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Response of Sensitive and Resistant Snap Bean Genotypes to Nighttime Ozone Concentration

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Abstract. **Effects of nighttime (2000 to 0700 hr) O_3 on the pod mass of sensitive (S156) and resistant (R123) snap bean (*Phaseolus vulgaris*) genotypes were assessed using continuous stirred tank reactors located within a greenhouse. Two concentration-response relationship trials were designed to evaluate yield response to nighttime O_3 exposure (10 to 265 ppb) in combination with daytime exposure at background levels (44 and 62 ppb). Three replicated trials tested the impact of nighttime O_3 treatment at means of 145, 144, and 145 ppb on yields. In addition, stomatal conductance (g_S) measurements documented diurnal variations and assessed the effects of genotype and leaf age. During the concentration-response experiments, pod mass had a significant linear relationship with the nighttime O_3 concentration across genotypes. Yield losses of 15% and 50% occurred at nighttime exposure levels of ≈45 and 145 ppb, respectively, for S156, whereas R123 yields decreased by 15% at ≈150 ppb. At low nighttime O_3 levels of ≈100 ppb, R123 yields initially increased up to 116% of the treatment that received no added nighttime O_3, suggesting a potential hormesis effect for R123, but not for S156. Results from replicated trials revealed significant yield losses in both genotypes following combined day and night exposure, whereas night-only exposure caused significant decreases only for S156. The g_S rates ranged from less than 100 mmol·m^{-2}·s^{-1} in the evening to midday levels more than 1000 mmol·m^{-2}·s^{-1}. At sunrise and sunset, S156 had significantly higher g_S rates than R123, suggesting a greater potential O_3 flux into leaves. Across genotypes, younger rapidly growing leaves had higher g_S rates than mature fully expanded leaves when evaluated at four different times during the day. Although these were long-term trials, g_S measurements and observations of foliar injury development suggest that acute injury, occurring at approximately the time of sunrise, also may have contributed to yield losses. To our knowledge, these are the first results to confirm that the relative O_3 sensitivity of the S156/R123 genotypes is valid for nighttime exposure.**

Tropospheric O_3 pollution causes yield losses to sensitive plant species throughout the world. Analyses of historical data (1980–2011) produced loss estimates of 5% and 10% for soybean (*Glycine max*) and corn (*Zea mays*), respectively, in the United States (McGrath et al., 2015). Other projections have suggested that, globally, O_3 impacts on economically important crops may result in up to 15% yield losses (Ainsworth, 2017; Booker et al., 2009). Vulnerable horticultural species include grape (*Vitis vinifera*), potato (*Solanum tuberosum*), bean (*Phaseolus vulgaris*), tomato (*Solanum lycopersicum*), watermelon (*Citrullus lanatus*), and lettuce (*Lactuca sativa*) (Booker et al., 2009; U.S. Environmental Protection Agency, 2013). In addition to North America, other regions where ambient O_3 threatens vegetation due to emissions of chemical precursors and conducive climatic conditions include southern Europe, northern India, northwestern and eastern China, Korea, and Japan (Mills et al., 2018). Furthermore, successful efforts by the Chinese government to reduce particulate matter pollution that began 2013 have inadvertently increased O_3 levels by increasing the availability of precursor pollutants (i.e., particulate matter may react with precursor pollutants, thus preventing O_3 formation) (Li et al., 2018).

In most locations, ambient O_3 concentrations follow a diurnal pattern, with a peak at midafternoon, driven by incoming solar radiation, and a minimum after sunset as O_3 is continuously converted back to NO_2 and O_2 in the presence of NO (U.S. Environmental Protection Agency, 2013). However, rural areas and high elevations can experience relatively stable O_3 concentrations throughout the day (Emberson et al., 2000; Forlani et al., 2005; Musselman and Minnick, 2000; U.S. Environmental Protection Agency, 2013). Because a broad range of plant species has measurable rates of nighttime g_S (Caird et al., 2007; Dawson et al., 2007; Musselman and

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Minnick, 2000), ambient O_3 can enter the leaf and cause foliar injury outside of daylight hours. Recent research indicates that elevated O_3 during the daytime can impact gas exchange, thus causing increased rates of nighttime g_S (Hoshika et al., 2019), potentially increasing nocturnal O_3 flux into the leaf. However, a better understanding of the potential for nighttime O_3 injury is needed to establish dose-response models and direct air quality policies (U.S. Environmental Protection Agency, 2013).

Previous results have demonstrated that nighttime O_3 exposure can cause foliar injury and/or yield losses in multiple species (Goknur and Tibbitts, 2001; Günthardt-Goerg, 1996; Lee and Hogsett, 1999; Matyssek et al., 1995; Winner et al., 1989). However, Lloyd et al. (2018) reported that treatment from 2000 to 0700 HR at concentrations ≤78 ppb O_3 for 21 d had no discernible impacts on yields of O_3-sensitive snap beans, even in combination with daytime O_3. In contrast, daytime (0800–1900 HR) O_3 exposure at ≥62 ppb caused foliar injury and significant yield decreases.

