Effects of Nighttime Ozone Treatment at Ambient Concentrations on Sensitive and Resistant Snap Bean Genotypes

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Additional Index Words. Phaseolus vulgaris, nocturnal stomatal conductance, R123, S156, biomonitor

Abstract. The effect of nighttime ozone (O₃) exposure, alone and in combination with daytime O₃ treatment, was tested on yield of an O₃-resistant (R123) and an O₃-sensitive (S156) snap bean (Phaseolus vulgaris L.) genotype. Three trials, with exposure durations ranging in length from 14 to 21 days, were conducted in continuous stirred tank reactors located within a greenhouse. The effects of day-only (0800–1900 HR = 11 hours day⁻¹) and day + night (0800–1900 HR + 2000–0700 HR = 22 hours day⁻¹) exposure timings were compared. The Fall 2014 trial also tested the effect of nighttime-only (2000–0700 HR = 11 hours day⁻¹) O₃ exposure. Nighttime O₃ exposure alone, at 62 ppb, did not cause foliar injury and had no effect on the yield of either genotype. In combination with daytime O₃ exposure, nighttime O₃ concentrations up to 78 ppb did not impact yields or show a consistent effect on nocturnal stomatal conductance (gₘn). When data were pooled across the day and day + night exposures times, mean daytime O₃ levels ≥62 ppb caused foliar injury and significant yield decreases in all three trials. Under control conditions, R123 and S156 produced similar pod masses in two of the three trials. In all three trials, R123 produced significantly greater yields by mass than S156 with elevated O₃. Nighttime conductance measurements suggested that S156 and R123 have inherently different gₘn rates and that cumulative O₃ exposure can increase gₘn in both genotypes.

Across the globe, tropospheric ozone harms human and environmental health (World Health Organization, 2006), while also reducing crop yields (Ashmore, 2005). In the United States, the U.S. Environmental Protection Agency (USEPA) regulates O₃ air pollution under the National Ambient Air Quality Standards (NAAQS). As a secondary air pollutant, O₃ forms through photochemical reactions involving nitrogen oxides (NOₓ) and volatile organic compounds (VOCs), which are produced by anthropogenic and natural processes. Both O₃ and its precursors migrate from urban sources to rural and remote locations, resulting in widespread impacts (USEPA, 2013).

Ambient O₃ exposure contributes to yield losses for economically important plant species, including O₃-sensitive cultivars of soybean [Glycine max (L.) Merr.], wheat (Triticum aestivum L.), potato (Solanum tuberosum L.), bean, tomato (Solanum lycopersicum L.), watermelon [Citrullus lanatus (Thunb.) Matsum and Nakai], onion (Allium cepa L.), turnip (Brassica rapa L.), and lettuce (Lactuca sativa L.) (Booker et al., 2009; USEPA, 2013). Ozone, an oxidant gas, diffuses into leaves through stomatal pores and can either dissolve in water or react directly with biochemical compounds. The resulting reactive oxygen species damage membranes and other cell components, reducing photosynthesis, accelerating aging, and negatively affecting growth and reproduction (Booker et al., 2009; USEPA, 2013). Ozone exposure can also reduce crop quality and nutritive value (USEPA, 2015).

Sensitivity to O₃ varies both within and among species. In general, plants that have higher stomatal conductance (gₘ), which allows a greater flux of O₃ into the leaf (Reich and Amundson, 1985; Temple, 1991), and lower levels of ascorbic acid, an antioxidant and key plant defense signaling molecule (Baier et al., 2005; Conklin and Barth, 2004), are more susceptible to injury. Within a species, different genotypes may vary in their molecular response to O₃ (Baier et al., 2005; Gravano et al., 2004; Kangasjärvi et al., 2005; Langebartels et al., 2002; Pell et al., 1997; Schraudner et al., 1998; Wohlgemuth et al., 2002). In addition, environmental factors, such as light level and water availability, can interact with O₃ stress and alter injury severity (Orvar et al., 1997; Wilkinson and Davies, 2010).

Booker et al. (2009) reviewed studies of O₃ impacts on agronomic and horticultural crops, reporting yield losses
ranging from ≈5% to 15% for O₃ sensitive plants. Based on a meta-analysis of 81 publications, Feng and Kobayashi (2009) estimated that mean ambient O₃ concentrations of 31–50 ppb, relative to base concentrations (<26 ppb), reduced the yield of six major crops as follows: bean (19%), rice [Oryza sativa L. (17.5%)], wheat (9.7%), barley [Hordeum vulgare L. (8.9%)], soybean (7.7%), and potato (5.3%). Notably, plants may have been exposed to actual O₃ concentrations in excess of 50 ppb following the diurnal cycle of O₃ over the 7- to 12-h averaging period. The USEPA’s Clean Air Scientific Advisory Commission suggested that O₃-induced crop yield reductions exceeding a threshold of 5% relative loss are “unacceptably high,” causing adverse impacts on public welfare (USEPA, 2015). Therefore, ambient O₃ levels pose a definite concern to food production, and standards designed to protect vegetation require continual review.

In the latest revision of the NAAQS for O₃, effective 28 Dec. 2015, the USEPA Administrator strengthened the secondary standard (i.e., protecting public welfare, including the yield and quality of agricultural crops) from 75 to 70 ppb. However, the legislation retains a “fourth-high metric” format and 8-h averaging time, identical to the primary standard used to protect public health (USEPA, 2015). In contrast to a cumulative exposure index, the fourth-high metric is based on the fourth-highest daily maximum O₃ concentration (i.e., averaged over an 8-h period within a 24-h window), averaged across three consecutive years.

