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RESEARCH PAPER

Diel trends in stomatal response to ozone and water deficit: a unique relationship of midday values to growth and allometry in Pima cotton?

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Keywords

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ABSTRACT

Plant responses to ozone (O_3) and water deficit (WD) are commonly observed, although less is known about their interaction. Stomatal conductance (g_s) is both an impact of these stressors and a protective response to them. Stomatal closure reduces inward flux of O_3 and outward flux of water. Stomatal measurements are generally obtained at midday when gas exchange is maximal, but these may not be adequate surrogates for stomatal responses observed at other times of day, nor for non-stomatal responses. Here, we find in Pima cotton that stomatal responses to O_3 observed at midday do not reflect responses at other times. Stomata were more responsive to O_3 and WD near midday, despite being at quasi-steady state, than during periods of active opening or closing in morning or evening. Stomatal responsivity to O_3 was not coincident with maximum gas exchange or with periods of active regulation, but coincident with plant sensitivity to O_3 previously determined in this cultivar. Responses of pigmentation and shoot productivity were more closely related to stomatal responses at midday than to responses at other times of day under well-watered (WW) conditions, reflecting higher stomatal responsivity, sensitivity to O_3, and magnitude of midday g_s. Under WD conditions, shoot responses were more closely related to early morning gs. Root responses were more closely related to early morning g_s under both WW and WD. Responses of stomata to O_3 at midday were not good surrogates for stomatal responses early or late in the day, and may not adequately predicting O_3 flux under WD or when maximum ambient concentrations do not occur near midday.

INTRODUCTION

In many crop production regions, as well as in unmanaged ecosystems, soil water deficit (WD) and tropospheric ozone (O_3) are increasing, and are projected to increase further, driven by rising CO_2, temperature and other elements of global change (Volz & Kley 1988; Meehl *et al.* 2007; Bates *et al.* 2008; IPCC 2014). Globally increasing background O_3, combined with O_3 resulting from rising emissions of locally generated precursors, will create novel and challenging conditions for production of food, fibre and bioenergy (Vingarzan 2004; Stevenson *et al.* 2006; The Royal Society 2008; Wilkinson *et al.* 2012). Efficient crop improvement and understanding ecosystem responses will require characterisation of traits and suites of traits that confer tolerance to combinations of these factors.

Current and anticipated O_3 causes direct damage to vegetation (Booker *et al.* 2009), suppresses agricultural yield (Heck *et al.* 1982; Lefohn *et al.* 1988; Emberson *et al.* 2009; Avnery *et al.* 2011) and inhibits growth of native vegetation (Skelly *et al.* 1983; Materna 1984). O_3 alters plant growth and structure (Heggestad *et al.* 1988; Miller 1988; Heggestad & Lee 1990) and reduces daily maximum stomatal conductance (g_s) and photosynthesis (Reich & Amundson 1984; Heath 1988), and the duration of these maximum rates on a daily and seasonal basis (Paoletti & Grulke 2005).

Plant WD has impacts in many ways similar to those of O_3, both reducing magnitude and duration of gas exchange and suppressing biomass production (Tingey *et al.* 1971; Miller 1988; Grantz & Yang 1996; Grantz *et al.* 2006). $O_3 \times$ WD interactions are common (Mansfield 1973; Fuhrer & Booker 2003; Hoshika *et al.* 2013), including antagonistic (protective) interactions (Temple 1986, 1990a; Temple *et al.* 1988a,b; Silim *et al.* 2009) and synergistic (deleterious) interactions (Heggestad *et al.* 1985; Wagg *et al.* 2012, 2013), both partly mediated by stomatal responses. Interactions of $O_3 \times$ WD for biomass productivity and morphological traits, as well as relationships of these responses with stomatal responses, remain poorly characterised.

Stomatal conductance (g_s) is closely correlated with photosynthetic carbon assimilation, both easily measured with modern instruments. In C_3 plants such as cotton, g_s effectively regulates gas exchange of water vapour, O_3 and carbon dioxide (CO_2) when radiation is not limiting. Stomatal closing responses are defences against both O_3 and WD, giving rise to potential interactions. Stomatal closing responses to WD reduce O_3 influx (Temple 1986, 1990a; Temple *et al.* 1988a,b;

Grantz *et al.* 1997; Grunhage *et al.* 1997; Massman *et al.* 2000; Musselman *et al.* 2006), while O_3-induced closure reduces transpiration and conserves soil moisture (Hayes *et al.* 2012). Additionally, there may be interactions due to biochemical cross-protection (Vannini *et al.* 2006; Biswas & Jiang 2011). Stomatal closure is also a direct response to both stressors mediated by hydrologic and hormonal factors, with impacts on productivity. Thus interpretation of stomatal responses to O_3 or WD, and their interaction, may be complex, and relationships with non-stomatal responses difficult to predict. Improved gas exchange traits (Long *et al.* 2006; Zhu *et al.* 2010; Ainsworth *et al.* 2012), including photosynthetic stability and stomatal regulation that minimises g_s during periods of high evaporative demand and elevated O_3, may contribute to stress tolerance (Dumont *et al.* 2013).

