indicated little sensitivity of SPAD to O_3 early in the photoperiod. The SPAD data exhibited very low variability.

In mid-afternoon (15:00 h; Fig. 2B), the sensitivity increased substantially and the slope became significant (m= –1.84 m^2 mmol^{-1}; *P*=0.0001). The absolute values of these slopes were taken as the sensitivity parameter, S_{SPAD}.

The g_s data were considerably more variable, but results were consistent with those obtained with the SPAD endpoint. The D–R of g_s versus D exhibited a non-significant relationship at 07:00 h (m=0.22 m^2 mmol^{-1}; *P*=0.38), near the beginning of the photoperiod (Fig. 3A). The slope increased

Fig. 1. Representative leaves photographed 7 d following midday exposure to a single 15 min pulse of O_3, with a dose of (A) 5.0 mmol m^{-2} or (B) 23.9 mmol m^{-2}.

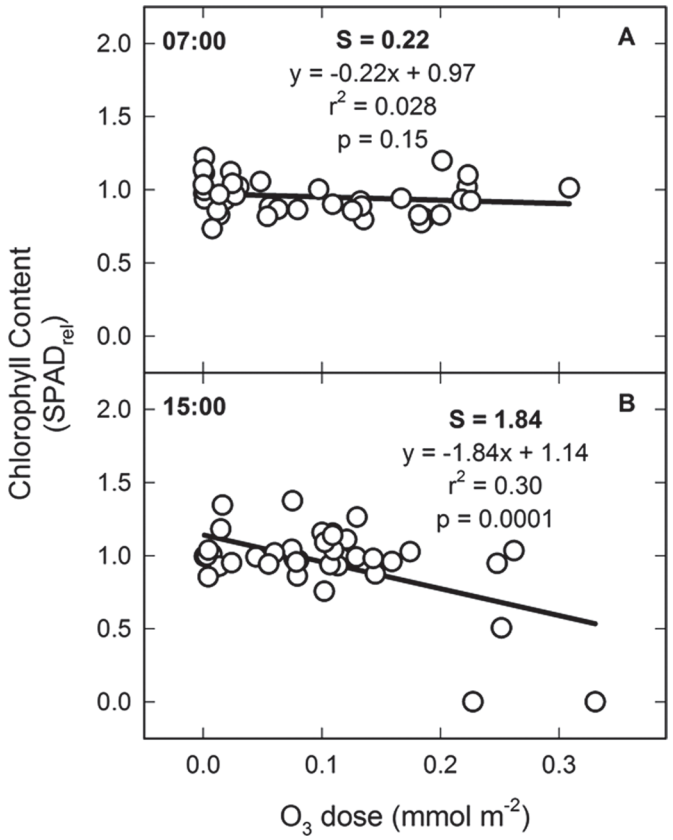

Fig. 2. Impact of O_3 as relative reduction in chlorophyll determined as SPAD, measured at 6 d post-pulse exposure, in response to O_3 dose (D) at (A) 07:00 h and (B) 15:00 h. The slope of the dose–response relationship yielded the sensitivity parameter (S_{SPAD}), determined at discrete 2 h intervals during the photoperiod. The best fit regression line was determined from the pooled data set obtained using two exposure technologies.

