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O_3 and Drought Effects on Steady State Conductance and Kinetics in Pima Cotton

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ABSTRACT: The degree to which ozone (O_3) exposure and drought affect stomatal control of water loss and respond to environmental stimuli such as varying light is poorly characterized. To that end, we exposed Pima cotton to chronic O_3 exposure (month-long daytime exposures) with and without sufficient water, as well as short term acute O_3 exposure and varying light levels to understand stomatal kinetics. Chronic, month-long exposure to moderately high O_3 (~114 ppb) reduced daytime steady state stomatal conductance (g_s), as did water deficit. Both stomatal opening and closing displayed dose specific, "sluggish" responses to step-changes in illumination with acute, 1-day, O_3 exposures of 0, 50, 100, and 125 ppb. At higher concentration (150 ppb), stomatal control of both opening and closing was degraded. Altered steady state and dynamic stomatal function suggest that elevated ambient O_3, expected to increase in the future, may increasingly influence field water management and appropriate crop choices.

KEYWORDS: Ozone effects, drought stress, stomatal kinetics, crop water use

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Introduction

Although it has been broadly documented that elevated ozone (O_3) exposure and uptake decreases plant productivity, alters within-plant resource allocation, and has economic repercussions in agronomic species (Tai & Val Martin, 2017), its effect on plant water balance is under-investigated. The frequency, severity, and global distribution of elevated ambient O_3, water limitation, and their intersection over agronomic regions are predicted to increase due to increases in tropospheric air pollutants and ongoing changes in regional climate (Bates et al., 2008; Emberson et al., 2018; ONS, 2014; Sitch et al., 2007; The Royal Society, 2008). Stomatal conductance (g_s) is sensitive to both O_3 exposure and water limitation and is the principal physiological regulator of carbon and water balance from plant to landscape scales. Understanding the roles of these stressors in plant water balance, particularly in crops, is important for planning and prediction of societal water consumption (Jackson et al., 2001). Further, stomatal conductance alone inadvertently regulates uptake of O_3 and other gaseous pollutants into the leaf, forming biologically important positive and negative feed-back loops.

Chronic and acute O_3 exposure (referring to sustained vs. brief duration) exert three distinct impacts on stomatal regulation: altered daytime rates of water loss (Nussbaum et al., 1995; Saurer et al., 1991; Vozzo et al., 1995), altered responsiveness to changes in other environmental stimuli (Leipner et al., 2001; McAinsh et al., 2002), and altered night time rates of water loss due to incomplete closure at night after daytime exposure (Grams et al., 1999; Grantz & Yang, 1996; Grulke et al., 2007b, 2007c; Hoshika, Omasa et al., 2013; Kellomaki & Wang, 1997; Kitao et al., 2009; Oksanen, 2003; Paoletti, 2005). All three regulatory processes have the capacity to impact total plant water use (Booker et al., 2004) and regional hydrology (McLaughlin, Nosal et al., 2007; McLaughlin, Wullschleger et al., 2007; Sellers et al., 1996; Sun et al., 2012).

Environmental effects on opening and closing kinetics appear to be closely related to each other (Paoletti & Grulke, 2010). Sluggish and or incomplete stomatal closure uncouples transpiration from carbon assimilation, degrades water use efficiency (WUE; Kirschbaum et al., 1988; Lawson & Blatt, 2014; Lawson et al., 2011), and depletes soil moisture (*reviewed in* Paoletti & Grulke, 2005). O_3-induced, sluggish stomatal response was suspected (Keller & Häsler, 1984; Reich & Lassoie, 1984; Skarby et al., 1987) and later confirmed in other gas exchange studies. Responses of g_s to many environmental stimuli were slowed by O_3 exposure including PPFD (Hoshika et al., 2012; Paoletti & Grulke, 2010; Tjoelker et al., 1995), VPD (Grulke et al., 2007a, 2007c; Hoshika, Omasa et al., 2013; Hoshika, Watanabe et al., 2013; Matyssek et al., 1995; Tjoelker et al., 1995; Uddling et al., 2009; Wieser & Havranek, 1995), soil moisture (Hayes et al., 2012), and CO_2 (Onandia et al., 2011).

The reduction of daytime g_s by water limitation has long been known (Hsiao, 1973). As evaluated within a meta-analysis (Wittig et al., 2007), g_s is also reduced by low to moderately high daytime O_3 exposure. Species with large midday g_s may limit plant water loss by rapid closing responses, reducing the potential for xylem cavitation (Caird et al., 2007; Drake et al., 2013; Grantz et al., 2019; Vialet-Chabrand et al., 2013; Vico et al., 2011). Both stressors provide proximate protection of the mesophyll from O_3 uptake at times of both peak O_3 concentration and evaporative demand in cotton (Temple, 1986, 1990;) and in a number of other species (Cavender-Bares et al., 2007; Grulke et al., 2003; Massman et al., 2000; Panek, 2004; Paoletti

& Grulke, 2005; Temple et al., 1988). Static and kinetic stomatal responses to O_3 and water limitation have been investigated separately, but their interactive effects on stomatal kinetics are not well characterized (Fuhrer & Booker, 2003; Hoshika, Omasa et al., 2013; Matyssek et al., 2006; Nikolova et al., 2010). Both antagonistic (ie, protective: Silim et al., 2009; Temple, 1986; Temple et al., 1988; Temple, 1990) and synergistic (ie, deleterious; Heggestad et al., 1985; Wagg et al., 2012, 2013) interactions of O_3 and water deficit have been described in some plant species. The net effect of $O_3 \times$ water limitation on stomatal response on the three distinct impacts on stomatal regulation (daytime, nighttime, and during changes in other environmental conditions) considered above remains unexplored.

