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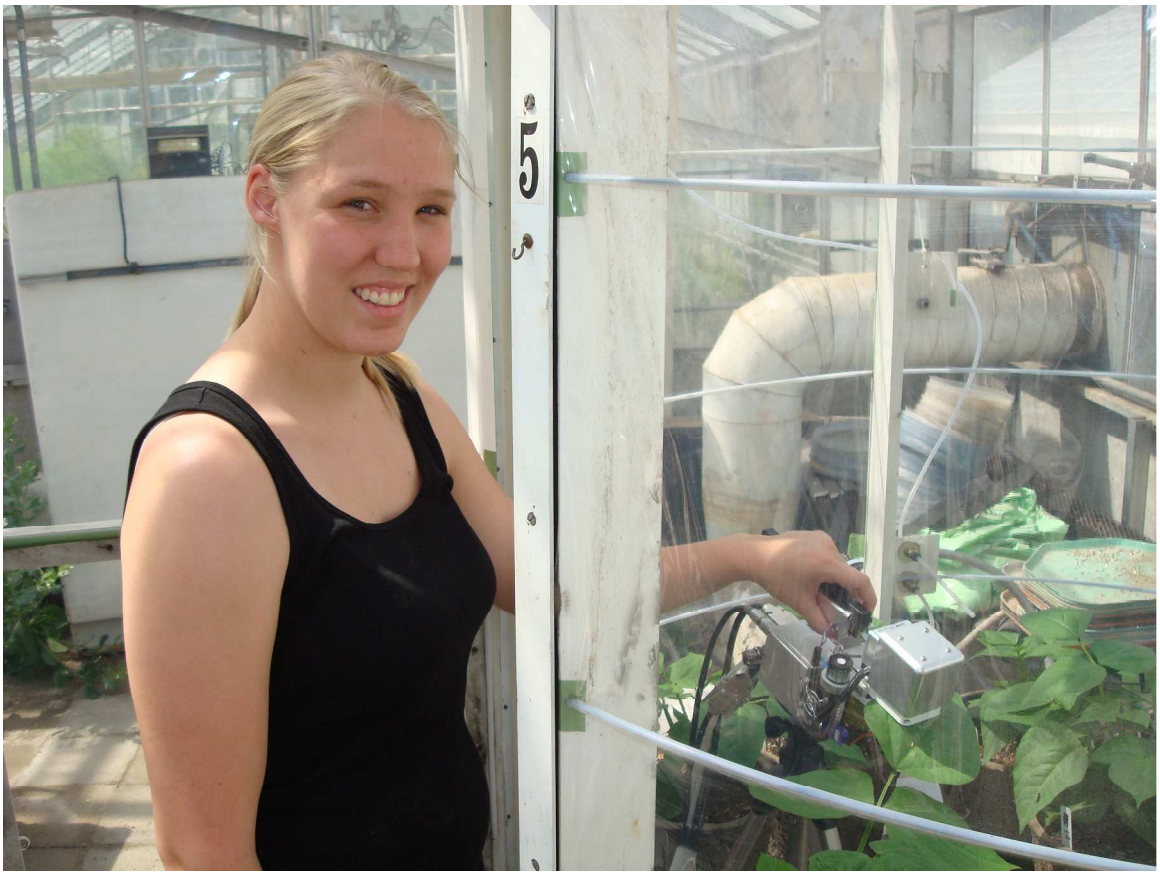
Plant physiological responses to nitric acid are evaluated against ozone for the first time.



Environmental Impact Statement

EM-ART-03-2014-000143 - Contrasting physiological responses of ozone-tolerant *Phaseolus vulgaris* and *Nicotiana tobaccum* cultivars to ozone and nitric acid

Cara M. Stripe, Louis S. Santiago, Pamela Padgett

This manuscript is the first report of our knowledge to study the leaf physiological responses of nitric acid under controlled conditions and relative to ozone. The work is novel in that we report the physiological responses to nitric acid and ozone of two agricultural species, each with known cultivars that are tolerant and sensitive to ozone. Nitric acid is an important co-pollutant of ozone, yet its physiological effects on crops have not been studied.

Contrasting physiological responses of ozone-tolerant *Phaseolus vulgaris* and *Nicotiana tobaccum* varieties to ozone and nitric acid

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Ozone (O_3) and nitric acid (HNO_3) are synthesized by the same atmospheric photochemical processes and are almost always co-pollutants. Effects of O_3 on plants have been well-elucidated, yet less is known about the effects of HNO_3 on plants. We investigated the physiological effects of experimental O_3 and HNO_3 fumigation on *Phaseolus vulgaris* (snap bean) and *Nicotiana tobaccum* (tobacco) varieties with known sensitivity to O_3, but unknown responses to HNO_3. Responses were measured as leaf absorptance, aboveground plant biomass, and photosynthetic CO_2-response curve parameters. Our results demonstrate that O_3 reduced absorptance, stomatal conductance and plant biomass in both species, and maximum photosynthetic rate in *P. vulgaris*, whereas the main effect of HNO_3 was an increase in mesophyll conductance. Overall, the results suggest that HNO_3 affects mesophyll conductance through increased nitrogen absorbed by leaves during HNO_3 deposition which in turn increases photosynthetic demand for CO_2, or that damage to epicuticular waxes on leaves increased diffusion of CO_2 to sites of carboxylation.

Keywords: air pollution, mesophyll conductance, photosynthetic CO_2 assimilation, nitrogen, urban ecology

Introduction

Air pollution is a process known to lower agricultural productivity because many components of polluted air react with plant biochemistry. Ozone (O_3) is a pollutant whose effects on plants have been well-documented, but far less is known about the effects of other pollutants that co-occur during contamination events. Ozone is one of the major gaseous pollutants that make up the tropospheric photochemical air pollution found throughout urban areas [1]. Increasing industrialization and urbanization has led to an average increase of 40 ppb O_3 over background levels in the last 30 years in the Northern Hemisphere [2], with current conditions in polluted areas on the United States and Europe in the range of 80-200 ppb [1]. Nitric acid (HNO_3) is a secondary pollutant that results from both the photochemical reactions that create O_3, and from non-photochemical reactions through the formation of N_2O_5 and NO_3 radicals [3]. In Southern California, the highest atmospheric concentrations occur during daylight hours [4]. In contrast to O_3, HNO_3 is more stable once it is formed, and deposits to exposed surfaces as dry deposition, or condenses into water to form an acid solution that falls as wet deposition. Nitric acid has a high deposition velocity and sticks to most substances resulting in short atmospheric residence times of 10 days or less [5]. Therefore, while O_3 and HNO_3 are generally co-pollutants, the proportion of each at any given time or location cannot be easily forecast [6]. Improved collection methods for HNO_3 [7-9], indicate atmospheric concentrations in highly polluted regions in the range of 13 ppb [10], far greater than the 0.81-1.7 ppb range observed in unpolluted wilderness areas [11], indicating that this highly reactive pollutant, which comprises the largest reservoir of reactive nitrogen in the lower troposphere [12], has a strong potential to influence plant productivity in agricultural lands near pollution sources.