Gas exchange measurements have become routine, and are more easily obtained than morphological and anatomical traits that may also be mechanistically linked to productivity or yield. Morphological characters such as harvest index and the spatial and temporal extent of leaf area display have been heavily selected in modern crop cultivars (Morrison *et al.* 1999, 2000; Long *et al.* 2006; Jin *et al.* 2010). There may be little scope for further improvement in these traits. However, maintenance of these traits under environmental stress may, itself, be a desirable phenotype (Hetherington & Woodward 2003; Ainsworth *et al.* 2012) that could contribute to yield stability (Leadley *et al.* 1990; Betzelberger *et al.* 2012). Relationships between stomatal response patterns to O_3 and WD at different times of day are not known. Similarly, relationships between other plant traits and gas exchange measurements at various times of day have not been characterised, although these may facilitate phenotypic screening for stress tolerance.

In the present study we use an O_3-sensitive cultivar of Pima cotton (*Gossypium barbadense* L.; cv. S-6; Grantz & Yang 1996), grown under controlled greenhouse conditions, to investigate the effects and interactions of O_3 exposure and mild WD. We contrast responses of daily maximum g_s obtained under the quasi-steady state conditions of midday, with responses of g_s early in the morning and evening, both periods of dynamic regulation and directional stomatal movements when ion transport capacities are fully activated. We characterise responses of productivity and allocation of biomass to O_3 and WD. We test three hypotheses: (i) the gas exchange responses observed at midday are similar to and thus surrogates for those occurring at other times of the day; (ii) g_s in a quasi-steady state at midday is less responsive to O_3 and WD than g_s during periods of dynamic stomatal adjustment; and (iii) that the responses of leaf pigmentation, productivity and allometric parameters may be uniquely related to responses of midday gas exchange when rates are maximal rather than to responses at other times of day when stomatal movements are more active.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of Pima cotton (cv. S-6) were obtained from foundation seed stock and germinated in a greenhouse in Parlier, CA, USA (36.6°N, 119.5°W). This cultivar of Pima cotton is O_3-sensitive (Grantz & Yang 1996). Seeds were planted in moist commercial seedling mix (Earthgro Potting Soil; Scotts Co., Marysville, OH, USA) in plastic pots (870 ml; 110 mm square × 125 mm deep; Dillen Products, Middlefield OH, USA). Emerged seedlings were thinned to one per pot.

The experiment was conducted twice (two runs). At 10 or 12 days after planting (DAP; Run 1 or 2, respectively), when cotyledons were fully expanded and the first true leaf was emerging, two pots were transferred to each of nine cylindrical, Teflon O_3 exposure chambers. These chambers (continuously stirred tank reactors, CSTRs; Heck *et al.* 1978) were located in the greenhouse, as previously described (Grantz *et al.* 2008, 2010) and illuminated with natural sunlight. Air with appropriate O_3 concentration (4, 59 and 114 ppb; 12-h means, 07:00–19:00 h) was introduced at one complete air exchange per minute into each CSTR. O_3 was dispensed from the time of transfer to the CSTRs as daily half-sine waves (7 days week^{-1}), with peak concentration at solar noon (13:00 h). Environmental conditions (T_{air}, RH, PPFD) in the chambers were stable from day to day, exhibiting a broad midday plateau (10:00–16:00 h) and distinct minima or maxima just prior to dawn (5:00–5:30 h). Mean values over these periods were: T_{air}, 31.9 ± 0.24 °C and 17.8 ± 0.34 °C; RH, $26.3 \pm 0.5\%$ and $58.5 \pm 0.9\%$; and PPFD 857 ± 33 μmol·m^{-2}·s^{-1} and 0.7 ± 0.3 μmol·m^{-2}·s^{-1}.

Ozone was generated 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) and regulated in a single CSTR with a dedicated O_3 monitor (Model 49C; Thermo Fisher Scientific, Waltham, MA, USA), with computerised feedback control (Grantz *et al.* 2008). The O_3 concentrations in the other CSTRs were maintained proportionally and determined every 15 min with a separate O_3 monitor (Model 49C) through continuously purged Teflon dust filters and tubing. O_3 monitors were calibrated against a factory certified calibration unit (Model 306; 2B Technologies; Boulder CO, USA).

There were two water status (W) treatments. Well-watered plants (WW) were irrigated with 600 ml·day^{-1} (about field capacity, 21% by volume; determined gravimetrically after 3 h drainage). Water deficit plants (WD) received one third this amount, as 200 ml·day^{-1} (Run 1) or 600 ml every third day (Run 2). No differences were observed in plant growth or gas exchange, or in soil water content prior to irrigation (9–11% in WD; 15–18% in WW). Pots were fertilised twice a week with a complete fertiliser solution (Miracle Gro; Scotts Miracle-Gro Products Inc., Port Washington, NY, USA).

Stomatal conductance and responsivity

Stomatal conductance was measured at 1.5-h intervals, from 07:30 to 18:00 h, to calculate the responsivity index. Values obtained at 10:30 and 12:00 h were averaged to represent maximal diel rates of gas exchange. All measurements were obtained on the youngest fully expanded leaf, on three occasions between 30 and 45 DAP, using a cycling leaf porometer (AP-4; Delta-T Devices, Cambridge, UK). The same leaf was used for all measurements on each day, avoiding major veins, and the next youngest leaf as it became fully expanded was used on subsequent measurement days.

An index of relative stomatal responsivity to O_3 was determined at each measurement time point from mean g_s in each

O_3 × water treatment (WW or WD). The index was calculated as the difference between stomatal conductance at low O_3 and high O_3, with this difference normalised by g_s at low O_3. This value was multiplied by 100 to provide a convenient range of index values.

Leaf pigmentation

A relative measure of chlorophyll content was determined as SPAD units on the same area of each leaf used to measure g_s. Measurements were obtained with a portable chlorophyll meter (Minolta SPAD-502; Spectrum Technologies, Plainfield, IL, USA).