Using a pair of O_3-sensitive (S156) and O_3-resistant (R123) snap beans bred by the U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS) (Flowers et al., 2007; Reinert and Eason, 2000), we attempted to determine how the genotypes respond to a wide range of nighttime O_3 levels. First, two separate trials were performed to determine the relationship between nighttime O_3 and pod mass at concentrations ranging from 10 to 265 ppb O_3 in combination with realistic levels of daytime O_3. Based on those results, our second objective was to confirm whether nighttime O_3 treatment consistently impacts snap bean yields and to determine how g_S rates vary diurnally and in response to O_3. Therefore, the effects of nighttime O_3 treatment (≈145 ppb) and genotype on pod mass and g_S were tested in three replicated trials (n = 6–8). The first trial included daytime O_3 exposure (56 ppb) and the nighttime treatment. Plants were exposed to O_3 during the nighttime only during the subsequent two trials. The "nighttime" treatment (2000–0700 HR) included the overnight period excluded by many exposure metrics, such as the 12-h W126 index, which considers cumulative O_3 concentrations from 0800 to 2000 HR (U.S. Environmental Protection Agency, 2015). Finally, because foliar injury symptoms suggested rapidly growing leaves may be more sensitive to nighttime O_3 than fully expanded leaves, the effect of leaf "age" (i.e., young vs. mature) as a proxy for developmental stage on g_S rates was evaluated.

Materials and Methods

Experiments were conducted within a greenhouse at the University Park campus of The Pennsylvania State University (lat. 40.805640°N, long. 77.852356°W). Ozone treatments were administered in 16 continuous stirred tank reactors (CSTR) (Heck et al., 1978), as described by Lloyd et al. (2018). Natural light entered the CSTRs through 76.2-µm transparent polytetrafluoroethylene film, supplemented on cloudy days by a 1000-W lamp positioned over each CSTR (Lumalux; GTE Products Corp., Danvers, MA). Charcoal filtration reduced ambient O_3 levels within the greenhouse. Ozone for the experimental treatments was generated from dried air using an electric current (Z-08; Zentox Corp., Newport News, VA), distributed to the CSTRs via polytetrafluoroethylene tubing and monitored as described by Lloyd et al. (2018). Results from calibration trials using dried air as a feed gas for the O_3 generator showed that at O_3 concentrations in the CSTRs up to 330 ppb, total N oxidant (i.e., the sum of NO, NO_2, N_2O_5, and HNO_3) concentrations did not exceed 9.2 ppb (Lloyd, 2019). Therefore, only O_3 was present at phytotoxic levels (Stripe et al., 2014; U.S. Environmental Protection Agency, 1993, 2012). Ozone concentrations (Model 49 Photometric Ozone Analyzer; Thermo Environmental Corp., Franklin, MA) as well as air temperature and relative humidity (RH) measurements (HX93BC; Omega Engineering, Stamford, CT) were recorded for each CSTR and the ambient greenhouse space using custom data acquisition software (REAL Controls, Salix, PA). Photosynthetically active radiation (*PAR*) was determined from quantum sensors (LI-190R; LI-COR, Lincoln, NE) that were positioned at canopy height in eight CSTRs. On hot, sunny days, air temperature was moderated by closing the overhead greenhouse shadecloth, thereby reducing *PAR* levels.

Plant material. Kent O. Burkey (USDA-ARS, Raleigh, NC) provided seeds of O_3-sensitive (S156) and O_3-resistant (R123) genotypes. Under ambient greenhouse conditions, three seeds were sown on the dates shown in Table 1 in 3.8-L pots (height, 20 cm; width, 18 cm), except for the night-only trial in Fall 2017, which used 2.8-L pots (height, 17 cm; width, 18 cm). When the first trifoliate leaves began to expand, plants were thinned to one per pot. The growth medium was a commercial potting mix containing 63% to 73% peatmoss, perlite, and dolomitic limestone (Sunshine Mix #4; Sun Gro Horticulture, Agawam, MA), supplemented with 15 g 15N–3.93P–9.96K fertilizer (Osmocote Plus; The Scotts Co., Marysville, OH). Plants were watered manually to pot capacity before and after O_3 treatments during the experimental period.

Concentration-response trials. Two experiments evaluated a range of nighttime O_3 levels (10–265 ppb) in combination with daytime exposure at 44 and 63 ppb for Summer 2016 and Spring 2017, respectively (Table 2). Plants were treated with O_3 for 15 d in 2016 and for 20 d in 2017 (Table 1). During the day, plants were randomly placed in one of five adjacent CSTRs and exposed to the same target O_3 concentration. One additional CSTR served as a control (ambient, no added day or night O_3). In Summer 2016, the mean daytime exposure duration was 7.8 h; it started between 0830 and 1030 HR each day. In Spring 2017, the mean duration was 7.2 h; it started between 0900 and 1145 HR each day (Table 1). In Spring 2017, before the 20-d treatment, plants were acclimated to increasing O_3 levels over the course of 3 d for 8 h·d^{-1} at mean concentrations 30, 42, and 39 ppb, consecutively, to decrease the chance of acute foliar injury.

For the nighttime treatments, plants were transferred to 11 CSTRs that had been randomly assigned and calibrated to encompass a range of O_3 levels from ambient (no added O_3) to 265 ppb (Table 2). In both trials, nighttime treatments began at 2000 HR, and O_3 concentrations were increased gradually to prevent acute injury. In Summer 2016, CSTRs reached target O_3 levels within 0.75 h, and treatments ended the next morning at 0730 HR. For all subsequent trials, nighttime treatments concluded at 0700 HR to minimize the risk of acute injury with increasing morning *PAR* levels. In Spring 2017, O_3 concentrations reached target levels within 1 to 1.25 h and ended the next morning at 0700 HR. Total treatment hours are summarized in Table 1. Mean temperature, RH, and *PAR* values across CSTRs are provided in Table 3.

Targeted reduction trials. Three replicated trials tested the effect of nighttime O_3 exposure at 145 ppb O_3; the target was to reduce yields of R123 and S156 by 15% and 50%, respectively, based on the results of the concentration-response experiments. The two nighttime O_3 treatments, a control

(ambient levels with no added O_3) and 145 ppb O_3, were applied from 2000 to 0700 HR. In 2017, experiments included a day and night trial (Fall 2017a) and a night-only trial (Fall 2017b). Spring 2018 treatments were applied at night only.