Agricultural plants are often exposed to O_3 levels in excess of 40 ppb, which is known to affect physiology, productivity, and yield [13]. Specific effects of O_3 on crops are often dependent on species, variety, or agricultural management [13]. However, negative effects generally increase with O_3 dose. On the cellular level, the oxidizing nature of O_3 affects the ability of plants to function to full capacity [14-18]. Ozone enters the leaf primarily through stomata, and reacts with essential cellular components causing a complex cascade of reactions that include induction of phytohormones to protect the plant from the reactive oxygen species (ROS) that can alter cellular components [19]. These processes lead to reductions in stomatal conductance (g_s) [20], and reduction in carbon dioxide assimilation (A) thought to be caused by decreased Rubisco concentration and activity. This response is due, in part, to the oxidation of proteins caused by ozone [13]. The up-regulation of ethylene and ABA also induce stomatal closure, further reducing gas exchange [20]. The inhibition of CO_2 uptake results in measurable losses in productivity and yield for crop plants. Ozone is also known to reduce the light absorption ability of chloroplasts [21], with internal damage often, but not always appearing as necrotic lesions on the leaf surface [22]. It has been estimated that some parts of Asia could see crop yield losses of 5-20% by 2030, for plants exposed to high levels of O_3 [23]. While O_3 levels in many urban areas have decreased from acute episodes of 600 ppb near Los Angeles, CA in the 1970's to more moderate concentrations of 180 ppb during the 1990's [24], O_3 is still a chronic problem for crops in mixed suburban-agricultural areas, and is reemerging as a serious issue given the recent rise in urban agriculture [25].

In contrast to O_3, the effects of HNO_3 air pollution on agricultural plants have been little studied. Most of the research regarding deposition of nitrogen in general and HNO_3 in particular, has been focused on natural terrestrial ecosystems and to some extent aquatic ecosystems. The basis for this separation in focus between natural and managed ecosystems goes back to nitrogen

saturation theory [26], where it was postulated that the early response to nitrogen deposition would be a positive growth response to increased nitrogen availability. Recent literature, however, has demonstrated that dry deposition of HNO_3 results in superficial wounding of the epicuticular waxes of leaves and direct foliar absorption and assimilation of nitrogen, thus bypassing conventional nitrogen assimilation regulatory pathways of roots [5, 27]. Yet the consequences of superficial wounding for plant physiology and crop production are unknown because it is difficult to discern whether the N-fertilization aspect or the strong oxidizing properties of HNO_3 are the dominant factors for plants. Another part of the difficulty in determining the effects of HNO_3 on plants, besides the stickiness of the substance, and the difficulty in distinguishing atmospheric HNO_3 from all other nitrogen oxides in real time, is that phytotoxic damage due to air pollution can be difficult to ascribe to a specific pollutant under field conditions. For example, for many years declines in lichen populations in polluted forests were ascribed to O_3 toxicity, and it was not until careful fumigation studies demonstrated that many of the species known to be sensitive to air pollution were in fact responding to HNO_3, O_3's co-contaminant rather than O_3 itself [28]. In the current study, we employ similar fumigation approaches to study two model crop species often used as O_3 bioindicators, *Phaseolus vulgaris* [29], and *Nicotiana tobaccum* [30] to compare and contrast physiological responses to O_3 and HNO_3 pollution. We utilized varieties of these species with known sensitivity and tolerance to O_3, but unknown responses to HNO_3. Our main questions were: 1) How does HNO_3 deposition affect plant productivity and leaf gas exchange relative to the well-known effects of O_3? 2) Does physical leaf damage interact with photosynthetic processes to influence plant function and productivity? 3) Does genetic tolerance to O_3 alter the response of *P. vulgaris* and *N. tobaccum* to HNO_3 deposition?

Materials and Methods

Plant material

Plant responses to O_3 and HNO_3 were evaluated using two plant species with known sensitivity to O_3. We used *Phaseolus vulgaris* (snap bean) tolerant (R331) and sensitive (S156) varieties and *Nicotiana tobaccum* (tobacco) tolerant (BelB) and sensitive (BelW3) varieties, which have been demonstrated to differ in their responses to O_3 [31-33]. *P. vulgaris* seeds were planted directly into 8-l molded fiber containers (Western Pulp Products Co., Corvallis, OR) containing commercial media (Sunshine Mix #1; Sun Gro Horticulture, Bellevue, WA). *N. tobaccum* seeds were germinated in 10-cm pots containing a mixture of fertilized sand, peat moss and dolomite (UC Mix #3), thinned to one or two plants per pot and transplanted into 8-l pots once they had developed 2 or 3 sets of true leaves. All plants were fertilized with slow release fertilizer (Osmocote 19-6-12:N-P-K, Scotts-Sierra Horticultural Products, Marysville, OH). Irrigation was provided by an automatic system, which was adjusted according to weather conditions and plant growth. Pots were irrigated to saturation, and then allowed to dry to approximately half of field capacity before the next irrigation.

Experimental design

The two experiments were performed from 2 August to 14 September, 2009 for *P. vulgaris* and from 20 September to 1 November 2009 for *N. tobaccum* in a charcoal-filtered, climate-controlled greenhouse at the University of California, Riverside. Seedlings were transferred into the fumigation chambers and exposed to pollutants once they had developed two or three sets of leaves. Plants were exposed to pollutants using a continuously stirred tank reactor (CSTR)

fumigation system [34]. CSTR chambers were 1.35 m dia × 1.35 m tall, made of clear Teflon and fitted with a 0.6 × 1.2 m door. The air exchange rate was approximately 1.5 air exchanges per minute. Ten plants, five of each variety, were placed in each chamber. The plants were rotated within chambers weekly. Ten CSTRs in the greenhouse were organized on two benches with five chambers on each bench. Eight of the chambers were established with levels of pollutants following typical diurnal patterns: very low concentrations overnight, increasing concentration with sunrise reaching a peak in the afternoon, followed by a decline in concentration as the sun sets for eight hours of total exposure. Treatments were distributed across chambers as two at low O_3 concentrations (~40 ppb), two at high O_3 concentrations (~80 ppb), two at low HNO_3 concentrations (30 – 40 ppb peak midday) and two at high HNO_3 concentrations (80 – 100 ppb peak midday; Fig. 1). Daily concentrations in each chamber fluctuated to some extent due to changes in temperature and humidity, which affected the synthesis and delivery of both pollutants. One chamber was designated as a control with no pollutants. The tenth chamber housed a weather station to determine microclimate conditions within the chambers in the absence of plants. Temperature and relative humidity were measured using a shielded temperature/humidity sensor (Model HMP35C, Vaisala, Helsinki, Finland). Photosynthetically active radiation (PAR) was measured using a quantum sensor (Model 190S, Li-Cor, Biosciences, Lincoln, NE, USA). Microclimate data were measured every minute with a micrologger (CR1000; Campbell Scientific Inc., Logan, Utah USA).

Ozone was synthesized from compressed oxygen by an O_3 generator (Superior Electric Co., Bristol, CT, USA). The amount of O_3 delivered to each chamber was controlled by a flow meter (Model 602, Matheson Gas Products, Edmonton, Alberta, Canada) and was delivered to the CSTR bulk air input tube through Teflon tubing. Ozone was delivered to the chambers 1000

– 0100 h daily to mimic southern California diurnal ambient ozone patterns. HNO_3 vapor was synthesized by diluting concentrated HNO_3 at a ratio of 1:50 with distilled water. A piston-type pump (Fluid Metering Inc., Oyster Bay N.Y., USA) delivered the HNO_3 solution drop-wise in to a volatilization chamber submerged in a 95°C water/antifreeze (50:50) bath. The volatilization chamber consisted of a glass cylinder (6× 20 cm) filled with glass beads. A heatless air dryer (HF200-12-143; MTI Puregas, Denver, CO, USA) introduced dry air into the bottom of the volatilization chamber, which forced the vaporized HNO_3 into a glass manifold, delivering HNO_3 gas to the CSTRs via Teflon tubing. The amount of HNO_3 delivered was controlled by flow meters located at the chamber. Nitric acid was delivered to the chambers between 0900 and 1600 h daily to replicate southern California ambient pollution patterns with HNO_3 concentrations peaking in the late afternoon.