Productivity and allocation

Plants were destructively harvested at 45 or 46 DAP. Plants were separated into leaves (including the petioles), stems and roots. Roots were washed in cool water to remove potting material. Leaf area was determined on pooled leaves from individual plants using a belt-driven leaf area meter (LI 3100; LiCor Inc. Lincoln NE, USA). Dry weights of plant components were determined using an electronic balance (model 200; Mettler-Toledo, Columbus, OH, USA; capacity 200 g, precision 1×10^{-4} g) after drying with forced air at 60 °C to constant weight (about 1 week). Allometric parameters were calculated on an individual plant basis.

Experimental design

The experiment was replicated in three blocks of CSTRs aligned parallel to windows and cooling fans in the greenhouse to isolate location effects, and performed twice (two runs) during the summer of 2013. One CSTR in each block was exposed to each of three O_3 concentrations. Runs yielded consistent results and were pooled for analysis as a split plot, randomised complete block design, with a main treatment of O_3 concentration and a sub-treatment of water application rate (W). The unit of replication was the individual CSTR. Data were analysed using SAS for Windows (version 9.2.1; SAS Institute Inc., Cary, NC, USA). Data that failed to meet the normality assumptions of ANOVA were log-transformed prior to analysis using PROC GLM. This was only necessary for the stomatal conductance data. O_3 treatments and W sub-treatments were taken as fixed factors, with block as a random factor. Mean separation was performed using Fisher's least significant difference (LSD) test. The interaction of W × O_3 on g_s at midday was significant (Fig. 1), so that effects of W and of O_3 were analysed within each level of the other factor (Table 1). For all other response parameters, the interaction was not significant, and the effects of W and O_3 were evaluated across all levels of both factors. All data are presented as means ± 1 SE, calculated from non-transformed data.

RESULTS

Midday stomatal conductance

Stomatal conductance at midday (10:00–13:00 h), when stomata approach steady state, was sensitive to both elevated O_3 and to water deficit (WD; Fig. 1). The interaction between them yielded significant protective antagonism. In the WD treatment, g_s at moderate O_3 was increased by ~25% relative to g_s in low O_3 (Fig. 1 open symbols; Table 1). Under WW conditions, there was no significant difference between g_s at low (4 ppb) and moderate (59 ppb) O_3 exposure, while high O_3 (114 ppb) decreased g_s by ~60%.

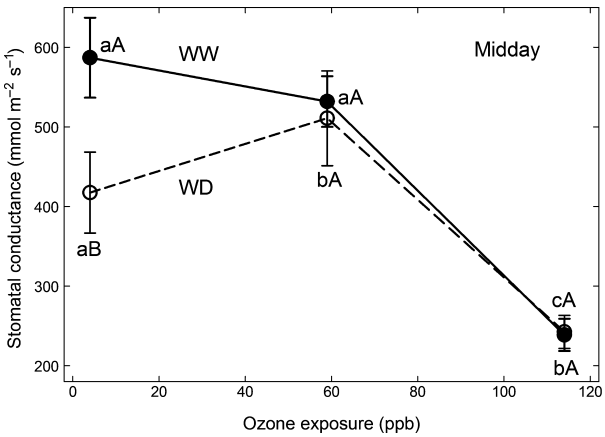

Fig. 1. Response of stomatal conductance (g_s) measured at midday (10:30–12:00 h) to 12-h mean O_3 under well-watered (WW; solid circles) or water deficit (WD; open circles) conditions (means ± SE; n = 18). The O_3 × W interaction effect was significant. Points within each line with different lowercase letters differ at $P \leq 0.05$. Points within each level of O_3 with different uppercase letters differ at $P \leq 0.05$.

The WD treatment had a significant inhibitory effect on g_s at midday (Table 1), reducing g_s by ~30% at low O_3 (Fig. 1), but exerting little effect at either moderate or high O_3. The interaction between the water treatment and O_3 exposure was not significant (Table 1). The relative O_3 impact at high O_3 *versus* low O_3 was smaller in WD (~40%) than in WW plants (~60%), because g_s at low O_3 was substantially depressed by the WD treatment (Fig. 1 open symbols). These O_3 effects were persistent throughout the 45 day growth period (not shown).

Non-steady state stomatal conductance

In early morning, around 07:30 h when PPFD was increasing rapidly, and early evening, around 18:00 h when PPFD was declining rapidly, stomata were actively opening and closing, respectively. Stomatal regulation and the underlying ion transport processes were dynamic, with no approximation of steady state. In the WW treatment, the response of g_s in the early morning to O_3 was proportional over the entire exposure range (Fig. 2A solid symbols), with a reduction of ~25% at moderate O_3 and ~50% at high O_3. Analysis within the WW treatment indicated a significant effect of O_3 (Fig. 2A), however, for the overall split-plot design, the effect of O_3 was not significant (Table 1). Early morning g_s was ~35% of midday g_s (cf. Figs 1 and 2A) under WW conditions at low O_3.