In Fall 2017a, plants received day O_3 treatments and were acclimated from 1000 to 1800 HR over the course of 2 d at 38 and 51 ppb O_3, consecutively, before starting day and night treatments. During the day, O_3-treated plants were randomly divided among four adjacent CSTRs that had been calibrated to the same target O_3 concentration. Daytime O_3 treatments were 6 h in duration (mean, 56 ppb), between 0900 and 1800 HR, over the course of 15 d (Table 1). For the night-only trials (Fall 2017b and Spring 2018), plants were located in a control CSTR (ambient, no added O_3) during the day and moved to their assigned nighttime treatment CSTR in the evenings.

Plants were exposed to nighttime O_3 for 20 d in Fall 2017 and for 27 d in Spring 2018 (Table 1), and treatment means were 145 ± 1 ppb (Table 2). Temperature, RH, and *PAR* values across CSTRs are reported in Table 3.

Foliar injury. The date of onset for foliar injury symptoms and the number of plants with symptoms, along with qualitative descriptions, were recorded for all trials. On selected dates, in coordination with g_S measurements, the percent injured leaf area was visually estimated for individual leaves.

Yield. At the conclusion of the O_3 treatment, pods were left on the plants to mature. Irrigation was stopped after the pods began to yellow. Pods were harvested when they were brown or yellow; then, they were dried at 66 °C to a constant weight. Pods with at least one fully developed seed were included in yield measurements.

Stomatal conductance. The g_S was measured in ambient conditions

Table 1. Seeding dates for snap bean with the number of days until flowering and the start and end of each O_3 treatment period for five long-term trials of yield response to O_3.

						Duration of O_3 exposure[z]			
			Time after seeding (d)			(d)		(h)	
Trial	Trial no.	Seeding date	Start O_3	Flower	End O_3	Day	Night	Day	Night
Summer 2016	1	27 June	21	28	39	15	15	117	172
Spring 2017	2	24 Mar.	22[y]	38	48	20	20	144	220
Fall 2017									
Day + night	3	28 July	22[x]	30	49	15	20	90	220
Night	4	8 Aug.	22	33	48	0	20	0	220
Spring 2018	5	27 Mar.	27	41	59	0	27	0	297

[z]Cumulative number of O_3 treatment days and hours at the target level for each trial.
[y]Plants were exposed to O_3 at 30, 42, and 39 ppb, consecutively, during the daytime for 3 d before the start of treatments.
[x]Plants were exposed to O_3 at 38 and 51 ppb, consecutively, during the daytime for 2 d before the start of treatments.

Table 2. Mean, SD, and maximum (max) concentrations for ambient (control) and O_3 treatments during day and night periods over the duration of five long-term trials, including Fall 2017a (day + night O_3) and Fall 2017b (night-only O_3), evaluating the response of snap bean to O_3 concentrations.

		O_3 concn (ppb)[z]									
		Summer 2016		Spring 2017		Fall 2017a[y]		Fall 2017b[x]		Spring 2018[x]	
Time		Amb[w]	+O_3	Amb[w]	+O_3	Amb	+O_3	Amb[w]	+O_3	Amb[w]	+O_3
Day	$\bar{X}$	9	44	11	63	—	56	6	—	10	—
	SD	—	1	—	1	—	2	1	—	2	—
	max	27	71	31	86	—	75	19	—	32	—
Night	$\bar{X}$	10	10–265[v]	8	16–260[v]	7	145	6	144	13	145
	SD	—	—	—	—	2	2	2	2	2	3
	max	24	305	23	310	23	172	21	175	40	181

[z]Ozone levels represent the mean across replications.
[y]Plants were treated with a mean of 56 ppb O_3 during the day.
[x]Plants were subjected to ambient conditions during the day.
[w]Amb = ambient (no added O_3) controls.
[v]Reflects range of 11 nighttime O_3 treatments for concentration-response trials.

Table 3. Mean, SD, minimum (min), and maximum (max) values across all treatment chambers for air temperature, relative humidity (RH), and photosynthetically active radiation (*PAR*) reported for day and night O_3 exposure periods in four long-term trials that evaluated the response of snap bean to O_3 concentrations.

		Air temp (°C)				RH (%)				*PAR* (μmol·m^{-2}·s^{-1})[z]			
Period		Sum 16	Spr 17	Fall 17[y]	Spr 18	Sum 16	Spr 17	Fall 17[y]	Spr 18	Sum 16	Spr 17	Fall 17[y]	Spr 18
Day	$\bar{X}$	29	24	27	24	62	55	55	55	376	546	460	409
	SD	0.4	0.6	0.6	0.6	1	1	2	3	57	72	74	68
	min	22	17	20	15	41	32	21	21	12	0	35	0
	max	36	33	41	34	92	79	84	91	1672	2265	1617	2096
Night	$\bar{X}$	22	19	18	18	80	62	79	67	8	1	0.5	2
	SD	0.2	0.3	0.3	0.6	2	3	2	2	1.4	0.8	0.6	0.8
	min	17	16	9	14	51	31	57	35	0	0	0	0
	max	28	23	25	25	96	90	95	92	220	93	35	122

[z]*PAR* data were available for half of the chambers (n = 8).
[y]Represents values for the Fall 2017 day and night trial. Values for the Fall 2017 night-only trial were either equivalent or within 5%.
Sum 16 = Summer 2016; Spr 17 = Spring 2017; Fall 17 = Fall 2017; Spr 18 = Spring 2018.