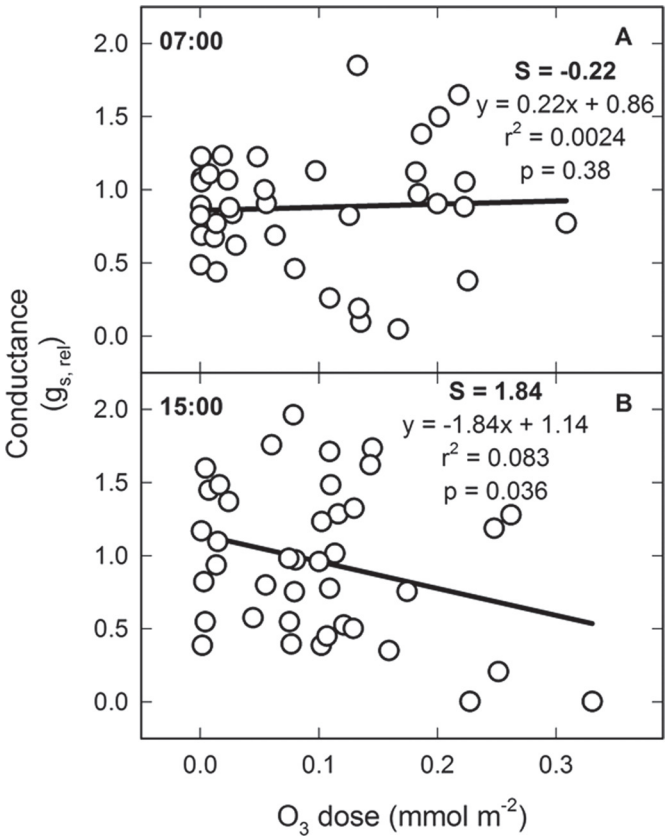

Fig. 3. Ozone impact as relative reduction in stomatal conductance (g_s), measured at 6 d post-pulse exposure, in response to O_3 dose (D) at (A) 07:00 h and (B) 15:00 h. The slope of the dose–response relationship yielded a second sensitivity parameter (S_{COND}), determined at discrete 2 h intervals during the photoperiod. The best fit regression line was determined from the pooled data set obtained using two exposure technologies.

throughout the day until mid-afternoon. At 15:00 h (Fig. 3B), the slope indicated a substantial sensitivity to O_3 (m= –1.84 m^2 $mmol^{-1}$), exhibiting the same regression equation as observed for SPAD at the same time point, and became statistically significant (P=0.036).

The D–R relationships for the two independent measures of O_3-induced injury were evaluated at 2 h intervals. The estimates of S derived from the slopes were consistent in magnitude at each of these sampling points. This led to a strong correlation between S_{COND} and S_{SPAD} (Fig. 4). The modest negative intercept reflects the generally observed lower sensitivity of g_s than SPAD early and late in the photoperiod.

The D–R relationship for digitally assessed non-injured leaf area exhibited similar results (Fig. 5) for a third measure of sensitivity ($S_{NON\text{-}INJ}$). Early in the photoperiod, at 07:00 h, the slope was shallow but in this case highly significant. The slope and its significance increased to maxima at 17:00 h. In contrast to the strong positive correlation between S_{COND} and S_{SPAD} (Fig. 4), the correlations between $S_{NON\text{-}INJ}$ and both S_{COND} and S_{SPAD} were weak (r=0.1) but positive trending in both cases.

Diel time course of sensitivity to O_3

All three measures of sensitivity, S_{SPAD}, S_{COND}, and $S_{NON\text{-}INJ}$, exhibited a clear diel time course. Sensitivity was minimal in the early morning, at the onset of the photoperiod, and increased toward a maximum in mid-afternoon (Fig. 6; circles). After the mid-afternoon peak, all three measures of S declined rapidly toward the end of the photoperiod.

The time course of S_{SPAD} (Fig. 6A) was very similar to that of S_{COND} (Fig. 6B). Sensitivity was minimal in the early morning, slightly lower for S_{COND} than for S_{SPAD} at the onset of the photoperiod. S_{COND} increased toward a maximum at 15:00 h (cf. Fig. 6A) that was nearly identical to the maximum value of S_{SPAD}, occurring at the same time (Fig. 6B). The high mid-afternoon value of S_{SPAD} differed significantly from the lower value observed in the early morning. While S_{COND} exhibited a sharper maximum at 15:00 h than did S_{SPAD}, due to greater variability in the porometric measurements (cf. Figs 2, 3), this value was not significantly different from the early morning value. After the mid-afternoon peak, S_{COND} declined more rapidly than S_{SPAD}, returning to near its