The 70-ppb level of the fourth-high metric was correlated with the cumulative, concentration-weighted W126 index. The W126 takes into account all hourly O₃ concentrations within the 12-h window from 0800 to 2000 HR (Lefohn et al., 1988; Lefohn and Runkeles, 1987; USEPA, 2015). Despite evidence showing injury due to nighttime O₃ exposure (Goknur and Tibbitts, 2001; Lee and Hogsett, 1999; Matyssek et al., 1995; Winner at al., 1989), the 12-h W126 index does not include elevated O₃ episodes occurring between 2000 and 0800 HR (USEPA, 2015). Public comment on the proposed NAAQS for O₃ suggested that the W126 cumulative index, as the sole calibration for the hourly maximum fourth-high metric, should include concentrations for the entire 24-h period within 1 d (USEPA, 2015; also see A.S.L. & Associates, n.d.). Therefore, the 12-h W126, when calculated from 0800 to 2000 HR, may not adequately describe exposure–response relationships to protect sensitive vegetation where nighttime O₃ exposure is a factor (Musselman et al., 2006).

Nocturnal stomatal conductance has been reported for a wide range of species (Caird et al., 2007; Dawson et al., 2007; Musselman and Minnick, 2000), demonstrating the potential for O₃ uptake from ambient air at night. Furthermore, researchers observed injury resulting from nighttime or dark period O₃ exposure, using both indoor (Goknur and Tibbitts, 2001) and outdoor chambers (Günthardt-Goerg, 1996; Lee and Hogsett, 1999; Matyssek et al., 1995). However, no clear nighttime exposure–response relationships have been developed for O₃ to date, and evidence documenting the cumulative effects of nighttime O₃ exposure remains limited (USEPA, 2013).

Nighttime O₃ exposure occurs when O₃ concentrations remain elevated in the surface layer of the atmosphere because of limited destruction via dry deposition and NO scavenging. Based on available monitoring data, the USEPA (2013) identified mountainous areas of southern California, the front-range of Colorado, and the Great Smoky Mountains as potential locations of concern for elevated nocturnal O₃ levels. However, nighttime O₃ exposure may cause injury in other locations. Typically, O₃ is produced photochemically during daylight hours, reaching a peak in midafternoon and decreasing rapidly, often to near 0, after sunset. Rural areas downwind of urban pollution sources can experience a delay in this cycle, leading to more uniform daily O₃ concentrations. This attenuation of the typical diurnal pattern results from additional time required to transport O₃ and precursors from urban sources, along with continued O₃ production during transport (USEPA, 2013).

In the residual layer of the atmosphere, which forms above a stable surface layer when vertical mixing is limited (Stull, 1988), O₃ concentrations can also remain relatively constant throughout the day and night (Emberson et al., 2000; Forlani et al., 2005; Musselman and Minnick, 2000; Orendovic-Best et al., 2010; USEPA, 2013; Winner et al., 1989). Therefore, vegetation growing at higher elevations may be exposed to greater nighttime O₃ levels. For example, at a mountainous site in rural central Pennsylvania, the mean O₃ concentration from 9 June to 9 Sept. 2003 was 45 ppb (Orendovic, 2005, see Table 9). However, nighttime O₃ levels often exceeded daytime concentrations, with maximum values around 100 ppb (Orendovic, 2005, see Fig. 9). Given the potential for nighttime O₃ exposure, particularly in rural areas and high-elevation sites (Emberson et al., 2000; Forlani et al., 2005; Musselman and Minnick, 2000; USEPA, 2013), it is important to consider effects on plants.

To determine if nighttime O₃ exposure causes injury and influences yield of O₃-resistant (R123) and O₃-sensitive (S156) snap bean genotypes (Burkey et al., 2005, 2012; Flowers et al., 2007; Reinert and Eason, 2000), the effects of day-only (0800–1900 HR = 11 h·d⁻¹) and day + night (0800–1900 HR + 2000–0700 HR = 22 h·d⁻¹) exposure times at control (3–15 ppb), low (25–49 ppb), and high concentrations (up to 78 ppb) were compared. Ozone treatments represented realistic nighttime levels based on observations from central Pennsylvania (Orendovic, 2005). The S156 and R123 genotypes were developed by the USDA-ARS Plant Science Research Unit in Raleigh, NC (Flowers et al., 2007; Reinert and Eason, 2000), and have been used to study flux-effect relationships by the International Cooperative Program on Effects of Air Pollution on Natural Vegetation and Crops (ICP Vegetation, 2012).

**Materials and Methods**

The experiments were conducted within a greenhouse on the University Park campus of The Pennsylvania State University (lat. 40.805640°N, long. 77.852356°W). Ozone treatments were administered in continuous stirred tank reactors (CSTR [Heck et al., 1978]). The cylindrical CSTR chambers measured 1.5 m diameter by 1.5 m height and were enclosed with 72.6-μm transparent polytetrafluoroethylene (Teflon; Chemours, Wilmington, DE) film. Each chamber was equipped with an overhead 1000-W lamp having a spectral distribution of 350–700 nm, with peaks at 550 and 650 nm (Lumalux; GTE Products Corp., Sylvania Lighting Center, Danvers, MA). Outside air was passed through activated charcoal filters to reduce O₃ levels within the greenhouse. Mechanical blowers pulled the filtered greenhouse air into the CSTR chambers, with the full chamber volume exchanging about once per minute and exhaust released to the outside. An O₃ generator (Z-08; Ozone...
Solutions, Hull, IA) was used to produce O$_3$ from dry air via electric current. Ozone was distributed to the CSTRs via manually adjusted valves and polytetrafluoroethylene tubing. Ozone levels in each chamber were monitored at 10-min intervals and adjusted using computerized feedback to the O$_3$ generator. Air samples were collected at a rate of $\approx1$ L min$^{-1}$ using a network of computer-controlled solenoid valves and measured with two photometric ozone analyzers (model 49; Thermo Environmental Corp., Franklin, MA). Ozone measurements were recorded via custom data acquisition software (REAL Controls, Salix, PA). The software also tracked air temperature and relative humidity (RH) data from sensors (HX93BC; Omega Engineering, Stamford, CT) installed in each chamber. Photosynthetically active radiation ($\text{PAR}$) (model 49; Sun Gro Horticulture, Agawam, MA) was used to measure abaxial g$_{\text{sn}}$. Leaflet of a mature trifoliate leaf (i.e., leaf diameter ranging from 18 to 30 cm) was used for measurements. Leaves with visible injury symptoms exceeding 20% of the leaf area were excluded from the analysis. At the time of g$_{\text{sn}}$ measurements, mature leaves of S156 were consistently smaller than R123 in the high O$_3$ treatments. Conductance was measured following daytime O$_3$ treatment on 30 Apr. and 7 May (33 and 40 DAS, respectively) in Spring 2015 and on 14 and 27 Oct. (40 and 53 DAS, respectively) during Fall 2015. Data reported for 14 Oct. 2015 represent the mean of two plants per replication. On the other three dates, one plant per replication was measured.