Incomplete stomatal closure increases nighttime transpiration and uptake of O_3. Downwind of urban centers, O_3 remains elevated at night (Gregg et al., 2003; Matyssek et al., 1995; Miller et al., 1972) and has been shown to inhibit growth of cottonwood, birch, and ponderosa pine (respectively). It was also suspected in the collapse of viticulture downwind of Los Angeles in the late 1950′ s (P.R. Miller, personal communication). O_3 is also elevated at night downwind from heavily fertilized agricultural fields due to NO emissions and NO_x interconversions to O_3 in sunlight. These emission plumes are transported into other agricultural areas as well as to natural ecosystems (Almaraz et al., 2018; Matson et al., 2002; Miller et al., 1972) where their effects are often unrecognized. The effects of O_3 on nighttime transpiration has been reported in a number of species (Grulke et al., 2004, 2007a; Hoshika et al., 2012; Matyssek et al., 1995; Wieser & Havranek, 1993;). In birch, nighttime g_s contributed about 10% of total transpiration in low O_3 (Matyssek et al., 1995), and 15% of total transpiration in high O_3. In blue oak and black oak, chronic daytime O_3 exposure increased nighttime g_s to about 16% and 30% (respectively) of daytime maxima (Grulke et al., 2007b).

The study presented here evaluates O_3 impacts on the three aspects of stomatal regulation: altered daytime g_s, incomplete stomatal closure at night with chronic exposure, and sluggish stomatal response to stepped increase and decrease of illumination during acute exposure of previously un-exposed ("naïve") plants. We evaluate a perennial species of economic importance, Pima cotton (*Gossypium barbadense* L.; cv. S-6), that has been characterized with respect to responses to O_3 and water limitation separately (Grantz, 2016; Temple & Grantz, 2010).

Materials and Methods

Plant material

Seeds of Pima cotton (*Gossypium barbadense* L.) from foundation seed stock were germinated in moist commercial potting mix (Earthgro Potting Soil, Scotts Company, Marysville, OH[1]) in plastic pots (870 ml; 110 mm × 110 mm × 125 mm). Plants were grown as described previously (Grantz et al., 2015) in a greenhouse at the University of California, Kearney Agricultural Center (Parlier, CA, USA; 103 masl; 36.598°N, 119.503°W). Pots were thinned to a single plant 10 to 12 days after planting (DAP). Plants remained on the greenhouse bench in filtered air until they developed five to six leaves (May 15 and June 21, 2014, in runs 1 and 2, respectively). Single-plant pots were then available for two types of experiments: stomatal kinetics of cotton with (1) chronic O_3 exposure at two water availabilities in exposure chambers; and (2) bench-top experiments with naïve (previously unexposed to O_3) plants, using stepped high and stepped low light level to drive kinetics in different background O_3 concentrations.

Chronic O_3 exposure × water availability: steady state conductance

Eighteen pots were distributed among nine cylindrical, Teflon-walled, O_3 exposure chambers (Continuously Stirred Tank Reactors; CSTRs; Grantz et al., 2008; Heck et al., 1978) located in the same greenhouse. The CSTRs were aligned in three blocks parallel to windows with cooling fans to reduce location effects. One CSTR per block was exposed to each of the three O_3 concentration profiles delivered as daily half-sine waves with peak concentrations at 1,300 PDT; 12 hours daytime, mean O_3 concentrations were 4, 59, and 114 ppb. O_3 was generated by corona discharge (Model SGC-11, Pacific Ozone Technology, Brentwood, CA) from purified oxygen (Series ATF-15, Model 1242, SeQual Technologies Inc., San Diego, CA). The flow rate through the CSTRs was one air exchange min^{-1}; air temperature was 20°C to 35°C, relative humidity was 34% to 60%, and midday PPFD from sunlight was 1,275 mmol m^{-2} s^{-1} (Grantz et al., 2008; Paudel et al., 2016).

Plants in the CSTRs were grown under well-watered (WW) or water-deficit (WD) conditions. Field capacity of the soil was 21% by volume (VWC). Plants were irrigated to maintain a VWC of 15% to 18% (WW, ~80% of pot "field" capacity), and 9% to 11% (WD, ~50% of pot field capacity). All pots were fertilized twice a week with a complete fertilizer solution (Miracle Gro, Scotts Miracle-Gro Products Inc., Port Washington, NY, USA). The two runs of the experiment yielded consistent results and were pooled. The data for chronic O_3 exposures in CSTRs were analyzed as a split plot, randomized complete block design. O_3 was the main treatment, water application rate was the sub-treatment, and CSTR was the unit of replication. Values of g_s were log-transformed prior to analysis using PROC GLM (SAS for Windows; v. 9.2.1).

Stomatal conductance (g_s) was measured on the youngest, fully expanded leaf, on 3 to 4 dates per experimental run, between 30 and 45 DAP ($n = 125$). Diurnal and nighttime g_s measurements were made, between 0730 and 1800 at 90 minutes intervals, and at 0200, respectively, within the CSTRs. Based on previous research, nighttime gas exchange minimas are achieved ~2 hours after full darkness is achieved (Grulke et al., 2004, 2007b). The intent of the nighttime g_s measurements was to capture the impact of daytime O_3 exposure on g_s

at night. Measurements of g_s were conducted with a cycling leaf porometer (AP-4; Delta-T Devices, Cambridge, UK).