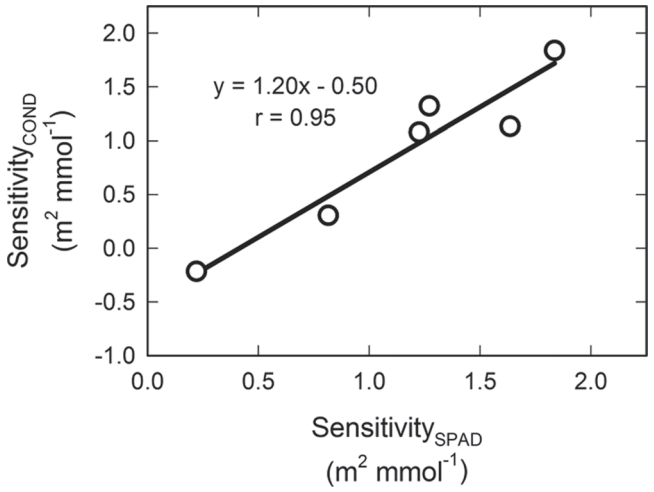

Fig. 4. Correlation between sensitivity to O_3 assayed as stomatal conductance (S_{COND}) and sensitivity assayed as SPAD (S_{SPAD}), both relative to control values at 6 d after the pulse exposure to O_3.

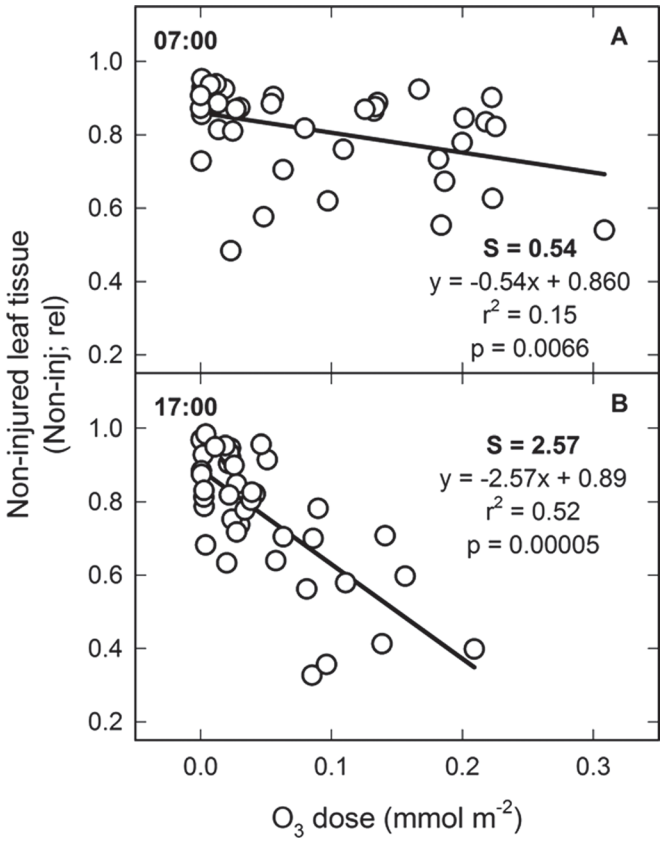

Fig. 5. Ozone impact as relative reduction in non-injured leaf tissue, measured at 7 d post-pulse exposure, in response to O_3 dose (D) at (A) 07:00 h and (B) 17:00 h. The slope of the dose–response relationship yielded a third sensitivity parameter ($S_{NON-INJ}$), determined at discrete 2 h intervals during the photoperiod. The best fit regression line was determined from the pooled data set obtained using two exposure technologies.

initially lower value late in the photoperiod as observed in the early morning.