using a leaf porometer (SC-1; Meter Group, Pullman, WA) for the targeted reduction trials in Fall 2017 and Spring 2018. Abaxial and adaxial g_S were measured on opposite sides of the midvein of the terminal leaflet and summed to obtain the total g_S. Although contrary to the parallel resistance law (Kirkham, 2014), empirical measurements have shown that the sum of the conductances for the abaxial and adaxial leaf surfaces provides a reliable estimate of total leaf conductance (Richardson et al., 2017). Therefore, the use of summed abaxial and adaxial conductance provides a valid parameter for comparing relative conductance rates among times of day, genotypes, and leaf ages. Measurements were performed multiple times during the day, including during light, dark, and transitional (i.e., sunrise and sunset) periods, to document diurnal variations. To determine the effects of genotype and O_3, one leaf per plant was measured during different growth stages. Measurements targeted the youngest fully expanded leaf (typically the second or third leaf from the plant apex). In Spring 2018, two leaves per plant subjected to the control treatment were measured during the pod-filling state to determine the effects of genotype and relative leaf age (i.e., young vs. mature) as a proxy for the developmental stage. Rapidly growing leaves were considered "young," and "mature" leaves were fully expanded in size.

Statistical analyses. Yields reported for each nighttime O_3 concentration and genotype combination in the concentration-response trials represent the mean of two plants per CSTR in Summer 2016 and one plant per CSTR in Spring 2017. Least-squares regression was used to analyze the relationship between the pod mass and nighttime O_3 concentration. For each genotype, the null hypothesis was that the slope of the response was equal to 0. Linear regression with an indicator variable and interaction term, genotype × ozone, was used to test the null hypothesis that the slopes of the responses were equal for the two genotypes. The control (no added day or night O_3) values represent the mean of four plants per genotype from the same CSTR; they are reported separately from the results of the regression analysis (Fig. 1).

Targeted reduction trials were designed as split-plot experiments, where O_3 was the whole plot and genotype was the split plot. Two CSTRs were assigned to each block with six (Fall 2017) or eight (Spring 2018) replications. Data for pod yields and g_S from the three trials were subjected to an analysis of variance using a mixed model with restricted maximum likelihood methodology and Satterthwaite estimation for df (Satterthwaite, 1946). To test the effects of genotype and leaf age (i.e., young vs. mature) on g_S in the control treatments during Spring 2018, each plant was an experimental unit, with genotype and age as the whole and split plot factors, respectively. Statistical analyses were conducted using JMP Pro 12 software (SAS Institute, Cary, NC). Results were considered significant at $P \leq 0.05$.

Results and Discussion

Concentration-response trials. In Summer 2016 (no pre-exposure; 44 ppb daytime mean O_3), the first foliar injury symptoms appeared in S156 after the second day of treatment on plants exposed to ≥200 ppb O_3 at night. After 4 d of treatment (DOT), in combination with a mean 44 ppb O_3 during the day, both genotypes exhibited foliar injury, with more severe symptoms on S156. In Spring 2017 (65 ppb daytime mean O_3), the 3-d pretreatment from 0900 to 1700 HR with mean O_3 levels ranging from 30 to 42 ppb caused minor injury to the primary leaves of both S156 and R123, with stippling on some trifoliate leaves of S156 only. In both trials, high nighttime O_3 treatments caused stippling, bifacial necrosis, and marginal curling. Premature leaf abscission occurred with nighttime O_3 levels ≥200 ppb for S156 and ≥250 ppb for R123.

In Summer 2016 and Spring 2017, pod mass for both genotypes had a negative linear relationship with nighttime O_3 concentration (Fig. 1). In all cases, slopes were statistically significant ($P < 0.0001$ for S156; $P < 0.02$ for R123). However, considering the observed variation and lack of replication, the effect of O_3 on R123 in Summer 2016 ($P = 0.02$) may not indicate biological significance at the experimental O_3 levels. Nighttime O_3 treatment explained more variation in S156 yields ($r^2 = 0.89$ to 0.91) than in R123 yields ($r^2 = 0.47$ to 0.59) and had a greater effect on S156 pod mass, with slope coefficients 2.2- and 2.5-times larger than that of R123 in 2016 and 2017, respectively. As indicated by the interaction of genotype and O_3, the slope coefficients of S156 and R123 were significantly different from each other in both 2016 ($P = 0.0099$) and 2017 ($P = 0.0013$). To our knowledge, these are the first concentration-response trials of nighttime O_3 for snap