Pollutant concentrations were monitored in real-time using an Ozone monitor (Model 1003-AH, Dasibi Environmental Corp., Glendale, CA), and a Thermo Instruments Nitrogen Oxide Monitor (Model 8840, Monitor Labs, Inc., Englewood, CO, USA). Each chamber was sampled for six minutes every hour, through a modified scanivalve (Scanivalve Corp., San Diego CA, USA). Ozone concentrations were sampled directly from the chamber and transmitted to the Ozone monitor. Nitric acid was monitored by converting air samples into NO with a molybdenum converter (Molycon, Monitor Labs Inc., Englewood, CO, USA) mounted just outside each CSTR in order to decrease the HNO_3 losses and all NO in the sample was assumed to come from HNO_3 [34]. Pollutant concentration data was stored on a micrologger (CR21X, Campbell Scientific, Inc. Logan Utah, USA), and downloaded daily to a computer. Ambient greenhouse levels of O_3 and HNO_3 were monitored alongside the chamber levels.

For *P. vulgaris*, the temperature range during the experiment was 17.9-40.5 °C, the relative humidity range during the experiment was 24.2-78.1%, and PFD averaged 8.59 mol day^{-1}. For *N. tobaccum*, the temperature range during the experiment was 14.3-34.2 °C, the relative humidity range during the experiment was 24.5-70.0%, and PFD averaged 5.99 mol day^{-1}.

Leaf nitrogen deposition

We used leaf washes for nitrate (NO_3^-) to verify HNO_3 deposition on leaves. Plants were thoroughly rinsed with nanopure water at the beginning of the experiment. At the beginning of the experiment and in week six, one leaf was removed from each plant and placed in a 50 mL centrifuge tube; 40 mL nanopure water was added and the tube was shaken by hand for 30 seconds. Wash solutions were stored in a freezer until NO_3^- concentration was analyzed with a continuous flow analyzer (ALPKEM 320, College Station, TX, USA). For the final leaf wash of *N. tobaccum*, a leaf was removed from each plant and washed using nanopure water in a garden sprayer due to large leaf size, and water was collected in 250 ml plastic containers. We measured the area of each washed leaf with an area meter (Li-Cor LI-3100C, Li-Cor Biosciences).

Plant physiological measurements

Gas-exchange was measured on three plants of each variety in each chamber per week on the youngest fully expanded leaf on each plant. Concurrent measurements of photosynthesis and chlorophyll fluorescence were performed with an open-system infrared gas analyzer (Li-6400, Li-Cor Biosciences) equipped with a leaf chamber fluorometer (Li-6400-40, Li-Cor Biosciences). Photosynthetic CO_2 assimilation (A), stomatal conductance to water vapor (g_s) and transpiration (E) were measured at eight concentrations of atmospheric CO_2 (C_a) between 100

and 1200 μmol mol^{-1} using the CO_2 mixing system (Li-6400-01, Li-Cor Biosciences), at a flow rate of 500 μmol s^{-1}, photon flux density of 1200 μmol m^{-2} s^{-1} with 10% blue light, and cuvette temperature of 27˚C. The maximum rate of carboxylation of Rubisco (Vc_{max}), maximum electron transport rate (J_{max}), triose phosphate utilization (TPU), day respiration (R_d) and mesophyll conductance to CO_2 (g_m) were calculated and normalized to a standard temperature of 25˚C using an A-C_i curve fitting utility, version 4.0 [35]. At the end of each experiment absorptance (α) of photosynthetically active radiation (400-700 nm) was determined from one leaf from each plant with an integrating sphere interfaced with a spectroradiometer (LI-1800, Li-Cor Biosciences). Visible examination of leaf damage was conducted.

Plant biomass

We determined aboveground biomass at the end of each experiment by cutting plants at the bases of their stems and placing entire shoots in paper bags. Plants were dried in an oven at 65°C until constant mass and weighed for total dry biomass.

Statistical Analysis

We first tested for the effects of chamber on response variables using a general linear model (GLM) with chamber as a main effect. Chambers with the same treatment were not significantly different for any parameter, so plants in the same treatment in different chambers were pooled. A GLM was then used to determine effects of date, pollution level and variety tolerance on dissolvable nitrates on leaf surfaces. To determine responses of leaf optical properties, physiological variables and plant biomass to pollutant level, we used a GLM with pollutant level and variety tolerance as main effects. For physiological measurements that were conducted

weekly, data from all six weeks were pooled because the effect of time was consistent across treatments. This was determined by first conducting a GLM with pollutant level, variety tolerance and week as main effects. In these analyses, there were no significant interactions involving week and significance levels were found to be the same as when weeks were pooled, so week was removed as a main factor for subsequent analyses. Differences in plant responses among variety, tolerance and pollutant levels were evaluated with post hoc Duncan's multiple range tests. ANOVAs were performed separately for each pollutant. The bivariate relationship between maximum photosynthetic rate and mesophyll conductance was evaluated using linear regression. All statistical analyses were conducted in SAS version 9.3.

Results

Leaf nitrogen deposition

Nitrate measured from the leaf wash showed a significant treatment × date interaction in which leaf wash nitrates were similar among plants in all treatments during week 0, but increased significantly in the low and high HNO_3 treatments during week 6 in *P. vulgaris* ($F = 10.26$, $P \leq 0.0001$; Fig. 2a) and in *N. tobaccum* ($F = 40.37$, $P \leq 0.0001$; Fig. 2b), indicating that HNO_3 was deposited on leaf surfaces in chambers fumigated with HNO_3.

Plant physiological measurements

In response to O_3, *P. vulgaris* had leaf absorptance (α) values that were significantly reduced in low O_3 compared to control and high O_3 treatments ($F = 18.19$, $P \leq 0.0001$), but α was statistically indistinguishable between tolerant and sensitive varieties ($F = 0.01$, $P = 0.9377$; Fig. 3a). In response to HNO_3, *P. vulgaris* showed greater α in high HNO_3 treatments than in low

HNO_3 and control treatments ($F = 9.54$, $P \leq 0.0001$), and greater α in sensitive than tolerant varieties ($F = 78.0$, $P \leq 0.0001$). *N. tobaccum* had α values that were greatest in the control treatment and decreased significantly in the low and high O_3 treatments for sensitive varieties, but not for tolerant varieties, causing a significant treatment × tolerance interaction ($F = 10.63$, $P \leq 0.0001$; Fig. 3b). In response to HNO_3, *N. tobaccum* showed no significant differences in α in among treatments ($F = 0.56$, $P = 0.5737$), or between varieties ($F = 0.40$, $P = 0.5247$). Visible leaf damage was evident in sensitive, but not tolerant varieties of both species in O_3 treatments, but not in HNO_3 treatments.

High O_3 treatments caused lower A_{max} and g_s in *P. vulgaris* relative to control and low O_3 treatments (Table 1; Figs. 4a, c). In *N. tobaccum*, high O_3 caused lower g_s relative to control and low O_3 treatments (Table 1, Fig. 4d), but there were no significant differences in A_{max} among O_3 treatments (Table 1, Figs. 4b, d). There were no significant differences in g_m in either species in response to O_3 (Table 1, Figs. 4 e, f), and there were no significant differences in A_{max} or g_s in response to HNO_3 for either *P. vulgaris* or *N. tobaccum* (Table 1, Figs. 5a-d). However, g_m increased with high HNO_3 in *P vulgaris* and with high and low HNO_3 in *N. tobaccum* (Table 1, Figs. 5e-f). The only other physiological responses to pollutants were lower J_{max} in the high O_3 treatment compared to control and low O_3 treatments for *P. vulgaris* (Table 1), and greater respiration in sensitive than tolerant varieties in response to O_3 in *N. tobaccum* (Table 1). There was significant positive correlation between A_{max} and g_m across all study plants demonstrating the functional interdependence of these two variables (Fig. 6).