Acute O_3 exposure of naïve cotton: Dynamic stomatal responses to illumination

Our experiments were conducted with previously unexposed (ie, "O_3-naïve"), WW potted plants in the laboratory, using a custom gas exchange system modified from a system previously described (Grulke & Paoletti, 2005; Grulke et al., 2007b, 2007c). To drive stomatal response kinetics within the context of differing concurrent O_3 concentrations (0, 50, 100, 125, or 150 ppb), stepped changes in PPFD were imposed from low to saturating light levels (100–1600 $\mu mol\, m^{-2}\, s^{-1}$) and the reverse, randomizing which light level was applied first. After a 1-hour equilibration at each O_3 concentration at the beginning of the day, g_s was allowed to come to steady state at the initial PPFD level, and illumination was increased or decreased depending on the initial light level. O_3 exposure was maintained as a constant throughout the experiment.

The gas exchange system consisted of matched, custom-designed, sample and reference cuvettes (i.d. $2 \times 3 \times 1$ cm) constructed of acrylic and lined with teflon film, fitted with low vibration micro-fans to ensure air mixing. The cuvettes were in parallel, receiving the same gas stream through the same length and diameter of Teflon tubing. Air for this open (flow-through) system was drawn from two large buffer volumes placed in series. Part of the gas stream was ozonated using an adjustable ultraviolet lamp, then cooled by passing across electronically controlled Peltier blocks, then humidified to maintain leaf to air VPD at ~2 kPa using a dewpoint generator (LI-610; LiCor Inc., Lincoln, NE, USA). Leaves were illuminated from above with a red and blue LED light array (LI-6400-18; LiCor Inc.; Lincoln, NE. USA); PPFD was measured at leaf level within the cuvette. The rest of the plant was illuminated uniformly using a bank of LEDs with a similar wavelength intensity profile (EcoSmart ECS 38 V2; 300 K, 24 W; 120 W tungsten bulb equivalent). A portion of the youngest fully expanded leaf was exposed to the experimental O_3 concentrations in the sample cuvette, one concentration per day. Individual leaves were measured only once and individual plants were not measured on consecutive days.

Water vapor, CO_2 and O_3 were recorded at 15 seconds intervals, using the sample and reference cells of a steady state gas exchange system (LI-6400) in parallel with two cross-calibrated ultraviolet O_3 monitors (Model 41C; Thermo Fisher Scientific Inc.; Waltham, MA, USA). Leaf temperature was determined with a contact thermocouple (Type E, 76 μm dia) appressed to the abaxial surface of the leaf and monitored directly by the LI-6400 cuvette. This temperature, ~30°C, was lagged by 30 seconds in calculations of VPD and g_s to be in sync with flow from cuvettes to analyzers.

Half times ($t_{½}$) for stomatal response to stepped changes in PPFD were calculated by fitting single exponential equations to g_s data obtained at the 15 seconds intervals. For stomatal opening:

$$g_s(t) = a - (b)\, e^{-\lambda t} \quad (1a)$$

and for stomatal closing:

$$g_s(t) = a + (b)\, e^{-\lambda t} \quad (1b)$$

where a and b are fitted parameters related to the initial and final magnitude of g_s; λ is the fitted time constant, and t is time after the stepped change.

The half time was calculated from:

$$t_{½} = \ln(2)/\lambda) \quad (2)$$

Statistical analyses

The data for chronic O_3 exposures in CSTRs were analyzed as a split plot, randomized complete block design. O_3 was the main treatment, water application rate was the sub-treatment, and CSTR was the unit of replication. Statistical analyses and graph preparation were performed with SAS for Windows, v. 9.2.1, SigmaPlot 11.0, and Systat 14.0. Statistical tests and their significance are as described in Results. Except as noted, significance is reported at $p < .05$.

Results

Daytime steady state conductance under chronic O_3 exposure

In all treatments, low O_3 (4 ppb, LO_3) and moderately high O_3 (114 ppb, HO_3), with adequate water (80% of "field" capacity in pots, WW) and limited water (50% of capacity, WD), peak daytime g_s values occurred at ~1,030 (Figure 1); end-of-day g_s was low. The peak level of g_s differed, with O_3 level, water regime, and their interaction significant ($p = .010$; .002; and .037, respectively). In the WW LO_3 treatment, diurnal g_s was characterized by a midday plateau (1030–1400). For plants in the three other treatments, the midday high was a peak diurnal value and the plateau as observed for WW LO_3 was truncated. WD reduced peak midday g_s by ~25% relative to WW LO_3 plants. High mean O_3 exposure (HO_3, 114 ppb) reduced the midday peak g_s by ~55%, relative to WW LO_3, whether plants were WW or WD.

