Elevated Atmospheric Carbon Dioxide and O_3 Differentially Alter Nitrogen Acquisition in Peanut

Cong Tu, Fitzgerald L. Booker,* Kent O. Burkey, and Shuijin Hu

ABSTRACT

Elevated atmospheric CO_2 and ozone (O_3) may affect productivity of legumes in part by altering symbiotic N_2 fixation. To investigate this possibility, measurements of plant biomass, N levels and natural ^{15}N abundance ($\delta^{15}N$) were used to examine the effects of elevated CO_2 and O_3 on N acquisition in field-grown peanut (*Arachis hypogaea* L.) using open-top chambers. Seasonal 12-h daily average CO_2 treatment concentrations were 376, 550, and 730 μmol mol^{-1}. Carbon dioxide treatments were applied in reciprocal combinations with seasonal 12-h daily average O_3 concentrations of 21, 49, and 79 nmol mol^{-1}. At mid-vegetative growth, elevated CO_2 significantly reduced leaf N concentrations by up to 44%, but not $\delta^{15}N$ values. Elevated O_3 did not significantly affect N concentrations or $\delta^{15}N$ values. At harvest, plant N concentrations were similar among treatments except for a 14% reduction in the highest-level CO_2–O_3 treatment. Plant N accumulation varied in proportion with treatment effects on biomass production, which was increased with elevated CO_2 when averaged over the O_3 treatments and suppressed by high-level O_3 at ambient CO_2. Elevated CO_2 reduced plant $\delta^{15}N$ values in low- and mid-level O_3 treatments while mid- and high-level O_3 increased them at ambient CO_2. The changes in $\delta^{15}N$ values suggested that N_2 fixation activity was stimulated with elevated CO_2 and inhibited by elevated O_3. Elevated CO_2 ameliorated detrimental O_3 effects to varying extents depending on the concentrations of the two gases. These results indicated that interactions between CO_2 and O_3 on plant physiology can alter N acquisition processes, with impacts on peanut productivity likely dependent in part on these changes.

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Abbreviations: *A*, net photosynthetic rate; AA, ambient air; CF, charcoal-filtered; NF, nonfiltered; OZ, 1.6 × ambient O_3.

The concentration of CO_2 in the atmosphere has increased by approximately 36% from about 280 μmol mol^{-1} in 1800 to 379 μmol mol^{-1} in 2005, with the greatest increase in historic times occurring since the 1940s (Prather et al., 2001). In parallel, tropospheric O_3 concentrations have increased from the natural background of 10 to 20 nmol mol^{-1} to the present level of 35 to 40 nmol mol^{-1} (Vingarzan, 2004). In addition, regional air pollution episodes during the growing season can increase ground-level O_3 concentrations to phytotoxic levels in major crop production areas in the United States, Mexico, Europe, China, south Asia, west Africa, and India (Booker et al., 2009; Mills et al., 2007; Wang and Mauzerall, 2004). Moreover, atmospheric CO_2 and O_3 concentrations are expected to continue to rise during this century (Dentener et al., 2006; Meehl et al., 2007; Prather et al., 2001; Vingarzan, 2004). These gases co-occur in the atmosphere and both have demonstrated, although generally antagonistic, effects on net photosynthesis, growth and yield (Fiscus et al., 2005; Fuhrer, 2003; Olszyk et al., 2000).

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Many studies have shown that elevated CO_2 concentrations increased photosynthesis rates, biomass accumulation, and seed production in C_3 plants (Jablonski et al., 2002; Kimball et al., 1993; Reddy and Hodges, 2000). Positive responses have been documented in crops such as cotton (*Gossypium hirsutum* L.), peanut (*Arachis hypogaea* L.), potato (*Solanum tuberosum* L.), rice (*Oryza sativa* L.), soybean [*Glycine max* (L.) Merr.], and wheat (*Triticum aestivum* L.) (Nösberger et al., 2006; Prasad et al., 2005; Reddy and Hodges, 2000). In contrast to CO_2, O_3 concentrations above natural backgrounds levels can be phytotoxic (Fuhrer and Booker, 2003; Heagle, 1989; USEPA, 2006). Chronic exposure to elevated O_3 inhibits photosynthesis, accelerates senescence, and leads to reduced plant growth and yield in sensitive crop species such as cotton, peanut, potato, rice, soybean, and wheat (Booker et al., 2009; Fiscus et al., 2005; Heagle, 1989; Mills et al., 2007).