Plant biomass

For *P. vulgaris*, there was a significant negative effect of high O_3 on biomass for both tolerant and sensitive varieties, but overall tolerant varieties had greater biomass than sensitive varieties (Table 1, Fig. 7). HNO_3 did not have an effect on plant biomass in *P. vulgaris*, but tolerant varieties exhibited greater biomass than sensitive varieties (Table 1). For *N. tobaccum*, biomass decreased with high O_3 in sensitive but not in tolerant varieties producing a significant O_3 effect and a significant tolerance × O_3 interaction. HNO_3 did not have any significant effects on biomass of *N. tobaccum* (Table 1).

Discussion

Our data indicate that although HNO_3 is a powerful oxidant, at the applied levels it does not appear to induce oxidative stress in the same way that O_3 has been shown to affect crop productivity. These results build on previous work in which leaves that had been exposed to HNO_3 were examined microscopically and for changes in N concentration [5, 27]. In previous studies, HNO_3 was shown to cause oxidative damage of epicuticular waxes, induce up-regulation of nitrate reductase and increase foliar N concentration [28, 36, 37]. In the current study, two species, each with varieties of known sensitivity to O_3 were cultivated under contrasting levels of pollutants so that the effects of HNO_3 on plant function and productivity could be determined relative to the better known effects of O_3. We were thus able to isolate the implications of HNO_3 deposition in agricultural plants in or near sources of high pollution, and assess the degree to which HNO_3 causes alterations in photosynthesis and productivity.

Our results are the first to demonstrate that HNO_3 at the applied levels does not cause the same oxidative stress to photosystems as O_3. In contrast, HNO_3 appears to have two main effects on leaf-scale physiology. The first effect is a large increase in available nitrogen. This

294 phenomenon has been confirmed through analysis of the amount of nitrogen deposited on leaves
295 through leaf washes and ^{15}N tracer techniques [5, 38], and inferred through measurement of up-
296 regulation of nitrate reductase in leaves that had been exposed to HNO_3 [36, 37]. The second effect
297 is an increase in g_m, which was found in the current study in O_3-sensitive and -tolerant varieties
298 of two agricultural species. These increases in g_m indicate that photosynthesis is less limited by
299 the ability of CO_2 to diffuse to the chloroplast under HNO_3 exposure relative to control
300 treatments [39], and is consistent with enhanced leaf nitrogen and greater CO_2 demand if greater
301 allocation to photosynthetic enzymes is indeed powered by excess nitrogen deposited on the leaf.
302 However, we did not observe an increase in A_{max} under HNO_3 fumigation (Fig. 5), suggesting
303 that the stimulatory effect of added N on plant photosynthesis under HNO_3 fumigation is small
304 or that enhanced g_m functions to make photosynthesis more efficient rather than producing high
305 rates. The second possibility is that increased g_m in plants fumigated with HNO_3 is related to
306 degradation of epicuticular waxes found in previous studies [27, 40]. Yet, the severe damage to
307 cuticles that could increase g_m would likely also increase water vapor fluxes from the leaf, which
308 was not observed as greater g_s or E from plants in HNO_3 treatments, suggesting that if g_m is
309 enhanced by ruptures in leaf cuticles, then these are small fissures and that the diffusion process
310 is complex. The extent of alterations of leaf N concentration, cuticular integrity, and g_m in
311 response to HNO_3 across other species of plants is unknown, but these parameters clearly have
312 the potential to influence carbon and water exchange from vegetation and the atmosphere, as
313 well as crop productivity.

314 The effects of O_3 on plant productivity have been studied for relatively longer than HNO_3
315 and research has generally shown that O_3 has negative effects on A_{max}, g_s, and other gas-
316 exchange-related variables due to O_3 interaction with Rubisco [13]. Our results are consistent with

this pattern, as high O_3 treatments reduced A_{max} in one species and reduced g_s in both. However, there were also negative effects of O_3 on leaf absorptance in *P. vulgaris* under low O_3 levels and *N. tobaccum* under low and high O_3 levels. These results suggest that the blotching and chlorosis that accompany chronic O_3 exposure in some species represents a reduction in absorptance which would likely increase albedo and affect surface energy balance in agricultural fields near large pollution sources [41]. Furthermore, although we measured a reduction in growth under high O_3 in *P. vulgaris*, low O_3 actually stimulated growth. Some research has suggested that low levels of O_3 may in some way be beneficial to the plant due to stimulation of anti-oxidant defenses [31]. The significant increase in biomass in low O_3 compared to the control treatment found in the tolerant variety of *P. vulgaris* is consistent with this idea, but no other results from *P. vulgaris* suggest beneficial impacts from O_3 fumigation.

In addition to the contrasting effects of O_3 and HNO_3, responses to fumigation differed between varieties. The most striking difference between varieties was observed in aboveground biomass which was greater in tolerant than sensitive varieties in both species and in both O_3 and HNO_3 treatments (Fig. 7; Table 1), which likely results from a coincidence in breeding because biomass was not the selection criterion. Leaf absorptance showed an overall greater absorptance in tolerant varieties in *P. vulgaris* with high HNO_3 fumigation, consistent with greater light harvesting enzymes and increased N, whereas *N. tobaccum* showed no responses of absorptance to HNO_3. Reductions in leaf absorptance of sensitive varieties under O_3 fumigation reflect the visible damage observed in leafs.

Conclusions

Ozone has been shown to decrease productivity, yield and photosynthesis in agricultural plants, and genetic lines have been established that are tolerant to O_3. Understanding the reason for this tolerance will create the ability to develop other agricultural plants that can withstand excess pollutant deposition. This research has emphasized that the difference between the O_3 sensitive and tolerant varieties is a genetic compensation to O_3 exposure. We demonstrate that leaf gas exchange responses to HNO_3 were different than the responses to O_3, but HNO_3 did not affect plant biomass. Furthermore, leaf damage appeared to interact with photosynthetic processes through a reduction in leaf absorptance with O_3 fumigation in sensitive varieties and possible effects of damage to leaf cuticular waxes on g_m with HNO_3 fumigation. Finally, genetic tolerance interacted with HNO_3 treatments in leaf absorptance and g_m responses, indicating that O_3 sensitive and tolerant varieties may respond differentially to other stresses besides O_3. Overall, the necessity to understand how pollutants affect plants is vital as increased dry deposition of O_3 and HNO_3 and other chemicals on agricultural and native species in surrounding areas is increasing.

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447

448 **Table 1.** *F*-values resulting from analysis of variance for effects of O_3 tolerance and exposure to
449 low and high levels of O_3 and HNO_3 relative to control on plant biomass, photosynthetic and leaf
450 optical properties, for *Phaseolus vulgaris* and *Nicotiana tobaccum* varieties that are sensitive and
451 tolerant to O_3.

	Tolerance	O_3	Tolerance×O_3	Tolerance	HNO_3	Tolerance×HNO_3
Phaseolus vulgaris						
Biomass (g)	68.25***	18.40***	1.10	27.00***	2.86	1.28
α (proportion)	0.01	18.19***	16.80***	78.00***	7.54***	9.58***
A_{max} (μmol m^{-2} s^{-1})	0.04	5.84**	0.26	0.78	0.34	0.38
g_s (mol m^{-2} s^{-1})	0.08	2.79	0.23	0.64	0.81	0.24
E (mmol m^{-2} s^{-1})	0.01	2.16	0.16	0.09	1.21	0.36
Vc_{max} (μmol m^{-2} s^{-1})	0.00	1.70	0.02	1.79	1.25	0.44
J_{max} (μmol m^{-2} s^{-1})	0.48	4.45*	0.08	2.15	0.96	0.19
TPU (μmol m^{-2} s^{-1})	0.21	2.15	0.79	0.45	1.48	0.31
R_d (μmol m^{-2} s^{-1})	3.02	0.95	0.69	0.31	2.27	0.10
g_m (μmol m^{-2} s^{-1} Pa^{-1})	0.08	1.44	0.06	0.39	3.37*	0.31
Nicotiana tobaccum						
Biomass (g)	1.88	4.86**	4.65**	0.02	1.41	0.57
α (proportion)	64.49***	15.61***	10.63***	0.40	0.56	0.95
A_{max} (μmol m^{-2} s^{-1})	2.35	0.55	0.81	0.79	0.14	0.27
g_s (mol m^{-2} s^{-1})	0.20	8.91***	3.80*	0.68	0.43	0.12
E (mmol m^{-2} s^{-1})	0.79	0.88	1.17	0.07	0.14	0.04
Vc_{max} (μmol m^{-2} s^{-1})	0.00	0.89	1.58	0.28	0.07	1.25
J_{max} (μmol m^{-2} s^{-1})	0.12	0.83	1.83	0.36	0.21	1.93
TPU (μmol m^{-2} s^{-1})	1.93	0.36	0.26	1.17	0.68	0.54
R_d (μmol m^{-2} s^{-1})	5.92*	0.94	0.03	3.25	1.54	0.65
g_m (μmol m^{-2} s^{-1} Pa^{-1})	1.57	1.28	2.82	1.07	7.46***	0.74