The time course of $S_{NON-INJ}$ exhibited a similar minimum early in the photoperiod (Fig. 6C) and a maximum in mid-afternoon. However, the peak sensitivity was observed one measurement period later than the peak sensitivity of SPAD and g_s. For $S_{NON-INJ}$, the peak was quite distinct and significantly different from values earlier in the day. The final measurement time point at 19:00 h was subject to extreme variability for all measurement endpoints and did not differ from any other time point.

Relationship with gas exchange and antioxidant metabolites

Leaf gas exchange determined as stomatal conductance exhibited a maximum in late morning at 11:00 h (Fig. 7). The same pattern was observed during both runs.

Leaf defence capacity was assayed as whole tissue antioxidant content. Ascorbate and total antioxidant capacity were determined on plants that were not exposed to pulses of O_3, but that were taken from the same population, and at the same 2 h intervals. This is equivalent to sampling prior to the pulse to ascertain the intrinsic antioxidant status at each time of day.

Whole leaf content of ascorbate was variable (Fig. 8A) and did not differ significantly throughout the day. AA content appeared to be elevated at the earliest sampling time (07:00 h; Fig. 8A) and depleted following the onset of the photoperiod, but relatively constant throughout the midday and mid-afternoon period. Ascorbate remained >90% in its reduced form, and thus protective, at all times of the day (data not shown).

In contrast to ascorbate, total antioxidant capacity (Fig. 8B) did not exhibit elevated concentrations at 07:00 h, but rather increased throughout the morning, peaking near solar noon. Total antioxidant capacity exhibited a sharp decline at 15:00 h, coincident with the peak in sensitivity observed in SPAD and conductance. The concentration of total antioxidant capacity (in ascorbate equivalent units) was much greater than of ascorbate itself. Throughout the day ascorbate accounted for ~10% of total antioxidant capacity.

Over the course of the day, the relative increases in whole leaf contents of ascorbate and of total antioxidants were similar. No measure of sensitivity was significantly correlated with ascorbate or with total antioxidant capacity. However, for all measures of sensitivity, the trends were positive with AA (i.e. sensitivity increased with total ascorbate), but negative with antioxidant capacity.

Discussion

Diel trends of S

A plant sensitivity parameter (S) is presented, relating injury (I) to cumulative O_3 flux (dose, D) during a brief exposure. By restricting O_3 exposure to a 15 min pulse, an attempt was made to isolate primarily passive, constitutive, defence mechanisms, although induction of defensive signalling cascades is known to occur within this time frame (e.g. Kangasjarvi *et al*., 2005). The resulting data disprove the hypothesis that plant