Under WD conditions the response of g_s in the morning was nearly proportional to O_3 (Fig. 2A open symbols; Table 1), with g_s consistently reduced by ~5% and ~26% at moderate and high O_3, respectively, but the effect was not significant. The effect of the WD treatment was significant (Table 1), reducing early morning g_s by ~40% at low O_3, similar to the

Table 1. ANOVA of response parameters.

response parameter	number of observations (n)	*P*-values ozone (O_3)	water status (W)	W × O_3 interaction
midday g_s (mmol·m^{-2}·s^{-1})	18	**0.0412** WW < 0**.0001** WD = 0**.0005**	**0.0030** Lo O_3 = 0**.0016** Med O_3 = 0.1184 Hi O_3 = 0.8151	**0.0083**
morning g_s (mmol·m^{-2}·s^{-1})	18	0.290	**0.0034**	0.253
evening g_s (mmol·m^{-2}·s^{-1})	18	0.986	**0.0518**	0.614
SPAD	18	**0.0387**	**0.0371**	0.856
shoot biomass (g)	6	**0.0148**	0.6535	0.641
leaf area (cm^2)	6	**0.0006**	0.7111	0.827
leaves/plant	6	**0.0067**	0.6725	0.519
plant height (cm)	6	**0.0123**	0.7558	0.368
leaf insertion rate (leaves per cm stem)	6	**0.0317**	0.6867	0.867
leaf biomass (g)	6	**0.0043**	0.691	0.589
stem biomass (g)	6	**0.0359**	0.6254	0.699
total biomass (g)	6	**<0.0001**	0.6576	0.621
root biomass (g)	6	**0.0182**	0.6686	0.380
root to shoot ratio	6	**0.0015**	0.3363	0.460
root mass/leaf area	6	**0.0002**	0.4173	0.530

Values in bold are statistically significant.

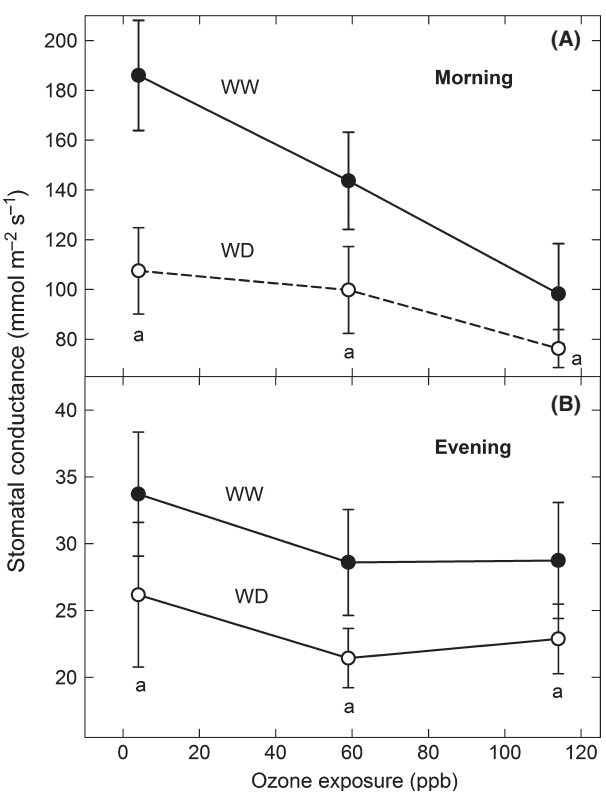

Fig. 2. Response of stomatal conductance (g_s) measured (A) in early morning (07:30 h) and (B) in early evening (18:00 h) to 12-h mean O_3 under well-watered (WW; solid circles) or water deficit (WD; open circles) conditions (mean ± SE; n = 18). The effect of water across all O_3 levels was significant but there was no significant O_3 × W interaction. Levels of O_3 (across both W treatments) did not differ at $P \leq 0.05$. In (A), within the WW treatment levels of O_3 with different lowercase letters differ at $P \leq 0.05$.

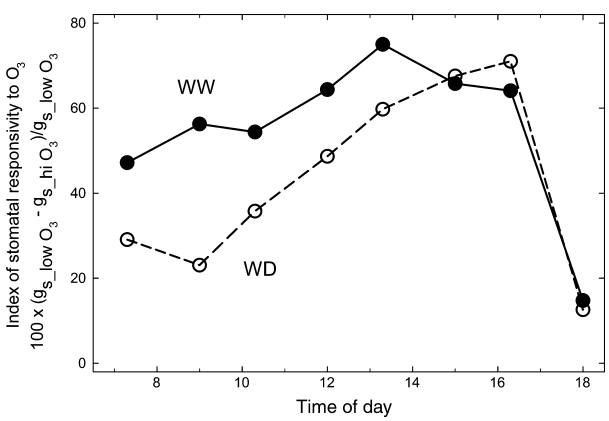

Fig. 3. The diel time course of the index of stomatal responsivity to chronic O_3 exposure under well-watered (WW; open circles) or water deficit (WD; solid circles) conditions.

~30% reduction by WD observed at midday (cf. Fig. 1). At moderate O_3, WD reduced g_s by ~35%, and at high O_3 by ~20%. The O_3 × WD interaction was not significant (Table 1).

In the evening (Fig. 2B), in both WW and WD, the stomatal response to O_3 exposure was flat, and similar to that for WD in the morning. The magnitude of g_s was considerably lower than at other times of the day, ~6% of midday values (cf. Figs 1 and 2B) under WW conditions at low O_3. The WD treatment decreased g_s ($P = 0.0518$; Table 1) by ~20% at all levels of O_3 relative to the WW treatment.

Stomatal responsivity

The stomatal responses to both O_3 and WD differed substantially throughout the day (*e.g.* Figs 1 and 2). These differences

were captured as an index of responsivity, calculated as the relative stomatal response between low and high O_3 (Fig. 3). Stomatal responsivity to chronic O_3 exposure exhibited a clear diel pattern, increasing from early morning through mid-afternoon and declining sharply into evening. The diel pattern was similar under WW and WD, but ~45% higher in WW from 07:00 to 10:00 h, and ~30% higher in WW from 10:00 to 14:00 h. Responsivity was similar in both water treatments from 15:00 to 18:00 h. Peak responsivity occurred several hours earlier in WW.