452

453 *P<0.05, **P<0.01, ***P<0.001

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455

456 **Figure Legends**

457

458 **Fig. 1** Diurnal concentrations of O_3 and HNO_3 for the 6-week experimental period of *Nicotiana*
459 *tobaccum*, conducted between 20 September – 1 November 2009 in continuously stirred tank
460 reactor chambers with controlled levels of Ozone (O_3) and nitric acid (HNO_3). Control chambers
461 (not shown) had averages of 13.1 ppb O_3 and 0.1 ppb HNO_3 over the same period.

462

463 **Fig. 2** Mean (± 1 Standard Error) nitrate concentration washed from leaf surfaces normalized by
464 leaf area at the initiation (Week 0) and end (Week 6) of 6-week experiments with *Phaseolus*
465 *vulgaris* and *Nicotiana tobaccum*) varieties that are sensitive (S) or tolerant (T) to ozone (O_3),
466 growing in chambers with controlled levels of O_3 and nitric acid (HNO_3). Elevated nitrate on
467 leaves indicates deposition by HNO_3 treatments. n = 5 for control treatments and 10 for low and
468 high ozone and nitric acid treatments.

469

470 **Fig. 3** Mean (± 1 Standard Error) leaf absorptance of 400 – 700 nm light for *Phaseolus vulgaris*
471 and *Nicotiana tobaccum* varieties that are sensitive (S) or tolerant (T) to ozone (O_3), growing in
472 chambers with controlled levels of O_3 and nitric acid (HNO_3).

473

474 **Fig. 4** Mean (± 1 Standard Error) photosynthetic responses to O_3: (a-b) Maximum
475 photosynthetic rate (A_{max}); (c-d) stomatal conductance at A_{max} (g_s); (e-f) mesophyll conductance
476 to CO_2 (g_m) for *Phaseolus vulgaris* and *Nicotiana tobaccum* plants growing in chambers with
477 controlled levels of ozone (O_3). Varieties that are sensitive (S) or tolerant (T) to ozone (O_3) were

pooled for this analysis because there were no significant differences. Values with the same letter are not significantly different at a *p*-value of 0.05.

Fig. 5 Mean (± 1 Standard Error) photosynthetic responses to HNO_3: (a-b) Maximum photosynthetic rate (A_{max}); (c-d) stomatal conductance at A_{max} (g_s); (e-f) mesophyll conductance to CO_2 (g_m) for *Phaseolus vulgaris* and *Nicotiana tobaccum* plants growing in chambers with controlled levels of nitric acid (HNO_3). Varieties that are sensitive (S) or tolerant (T) to ozone (O_3) were pooled for this analysis because there were no significant differences. Values with the same letter are not significantly different at a *p*-value of 0.05.

Fig. 6 Maximum photosynthetic CO_2 assimilation per area (A_{max}) as a function of mesophyll conductance to CO_2 (g_m) for *Phaseolus vulgaris* and *Nicotiana tobaccum* varieties that are sensitive (S) or tolerant (T) to ozone (O_3), growing in chambers with controlled levels of O_3 and nitric acid (HNO_3). Values are mean (± 1 Standard Error).

Fig. 7 Mean (± 1 Standard Error) aboveground biomass of *Phaseolus vulgaris* and *Nicotiana tobaccum* varieties that are sensitive (S) or tolerant (T) to ozone (O_3), growing in chambers with controlled levels of O_3 and nitric acid (HNO_3). The graph shows a significant negative effect of high O_3 on biomass for both tolerant and sensitive varieties of *Phaseolus vulgaris* and that biomass decreased with high O_3 in sensitive but not in tolerant varieties of *Nicotiana tobaccum* producing a significant O_3 effect and a significant tolerance × O_3 interaction. HNO_3 did not have any significant effects on biomass for either species. Statistical results in Table 1.

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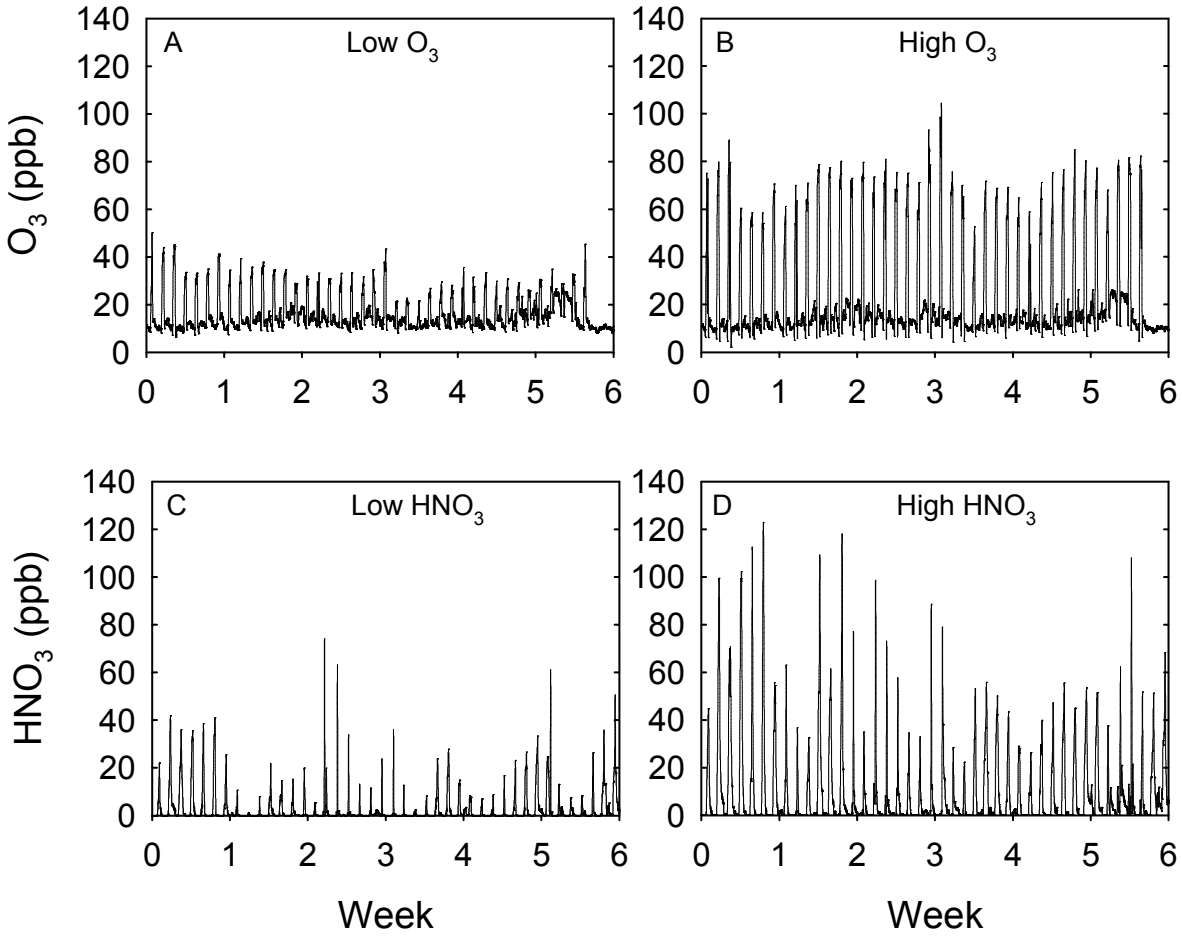