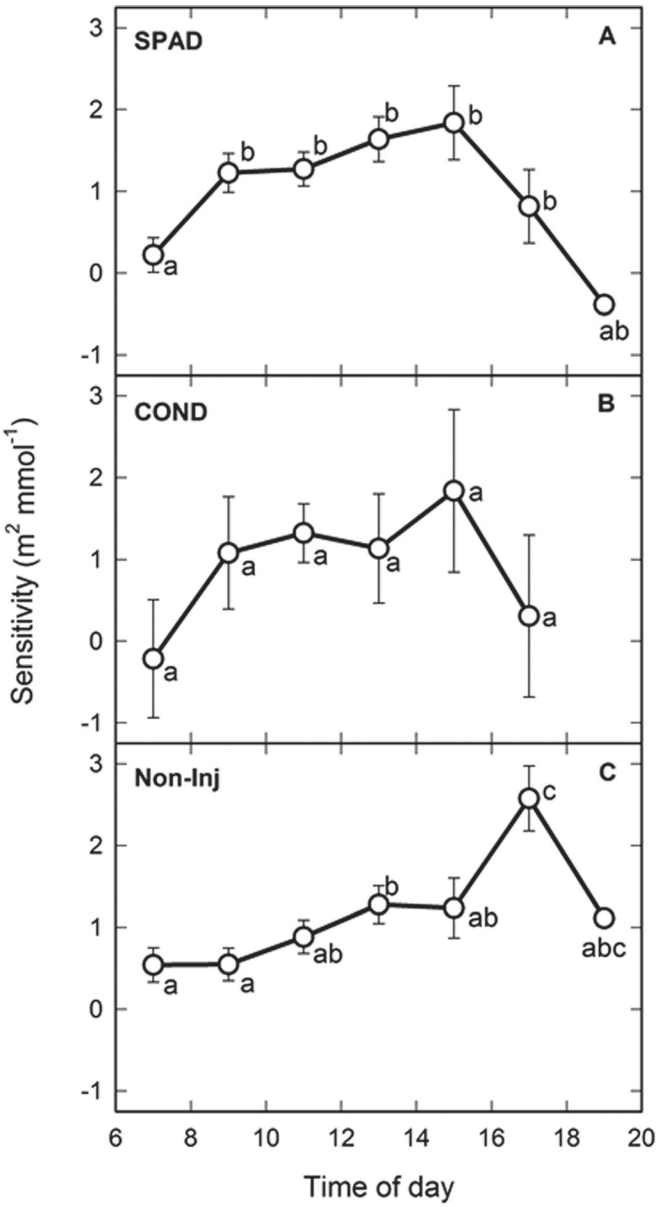

Fig. 6. The diurnal course of plant sensitivity to ozone in Pima cotton. Sensitivity (S) is characterized as (A) chlorophyll pigmentation (S_{SPAD}), (B) stomatal conductance (S_{COND}), both relative to control values at 6 d after the pulse exposure to O_3, and (C) non-injured leaf area ($S_{NON\text{-}INJ}$) relative to total leaf area at 7 d after the pulse exposure. Points with the same letter do not differ at $P < 0.05$. Error bars are omitted from the 19:00 h data points in (A) and (C) as they were very large.

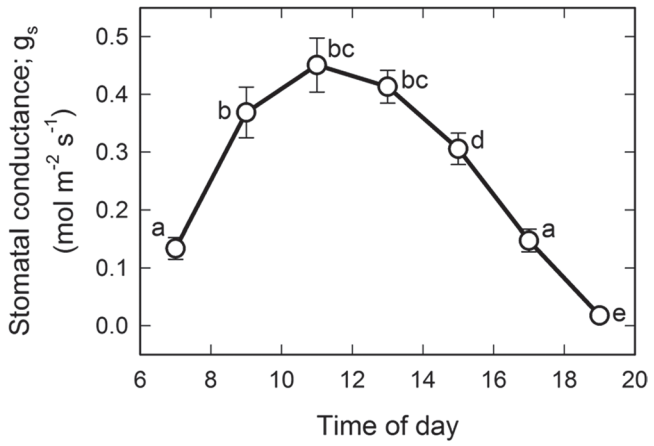

Fig. 7. The diurnal course of leaf stomatal conductance determined on the growth bench prior to the pulse exposure to O_3, characterized as the mean over both runs. Points with the same letter do not differ at $P < 0.05$.