Leaf properties

Leaf chlorophyll content (SPAD units) responded significantly to O_3 (Fig. 4, Table 1). The responses were similar under WW and WD conditions, with little impact at low and moderate O_3, and a substantial decline at high O_3. At high O_3, SPAD was reduced by ~15% in both WW and WD treatments. These response patterns were similar to those observed in midday g_s.

The effect of WD on SPAD was significant (Table 1), although in contrast to g_s, leaf pigmentation was higher in WD than in WW at all levels of O_3. There was no convergence at high O_3, and the interaction of W × O_3 was not significant. The response of SPAD was not mediated by responses of leaf density (leaf mass per area, LMA; not shown), which did not respond consistently to O_3 or WD. Some bronze discoloration was observed visually on lower leaves in high O_3, which is an indication of O_3-induced early senescence. Neither necrosis nor accelerated abscission was observed in the experiment.

Shoot growth

Total plant biomass (not shown) was dominated by shoot biomass (Fig. 5A; between 84% and 89% of total biomass over all treatments). Both parameters exhibited responses to O_3 similar to that observed in midday g_s. Moderate O_3 caused little response, but shoot biomass declined substantially at high O_3. The effect of WD was not significant (Table 1, Fig. 5A). Shoot biomass was approximately evenly divided among stem and leaf components. Leaf biomass (not shown) and leaf area per plant (Fig. 5B) exhibited responses similar to those of shoot biomass, declining substantially at high O_3.

The number of leaves per plant was also significantly affected by O_3 exposure (Fig. 5C) driven by the response to high O_3 relative to that in low O_3 and moderate O_3. The response was considerably more pronounced in the WW treatment. Plant height (not shown) increased significantly at moderate O_3, relative to both low O_3 and high O_3 (Table 1). The WD treatment consistently, but not significantly (Table 1), reduced plant height and number of leaves per plant at low and moderate O_3, but provided apparent protection at high O_3, where WD exceeded WW for both parameters.

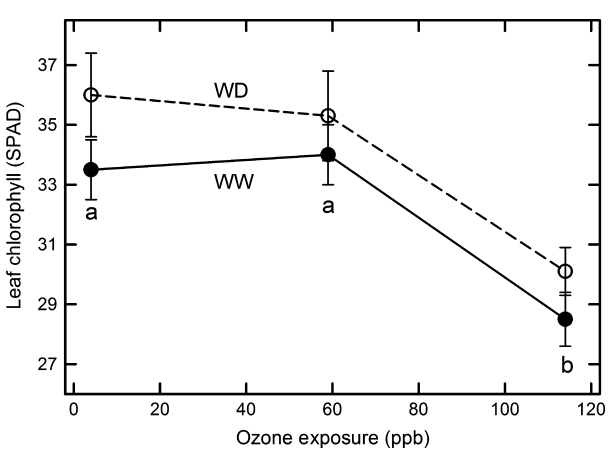

Fig. 4. Response of leaf chlorophyll content (SPAD units) to 12-h mean O_3 under well-watered (WW; solid circles) and water deficit (WD; open circles) conditions (means ± SE; n = 18). The effect of water across all O_3 levels was significant but there was no significant O_3 × W interaction. Levels of O_3 (across both W treatments) with different lowercase letters differ at $P \leq 0.05$.

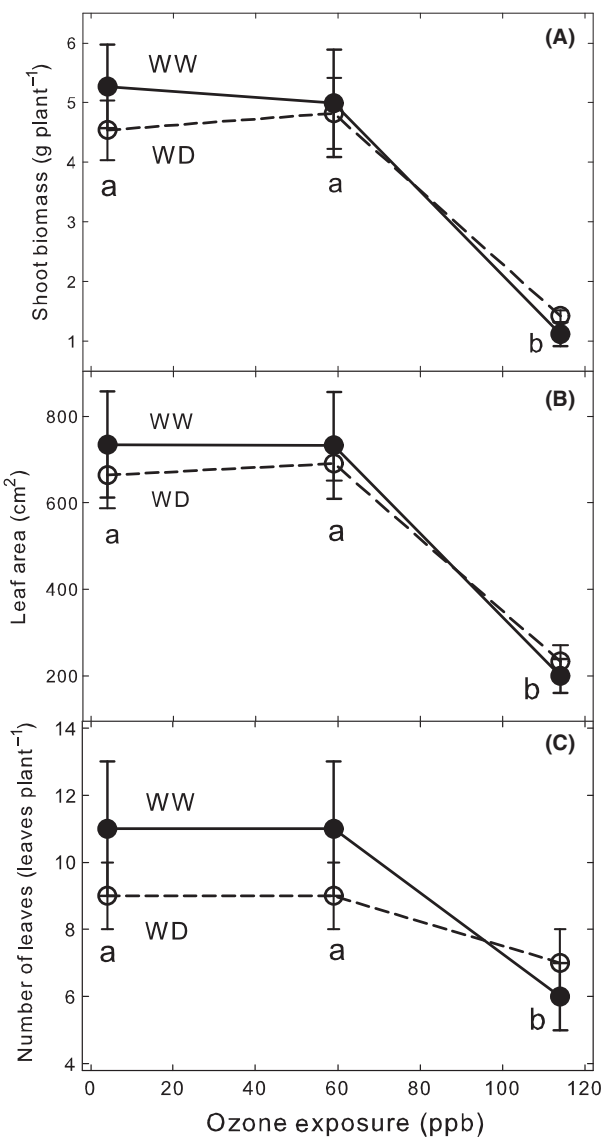

Fig. 5. Response of (A) shoot biomass, (B) leaf area and (C) number of leaves per plant to 12-h mean O_3 under well-watered (WW; open circles) and water deficit (WD; solid circles) conditions (means ± SE; n = 6). The W and W × O_3 effects were not significant. Levels of O_3 (across both W treatments) with different lowercase letters differ at $P \leq 0.05$.