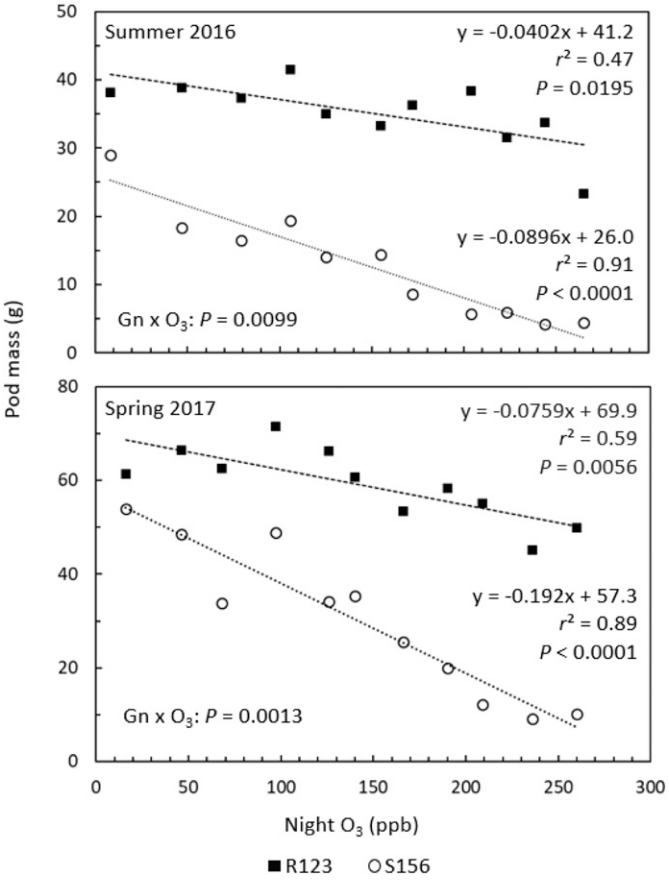

Fig. 1. Relationship between the pod mass and nighttime O_3 concentration for sensitive (S156) and resistant (R123) snap bean genotypes in Summer 2016 and Spring 2017. Values for the controls (ambient O_3, n = 1 CSTR, mean of 4 plants per genotype) are not shown and were 38.22 and 37.76 g for S156 and R123, respectively, in Summer 2016, and 68.56 and 75.39 g, respectively, in Spring 2017. The interaction of genotype (Gn) × O_3 tests the null hypothesis that the slopes of the best-fit lines for S156 and R123 are equal.

bean yield. The results establish that the relative O_3 sensitivity of the S156/R123 genotype pair is valid for nighttime exposure.

Pod mass ratios (S156:R123) for plants growing in a single control CSTR (no added day or night O_3; mean of four subsamples) were 1.01 and 0.91 for Summer 2016 and Spring 2017, respectively (Fig. 1). These ratios agree with those of other studies (Burkey et al., 2005; Flowers et al., 2007), although environmental factors, such as heat stress, can affect pod yields differentially between genotypes (Agathokleous et al., 2017). Comparatively, the Y-intercepts (Fig. 1) at no added night O_3 represent the relative effects of daytime O_3 treatment on the genotypes. Overall, pod masses were lower in 2016 than in 2017 despite greater daytime O_3 levels in Spring 2017 (63 vs. 44 ppb) (Table 2) and longer durations (20 vs. 15 d). Heat stress in Summer 2016, along with lower *PAR* levels (i.e., the overhead shadecloth was closed to reduce air temperatures) (Table 3), likely decreased yields and dampened the potential effect of nighttime O_3 treatment. Mean CSTR air temperatures were 29 and 24 °C in Summer 2016 and Spring 2017, respectively (Table 3).

These results indicate that sensitive genotypes may experience economically significant yield losses (≥5%) when exposed to nighttime O_3 in combination with realistic daytime levels. Across both trials, expected yields for S156 decreased by 5% at ≈15 ppb nighttime O_3. Losses of 15% and 50% occurred at nighttime exposure levels of ≈45 and ≈145 ppb, respectively. In contrast, R123 yields initially increased at low nighttime O_3 levels, reaching maxima at 106 and 97 ppb in Summer 2016 and Fall 2017, respectively (Fig. 1). Relative to the treatment that received no added nighttime O_3, R123 yields reached 109% in Summer 2016 and 116% in Fall 2017. These results suggest that nighttime O_3 may have stimulated the productivity of R123. Agathokleous et al. (2019a) showed that O_3 can induce nonlinear responses with an initial growth enhancement known as hormesis. The authors pointed out that, in such cases, a linear model does not accurately predict the response of vegetation to O_3. Instead, a toxicological threshold may be determined as the point where O_3 begins to inhibit growth responses. To detect hormetic (biphasic) relationships, particularly at levels in the range of 10% stimulation, experiments must be designed with narrow spacing between O_3 treatments and replication to increase statistical power (Agathokleous et al., 2019b). As O_3 concentrations increased, at ≈150 ppb, R123 yields decreased by 15%. Therefore, the response of R123 to nighttime O_3 treatment appeared to follow a threshold model (Agathokleous et al., 2019b). Notably, nighttime O_3 concentrations ≥50 ppb occur in the United States (e.g., central Pennsylvania) (Orendovici, 2005).

Targeted reduction trials. High air temperatures in Fall 2017 (Table 3) caused minor heat stress symptoms, mainly before O_3 treatment. Similar to the concentration-response experiments, foliar injury appeared after the 2-d pretreatment (means of 38 and 51 ppb O_3 for 8 $h \cdot d^{-1}$) in Fall 2017a. After 1 DOT, injury was apparent on the oldest two trifoliate leaves of all S156 plants, including the controls, indicating that daytime exposure alone (56 ppb) caused injury. R123 showed a lower occurrence of injury, with symptoms on 67% of control plants (56 ppb day; no added nighttime O_3) and on 17% of plants treated with nighttime O_3 (data not shown). Nighttime O_3 treatment (145 ppb) incited bifacial necrosis on some plants, which appeared after 2 and 10 DOT in S156 and R123, respectively. Exposure to 56 ppb O_3 during the day caused premature leaf abscission in both genotypes. After 15 DOT, injured leaf areas on the youngest fully expanded leaf of S156 ranged from 25% to 60% and 95% to 98% for control and nighttime O_3-treated plants, respectively. R123 injured leaf areas ranged from 5% to 15% for the control and 10% to 90% when exposed to nighttime O_3 (data not shown). Notably, bifacial necrosis was observed only on plants receiving nighttime O_3, indicating potential acute injury during the 2000 to 0700 HR treatment period.