**YIELD.** Before harvesting, pods remained on the plants to dry. In a few cases, pods on S156 plants remained yellowish-green, whereas most of the R123 pods were brown. Any pod with one or more fully developed seeds was considered mature (sensu Burkey et al., 2005).

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**Table 1. Seeding dates for snap bean with the number of days until flowering, harvest, and the start and end of O$_3$ treatment for Fall 2014, Spring 2015, and Fall 2015 trials.**

| Trial      | Seeding date | Start O$_3$ | Flower | End O$_3$ | Harvest | Total O$_3$ exposure (d)$^a$ |
|------------|--------------|-------------|--------|-----------|---------|-----------------------------|
| Fall 2014  | 16 Sept.     | 15          | 29     | 38        | 92      | 18                          |
| Spring 2015| 28 Mar.      | 26          | 33     | 45        | 102     | 14                          |
| Fall 2015  | 4 Sept.      | 28          | 34     | 53        | 79      | 21                          |

$^a$Cumulative number of O$_3$ treatment days; exposure times were $\approx11$ h d$^{-1}$ for the individual day and night treatments and 22 h d$^{-1}$ for the day + night treatments.
For each plant, the number of mature pods was recorded. In Fall 2014, each pod was opened and threshed by hand on 17 Dec., and the number of seeds per pod was counted. Seeds were dried at 21 °C for 24 d before weighing. In Spring and Fall 2015, pods harvested on 8 July and 23 Nov. were oven-dried at 66 °C for 48 h and weighed.

**Statistical analysis.** The experimental design was a randomized complete block with four chambers per block and either two (Fall 2014) or four replications (Spring and Fall 2015). Chambers were the experimental units. The treatments followed an augmented factorial structure (Lentner and Bishop, 1993), with a factorial combination of two genotypes (i.e., S156, R123) and four day/night O3 combinations (i.e., low/ambient, high/ambient, low/low, and high/high), along with a control (ambient/ambient). The Fall 2014 trial included two additional night-only O3 treatments (i.e., ambient/low and ambient/high).

Data from the three trials were analyzed separately. Response variables recorded in Fall 2014 were pod number, seed number, seed mass, seeds per pod, and mean seed mass (grams per seed). Pod number, pod mass, and $g_s$ were recorded for Spring and Fall 2015. Values for multiple plants within an experimental unit were averaged before analysis. Block was considered a random effect. All data were subjected to analysis of variance in JMP Pro 12.1.0 (SAS Institute, Cary, NC) using the mixed model platform with restricted maximum likelihood methodology (REML) and Satterthwaite estimation for df. Single degree-of-freedom contrasts were used to test the significance of the main effects genotype, O3 level, and time (i.e., day vs. day + night), as well selected interactions (Marini, 2003). Differences were considered significant at $P = 0.05$.

**Results and Discussion**

**Ozone treatments.** Table 2 provides a summary of mean O3 concentrations, separated into day and night exposure periods, for each of the three trials. Mean O3 levels in the control and spare chambers (greenhouse ambient + 0 ppb added O3) ranged from 3 to 15 ppb over the duration of the three experiments. In Fall 2014, target O3 concentrations for the low and high chambers were 30 and 60 ppb, respectively, and were lower in the added O3 treatment than ambient air (Bytnerowicz et al., 1995). Although pure O3 is the ideal source gas for high-voltage O3 generation, cost and maintenance issues are prohibitive for long-term studies (Mortensen and Jørgensen, 1996; Olszyk et al., 1990). Given the low O3 concentrations in this study, it is unlikely that incidental production of other trace oxidants impacted results.

**Greenhouse environment.** Mean daytime air temperatures in the CSTRs ranged from 24 to 25 °C during the three trials, with nighttime means in the range of 20 to 22 °C (Table 3). Maximum daytime air temperatures did not exceed 37 °C, and a minimum temperature of 12 °C occurred once in Fall 2015. Mean RH in the CSTRs ranged from 49% to 57% during the day and 46% to 56% at night for the three trials (Table 3). RH values near 50% represent realistic conditions for O3 exposure, given that O3 production is generally greatest during hot, dry weather conditions associated with stagnant high pressure systems (Ryan et al., 2000; USEPA, 2013).