The vertical density of leaves in the canopy is an important determinant of leaf area index and radiation interception. Leaf insertion rate (leaves·cm^{-1} stem; not shown) declined significantly with increasing O_3 in both WW and WD treatments. At low and moderate O_3 leaf insertion rate was higher in WW, but at high O_3 the WD was higher. Overall, the WD effect was not significant (Table 1). The response of leaf insertion rate to O_3 was not driven by extension growth (plant height) but rather by leaf initiation (leaves per plant; Fig. 5C).

Root growth

Root biomass exhibited a similar response to O_3 as the shoot biomass components. Under WW conditions, the response was proportional to increasing O_3 and was significantly inhibited by the O_3 treatments (Table 1, Fig. 6A). In WD the decline was non-linear, with little response between low and moderate O_3 and substantial decline at high O_3. Although WD reduced root biomass at low O_3, it protected root biomass against moderate O_3. Both WW and WD declined to the same low level at high O_3. This was similar to the response observed in midday maximum g_s.

Both the root to shoot biomass ratio (R/S; Fig. 6B) and the leaf specific root biomass (Fig. 7) declined proportionally with increasing O_3 exposure. The water treatments did not significantly differ and there was no W × O_3 interaction. The significant decline of both with increasing O_3 exposure (Table 1) suggests that water acquisition capacity was degraded by O_3 with no protective interaction with the WD treatment.

DISCUSSION

Stomatal regulation

The midday period from late morning through early afternoon represents maximum daily g_s. This is the period that most closely approximates a steady state. Despite perturbations by fluctuating environmental conditions, stomata are neither consistently opening nor closing. The duration and magnitude of the midday plateau determines if these maximal rates dominate integrated diel carbon assimilation. Most published gas exchange measurements are obtained at this time, at maximum magnitude and stability of g_s, and are implicitly assumed to represent responses throughout the day.

Average g_s during the midday period was sensitive to both O_3 exposure and to WD. This persisted throughout the entire plant growth period (45 days), similar to results obtained under field exposure conditions (*e.g.* Gillespie *et al.* 2012). This contrasts with transient declines in g_s following acute O_3 exposure (Chen *et al.* 2009) associated with a reactive oxygen burst in guard cells (Gadjev *et al.* 2006; Kollist *et al.* 2007; Vahisalu *et al.* 2010). These results obtained at midday are consistent with numerous previous observations (Reich & Amundson 1984; Temple 1986, 1990a; Heath 1988; Temple *et al.* 1988b; Reiling & Davison 1995; Grantz & Yang 1996; Grulke 1998).

The inhibition of maximum g_s is a response to O_3, but may also represent an adaptive defence mechanism that reduces O_3 uptake (Massman *et al.* 2000). At present, it is unclear whether this confounds interpretation of stomatal response. While restricting influx of O_3, reduced g_s also reduces transpiration and, in C_3 plants, reduces photosynthesis due to decreased intercellular CO_2 concentrations (Aphalo & Jarvis 1993; Paoletti & Grulke 2005). Under WD conditions, the moderate O_3 treatment provided some protection of midday maximum g_s

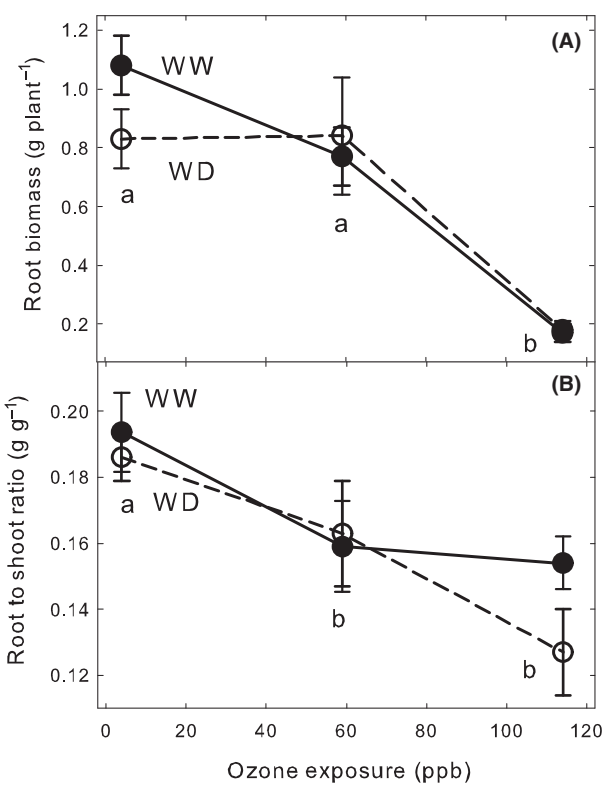

Fig. 6. Response of (A) root biomass and (B) root biomass to shoot biomass ratio to 12-h mean O_3 under well-watered (WW; open circles) and water deficit (WD; solid circles) conditions (means ± SE; n = 6). The W and W × O_3 effects were not significant. Levels of O_3 (across both W treatments) with different lowercase letters differ at $P \leq 0.05$.