Relative to combined day and night O_3 treatment in Fall 2017a, nighttime exposure alone (145 ppb) caused minor injury. In Fall 2017b, a small amount of dark stippling appeared near the base of the oldest trifoliate leaves of S156 after 2 nights of O_3 treatment. After 5 DOT, slight injury (≤5% leaf area) occurred on leaves of several S156 plants, with symptoms more frequent on younger trifoliate leaves. No conclusive symptoms were detected on R123 or control plants. After 16 DOT, symptoms on S156 increased, ranging from 2% to 50% leaf area, with no symptoms on R123 (data not shown). Ozone-induced leaf abscission occurred only in S156.

In Spring 2018, foliar injury appeared later on rapidly expanding S156 trifoliate leaves (all eight replications) after 14 DOT. Symptoms included chlorosis and dark, punctiform (i.e., dot-like) lesions on the adaxial surface affecting ≤5% of the leaf area, with two to three injured leaves per plant. Foliar injury was not present on older leaves. Minor foliar injury appeared on R123 after 18 DOT. Symptoms progressed to maroon stipple and bifacial necrosis, with the latter occurring in S156 only. At the conclusion of the O_3 treatments, younger leaves, which were rapidly growing during the treatments, showed the highest injury levels. The maximum amount of the symptomatic leaf area occurring on individual leaves was visually estimated as 55% and 15% for S156 and R123, respectively (data not shown).

Across the three trials, nighttime O_3 treatment at 145 ppb significantly reduced pod mass in S156 relative to the controls (Fig. 2). The decrease in S156 pod mass was greater in combination with daytime O_3 treatment at 56 ppb (Fall 2017a) than for night O_3 alone (Fall 2017b and Spring 2018).

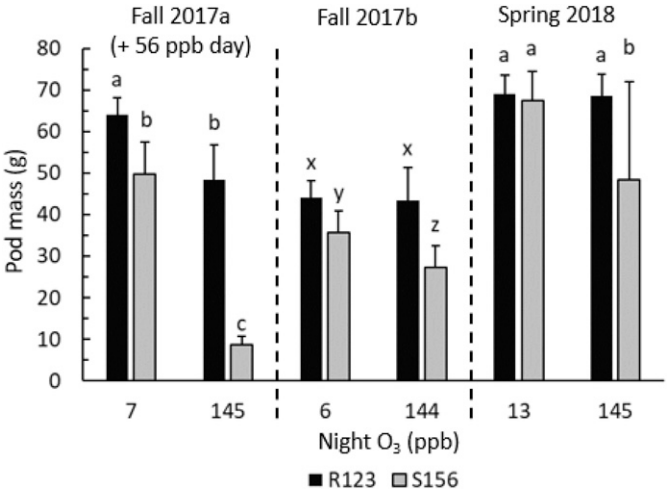

Fig. 2. Pod mass as influenced by nighttime O_3 treatment for sensitive (S156) and resistant (R123) snap bean genotypes in three replicated trials. Fall 2017a included daytime treatment at a 56 ppb O_3. Durations of nighttime O_3 treatment were 20 and 27 d in Fall 2017 and Spring 2018, respectively. The main effects, O_3 and genotype, and the interaction were significant in all trials. Within a trial, least-squares means accompanied by the same lowercase letter are not significantly different ($P \leq 0.05$) according to Tukey's honestly significant difference. Error bars represent 1 SD from the mean.

Table 4. Stomatal conductance (g_S) values for the youngest fully expanded leaf of snap bean in three trials as influenced by night O_3 treatment from 2000 to 0700 HR (ambient control vs. mean of 144 to 145 ppb of added O_3), genotype (sensitive S156 vs. resistant R123), and the interaction of O_3 and genotype (Gn), with time periods given for morning and evening measurements.

		Fall 2017: day + night O_3[z]						Fall 2017: night O_3[z]					Spring 2018: night O_3[y]				
	DAS[x]	24	25	26	27	28	22	23	30	39	44	49	35	36	44	45	45[v]
	DOT[w]	0	1	2	3	4	0	1	6	13	16	19	8	9	15	16	16
	Start[u]	11:45	12:00	19:00	7:30	11:45	20:00	5:45	13:00	12:30	6:00	20:00	13:00	18:15	7:00	22:00	5:15	12:15
	End[u]	12:45	13:30	20:00	9:00	13:00	21:00	7:00	14:00	13:45	7:00	21:00	14:00	20:00	8:00	23:30	6:45	13:00
Treatment										g_S (mmol·m^{-2}·s^{-1})								
R123	CTRL[t]	776	739	87	323	738	143	292	771	1028	167	63	275	115	213	70	208	551
	+ O_3	742	755	95	235	721	92	236	729	1046	208	67	275	112	—	106	202	505
S156	CTRL	853	832	130	391	691	140	255	736	1079	216	85	336	182	273	105	239	616
	+ O_3	706	788	146	304	720	124	227	749	1053	242	116	201	165	—	103	236	548
										P value								
Ozone		0.14	0.86	0.51	0.052	0.89	0.001	0.044	0.86	0.95	0.061	0.28	0.13	0.20	—	0.15	0.63	0.27
Genotype		0.77	0.26	0.016	0.017	0.60	0.52	0.31	0.89	0.25	0.004	0.008	0.75	0.0001	0.004	0.23	0.003	0.069
O_3 × Gn		0.43	0.58	0.81	0.99	0.62	0.42	0.51	0.62	0.36	0.51	0.23	0.12	0.36	—	0.15	0.87	0.68

[z]For the Fall 2017 trials (n = 6), df for ozone, genotype, and the interaction were 5, 10, and 10, respectively; the day O_3 treatment was 56 ppb.
[y]For Spring 2018 (n = 8), df for ozone, genotype, and the interaction were 7, 14, and 14, respectively, except as noted.
[x]DAS = days after seeding.
[w]DOT = days of treatment; in Fall 2017, the day + night trial included an additional 2 d of pretreatment at 38 and 51 ppb O_3, consecutively.
[v]For this date/time (n = 4), dfden for ozone, genotype, and the interaction were 3, 6, and 6, respectively,
[u]Measurement start and end times.
[t]CTRL = control (no added O_3).