A CO_2 stimulation of plant growth in theory requires a corresponding increase in nutrient acquisition to maintain the plant C to nutrient balance, whereas inhibition of plant growth by O_3 may restrain soil nutrient uptake by plants. With elevated CO_2, decreases in soil nutrients such as N, P, Ca, and K induced by stimulated plant growth have been observed in forest and grassland systems (Hu et al., 2006). The situation may differ for agricultural soils where significant amounts of nutrients are added through fertilization. Also, many legume crops can acquire additional N through symbiotic N_2 fixation. Several studies on N_2–fixing plants such as ladino clover (*Trifolium repens* L.), alfalfa (*Medicago sativa* L.), pea (*Pisum sativum* L.), and soybean have shown an increase in symbiotic N_2 fixation with elevated CO_2 (Lüscher et al., 2000; Phillips et al., 1976; Rogers et al., 2006; Torbert et al., 2004; Zanetti et al., 1996). In contrast, O_3 has been found to reduce nodulation in legumes such as ladino clover, pinto bean (*Phaseolus vulgaris* L.), and soybean, potentially reducing symbiotic N_2 fixation (Blum and Tingey, 1977; Letchworth and Blum, 1977; Manning et al., 1973). Nitrogen fixation and total nodule activity were suppressed by added O_3 in soybean (Flagler et al., 1987; Pausch et al., 1996). However, no information is available regarding whether O_3 affects symbiosis-mediated N acquisition in legumes at elevated atmospheric CO_2 concentrations.

We hypothesized that elevated CO_2 and O_3 differently altered C allocation to roots and symbiotic N_2–fixing bacteria, thereby modifying N_2–fixing activities. Consequently, a change in N_2 fixation may alter the relative contribution of soil- and air-derived N to the plant. Stable isotope ratios can be used to partition N sources. This is possible because the natural ^{15}N abundance in the atmosphere (0.3663 atom %; $\delta^{15}N = 0$ ‰) is different from soil $\delta^{15}N$ values, which range from 0 to +13 ‰ (Högberg, 1997). Plants that solely rely on soil N sources have $\delta^{15}N$ values similar to those of the soil N, whereas plant species that can obtain atmospheric N via symbiotic N_2 fixation exhibit $\delta^{15}N$ values closer to 0 ‰ (Shearer and Kohl, 1986). Thus, the effects of elevated CO_2 and O_3 on N acquisition during development can be dissected through determinations of $\delta^{15}N$ values in vegetative and reproductive plant tissues.

In this paper, we present results from an open-top chamber field experiment that examined N levels in peanut treated with reciprocal mixtures of CO_2 and O_3. Our objectives were (i) to quantify how elevated CO_2 and O_3 affected N concentrations in leaves during vegetative growth as well as in leaves, stems, and seeds at harvest; and (ii) to determine whether elevated CO_2 and O_3 altered the relative contributions of soil N and N_2 fixation to various plant tissues, as indicated by $\delta^{15}N$ analysis. Average midday net photosynthesis during the growing season and harvest biomass were measured to evaluate overall gas treatment effects on C assimilation and plant productivity, while biomass measurements provided the basis for determinations of total N per leaf, stem, and seed components.

MATERIALS AND METHODS

Plant Culture Conditions and Gas Treatments

The experiment was conducted with peanut ('NC-V11') during 2002 at a site 5 km south of Raleigh, NC (35°44′ N, 78°41′ W) (Booker et al., 2007). The soil consisted of about 30 cm of Norfolk sandy loam (fine-loamy, kaolinitic, thermic Typic Kandiudult) overlying an Appling sandy loam (fine, kaolinitic, thermic Typic Kanhapludult). The field was treated with granular gypsum (86% $CaSO_4$) at a rate of 897 kg ha^{-1} and fertilized according to soil test recommendations with 94 kg K ha^{-1} on 15 April. Analysis of soil samples (0 to 20 cm depth) indicated that average total N concentration in the field was 0.6 g kg^{-1} with a $\delta^{15}N$ value of 4.4 ± 0.2 ‰ (mean ± SD).