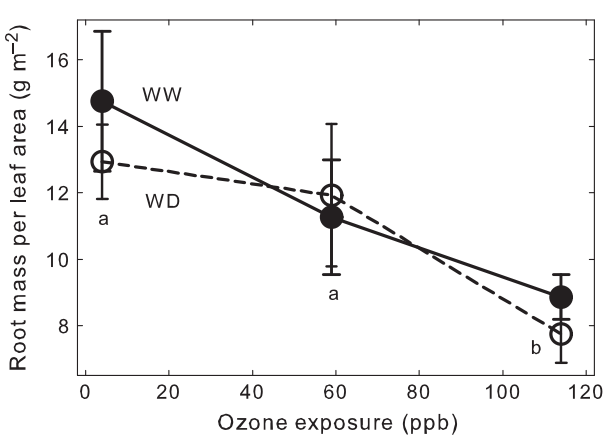

Fig. 7. Response of leaf area specific root mass to 12-h mean O_3 under well-watered (WW; solid circles) and water deficit (WD; open circles) conditions (means ± SE; n = 6). The W and W × O_3 effects were not significant. Levels of O_3 (across both W treatments) with different lowercase letters differ at $P \leq 0.05$.

but limitations to maximum g_s appeared to be dominated by stomatal sensitivity to O_3 rather than to WD.

Stomatal responses to O_3 and WD in the morning and evening were not similar to each other or to responses at midday. The diel differences in stomatal responses to O_3 were captured in the diel responsivity index. Responsivity was maximal during the nearly steady state midday period (11:00 to 14:00 h), with the lowest responsivity observed during periods of rapid stomatal movement early in the morning and evening. Hypothesis 1, that the gas exchange responses observed at midday are similar to and thus surrogates for those occurring at other times of the day, was not supported. This is significant for directing experimental investigations to periods of maximum conductance and transpiration. There are substantial other advantages to measurements at this time, including convenience and optimal signal to noise ratio. These results suggest no attenuation of stomatal response during the midday period of reduced regulatory activity and of relative stomatal stability, which might have discouraged mechanistic studies of signalling and ion transport at midday. In future studies a diel profile of the responsivity index may be used to confirm these features in other environments with contrasting species.

Early morning and evening are characterised by directional changes in irradiance and stomatal movements. Magnitudes of gas exchange at these times were lower and radiation-limited. In the present study, both O_3 and WD truncated afternoon profiles of g_s, with maxima observed prior to solar noon, leading to abbreviated periods of daily maximum gas exchange (Paoletti & Grulke 2005). The protection of g_s observed at midday with moderate O_3 was not observed in early morning or evening, when suppression by WD was observed at all O_3 exposure levels. There are fewer descriptions of stomatal responses to O_3 and WD during these non-steady state conditions. Stomatal response kinetics during periods of dynamic regulation have substantial impact on net carbon gain (Cardon *et al.* 1994; Leakey *et al.* 2002; Lawson & Blatt 2014). Assimilation under radiation-limited conditions may account for a substantial fraction of daily integrated carbon acquisition (Long 1993). Slower response kinetics during non-steady state conditions do not appear to affect diel water balance as much as altered kinetics at midday, when g_s is maximal (Paoletti *et al.* 2011).

Responsivity was not enhanced during periods of active guard cell ion transport associated with directional adjustment of g_s. This falsified Hypothesis 2, since g_s was not more responsive during periods of dynamic stomatal adjustment than at quasi-steady state. The effects of WW and WD on responses of g_s to O_3 were more distinct during these dynamic periods, particularly during opening in the morning, than during periods of more stable maximum g_s at midday. This is consistent with previous observations of substantial differences in morning opening responses in mature ponderosa pine (*Pinus ponderosa*) exposed to 40–80 ppb O_3 (Grulke 1998) and with attenuated stomatal responsivity (to CO_2) early and late in the day in soybean (*Glycine max* L.; Bernacchi *et al.* 2006). Maximum values of responsivity did not coincide with maximum g_s, but rather with the previously determined maximum plant sensitivity to short-term pulse exposures to O_3 in these plants (Grantz *et al.* 2013; Grantz 2014). Under WW conditions, peak responsivity occurred just after the period of peak g_s and in WD plants somewhat later. This suggests a potential commonality of mechanism (Hills *et al.* 2012; Drake *et al.* 2013).

Relationships with other parameters

Responses of SPAD to O_3 under WW conditions were closely related to responses of g_s at midday ($r^2 = 0.99$), and less so to responses of g_s in early morning or evening ($r^2 = 0.65$, 0.0; respectively). SPAD was not driven by leaf anatomical changes. Leaf density (LMA) was not closely related to midday g_s and was less responsive to O_3 than was SPAD, particularly in the WD treatment.

Leaf pigmentation was higher in WD than in WW leaves at all levels of O_3, in contrast to g_s. This pattern suggests that the WD treatment was relatively mild, as more severe WD has been observed to decrease leaf chlorophyll (*e.g.* Parida *et al.* 2007). No necrotic lesions were observed in this experiment, but some bronze stippling and accelerated senescence were observed on lower leaves in high O_3, as is commonly observed in shaded leaves (Pell *et al.* 1997; Chappelka *et al.* 2003; Goumenaki *et al.* 2010; Sarkar *et al.* 2010). These results suggest that SPAD represents a useful surrogate for O_3-induced changes in midday g_s but not for WD-induced changes, nor for stomatal responses observed in morning or evening. Under WD conditions, SPAD was more closely related to g_s in early morning ($r^2 = 0.97$) than at midday or evening ($r^2 = 0.87$, 0.0).