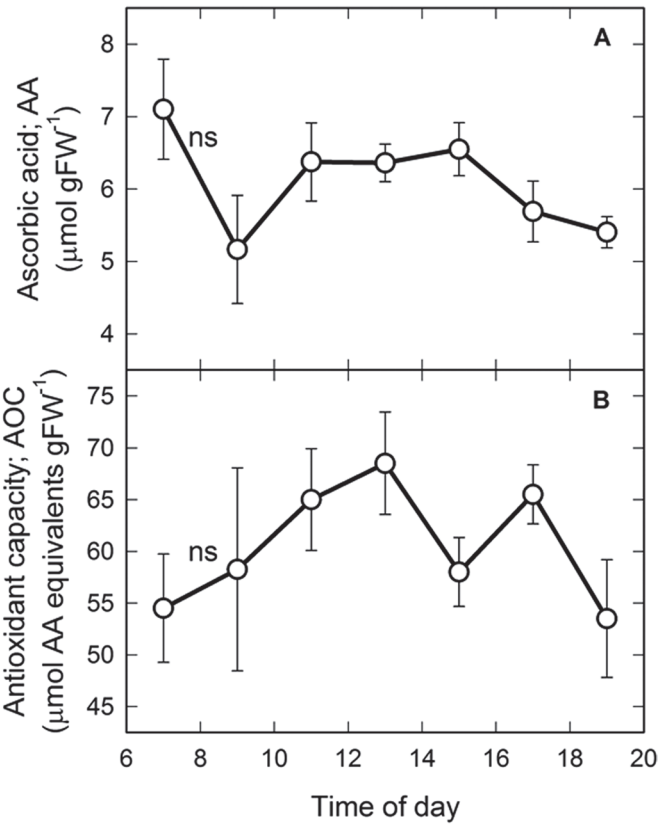

Fig. 8. The diurnal course of (A) ascorbic acid and (B) total antioxidant capacity in whole leaf extracts, expressed as ascorbic acid (AA) equivalents, averaged over both runs. Points with the same letter do not differ at $P < 0.05$.

sensitivity is invariant over diurnal time frames. It is speculated that sensitivity may also vary seasonally and between species. The central conclusion of this work is that S in Pima cotton varies in a characteristic diel fashion, and was greatest in mid-afternoon. This is demonstrated by significant differences between time points in two sensitivity parameters and a parallel diel trend in a third parameter. Early in the photoperiod the I versus D dose–response (D–R) relationships were insensitive and not significant, while in mid-afternoon the D–R relationships were significant, reflecting elevated sensitivity to O_3. These represent the first direct measurements of the diel course of a weighting factor for O_3 exposure or flux that incorporates plant sensitivity to O_3.

The experimental protocols minimized confounding of O_3-induced injury with naturally progressing senescence.

Controls that were enclosed in the exposure chambers but exposed to low D did not exhibit injury at 6 d post-exposure. All measurements were made on the YFEL, an age class that is characterized by robustness to manipulation and is reproducible between plants. As noted in *Populus* (Bohler *et al*., 2010), younger expanding leaves are relatively insensitive to O_3 due to their unique metabolism. Sensitivity develops at the time of full expansion. Thus the YFEL in these experiments remained physiologically active and injury free for several weeks in the absence of exposure to O_3 and exhibited clear injury in response to appropriate D.

Three independent measures of O_3-induced injury were employed, SPAD (Fig. 2) and g_s (Fig.3), both determined at 6 d post-exposure, and digital assessment of non-injured (non-chlorotic plus non-necrotic) leaf area (Fig. 5), determined at 7 d post-exposure. The two endpoints, S_{SPAD} and S_{COND}, were consistent in suggesting a peak sensitivity at ~15:00 h. These measures sampled only a small fraction of the YFEL surface, and not necessarily the same area, yet these endpoints were strongly and positively correlated (Fig. 4), similar in magnitude and diel trend, and consistent with the digital photographic method which assayed the entire leaf lamina. The photographic assessment of non-injured tissue, in particular, has long been used to document and confirm O_3 damage observed visually (e.g. Flagler, 1998). This method indicated that peak sensitivity occurred one time point later in the photoperiod than the other two measures. However, the timing of peak sensitivity could not be defined with precision from the g_s and SPAD data because midday values of S did not differ significantly across a broad midday plateau. Both exhibited consistent peaks at 15:00 h, for SPAD significantly different from the early morning. In contrast, the photographic non-injury endpoint indicated a more distinct and significant peak at 17:00 h. There are many additional sensitivity endpoints that could be utilized in this context. While it is likely that all will reflect a similar suite of metabolic determinants of sensitivity, one or another may prove to be most useful in different biological systems or environmental conditions.