**Phenology and onset of injury.** In Fall 2014, O3 treatments began at 15 DAS, before full expansion of the second trifoliate leaf (19 DAS for all plants). Plants bloomed by 29 DAS and began pod fill at 35 DAS. At 37 DAS, the second to last day of O3 treatment, the largest pod was ≈5 cm in length. Plants grew more slowly in Spring 2015, with the second trifoliate leaf expanded at 24 DAS. Ozone treatments also began later, at 26 DAS, and all plants flowered by 33 DAS. The largest pods reached ≈5 cm at 37 DAS. In Fall 2015, plants had at least three to four expanded leaves when O3 exposures began at 28 DAS. All plants flowered by 34 DAS, and pods were 12–15 cm in length at 44 DAS.

No injury was observed for the night-only O3 treatments in Fall 2014. By contrast, daytime O3 exposure produced stippling, bleaching, and mesophyll collapse on foliage of both genotypes, with curling on the leaf margins of S156 exposed to high O3 levels. In Fall 2014, foliar injury symptoms appeared 5 DAT in the high O3 treatments. Injury progressed in both genotypes with cumulative exposure to high O3, but no symptoms were apparent at low O3 (mean = 25 ppb). At the conclusion of the trial, S156 plants exposed to 62 ppb mean O3 during the day appeared stunted, with smaller leaves relative to R123 plants, and developed greater foliar injury. Injury symptoms appeared on plants treated with high O3 (mean = 74 ppb) on 2 DAT in Spring 2015. At 3 DAT, stippling was also evident on plants in the 45-ppb treatments. By 17 DAT, early leaf abscission occurred in the 75-ppb treatments. In Fall 2015, O3 injury appeared in the high and low O3.

| Time  | Fall 2014 | Spring 2015 | Fall 2015 |
|-------|-----------|-------------|-----------|
| Day   |           |             |           |
|       | Amb$^a$   | Low         | High      | Amb$^a$   | Low         | High      |
|       | 3         | 25          | 62        | 9         | 44          | 74        |
|       | sd        | 2           | 4         | 8         | 3           | 5         | 11        |
|       | Max       | 14          | 38        | 91        | 22          | 61        | 426       |
| Night |           |             |           |           |             |           |           |
|       | Amb$^a$   | Low         | High      | Amb$^a$   | Low         | High      |
|       | 3         | 27          | 62        | 8         | 46          | 75        |
|       | sd        | 3           | 4         | 9         | 4           | 4         | 6         |
|       | Max       | 12          | 41        | 91        | 20          | 61        | 94        |

$^a$Ozone levels represent the mean across chambers for each treatment.

$^b$Amb = ambient (no added O3) includes means for the control and spare chambers.
treatments 1 and 5 DAT, respectively. At 16 DAT, premature leaf abscission occurred on plants of both genotypes exposed to 75 ppb O₃. Across the three trials, mean daytime O₃ exposures greater than 45 ppb caused foliar injury to both genotypes. However, only the high O₃ treatment (mean daytime O₃ ≥ 60 ppb) caused early leaf abscission.

FALL 2014: YIELD. Given the absence of visual injury in the Fall 2014 night-only (2000–0700 HR) O₃ exposures, it was not surprising that night O₃ treatment (high O₃ mean = 62 ppb) did not significantly affect any yield parameter (Table 4). Among the control and night-only O₃ treatments, seed mass for R123 and S156 varied by only 1.6% and 5.7%, respectively. Other studies have also failed to demonstrate significant injury from night-only O₃ exposure. Günthardt-Goerg (1996) found that night-only O₃ treatment at 75 ppb did not produce foliar injury on a sensitive tobacco (Nicotiana tabacum L.) cultivar (BelW3). For turnip plants grown in open-top chambers, there was no significant difference in total plant biomass for plants exposed to ambient O₃ during either the day or night in three separate trials (Winner et al., 1989, see Table 1 therein). Using Plexiglas chambers, Goknur and Tibbitts (2001) found that a dark period O₃ concentration of 300 ppb...
O$_3$ was required to induce similar foliar injury to potato as exposure to 100 ppb in the light, over 3-h time periods (see Fig. 5 therein).

Under control conditions, S156 produced significantly more mature pods than R123 in Fall 2014 ($P = 0.031$ (Table 4)). However, R123 had significantly more seeds per pod than S156 ($P < 0.001$), with means of 4.55 and 3.52, respectively. Therefore, although S156 produced more mature pods than R123, R123 had a greater number of seeds in each pod, leading to similar yields by mass (S156:R123 = 1.03). Total seed number and mean seed mass (grams per seed) were not significantly different for the two genotypes. Similarly, Burkey et al. (2005) observed greater pod numbers with fewer seeds per pod in S156 than R123, leading to similar yields by mass, when plants were grown in open-top chambers with charcoal-filtered air. Therefore, mass-based yield can be compared more directly than pod number for the two genotypes.

High O$_3$ treatment during the day (mean = 62 ppb) significantly reduced all yield parameters ($P < 0.001$), except mean seed mass, relative to both the control and low O$_3$ (mean = 25 ppb) treatments (Table 4). However, yields under low O$_3$ were not significantly different from the control. Flowers et al. (2007) also found that both R123 and S156 tolerated O$_3$ concentrations less than 30 ppb. Notably, the interaction of genotype and O$_3$ (low vs. high) was significant for all yield parameters except mean seed mass.

Pooled across the day and day + night timings, the increase in mean daytime O$_3$ from 25 to 62 ppb decreased total seed mass by 39% for S156 but only 9% for R123. Similarly, total seed number declined by 63% (S156) and 13% (R123), with decreases of 22% (S156) and 4.5% (R123) in the number of seeds per pod (Table 4). Therefore, exposure to 62 ppb O$_3$ had a greater impact on both pod initiation and maturation in S156 than R123. Mass yield ratios (S156:R123) for the low and high O$_3$ treatments were 1.03 and 0.47, respectively, compared with 1.03 for the control.