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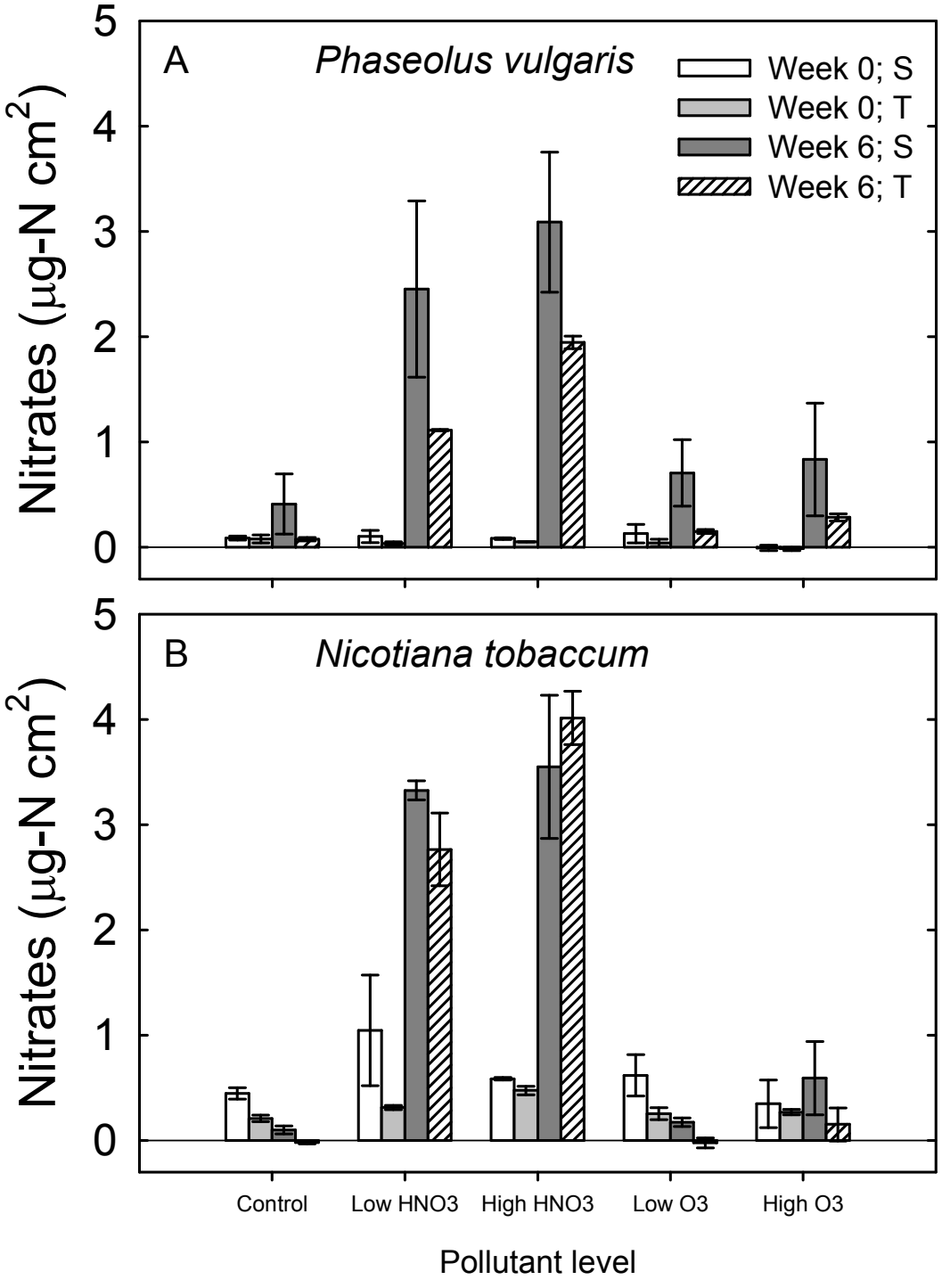

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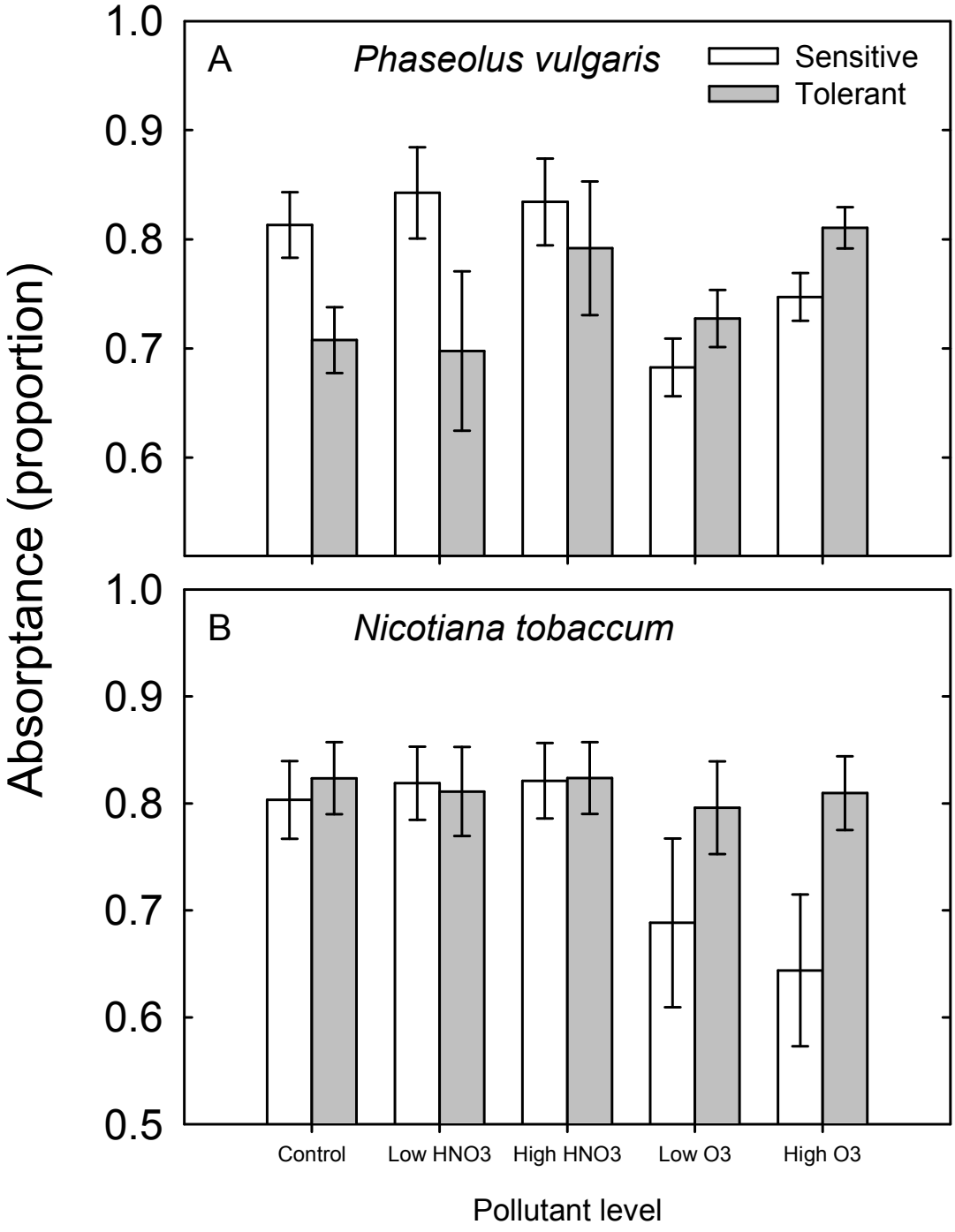