The responses of biomass and leaf area to O_3 and WD were similar to responses of g_s observed at midday. In WW, shoot biomass was more closely related to gs at midday ($r^2 = 0.96$) than in early morning or evening ($r^2 = 0.55$, 0.0). In WD, shoot biomass was more closely related to gs in early morning ($r^2 = 0.89$) than at midday or evening ($r^2 = 0.81$, 0.0). These observations are consistent with previous evidence in upland and Pima cottons (Oshima *et al.* 1979; Miller 1988; Temple *et al.* 1988b; Temple 1990b; Grantz & Yang 1996) and in other species (McLaughlin *et al.* 1982; Heggestad *et al.* 1988; Miller 1988; Pell *et al.* 1994). Responses of root biomass were also similar to those of the shoot and midday g_s (Cooley & Manning 1987; Kostka-Rick *et al.* 1993; Grantz & Yang 1996; Grantz *et al.* 2006). The WD treatment reduced root biomass at low O_3, but had no further effect at moderate or high O_3, suggesting dominance by the responses to O_3. In contrast to shoot parameters, root biomass under both WW and WD conditions was more closely related to g_s in early morning ($r^2 = 0.86$, 0.95) than at midday ($r^2 = 0.51$, 0.46) or evening ($r^2 = 0.52$, 0.0).

Plant height did not respond similarly to biomass, leaf area or g_s. It was increased by moderate O_3 in both WW and WD, relative to both low and high O_3, and was reduced by WD at low and moderate O_3 but protected by WD at high O_3. This reflects the combined effects of etiolation and reduced productivity; both are observed at moderate to high O_3 (Tingey *et al.* 1971; Leone & Brennan 1975; Kostka-Rick *et al.* 1993). In contrast, leaf insertion along the stem, the inverse of internode length, declined monotonically with O_3 exposure. This was driven by the rate of leaf initiation rather than by stem elongation, and responded similarly to midday g_s.

Root biomass, and the ratios of root biomass to shoot biomass and to transpiring leaf area were all reduced significantly and nearly proportionally with increasing O_3 exposure. None were significantly impacted by WD. Root growth is generally reduced by O_3 (Grantz *et al.* 2006) and enhanced by WD (Sharp & Davies 1989; Grulke & Balduman 1999), but in the present study no protective antagonism between O_3 and WD was observed, and potentially damaging synergy was observed

in the root to shoot ratio, as found previously in Pima cotton (Grantz & Yang 1996) and other species (Fiscus & Markhart 1979; Meinzer & Grantz 1990; Yang & Tyree 1993, 1994; Ren & Sucoff 1995). Responses of these functional parameters were similar to those of g_s observed at midday. These data support Hypothesis 3, that the responses of leaf pigmentation, productivity and allometric parameters may be uniquely related to responses of midday gas exchange when rates are maximal rather than to responses at other times of day when stomatal movements are dynamic. Midday stomatal measurements therefore may adequately reflect broader impacts of O_3 and WD on plant development.

Root biomass per shoot biomass (R/S), and more specifically root biomass per transpiring leaf area, are functionally relevant allometric parameters that reflect the balance between water acquisition and transpiration. Low root mass reduces the volume of soil explored and impairs root hydraulic conductance (McLaughlin *et al.* 1982; Heggestad *et al.* 1985; Lee *et al.* 1990; Grantz & Yang 1996). This might increase sensitivity to soil WD (Heggestad *et al.* 1985) and enhance xylem cavitation (Tyree & Sperry 1988), as observed in soybean (*Glycine max* L.; Heggestad *et al.* 1985). Strong positive relationships between stomatal and hydraulic conductance stabilise plant water potential as both change with development, WD or O_3 (Temple 1986, 1990a; Temple *et al.* 1988a; Lee *et al.* 1990; Meinzer & Grantz 1990; Grantz & Yang 1996; Grantz *et al.* 1999; Meinzer 2002). Reduced allocation to roots in this cultivar has been linked with reduced translocation of carbohydrates from source leaves (Grantz & Farrar 1999, 2000).

CONCLUSION

The present study demonstrates that the impact of O_3 and WD on g_s is subject to different regulatory processes at midday than during periods of active stomatal movement in early morning and evening. A stomatal responsivity index reflected diel trends in these processes, with maximum responsivity coinciding with maximum plant sensitivity, and not with maximum gas exchange. Shoot biomass and pigmentation under WW conditions were well predicted by midday measurements of g_s, as they varied in response to O_3 and WD, and were less closely related to measurements in early morning or evening. This may reflect the dominance of diel gas exchange by the larger values of g_s and O_3 uptake at midday. However, shoot parameters under WD, and root parameters under both WW and WD, were more closely related to g_s in early morning, reflecting the earlier diel peak of g_s under WD conditions, and a potential relationship between allocation to roots and responses to WD. Putative interactions of O_3 × WD were not generally significant, despite significant inhibition of gas exchange and consistent reduction of productivity by WD. We conclude that midday measurements of stomatal responses are justified as sensitive indicators of plant growth and developmental responses to O_3 and WD, and that responses of g_s at this time are predictive of these broader impacts. However, midday measurements are not adequate to generalise diel stomatal response patterns nor other responses under WD conditions or belowground. Under WD, and where maximum ambient O_3 concentrations are not observed during the midday period, midday stomatal measurements may not be suitable surrogates for integrated diel O_3 flux.

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