Nighttime steady state conductance under chronic O_3 exposure

Nighttime g_s was non-zero following daytime exposure to O_3 (Table 1). The effect of daytime LO_3 on nighttime g_s was minimal in both WW and WD treatments (both 1.9% of peak daytime g_s). Daytime moderately high O_3 exposure increased the subsequent nighttime g_s to 7.7% and 7.3% of peak daytime values in WW and WD treatments, respectively. Assuming that g_s at 0600 and 1800 were those as measured at 0200 (nighttime g_s, Table 1), mean daytime and nighttime g_s were calculated. In this case, nighttime g_s was 16% to 17% of daytime g_s in high O_3

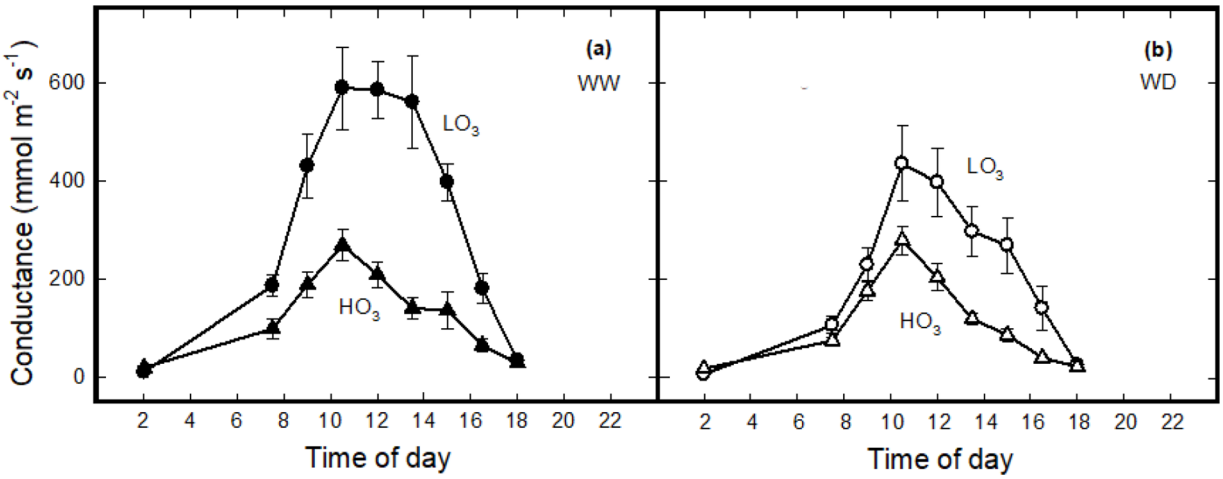

Figure 1. The effect of chronic ozone (O_3) exposure on the diel time course of stomatal conductance (g_s) in Pima cotton under (a) well-watered (WW, ~80% of capacity) or (b) water deficit (WD, ~50% of capacity) conditions. Plants were exposed to low O_3 (circles, LO_3; 4 ppb, 12 hours mean) or moderately high O_3 (triangles, HO_3, 114 ppb, 12 hours mean) for 30 to 35 days (first and second run, respectively and combined here).

Table 1. Effect of Ozone Exposure Level (LO_3, HO_3) and Water Availability (WW; WD as Described in Figure 1) on Daytime Peak and Nighttime Steady State Stomatal Conductance (g_s).

MEAN O_3	% "FIELD CAPACITY"	DAYTIME 1,000	NIGHTTIME 0200	DAYTIME WATER USE	NIGHTTIME WATER USE	TOTAL WATER USE
PPB		G_S, MOL $M^{-2}S^{-1}$	G_S, MOL $M^{-2}S^{-1}$	L/YEAR	L/YEAR	L/YEAR
4	80	0.590 ± 0.100	0.011 ± 0.001	59,494	1,130	60,624
4	50	0.430 ± 0.040	0.008 ± 0.001	38,074	990	39,064
114	80	0.260 ± 0.035	0.020 ± 0.004	21,001	924	21,925
114	50	0.260 ± 0.015	0.019 ± 0.004	19,277	578	19,855

Note. DAYTIME: O_3, p = .010; W, p = .002; $O_3 \times W$, p = .037. NIGHTTIME: O_3, p = .26; W, p = .057; $O_3 \times W$, p = .037. Acronyms as in Figure 1. Means ± 1 *SD*.

(WW and WD, respectively), and only 3.4% to 3.9% in low O_3 (WW and WD, respectively). O_3 (p = .025) and water availability (p = .057) individually had a significant effect on nighttime g_s, but the interaction term not significant ($O_3 \times W$, p = .535).

Stomatal response kinetics to acute O_3 exposure

The kinetics of stomatal opening (Figure 2a and c) and closing (Figure 2b and d) were evaluated following step increases (100–1600 µmol $m^{-2}s^{-1}$) and step decreases (1600–100 µmol $m^{-2}s^{-1}$) in PPFD during five levels of concurrent, acute (day of experiment) O_3 exposure, with cotton plants not previously exposed to O_3. Time courses of both stomatal opening (equation (1a)) and closing (equation (1b)) were well described by exponential relationships over two levels of O_3 exposure tested (50 and 100 ppb; Figure 2).

The mean $t_{½}$ of stomatal opening and closing in O_3-free air did not significantly differ between replicate runs. The $t_{½}$ for both stomatal opening and closing increased linearly with O_3 between 0 and 125 ppb (Figure 3). On average, the $t_{½}$ of stomatal opening was slower (eg, greater $t_{1/2}$ times), and thus more sensitive to O_3 exposure, than that for closing (Figure 3; significant differences in slope, p = .005). At 125 ppb the $t_{½}$ of stomatal opening increased 3-fold and of closing by 1.7-fold, relative to no O_3. The $t_{½}$ for both stomatal opening and closing was lower at 150 ppb than that at 125 ppb (Figure 3), reversing the increasingly sluggish g_s response and suggesting loss of stomatal control (Figure 3). Relative to the $t_{1/2}$ for stomatal opening at 125 ppb O_3, $t_{1/2}$ at 150 ppb decreased by ~35%, and for stomatal closure, decreased by 40% (Figure 3). The greater variability in g_s observed at 125 ppb (Figure 3) may reflect variability in individual plant response, with some having reached their threshold O_3 concentration and some not. Closing remained faster than opening at all non-zero O_3, even above 125 ppb.