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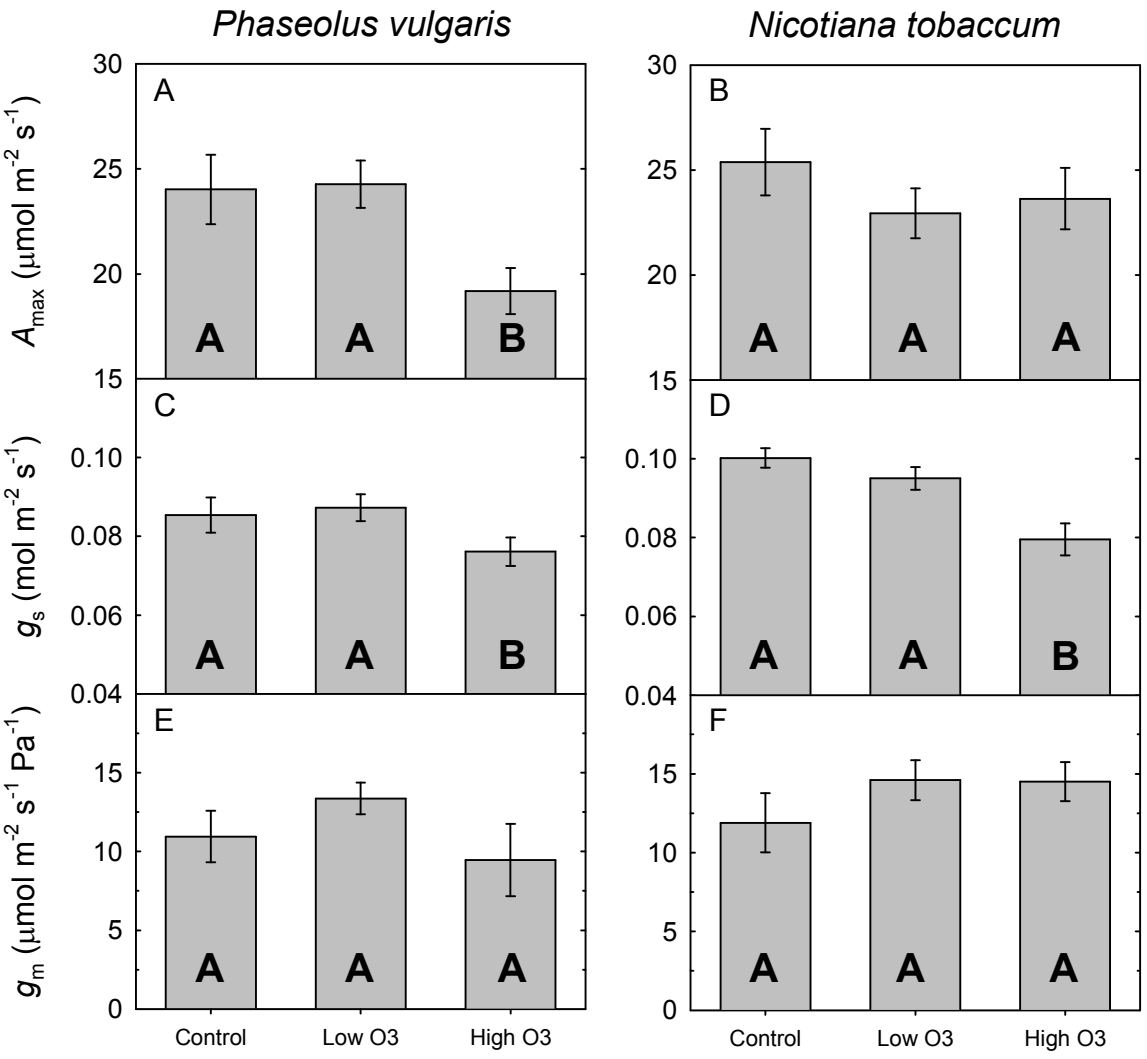

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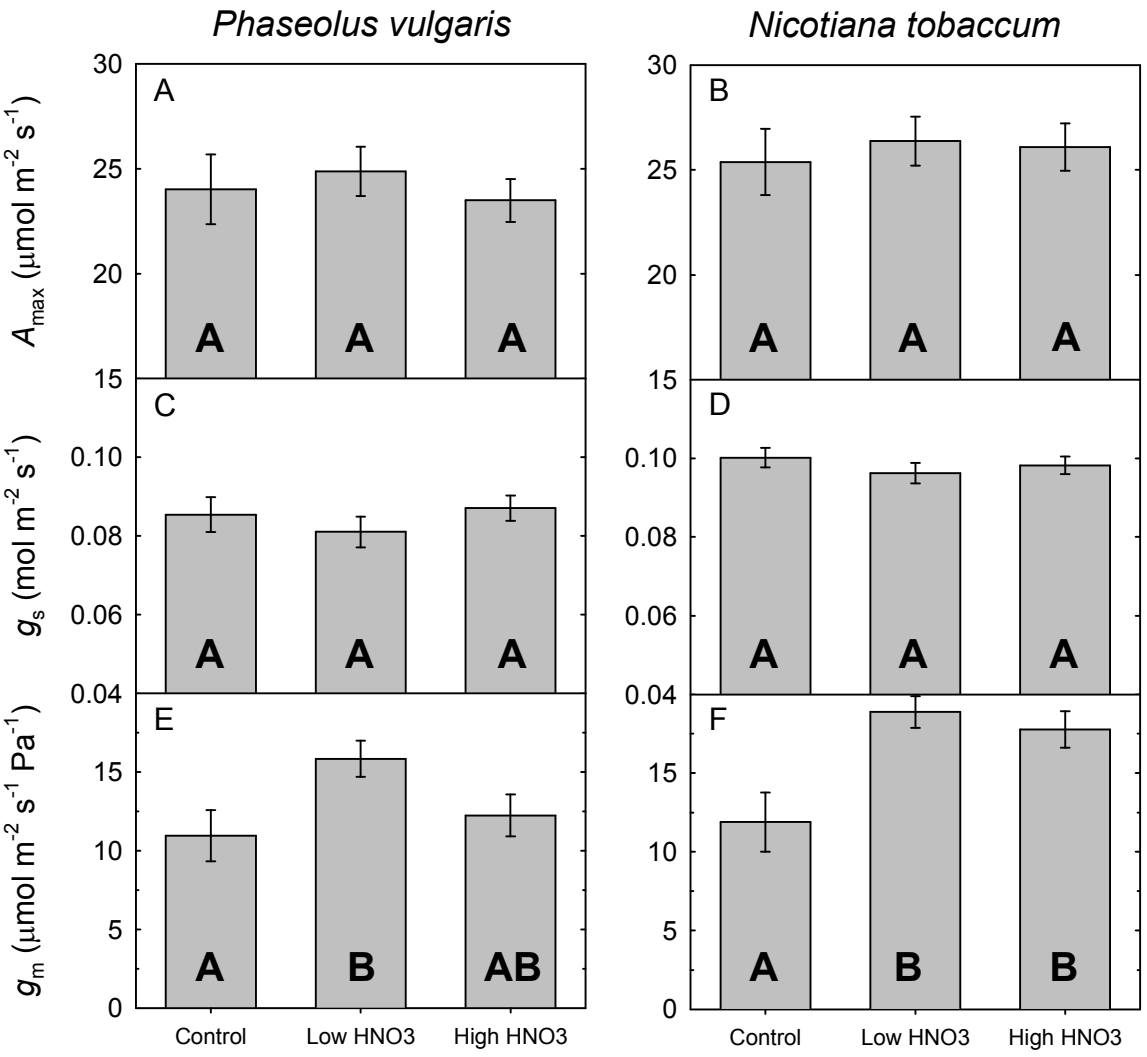