Before planting, peanut seeds were treated with a commercial *Bradyrhizobium* preparation (Rhizo-Flo, Becker Underwood Inc., Ames, IA) and then planted on 15 May. Plants were sown in rows with 1-m row spacing and 9-cm in-row plant spacing (11 plants m^{-2}). Plants were irrigated with soaker hoses installed parallel to each row.

Plants were treated with mixtures of CO_2 and O_3 in open-top field chambers (3-m diameter by 2.4-m tall), beginning on 30 May. Carbon dioxide and O_3 were dispensed and monitored as previously described (Booker et al., 2007). To mimic atmospheric O_3 dynamics, supplementary O_3 generated by electrostatic discharge in dry O_2 was dispensed 12 h d^{-1} (0800 to 2000 Eastern standard time) in proportion to ambient O_3 concentrations. Ozone concentration in the chambers was monitored at canopy height using UV photometric O_3 analyzers (model 49, Thermo Environmental Instruments, Franklin, MA). Carbon dioxide was dispensed 24 h d^{-1} from a 12.7-Mg liquid receiver and was monitored at canopy height with infrared CO_2 analyzers (model 6252, Li-Cor, Inc. Lincoln, NE). Dispensing of CO_2 was reduced by half at night (1900 to 0700 h Eastern standard time) to prevent excessive CO_2 concentrations in the chambers.

The experiment consisted of all combinations of three CO_2 and O_3 treatments (Table 1). Seasonal average CO_2 treatment concentrations were 376 (ambient CO_2, control), 550 (ambient plus 174), and 730 (ambient plus 354) μmol mol^{-1}. Average O_3 concentrations

were 21, 49, and 79 nmol mol^{-1}, which were respectively obtained with charcoal-filtered air (CF), nonfiltered air (NF), and NF air plus 1.6 times ambient O_3 (OZ). Replication of treatment combinations varied within the experiment. Open-top chamber replicates were three for each $CO_2 \times O_3$ combination at both high and low concentrations (CF-376, CF-730, OZ-376, OZ-730) and two for the treatments of CO_2 at 550 μmol mol^{-1} and O_3 at 49 nmol mol^{-1} (CF-550, OZ-550, NF-376, NF-550, NF-730).

Net Photosynthesis

Net photosynthesis (*A*) was measured on the second leaf down from a branch apex on three plants per chamber using a portable photosynthesis system (Model 6200, Li-Cor, Inc.) and a 1-L cuvette (Booker et al., 2007). Measurements were made at growth CO_2 and O_3 conditions in a chamber between 1000 and 1300 h when ambient photosynthetic photon flux was > 1000 μmol m^{-2} s^{-1}. Net photosynthesis was measured weekly between 1 July and 20 September in all chambers except the 550 μmol CO_2 mol^{-1} treatment combinations because of time limitations.

Collection and Analyses of Plant Samples

Each chamber consisted of two 3-m rows of plants. Leaf tissue samples were obtained from the third leaf down from a branch apex on four plants in each chamber on July 19 and at the final harvest on October 1. At harvest, plants in two 1-m row segments of each row were unearthed independently with a digging fork to assess biomass and yield from four separate 1-m^2 areas within each chamber. Three plants from each quadrant of a chamber were sampled for biomass measurements (Booker et al., 2007). Plants for biomass determinations were air-dried to a constant mass and weighed. For yield determinations, remaining plants in each quadrant were inverted and left to dry in the field for 1 wk (Burkey et al., 2007). Pods attached to plants as well as those left in the soil during the digging process were collected by quadrant, placed in mesh bags, and dried in a greenhouse. Pod mass was then measured. Pods from the yield harvest and biomass harvest were combined by quadrant and quadrant pod yields averaged to generate a chamber mean for statistical analyses. Pods from each quadrant within a chamber were then pooled, subsamples were shelled, and seed mass was calculated from pod mass and percentage total kernels measured during market grade assessment (Burkey et al., 2007).