**Spring and Fall 2015: Yield.** In contrast to Fall 2014 (Table 4) and Spring 2015, R123 produced significantly greater pod mass than S156 under control conditions in Fall 2015 ($P < 0.001$ (Table 5)). In Fall 2015, plants were relatively larger when transferred into the CSTRs, with three to four expanded trifoliate leaves. In addition, higher PAR levels and lower air temperature likely enhanced growth in Fall 2015 compared with Spring 2015 (Table 3). Ratios of S156:R123 pod mass in the controls for Spring and Fall 2015 were 0.91 and 0.77, respectively. For plants grown outdoors in charcoal-filtered air, over two subsequent years (seasonal mean O$_3$ = 25–31 ppb), the S156:R123 yield ratio was about 1.0 (Burkey et al., 2005). Similarly, Flowers et al. (2007) noted a seed yield ratio of ~1 for S156 and R123 plants grown in environmentally controlled field chambers (mean O$_3$ = 0 ppb). However, plants grown in the same facility, exposed to 0 ppb O$_3$ in combination with low and high vapor pressure deficit treatments, had seed mass ratios of about 0.72 and 0.70, respectively (Fiscus et al., 2012).

Agathokleous et al. (2017) showed that small increases in air temperature, particularly in the range of 31 to 34 °C, can substantially decrease pod number and affect the mass of pods and seeds for S156 and R123. Therefore, S156:R123 yield ratios may vary with environmental factors (e.g., temperature, RH, PAR, nutrient availability) and cultural practices (e.g., container volume, media composition) and should be interpreted with caution (Burkey et al., 2012).

Data for the day and day + night treatments were pooled to compare the effects of the low and high O$_3$ treatments relative to the control and each other. Across genotypes, the low O$_3$ treatments (mean = 44 ppb) significantly decreased pod number ($P < 0.001$) and mass ($P < 0.001$) in Fall 2015 only (Table 5). In Fall 2015, plants were 2-d older at the beginning of treatments, and the exposures lasted 7-d longer than in Spring (Table 1). High O$_3$ (mean = 74 and 75 ppb for Spring 2015 and Fall 2015, respectively) significantly reduced pod number and mass.

**Table 5.** Yield parameters of O$_3$ resistant (R123) and O$_3$ sensitive (S156) snap bean genotypes and single degree-of-freedom contrasts comparing the effects of day (0800–1900 HR) and day + night (0800–1900 HR + 2000–0700 HR) O$_3$ treatments in Spring 2015 and Fall 2015 ($n = 4$).

| Treatment | Genotype | Ozone$^a$ | Pod no. | Pod mass (g) |
|-----------|----------|----------|---------|-------------|
|           |          | Day      | Night   | Spring      | Fall     |
| 1 (Control) | R123 | AMB      | AMB     | 22.5        | 24.9     | 31.47 | 37.16 |
| 2         | R123    | LOW      | AMB     | 21.4        | 20.9     | 28.01 | 31.14 |
| 3         | R123    | LOW      | LOW     | 22.3        | 21.0     | 30.03 | 32.13 |
| 4         | R123    | HIGH     | AMB     | 19.3        | 16.9     | 27.26 | 21.23 |
| 5         | R123    | HIGH     | HIGH    | 18.6        | 17.1     | 24.55 | 21.44 |
| 6 (Control) | S156 | AMB      | AMB     | 28.1        | 26.8     | 28.62 | 28.73 |
| 7         | S156    | LOW      | AMB     | 26.6        | 22.5     | 29.28 | 23.54 |
| 8         | S156    | LOW      | LOW     | 24.9        | 21.0     | 27.01 | 22.95 |
| 9         | S156    | HIGH     | HIGH    | 18.9        | 14.5     | 19.51 | 13.45 |
| 10        | S156    | HIGH     | HIGH    | 18.9        | 14.8     | 18.19 | 11.75 |

Contrast ($P > F$$^y$)

| Control: Gn (1 vs. 6) | 0.044 | 0.197 | 0.265 | 0.001 |
| O$_3$ treatments$^x$ | 0.035 | 0.033 | 0.020 | 0.833 |
| Gn (2 + 3 + 4 + 5 vs. 7 + 8 + 9 + 10) | 0.004 | 0.280 | 0.004 | 0.001 |
| C vs. L (1 + 6 vs. 2 + 3 + 7 + 8) | 0.177 | 0.001 | 0.347 | 0.001 |
| C vs. H (1 + 6 vs. 4 + 5 + 9 + 10) | 0.000 | 0.001 | 0.001 | 0.001 |
| L vs. H (2 + 3 + 7 + 8 vs. 4 + 5 + 9 + 10) | 0.001 | 0.001 | 0.001 | 0.001 |
| Time (2 + 4 + 7 + 9 vs. 3 + 5 + 8 + 10) | 0.681 | 0.760 | 0.400 | 0.740 |
| Interactions | 0.035 | 0.033 | 0.020 | 0.833 |
| O$_3$ x Gn (4 + 5 + 7 + 8 vs. 2 + 3 + 9 + 10) | 0.312 | 0.425 | 0.237 | 0.500 |
| Time x Gn: L (2 + 8 vs. 3 + 7) | 0.312 | 0.425 | 0.237 | 0.500 |
| Time x Gn: H (4 + 10 vs. 5 + 9) | 0.808 | 1.000 | 0.696 | 0.416 |

$^a$Mean O$_3$ levels were AMB (no added O$_3$) = 9 ppb day, 8 (spring) and 15 (fall) ppb night; LOW = 44 ppb day, 46 (spring) and 49 (fall) ppb night; HIGH = 74 (spring) and 75 (fall) ppb day, 75 (spring) and 78 (fall) ppb night.