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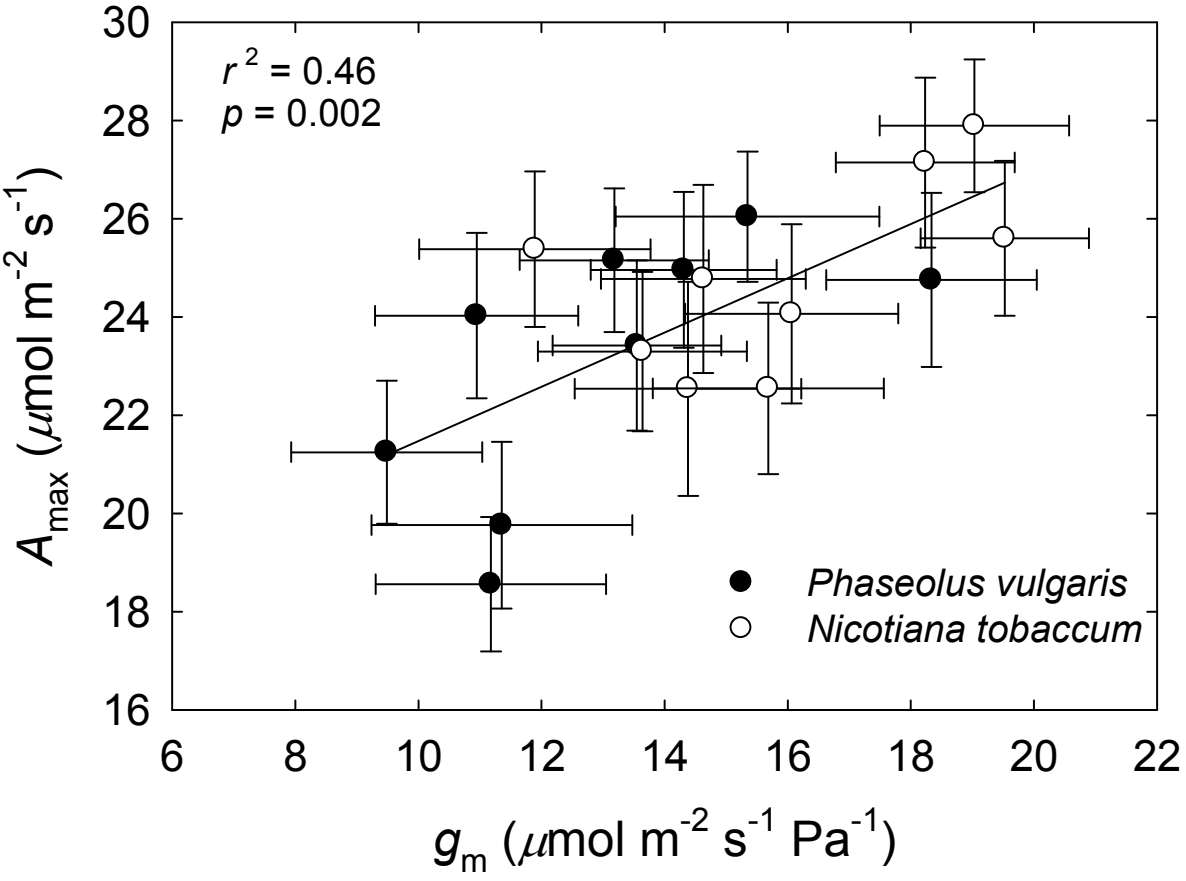

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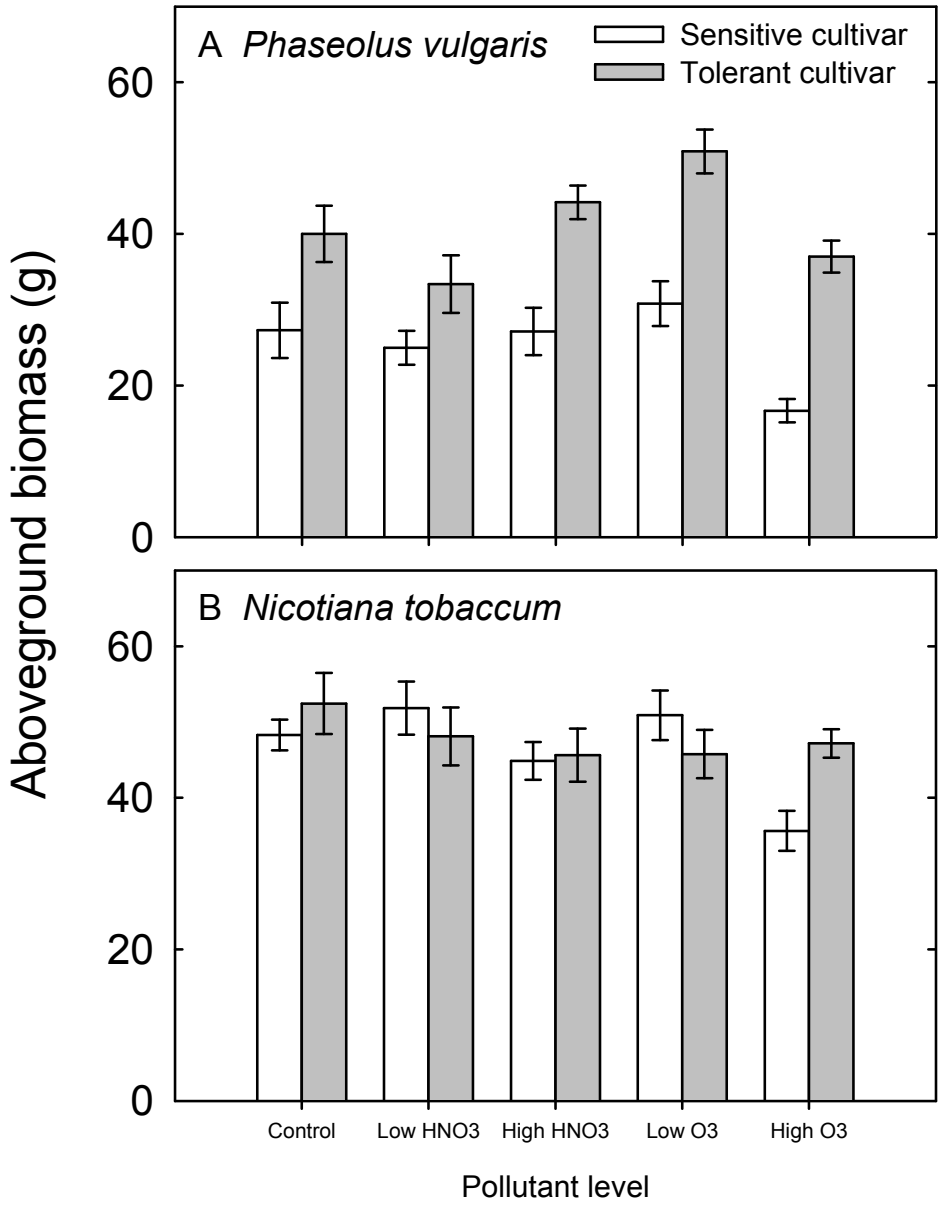

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