Plant samples used for N determinations were oven-dried for 72 h at 65°C, ground to fine powder using a SPEX CertiPrep 8000-D Dual Mixer mill (Metuchen, NJ), and analyzed in triplicate. Total N concentration was determined using a PerkinElmer 2400 CHNS/O elemental analyzer (Norwalk, CT). The $\delta^{15}N$ values in plant samples were measured using a Thermo Finnigan DELTA Plus continuous flow isotope ratio mass spectrometer (CF-IRMS, Bremen, Germany). Nitrogen mass in stem, leaf, and seed tissues was obtained by multiplying tissue sample N concentrations by biomass values for the respective plant component. Plant N mass was determined by summing N mass per plant component (stem, leaf and seeds). Plant N concentration was calculated by summing plant component N concentrations adjusted for the proportional biomass contribution of the component to plant biomass. Similarly, plant $\delta^{15}N$ value was calculated by summing plant component $\delta^{15}N$ values adjusted for the proportional N mass contribution of the component to plant N mass. Our objectives did not include an investigation of treatment effects on N acquisition in root and pod husk tissues.

Table 1. Treatment abbreviations, seasonal 12 h d^{-1} (0800 to 2000 h, Eastern standard time) average CO_2 and O_3 concentrations during the experiment (30 May to 30 Sept. 2002) and number of replicate chambers per treatment.

Treatment abbreviation†	O_3	CO_2	Treatment replicates
	nmol mol^{-1}	μmol mol^{-1}	
CF-376 (control)	21	376	3
CF-550	21	550	2
CF-730	21	730	3
NF-376	49	376	2
NF-550	49	550	2
NF-730	49	730	2
OZ-376	79	376	3
OZ-550	79	550	2
OZ-730	79	730	3

†CF: charcoal-filtered air; NF: nonfiltered air; OZ: nonfiltered air plus 1.6 times ambient O_3; 376: ambient CO_2; 550: ambient plus 174 μmol mol^{-1}; 730: ambient plus 354 μmol mol^{-1}.

Statistical Analyses

The treatments consisted of all combinations of three CO_2 levels and three O_3 levels. Treatments were assigned to chambers in a completely randomized design. Assay results of plant tissue samples obtained within a chamber were averaged for use as a chamber replicate value. Data were checked for homogeneity of variance before the statistical analysis. A ln transformation was applied to the net photosynthesis data before analysis. Treatment effects were statistically analyzed as a 3 × 3 factorial using analysis of variance techniques (SAS Proc GLM, SAS Systems for Windows, Ver. 9.1, SAS Institute, Cary, NC). Comparisons between the control and the other treatments were made using Estimate statements in one-way analysis of variance tests, and multiple comparisons among treatments and among N concentrations in stem, leaf, and seed tissues were performed using the Tukey–Kramer method. Mean values were expressed as LS means.

RESULTS

Net Photosynthesis and Harvest Biomass

Treatment effects were statistically significant for seasonal midday net photosynthesis rates (*A*) (Table 2, Fig. 1). Average *A* was 25% greater in the CF-730 treatment than in the CF-376 (control) treatment. In contrast, *A* was 24 and 46% less in the NF-376 and OZ-376 treatments, respectively, than in the control. Elevated CO_2 fully ameliorated the detrimental effect of O_3 on *A* in the NF-730 and OZ-730 treatments ($CO_2 \times O_3$ interaction $P \leq 0.001$, Table 2).

Treatment effects and interactions on harvest biomass were similar to those observed with *A*, with some exceptions (Table 2, Fig. 1). Elevated CO_2 generally increased biomass production while O_3 suppressed it, and CO_2 ameliorated the O_3 effect. However, biomass accumulation in roots, stems, leaves, and pods was not significantly greater at elevated CO_2 in the charcoal-filtered air treatments

Table 2. Probabilities of elevated CO_2 and O_3 treatment effects on seasonal average net photosynthesis (*A*) at midday and root, stem, leaf, pod and total biomass at harvest.

Source	*A*†	Root	Stem	Leaf	Pod	Total biomass
CO_2	***	**	***	***	***	***
O_3	***	NS‡	NS	*	***	**
$CO_2 \times O_3$	***	NS	NS	**	NS	*

*Significant treatment effects and interactions $P \le 0.05$.