$^b$Each contrast compares individual treatments or combinations of treatments as noted by treatment numbers. C = control; L = low O$_3$; H = high O$_3$; Gn = genotype; Time = day vs. day + night.

$^c$Low and high O$_3$ levels are pooled across day and day + night treatment times.

$^d$Compared response of genotypes to low and high O$_3$, pooled across day and day + night treatment times.
relative to both the control and low O3 treatments [all \( P < 0.001 \) (Table 5)]. However, the interaction of genotype and O3 (low vs. high) had a significant effect on pod number in Spring (\( P = 0.035 \)) and Fall (\( P = 0.033 \)). The decreases in pod number at high O3 during Spring and Fall were greater for S156 (27% and 33%, respectively) than R123 (13% and 11%, respectively). For pod mass, the interaction was significant in Spring (\( P = 0.020 \)) but not Fall 2015. In Spring 2015, R123 pod mass was 11% lower under high O3 than low O3, whereas S156 pod mass was 33% less. By contrast, R123 pod mass decreased by 33% in Fall 2015, with a 46% reduction for S156. Therefore, the high O3 treatment had greater effects on yield in Fall 2015, when exposures were 7-d longer in duration (Table 1). Flowers et al. (2007) also observed a significant decrease in the seed yield, by mass, of both genotypes at 60 ppb O3. Compared with the control, they reported 19% and 77% reductions for R123 and S156, respectively. In contrast to the present study, O3 treatments began 1 week after planting, starting at one-third of the target O3 level and increasing in two steps during the second week of treatment. Their approach provided more time for the plants to acclimate to O3 stress, as well as the opportunity to respond at a younger age than in the present study, which appeared to minimize R123 yield losses relative to S156. Ficus et al. (2012), using environmentally controlled field chambers, noted \( \approx 55\% \) and 72% reductions in seed yield for R123 and S156, respectively, at low vapor-pressure deficit (1.26 kPa) and 60 ppb O3. Using open-top field chambers, Heagle et al. (2002) observed a striking decline of 90% pod mass for S156 when exposed to 73 ppb relative to 23 ppb, but the study did not include R123.

In contrast to the present results, mature pod (>4 cm with seeds) mass decreases of only 28% and 30% for R123 and S156, respectively, were reported for plants exposed to a mean of 78 ppb O3 for 20 d in enclosed chambers (Salvatori et al., 2013). The relatively minor impact of O3 on yield was accompanied by overall low yields. For example, the researchers reported 13.8 and 19.2 pods per plant for R123 and S156, respectively, under control conditions. In comparison, the number of pods per plant for R123 and S156 in the present study ranged from 12.9 to 24.9 and 19.2 pods per plant for R123 and S156, respectively, under low vapor-pressure deficit (1.26 kPa) and 60 ppb O3. Using open-top field chambers, Heagle et al. (2002) observed a striking decline of 90% pod mass for S156 when exposed to 73 ppb relative to 23 ppb, but the study did not include R123.

Daytime O3 treatment (mean = 44–75 ppb) resulted in significantly lower pod mass for S156 relative to R123 in both Spring and Fall 2015 (Table 5). Pooled across treatment times, pod mass yield ratios of S156:R123 at low O3 were 0.97 and 0.73 in Spring and Fall 2015, respectively. At high O3, these ratios declined to 0.73 (Spring) and 0.59 (Fall). Similar to Fall 2014, the timing of O3 exposure (i.e., day vs. day + night) did not significantly affect yield responses in Spring or Fall 2015 (Table 5). For tobacco, there was no difference in the onset of foliar symptoms between day-only and continuous (i.e., day + night) O3 exposures, but biomass was not reported (Günthardt-Goerg, 1996). Winner et al. (1989) obtained inconsistent results for turnip grown in open-top chambers in which charcoal filters were used to reduce ambient O3 from between 50 and 100 ppb to \( \leq 25 \) ppb. Plants exposed continuously to ambient O3 had significantly lower biomass than plants exposed during the day only (i.e., charcoal-filtered air at night) in one of three trials, whereas treatments were not significantly different in the other two trials. Therefore, more evidence is required to support the hypothesis that nighttime O3 exposure, at realistic concentrations, exacerbates daytime O3 injury.

**Nocturnal stomatal conductance.** Nighttime conductance measurements in Spring and Fall 2015 showed significant differences between genotypes in the control (ambient + 0 ppb) on two of four observation dates (Table 6). On each date, S156 maintained higher \( g_{stn} \) ranging from 73.8 to 94.1 mmol m\(^{-2}\) s\(^{-1}\), relative to R123, with \( g_{stn} \) in the range of 48.0 to 61.9 mmol m\(^{-2}\) s\(^{-1}\). These results support the observation that S156 has higher \( g_{stn} \) relative to R123 (Salvatori et al., 2013), potentially leading to higher nighttime O3 fluxes per unit leaf area. However, compared with the present study, Salvatori et al. (2013) reported lower \( g_{stn} \) values for both genotypes under control conditions (mean O3 = 4 ppb) when measured from 39 to 42 DAS, with means of 28 and 12 mmol m\(^{-2}\) s\(^{-1}\) for S156 and R123, respectively. In the current experiment, plants were watered 2–3 h before measurement, which likely resulted in maximal \( g_{stn} \). In addition, daytime \( PAR \) values were higher, with means of 507–537 mmol m\(^{-2}\) s\(^{-1}\) in (Table 3), compared with the artificial lighting used by Salvatori et al. (2013), which supplied a constant 350 mmol m\(^{-2}\) s\(^{-1}\). Greater light availability in the CSTRs likely supported higher rates of photosynthesis and transpiration, as well as increased \( g_{stn} \), relative to enclosed growth chambers (Easlon and Richards, 2009).