The amplitude of stomatal opening (Δg_s from g_{t0} to g_{stf}) increased linearly from 0 to 125 ppb O_3 ($p < .001$; Figure 4, solid circles). An abrupt decrease in the amplitude of stomatal opening was observed when [O_3] increased from 125 to 150 ppb O_3 (Figure 4). The amplitude for stomatal closure with a stepped decrease in light was greater at each O_3 exposure than that for opening, but was less responsive to O_3, differing

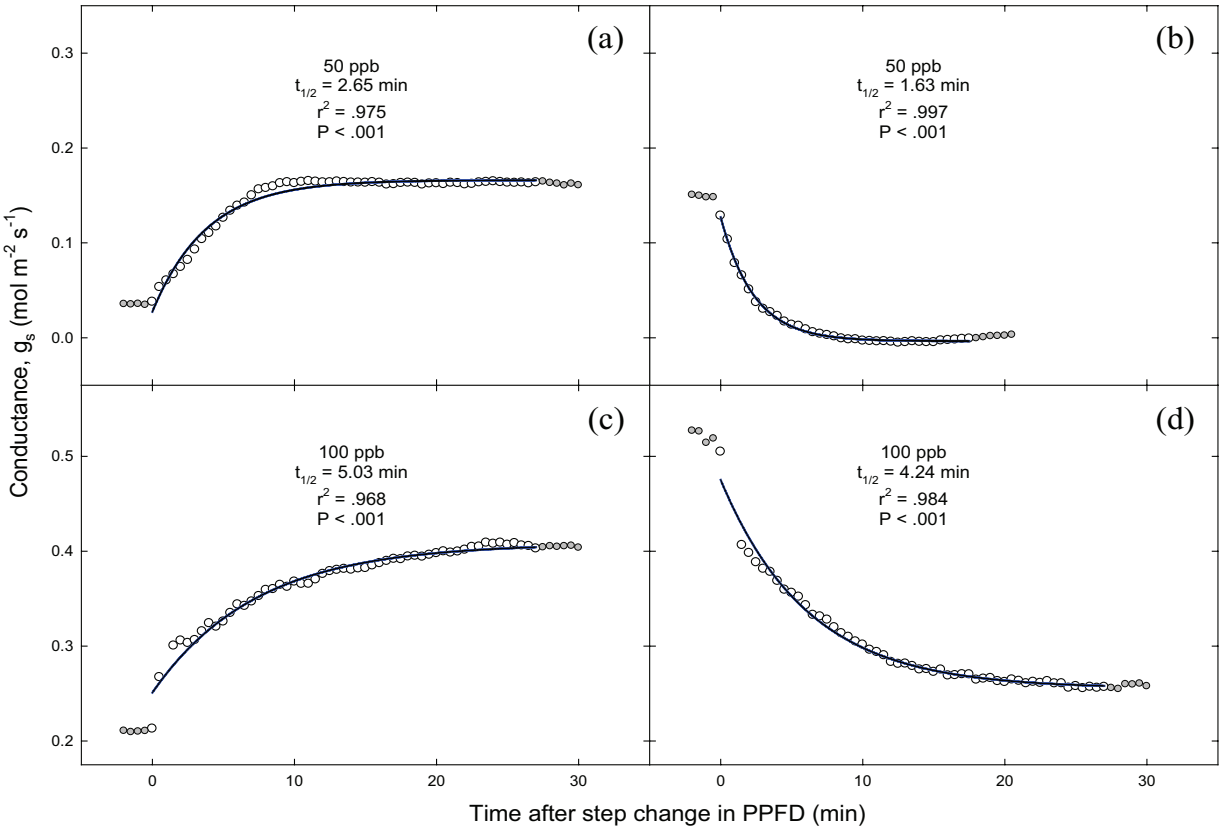

Figure 2. Representative stomatal responses to a stepped increase in PPFD (100–1,600 µmol m^{-2} s^{-1}; (a and c)), or a step decrease in PPFD (1600–100 µmol m^{-2} s^{-1}; (b and d)) in Pima cotton during acute exposure (same day exposure of naïve plants) to 50 ppb O_3 (a and b) or 100 ppb O_3 (c and d). Values of $t_{½}$ and solid lines were determined by fitting single exponential functions for opening (equation (1a)) and closing (equation (1b)). Curves were fitted to the open circles, beginning immediately after the stepped change in PPFD and ending when the new steady state was attained. Filled circles represent g_s before and after the response.

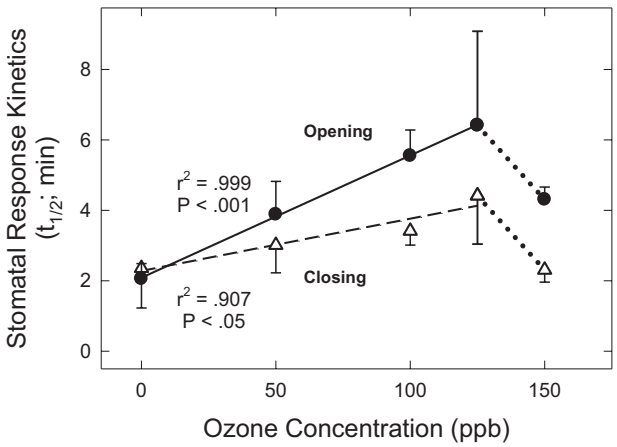

Figure 3. Effect of acute ozone (O_3) exposure in Pima cotton on the kinetics ($t_{1/2}$, half time response to PPFD stimulus or reduction) of stomatal opening (filled circles; solid line) and stomatal closing (open triangles; dashed line) (means ± 1 S.E.). Linear regressions were fitted for opening and closing separately for data between 0 and 125 ppb and was assumed to linearly decrease from 125 to 150 ppb O_3 (dotted lines).