In contrast, nighttime O_3 treatment decreased R123 yields only in combination with daytime O_3 exposure (Fall 2017a) (Fig. 2). Exposure to O_3 during the day may decrease net photosynthesis and antioxidant pools (Pell et al., 1997; Reich and Amundson, 1985), thus exacerbating the effect of nighttime O_3 treatment.

In contrast to previous results, which did not show significant yield losses for S156 or R123 at O_3 levels ≤75 ppb for periods ≤21 d (Lloyd et al., 2018), the current results revealed that nighttime O_3 exposure outside of the 0800 to 2000 HR window typically considered for air quality standards can significantly impact yields of sensitive species. Based on the regressions developed in the concentration-response trials, the 75-ppb nighttime treatment used by Lloyd et al. (2018) is predicted to cause ≈25% and ≈7% decreases in pod masses for S156 and R123, respectively. Although previous results showed a decrease in yields with nighttime treatment, limited replication (n ≤ 4) reduced statistical power to detect differences. Treatment concentrations in the present study (145 ppb) targeted a 50% decrease in S156 yields, and replication was increased (n = 6–8).

Maximum hourly O_3 observations recorded over 24-h intervals in severely polluted areas, such as China and India, exceed 145 ppb (Karthik et al., 2017; Wang et al., 2017). However, we are not aware of any locations in the United States where O_3 levels ≥145 ppb have been recorded throughout the night hours. Considering the potential for higher O_3 toxicity at night relative to the day (e.g., due to low levels of antioxidant defenses) (Musselman and Minnick, 2000), acute exposures occurring from 2000 to 0800 HR may cause injury to sensitive plants. Furthermore, analysis of ambient O_3 data from 1000 sites in the United States collected during 1990 to 2014 revealed a statistically significant trend of increasing mean nighttime (1900 to 0700 HR) O_3 concentrations, whereas peak daytime O_3 levels decreased (Yan et al., 2018). Increasing nighttime O_3 was attributed to reduced NO_x emissions and the resulting lower rates of O_3 destruction due to the reaction with NO.

Stomatal conductance. Reported values serve as a proxy for potential O_3 uptake and are not representative of absolute theoretical measurements under the parallel resistance law (Kirkham, 2014). Conductances for the abaxial and adaxial leaf surfaces were summed to obtain the total leaf conductance (Richardson et al., 2017) because O_3 may diffuse into the leaf from both surfaces (Musselman et al., 2006).

Measurements obtained in Fall 2017 and Spring 2018 during morning, midday, and night hours show a wide g_S range for S156 and R123 (Table 4), with values lowest after sunset [e.g., Fall 2017b: night only, 44 d after seeding (DAS)] and increasing before sunrise to midday maxima. Rates exceeded 1000 mmol·m^{-2}·s^{-1}, and greater g_S was associated with high *PAR* levels. The lowest midday g_S (201–336 mmol·m^{-2}·s^{-1}) occurred in the Fall 2017b night-only trial, at 49 DAS, when the shadecloth was closed due to high air temperatures. Daytime g_S rates were generally greater than measurements reported by studies with *PAR* levels less than 400 to 500 µmol·m^{-2}·s^{-1} (Hoshika et al., 2013; Li et al., 2017; Salvatori et al., 2013; Stripe et al., 2014; Wang et al., 2015). However, methodological differences may explain some variations. For example, steady-state porometer measurements exceeded values obtained using an IR gas analyzer by 2- to 3.5-fold (Toro et al., 2019). Nonetheless, plants grown under low light conditions, particularly with only supplemental lighting, may respond to O_3 differently.

Nighttime O_3 affected g_S the evening before ($P = 0.001$) and morning after ($P = 0.044$) the first O_3 treatment in the Fall 2017b (night-only) trial. If O_3 induced stomatal closure (Butler and Tibbits, 1979; Hoshika et al., 2013; Salvatori et al., 2013), then the effect would likely be observed following the nighttime O_3 exposure (to the contrary, Hucl et al., 1982). Therefore, the significance of O_3 before treatment (22 DAS, 0 DOT) was unexpected because plants for a given replication were located in the same (control) CSTR and removed in pairs immediately before measurement. Notably, rates of g_S measured on 0 DOT were small in magnitude and variable, with the SD ranging from 31 to 60 mmol·m^{-2}·s^{-1} among treatments, suggesting that the statistical difference occurred by chance. The morning after nighttime treatment (23 DAS, 1 DOT), g_S rates were higher for plants of both genotypes in the control treatments than for O_3-treated plants. Therefore, plants may have responded to O_3 treatment with stomatal closure, confirming the observations of Butler and Tibbits (1979), Hoshika et al. (2013), and Salvatori et al. (2013). The effect of O_3 was not significant on other dates, but control plants tended to have higher morning g_S at 27 DAS (3 DOT) in the Fall 2017a trial ($P = 0.052$).

Differences between the two genotypes were significant for multiple morning (Fall 2017a, 27 DAS; Fall 2017b, 39 DAS; Spring 2018, 36 and 45 DAS) and evening (Fall 2017a, 26 DAS; Fall 2017b, 44 DAS; Spring 2018, 35 DAS) measurements, with higher g_S for S156 than R123 on all dates. Salvatori et al. (2013) also reported higher g_S for S156 during the evening. However, genotype did not have a significant effect on midday measurements (Table 4).