At elevated O3, \( g_{stn} \) varied significantly between genotypes on all four dates, with higher \( g_{stn} \) for S156 in each O3 treatment (Table 6). Rates of \( g_{stn} \) for S156 ranged from 66.0 to 185.5 mmol m\(^{-2}\) s\(^{-1}\), and values for R123 were between 33.8 and 69.7 mmol m\(^{-2}\) s\(^{-1}\). Salvatori et al. (2013) reported \( g_{stn} \) for O3-treated S156 and R123 plants of \( \approx 88 \) and 44 mmol m\(^{-2}\) s\(^{-1}\), respectively, in agreement with the present study.

Across genotypes and exposure times, O3 had a significant effect on \( g_{stn} \) only on 27 Oct. (Table 6). There was no difference between the control and low O3 treatment, but \( g_{stn} \) rates in the high O3 treatment were significantly greater than values from the control (\( P = 0.009 \)) and low O3 treatment (\( P < 0.001 \)). The interaction of O3 (low vs. high) and genotype was also significant on 27 Oct. (\( P = 0.002 \)), with a much greater increase in \( g_{stn} \) for S156 (90%) in the high treatments than R123 (29%). In addition, the interaction was significant between the control and high O3 treatment on 14 Oct. (\( P = 0.032 \)) because of a decrease (–23%) in \( g_{stn} \) for R123 and increase for S156 (7%) with elevated O3. The effects of O3 treatment may have been more advanced on 27 Oct. (25 DAT) relative to the three earlier sampling times (7–14 DAT), leading to greater \( g_{stn} \) at high O3 in both genotypes.

Similarly, Salvatori et al. (2013) reported that exposure to 75 ppb O3 (7 h d\(^{-1}\) for 20 d) caused a significant increase in \( g_{stn} \) and dark respiration (\( R_d \)) compared with control conditions, when measured from 17 to 20 DAT, for both genotypes. However, the relative increases were greater for R123 (\( g_{stn} = 271\% \) and \( R_d = 29\% \)) than S156 (\( g_{stn} = 209\% \), \( R_d = 9\% \)). They hypothesized that
Table 6. Nocturnal stomatal conductance ($g_{sn}$) for O₃ resistant (R123) and O₃ sensitive (S156) snap bean genotypes and single degree-of-freedom contrasts comparing the effects of day (0800–1900 hr) and day + night (0800–1900 hr + 2000–0700 hr) O₃ treatments for Spring (April, May) and Fall 2015 (October) trials ($n = 4$).

| Treatment | Genotype | Day | Night | 30 Apr. | 7 May | 14 Oct.² | 27 Oct. |
|-----------|----------|-----|-------|---------|-------|----------|--------|
| 1 (Control) | R123 | AMB | AMB | 57.0 | 61.9 | 54.4 | 48.0 |
| 2 | R123 | LOW | LOW | 42.8 | 56.6 | — | 33.8 |
| 3 | R123 | LOW | LOW | 69.7 | 65.5 | — | 38.5 |
| 4 | R123 | HIGH | AMB | 62.0 | 56.9 | 39.0 | 50.1 |
| 5 | R123 | HIGH | HIGH | 55.7 | 46.2 | 45.2 | 43.1 |
| 6 (Control) | S156 | AMB | AMB | 94.1 | 73.8 | 88.3 | 88.9 |
| 7 | S156 | LOW | AMB | 72.2 | 95.8 | — | 89.2 |
| 8 | S156 | LOW | LOW | 73.6 | 66.0 | — | 67.5 |
| 9 | S156 | HIGH | AMB | 71.4 | 67.9 | 107.0 | 185.5 |
| 10 | S156 | HIGH | HIGH | 73.6 | 129.8 | 81.5 | 112.5 |

Contrast ($P > F$)³

Control: Gn (1 vs. 6) 0.004 0.403 0.067 0.025
O₃ treatments³

Gn (2 + 3 + 4 + 5 vs. 7 + 8 + 9 + 10) 0.014 0.001 0.001 0.001
C vs. L (1 + 6 vs. 2 + 3 + 7 + 8) 0.134 0.723 — 0.295
C vs. H (1 + 6 vs. 4 + 5 + 9 + 10) 0.176 0.403 0.769 0.010
L vs. H (2 + 3 + 7 + 8 vs. 4 + 5 + 9 + 10) 0.849 0.552 — 0.001
Time (2 + 4 + 7 + 9 vs. 3 + 5 + 8 + 10) 0.350 0.289 0.437w 0.009
Interactions

$O_3 \times Gn$ (4 + 5 + 7 + 8 vs. 2 + 3 + 9 + 10) 0.800 0.061 0.032w 0.002
Time $\times Gn$: L (2 + 8 vs. 3 + 7) 0.133 0.062 — 0.288
Time $\times Gn$: H (4 + 10 vs. 5 + 9) 0.612 0.001 — 0.011

³Mean O₃ levels were AMB (no added O₃) = 9 ppb day, 8 (spring) and 15 (fall) ppb night; LOW = 44 ppb day, 46 (spring) and 49 (fall) ppb night; HIGH = 74 (spring) and 75 (fall) ppb day, 75 (spring) and 78 (fall) ppb night.

²Only the control and high O₃ treatments were sampled. The number of contrasts was restricted to five to control familywise error rate.

³Each contrast compares individual treatments or combinations of treatments as noted by treatment numbers. C = control; L = low O₃; H = high O₃; Gn = genotype; Time = day vs. day + night.

⁴Value represents effect of time at high O₃ (4 + 9 vs. 5 + 10).

⁵Value represents interaction for C vs. H (1 + 9 + 10) vs. (4 + 5 + 6).