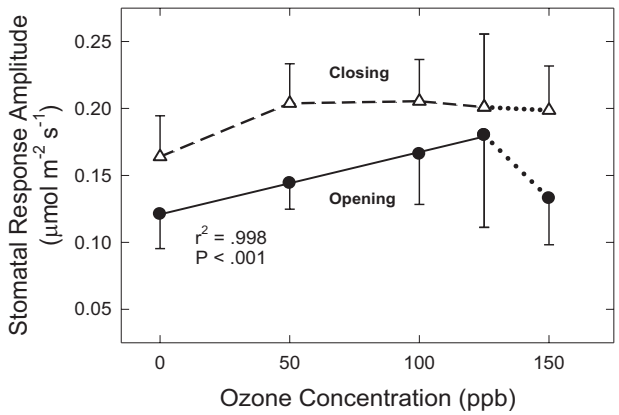

Figure 4. Effect of 1-day ozone (O_3) exposure of naïve Pima cotton on the amplitude (Δg_{st0} to g_{stf}) of stomatal opening (circles; solid line) and stomatal closing (triangles; dashed line) to a stepped change in PPFD from 100 to 1600 µmol m^{-2}s^{-1} (opening) or 1600 to 100 µmol m^{-2}s^{-1} (closing) (means ± 1 S.E.). A linear regression was fitted for stomatal opening for exposure between 0 and 125 ppb; a linear trend in stomatal amplitude was assumed between 125 and 150 ppb.

significantly from Δg_s for opening only at 50 ppb (Student's *t* test; Figure 4, open triangles). The amplitude of closing did not change significantly from 50 to 150 ppb. Greater $t_{½}$ was correlated with a greater response amplitude, both for stomatal opening ($p = .003$; Student's *t*-test) and closing ($p = .029$; Mann-Whitney *U* test).

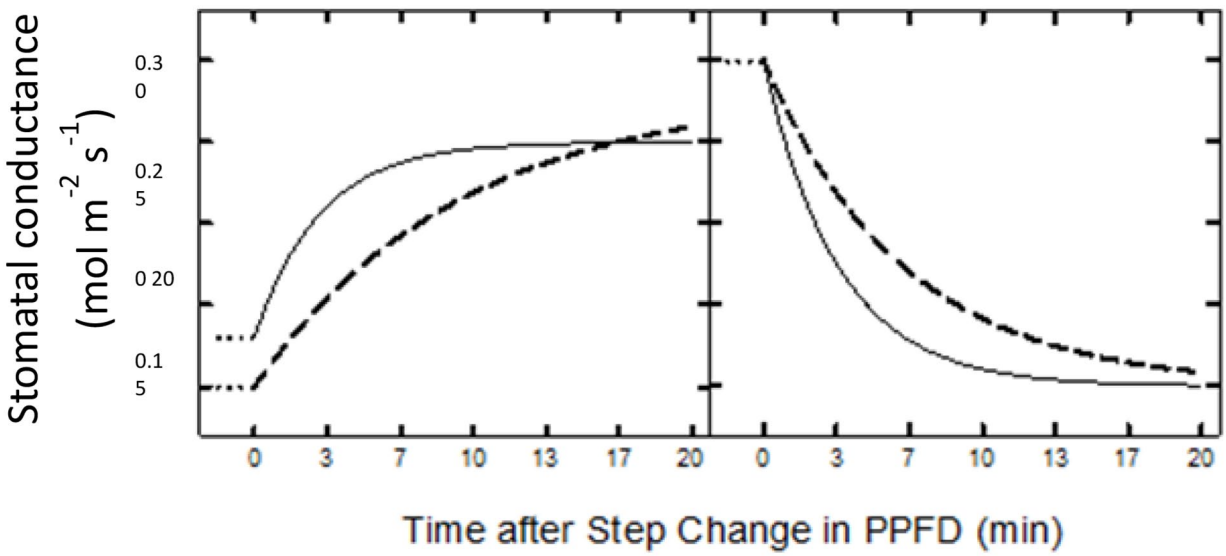

Figure 5. Mathematically fit instantaneous changes in stomatal conductance to increasing (left) and decreasing light (right) from experimentally derived mean initial, half-times ($t_{1/2}$) and final g_s, in 0 ppb (solid lines) and 125 ppb O_3 (dashed lines). Presented graphically here to 20 minutes. Transpirational water use calculated to 60 minutes presented in Table 2.

Table 2. O_3 Impact on the Half Time of Stomatal Response to Increasing or Decreasing Light, Cumulative g_s, and Resulting Effect on Integrated g_s Over Periods of 5 to 60 Minutes in Pima Cotton.