For control plants, younger, rapidly expanding leaves had significantly higher g_S rates than mature leaves at four times of the day in Spring 2018 (Fig. 3). Mean diameters of the mature trifoliate leaves measured (n = 8) were 26 and 30 cm for S156 and R123, respectively, and for both genotypes, young leaves averaged 16 cm wide (data not shown). The interaction between genotype and age was not significant, indicating that the effect of leaf age was similar for S156 and R123. Midday rates were highest among the measurement times, with g_S decreasing in the evening, minimal at night, and beginning to increase before sunrise (Fig. 3), similar to other observations (Table 4). Between the two genotypes, S156 had significantly higher g_S than R123 at dawn (0515 to 0630 HR) and in the evening (1945 to 2100 HR) (Fig. 3). Higher g_S rates indicate a greater potential O_3 flux into the leaf, providing one explanation for the more severe injury observed on younger leaves during the nighttime exposure (Lee and Bennett, 1982) and in S156.

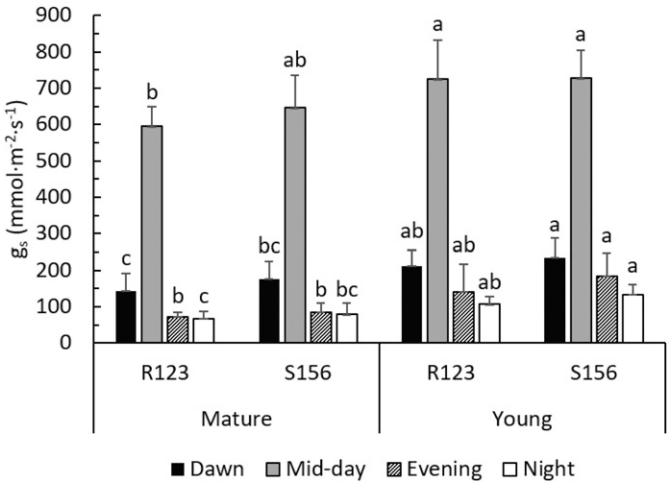

Fig. 3. The stomatal conductance (g_S) at four different times of the day (dawn = 0515–0630 HR; midday = 1130–1245 HR; evening = 1945–2100 HR; night = 2200–2300 HR) in snap bean control plants as influenced by genotype (resistant R123 vs. sensitive S156) and leaf age (mature and "fully expanded" vs. young and rapidly growing) during the pod filling stage in Spring 2018. Genotype was significant at dawn ($P = 0.003$) and evening ($P = 0.031$), and age was significant at all four times of the day ($P \leq 0.004$). The interaction was not significant. Within a measurement time, least-squares means accompanied by the same lowercase letter are not significantly different ($P \leq 0.05$) according to Tukey's honestly significant difference. Error bars represent 1 SD from the mean.

Injury observations from the concentration-response and targeted reduction trials suggest that daytime O_3 causes greater injury than nighttime exposure, as observed by others (Goknur and Tibbitts, 2001). Night O_3 treatment alone caused injury primarily on young, rapidly expanding leaves, which indicates that damage occurred, at least partly, from acute exposure to 145 ppb O_3 during the 2000 to 0700 HR treatments. Notably, O_3 levels more than 100 ppb are capable of affecting vegetation over short exposure durations (Ainsworth, 2017). Although g_S decreased to minimal levels after sunset, measurements recorded during nighttime O_3 treatment (2000 to 0700 HR) showed g_S rates reaching ≈300 mmol·m^{-2}·s^{-1} after sunrise (Table 4; see Fall 2017b, 23 and 39 DAS; Spring 2018, 45 DAS), permitting potentially injurious O_3 fluxes to enter the leaf. Because O_3 concentrations were increased gradually to target levels at the start of nighttime treatments, acute injury likely occurred in the morning, when target O_3 concentrations were maintained until 0700 HR. Although some yield loss may have resulted from high O_3 fluxes after sunrise, pod mass decreased linearly in response to increasing nighttime O_3 concentrations (Fig. 1).

Conclusions

Differences in g_S between young and mature leaves (Fig. 3) indicate that researchers may need to measure leaves of different developmental stages to accurately quantify mean O_3 flux. Similarly, the plant growth phase may impact exposure-response relationships, particularly at night. For example, two commonly cited studies of damaging nighttime O_3 impacts used rapidly growing plant material: 1-year-old container-grown *Pinus ponderosa* seedlings (Lee and Hogsett, 1999) and first-year hardwood cuttings (Matyssek et al., 1995). High growth and respiration rates likely increase O_3 flux into leaves at night, especially under favorable cultural conditions, potentially exaggerating injury levels. In addition, recent work by Grantz et al. (2018) showed that particulate pollution can enhance water loss from leaves. Therefore, in areas with elevated O_3, co-occurring particle pollution may increase O_3 flux via increased g_S.

For a given flux of O_3 entering the leaf, plant sensitivity varies diurnally as a function of detoxification capacity (Grantz, 2014; Grantz et al., 2013). Therefore, the "effective flux," which accounts for plant defense mechanisms (e.g., detoxification by antioxidants), is the most robust predictor of O_3 injury (Heath et al., 2009; Lefohn, n.d.; Musselman et al., 2006). Based on the concept of "effective flux," the development of separate models for night (dark) and day (light) periods may be necessary to develop standards that protect sensitive vegetation from injury. However, researchers must also define "nighttime" exposure to design relevant and comparable experiments and provide data that will allow policymakers to incorporate physiological (e.g., detoxification

capacity) and seasonal changes (e.g., variability in daylength) into regulatory standards.

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