The time of O₃ exposure had a significant effect on $g_{sn}$ only on 27 Oct. ($P = 0.009$), when rates were generally higher for the day than the day + night treatments (Table 6). However, the interaction of time × genotype was also significant ($P = 0.011$) for the high O₃ treatment, reflecting a larger difference in $g_{sn}$ between the day (185.5 mmol·m⁻²·s⁻¹) and day + night (112.5 mmol·m⁻²·s⁻¹) exposure times for S156 than R123 (50.1 vs. 43.1 mmol·m⁻²·s⁻¹). The opposite trend occurred for the significant time × genotype interaction on 7 May ($P = 0.001$), with greater $g_{sn}$ for S156 receiving the day + night timing (129.8 mmol·m⁻²·s⁻¹) than the day-only timing (67.9 mmol·m⁻²·s⁻¹) at high O₃. For R123, differences on 7 May in day and day + night rates at high O₃ were small (i.e., 56.9 vs. 46.2 mmol·m⁻²·s⁻¹, respectively). Therefore, the impact of exposure time on $g_{sn}$ was significant but inconsistent for S156.

To date, few studies have investigated the effects of nighttime O₃ exposure on $g_{sn}$. Skárby et al. (1987) reported increased $g_{sn}$ in a single shoot ($n = 1$) of scots pine (Pinus sylvestris L.) exposed to O₃ at night, relative to a shoot that was not exposed to O₃. The inconclusive effects observed here may have been a sampling artifact because of variability in leaf injury levels and/or differences in leaf maturity. Although data on $g_{sn}$ across leaf age are lacking, anatomically based estimates of conductance within mesophyll intercellular air spaces suggest that young S156 leaves allow greater gas exchange rates than adult leaves of both S156 and R123 (Villányi et al., 2013). In addition, epidermal cells in younger leaves of S156, those that developed after O₃ exposure, stopped dividing earlier than cells from mature leaves. Consequently, cells expanded for a longer period of time, producing larger guard cells and stomatal apertures as a result of prior O₃ exposure (Villányi et al., 2013). Therefore, conductance should ideally be measured on leaves of similar ages because O₃ exposure affects anatomical development.

Leaf injury and repair also likely affect $g_{sn}$ (Salvatori et al., 2013). Therefore, it would be informative to measure leaves of different age and injury classes to determine whether the impact of O₃ exposure on $g_{sn}$ is consistent. Other studies have focused on the first or second “fully expanded” trifoliate leaf from the top of the plant, shifting to younger leaves as the plant produces new growth over time (Elagöz et al., 2006; Flowers et al., 2007; Hoshika et al., 2013; Salvatori et al., 2013; Villányi et al., 2014). However, the definition of “fully expanded” does not appear uniform among studies. Importantly, leaf maturity has
been shown to influence sensitivity to O₃. Among different developmental stages, mature trifoliate leaves that had expanded to about 70% to 95% of full size were the most sensitive to O₃ (i.e., in terms of visual foliar injury), relative to expanding leaves at 50% to 70% of full size, which had intermediate injury levels, and young leaves, which were the least sensitive (Lee and Bennett, 1982). Therefore, sampling the youngest “fully expanded” trifoliate leaf may not be an objective measure of how leaf gas exchange responds to O₃ exposure.

**Conclusion**

Under control conditions, S156 and R123 produced similar yields by mass in Fall 2014 and Spring 2015 but not Fall 2015 (Tables 4 and 5). Yield mass ratios of S156:R123 ranged from 1.03 (Fall 2014) to 0.77 (Fall 2015) and were likely affected by environmental factors (Agathokleous et al., 2017). R123 produced significantly greater pod mass than S156 in the Fall 2015 control, and in all three trials, R123 had significantly greater mass yields than S156 under elevated daytime O₃.

Nighttime O₃ exposure alone, at 62 ppb, did not cause foliar injury and had no effect on the yields of S156 and R123. In combination with daytime O₃ exposure, nighttime O₃ treatment at up to 78 ppb did not impact yield or show a consistent effect on gₛₚ. Because plants generally have a lower capacity to detoxify O₃ at night, without a constant supply of metabolites from photosynthesis, an equivalent O₃ dose may cause greater injury at night than during the day (Musselman and Minnick, 2000). Therefore, the “effective flux,” which accounts for plant defense mechanisms and therefore overall sensitivity to O₃, has the greatest potential to accurately predict O₃ injury (Heath et al., 2009; Musselman et al., 2006). Recent work has demonstrated diurnal variation in plant sensitivity to O₃ (Grantz, 2014; Grantz et al., 2013), but more research is required to establish the effects of nighttime O₃ exposure.

Nighttime conductance measurements suggested that S156 and R123 have inherently different gₛₚ rates and that cumulative O₃ exposure can increase gₛₚ in both genotypes [i.e., results for 27 Oct. reflected the longest cumulative exposure (Table 6)]. However, exposure time (day vs. day + night) did not have a consistent effect on gₛₚ. High O₃ treatment caused a greater relative increase in gₛₚ for S156 than R123, contrary to the results reported by Salvatori et al. (2013). Notably, elevated gₛₚ may create a positive feedback when nighttime O₃ levels are high, increasing O₃ flux into the leaf (Salvatori et al., 2013). However, different sampling strategies and potential variation of gₛₚ with leaf maturity and injury confound results to date. Future experiments should test a larger range of night O₃ levels, and young leaves, which were the least sensitive (Lee and Bennett, 1982). Therefore, sampling the youngest “fully expanded” trifoliate leaf may not be an objective measure of how leaf gas exchange responds to O₃ exposure.

**Future experiments should test a larger range of night O₃ levels, and young leaves, which were the least sensitive (Lee and Bennett, 1982). Therefore, sampling the youngest “fully expanded” trifoliate leaf may not be an objective measure of how leaf gas exchange responds to O₃ exposure.**

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