While similar to concentration-based weighting schemes such as W126 (Lefohn and Runeckles, 1987; Lefohn *et al*., 1988), and the phenologically based gamma functions of Lee (1988), the physiologically based S is not related to concentration but to flux of O_3. It captures the potentially myriad diurnal, seasonal, and developmental changes that may occur in antioxidants, tortuosity of substomatal diffusion pathways, and even in the conformational sensitivity of specific ozone targets. Variability in susceptibility to $[O_3]$ has been linked to stomatal responses (Brosche *et al*., 2010), and this is the basis for protection against O_3 by drought (Temple *et al*., 1985, 1988; Fuhrer, 2009). These conclusions are independent of diel patterns of stomatal regulation and of the diel patterns of ambient $[O_3]$, since S is quantified in terms of F. S is an independent parameter that may be useful as a weighting factor in relating injury to F.

The S parameter may function well in combination with a sigmoidal weighting of flux, analogous to the W126 weighting of concentration, and perhaps with a phenological parameter as proposed by Lee (1988). The diel trend in S is consistent with evidence (e.g. Leuning *et al*., 1979*a*; Amiro *et al*., 1984; Lefohn and Runeckles, 1987) that higher $[O_3]$causes disproportionate damage. Linear D–R relationships are used to define S, to avoid overparameterization from the limited data set available. However, a linear relationship between injury and dose would suggest a single mechanism for O_3 injury. A non-linear D–R relationship is more likely, reflecting the diversity of receptors, defences, and signalling pathways.

Determinants of S

Gas exchange

It has been suggested that sensitivity may be minimal at midday, concurrent with maximal rates of gas exchange, photosynthetic generation of reactive oxygen species (ROS), antioxidant capacity driven by reducing metabolites such as NADPH, ascorbate-regenerating enzymes, and possible changes in carboxylase activities (Musselman *et al*., 2006; Dizengremel *et al*., 2008; Heath *et al*., 2009). Absence of photosynthetic generation of antioxidant metabolites and reduced sink demand for energetic metabolites has also suggested that plants may be more sensitive to O_3 nocturnally and early or late in the photoperiod (Musselman and Minnick, 2000).

In the Pima cotton system, maximum sensitivity occurred in mid- to late afternoon (Fig. 6), while maximum stomatal gas exchange occurred in late morning (Fig. 7). This pattern did not suggest a close relationship between gas exchange and S. While S remained relatively low during periods of peak gas exchange, it was at a minimum at both ends of the photoperiod, when gas exchange was minimal. While maximum S occurred during a period of substantial gas exchange activity, the diel course of both suggests that sensitivity was related to consumption of protective compounds during the photoperiod. The apparent reduction in S at the end of the photoperiod, despite substantial variability at this time of day, could reflect the renewal of protective metabolites, not necessarily captured as AA or antioxidant capacity (Fig. 8), or may indicate reduced competition for available detoxifying metabolites as ROS production from photosynthetic electron transport declines.

Antioxidants

A candidate antioxidant that has been linked to reduced sensitivity to O_3 is ascorbate, widely considered a first line of antioxidant defence. This concept is supported in some cases where leaf ascorbate content is lower in sensitive genotypes and thus negatively correlated with S (Burkey *et al*., 2003; Musselman *et al*., 2006). Differences in S among species have been attributed to levels of ascorbate (Conklin and Barth, 2004). Ascorbate has often been suggested as a surrogate for O_3 insensitivity (Plochl *et al*., 2000).

Ascorbate is responsive to radiation and temperature, as well as to O_3 exposure (Hofer *et al*., 2008). All forms of the ascorbate pool are maximal near solar noon in many systems, rising from an early morning minimum (Burkey *et al*., 2003), with late afternoon depletion (Kollist *et al*., 2000), and reduction by half at night (Peltzer and Polle, 2001). Typically the redox ratio of ascorbate declines at midday (Heath *et al*., 2009).