[O_3]	0 PPB				125 PPB			
Time, min	5	10	20	60	5	10	20	60
Stomatal opening								
Half time, $t_{1/2}$	2.06				6.42			
Amplitude range, Δ g_s	0.12 (0.13–0.25)				0.18 (0.10–0.28)			
Cumulative gs, Σ g_s	348	387	418	440	252	306	372	454
Σ g_s, 125–0 ppb					−96	−81	−46	14
Stomatal closing								
Half time, $t_{1/2}$	2.36				4.43			
Amplitude range, Δ g_s	0.30 (0.30–0.10)				0.30 (0.30–0.10)			
Cumulative gs, Σ g_s	372	297	242	200	432	363	290	218
Σ g_s, 125–0 ppb					60	66	48	18

Effect of sluggish stomatal response on transpiration

Mean values of initial g_s, $t_{½}$, and final g_s across O_3 exposures of 0 to 125 ppb O_3 were used to define responses of stomatal opening and closing to stepped increased and decreased light, respectively (Figure 5). The responses were integrated to calculate the effect of changes in light with no and moderately high O_3 exposure on cumulative transpiration over an hour. The cumulative values, and sign of the difference between opening and closing g_s measured in 125 versus 0 ppb, were similar to the differences in nighttime g_s in the two O_3 exposures: ~ 22 mmol $m^{-2}s^{-1}$ and 20 ± 0.4 mmol $m^{-2}s^{-1}$, respectively (Table 2). Up to 10 minutes following step change in PPFD, sluggish opening reduced cumulative g_s more than sluggish closure increased it. By 60 minutes, sluggish closure slightly increased cumulative g_s slightly more than did sluggish opening. As noted above, $t_{½}$ was correlated with the amplitude of stomatal response. Disregarding this influence underestimated the effect of O_3 on cumulative g_s and expected transpirational losses.

Discussion

Well-watered pima cotton grown with negligible O_3 exposure had a bell-shaped, diurnal g_s curve with a midday plateau,

consistent with the pattern described in the analysis of the diel sensitivity in the same variety of cotton (Grantz et al., 2015). Grown in elevated O_3 and or droughted, the maximum g_s occurred at 1030, and g_s then declined with incomplete stomatal closure by 1800. Moderately high O_3 exposure reduced g_s and expected transpirational water loss to a greater extent than did drought stress, and in combination, the two did not act synergistically. Similar effects of O_3 and WD were observed in the congeneric upland cotton, *G. hirsutum* (Temple, 1986, 1990; Temple et al., 1988). Although antagonistic (ie, protective) interactions (Silim et al., 2009; Temple, 1986, 1990; Temple et al., 1988) and synergistic (ie, deleterious) interactions (Heggestad et al., 1985; Wagg et al., 2012, 2013) have been described in this and other species (*Populus* spp.; *Glycine max* L. Merr., *Dactylis glomerata* L., *Ranunculus acris* L.).

Stomata did not completely close in any of the growth treatments tested (O_3 level × water availability) by the end of the day (1,800) or following several hours of darkness during the night (0200). Grown in LO_3, instantaneous nighttime g_s was <4% of daytime mean g_s; in HO_3, instantaneous g_s was <18% of daytime values and slightly lower with concurrent water deficit. Nighttime g_s has rarely been measured in crop species exposed to elevated O_3.

Pima cotton has much higher g_s than is observed in many forest species (Lu et al., 1994) which could alter the relative impact of daytime and nighttime stomatal responses. In trees, nighttime g_s was ~15% of daytime maximum g_s values (beech (*Fagus crenata*), Hoshika, Watanabe et al. (2013); spruce (*Picea abies*), Wieser and Havranek (1993); larch (*Larix decidua*), Wieser and Havranek (1993, 1995) birch (*Betula pendula*), Matyssek et al. (1995); ponderosa pine (*Pinus ponderosa*), Grulke et al. (2004); blue oak (*Quercus douglasii*), Grulke et al., 2007b). After daytime high O_3 exposure, nighttime g_s increased to 30% of daytime maxima in black oak (*Q. kelloggii*), Grulke et al., 2007b). Significant nighttime g_s has also been observed in the absence of O_3 (Dawson et al., 2007) with drought or in areas of low soil nutrient availability (Caird et al., 2007). Other pollutants such as NO_x (Grulke et al., 2004) may also result in nighttime transpirational losses. Similar to responses of daytime g_s, nighttime g_s also responds to VPD, CO_2, and WD (Cavender-Bares et al., 2007; Daley & Phillips, 2006; Dawson et al., 2007; Donovan et al., 2003; Zeppel et al., 2011), potentially enhancing nutrient and oxygen availability (Caird et al., 2007) but reducing hydraulic lift and water redistribution between soil horizons (Dawson, 1996).

In comparison to those grown in WW LO_3, plants exposed to high O_3 with either adequate water or a water deficit would lose less water despite the small increases due to sluggish closure and increased nocturnal g_s following O_3 exposure. Although this suggests that exposure to HO_3 would offer field water savings, both above- and below-ground biomass was significantly decreased in exposure levels comparable to the HO_3 treatment described here (Paudel et al., 2016), suggesting that O_3 toxicity is a greater determinant of growth than effects of water budgets. Decreased root biomass as reported would exacerbate the impact of soil water deficits (in cotton, Grantz et al., 2006; in ponderosa pine, Grulke & Balduman, 1999).

The lack of complete stomatal closure at the end of the day and at night suggested O_3-induced, mechanistic inhibition of stomatal kinetics (Caird et al., 2007; Paoletti & Grulke, 2010; Torsethaugen et al., 1999). The $t_{1/2}$ linearly increased from 0 to 125 ppb for both opening and closing in short term daytime O_3 exposures. At all exposures, $t_{1/2}$ times were greater for opening than closing in this controlled experiment, for example, less water was transpired with sluggish stomatal opening than with sluggish stomatal closure at the same O_3 exposure. Slower stomatal opening restricts CO_2 uptake under potentially more favorable conditions such as in the morning when VPDs may be lower, limiting transpirational losses. Slower stomatal closure potentially increases transpirational water losses under unfavorable conditions such as in the afternoon when VPDs are higher, increasing the rate of transpirational losses (Panek, 2004; Patterson & Rundel, 1989). An increase in the time for stomatal response to stimulus at high O_3 has been described as "sluggish" (Kaiser & Paoletti, 2014). The "sluggish" stomatal response with O_3 exposure reported here is consistent with previous results (Handley & Grulke, 2008; Hetherington & Woodward, 2003; Paoletti & Grulke, 2010).

High O_3 concentrations (150 ppb) decreased $t_{1/2}$, and if the change in $t_{1/2}$ from 125 to 150 ppb can be assumed to be linear, the rate of decline for both opening and closing is similar, suggesting that either antioxidant capacities were exceeded at that concentration (Wieser & Matyssek, 2007), and or that guard or subsidiary cell turgor was altered due to changes in membrane ion exchange (Dumont et al., 2013; Moldau et al., 1990). These data provide evidence of a fundamental change in stomatal regulation (or loss thereof) of a 1 day, high O_3 exposure in cotton. A similar threshold was observed in experimental exposures of deciduous oak (*Q. kelloggii*, Grulke & Paoletti, 2005), European beech (*Fagus sylvatica*, Paoletti et al., 2020), and hybrid poplar (*Populus, spp.*, Grantz, *unpubl. data*). In each case, an apparent loss of stomatal control occurs above a species-specific O_3 concentration.

Greater $t_{½}$ was correlated with a greater amplitude in stomatal response to a step change in PPFD as O_3 increased for stomatal opening, but the $t_{½}$ for stomatal closure was not related to the amplitude of response. These kinetic parameters can be sensitive to plant stress history (Assmann & Grantz, 1990; Lawson & Blatt, 2014; Pearcy & Way, 2012) and an interaction at the level of signaling has been suggested (Wilkinson & Davies, 2010), in which stress ethylene induced by O_3 antagonizes responses to abscisic acid (ABA). ABA is known to induce stomatal closure under conditions of soil or root water deficit. However, in this cultivar of Pima cotton ethylene emission was not induced by O_3 over this range of exposure (Grantz & Vu, 2012). Disregarding the amplitude of the change in g_s to environmental stimuli will underestimate transpirational losses.

The decline in g_s after a mid-morning peak to high O_3 and drought dominated the daytime integrated response so that effects of O_3 or WD on steady state stomatal responses do not provide a mechanism for increased stand water loss of Pima cotton. This contrasts with previous results in a deciduous forest (eg, McLaughlin, Nosal et al., 2007; McLaughlin, Wullschleger et al., 2007;). Our calculations were based on a well-characterized daytime course of g_s and representative nighttime g_s, both obtained under controlled conditions. Extrapolation to canopy-scale water loss or O_3 uptake requires more complete characterization of the diel course of g_s, environmental conditions, and plant species composition. Steady state conditions are not common outside of greenhouses or the laboratory, so that the impacts on steady state conductance do not reflect stomatal kinetics in variable environmental conditions (eg, Kaiser & Paoletti, 2014). Our estimates of cotton water use were estimated with "full sun" leaves which dominate gas exchange. Similarly, shade leaves of poplar also had altered stomatal kinetics but the steady state and kinetic g_s of full sun leaves dominated whole tree water balance (Paoletti et al., 2020). Response rate has been shown to be negatively related to stomatal size (Drake et al., 2013), and the stomata of Pima cotton are larger than those of many tree species.

Conclusions

The effects of O_3 exposure on fundamental processes of plant water balance were investigated in Pima cotton, an economically important species grown in the Central Valley of California, U.S.A. We report here that low to moderately elevated O_3 exposure (up to 125 ppb): (1) reduced daytime and nighttime steady state g_s responses; (2) increased sluggish stomatal opening with increasing light; (3) increased sluggish stomatal closure with decreased light; and (3) incomplete nighttime closure. As the magnitude of stomatal conductance is much larger in the daytime, and daytime stomatal closure was faster than that of opening, the net result of O_3 exposure was reduction of transpirational losses in Pima cotton up to 125 ppb. The current results suggest that neither nighttime stomatal opening nor sluggish stomatal closure are likely to increase whole plant, or agricultural field water use. Our results are consistent with those obtained with soybean under open-air O_3 exposure in field conditions, in which a consistent decrease in canopy water loss was observed with increasing O_3. However, acute O_3 exposures in the range of 125 to 150 ppb affected two aspects of stomatal kinetics: a decrease in half times for both stomatal opening and closing, and a decrease in amplitude of stomatal opening, suggesting a loss of stomatal control. If ambient or hourly spikes in O_3 concentrations were to increase, the water balance of agricultural fields would need to be further considered.

Author Note

This manuscript evaluates the effect of realistic tropospheric ozone exposure and drought stress on stomatal control of an important crop, Pima cotton, and how it may influence field water management.

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Author Contributions

Conceived and designed the experiments: DAG, NEG. Analyzed the data: DAG. Wrote the first draft of the manuscript: DAG. Contributed to the writing of the manuscript: DAG, NEG. Agree with the manuscript results and conclusions: DAG, NEG. Jointly developed the structure and arguments for the paper: DAG, NEG. Made critical revisions and approved final version: DAG, NEG. All authors reviewed and approved of the final manuscript.

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NOTE

1. Mention of commercial names are for convenience only and do not constitute promotion of product.

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