However, in the current study, S was relatively high throughout the midday period and was maximal in mid-afternoon. Whole leaf ascorbate content (Fig. 8) was not predictive of S. In contrast, total antioxidant capacity exhibited somewhat parallel diel courses to sensitivity. Ascorbate accounted for ~10% of total antioxidant capacity at all times of day and whole leaf ascorbate remained >90% reduced throughout the day, suggesting that S was not related to the redox status of the ascorbate pool.

Apoplastic antioxidant capacity may be more closely related to S than whole leaf levels. This was suggested by preliminary data in the current study (DAG, RLH, and KOB, unpublished DPPH analyses of apoplastic fluid). Concentrations of ascorbate in the apoplast are much higher than in the symplast (Turcsanyi *et al*., 2000), but total amounts are lower, perhaps only 1% (Lyons *et al*., 1999), and more oxidized. The apoplastic ascorbate pool is replenished by the cytoplasmic pool and by reduction *in situ* (Bichele *et al*., 2000; Kollist *et al*., 2001). However, the apoplastic ascorbate pool is rapidly depleted and in some cases is unlikely to intercept biologically meaningful quantities of O_3 (Turcsanyi *et al*., 2000; Booker *et al*., 2012).

Relationships between I and various measures of leaf antioxidant capacity have not consistently supported the suggestion that concentrations of these metabolites are directly related to sensitivity. The contribution of ascorbate in breaking down ozone may be balanced by the damaging first-order reaction products, including singlet oxygen and various peroxy-acids (Sandermann, 2008). Increased antioxidant metabolism may come at the expense of photosynthetic capacity, itself a predictor of yield in O_3-impacted environments (Betzelberger *et al*., 2010).

Sensitivity to O_3 has been associated with levels of glutathione (Conklin and Barth, 2004), carotenoids, flavonoids, and phenolics (Blochina *et al*., 2003; Heath *et al*., 2009), catalases and peroxidases, superoxide dismutases, and enzyme systems that maintain these materials (Eltayeb *et al*., 2007; Frei *et al*., 2010). Diel trends in xanthophyll cycle activity (Cheng and Ma, 2004) and signalling networks involving ethylene, jasmonic acid, and other metabolites are also involved (Overmyer *et al*., 2008; Guidi *et al*., 2010). However, similar to apoplastic ascorbate, apoplastic phenolics may also be insufficient to provide meaningful degradation of O_3 (Booker *et al*., 2012). It will probably be necessary to consider the full suite of antioxidant metabolism as well as structural modifications that may be associated with accumulation of phenolic compounds in assessing sensitivity to O_3.

Application of diel trends in sensitivity

The conclusion that injury is related to the product of S and F is model independent. Appropriate approaches have been suggested (Wesely, 1989; Emberson, 2000; Massman *et al*., 2000), though the practicality of this approach to regional ozone impacts will depend on improved stomatal models. These must include nocturnal conductance, increased spatial resolution of land use models, and accurate parameterization of horizontal and vertical distributions of ambient [O_3]. Most challenging may be development of fully parameterized indices of plant sensitivity to O_3. The simple use of the gas exchange rate as a surrogate for sensitivity is not supported by the current results. This should be a major focus of further vegetation research regarding impacts of O_3 on unmanaged and agricultural ecosystems.

Direct measurement of S as in the current study is impractical for the large number of potential target species. Parameterization of inherent defence capability will require metabolic information and mechanistic modelling of the underlying physiological processes, as they vary with phenology, species, and environment, as well as with time of day (Massman and Grantz, 1995; Chen *et al*., 1998; Barnes *et al*., 2002; Massman, 2004; Musselman *et al*., 2006). In the short term, it may prove useful to pursue careful measurements of diel and developmental time courses of S in a few key species, while development of a comprehensive mechanistic description is pursued. An important component of future research needs is to assess the power of S and F_{eff}, relative to that of [O_3] or F, to unify O_3 impacts across environments and vegetation types.

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