**Significant treatment effects and interactions $P \le 0.01$.

***Significant treatment effects and interactions $P \le 0.001$.

†Does not include the CF-550, NF-550, and OZ-550 treatments (see Table 1 for description).

‡NS, not statistically significant $P > 0.05$.

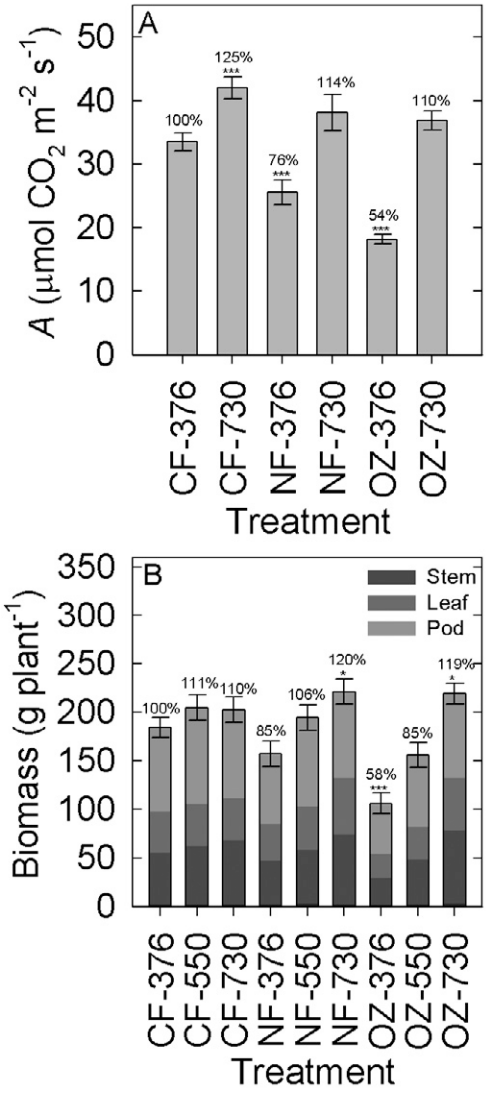

Figure 1. (A) Seasonal midday net photosynthesis (*A*) rates and (B) stem, leaf, pod, and plant biomass at harvest of peanut treated with reciprocal combinations of CO_2 and O_3 in open-top field chambers (see Table 1 for treatment descriptions). Values for root biomass were included in plant biomass determinations but are not discernable in the figure (see "Results" for treatment response descriptions). Values are means ± SE from two or three replicate chambers per treatment. Values above the bars indicate total biomass percentage of the control (CF-376) and asterisks indicate probabilities of statistical difference from the control. Statistical significance: $P \le 0.05$ (*), $P \le 0.001$ (***).

compared with ambient CO_2 (CF-550 and CF-730 versus CF-376). Also, unlike with *A*, biomass accumulation in the NF-376 treatment was not significantly different from the control (CF-376). Biomass in the midlevel CO_2 treatments (NF-550 and OZ-550) was not significantly different from the control, whereas the high-level CO_2 treatments (NF-730 and OZ-730) improved plant growth. Biomass was not significantly different among the high-level CO_2 treatments (CF-730, NF-730 and OZ-730) ($P > 0.05$).

When averaged across the O_3 treatments, biomass accumulation in roots, stems, leaves, and pods was increased by as much as 51, 69, 47, and 25%, respectively, in the elevated CO_2 treatments compared with controls ($P \le 0.01$) (Fig. 1). Elevated O_3 lowered the biomass of leaves and pods by 12 and 23%, respectively, when averaged over the CO_2 treatments ($P \le 0.001$). For leaves, the $CO_2 \times O_3$ interaction was statistically significant (Table 2). Among the individual treatments, root, stem, leaf, and pod biomass was 52 to 61% less in the OZ-376 treatment compared with the control (CF-376) ($P \le 0.05$). Root biomass (not discernable in Fig. 1) declined from 1.90 ± 0.23 g plant^{-1} (mean ± SE) in the CF-376 treatment to 1.02 ± 0.23 g plant^{-1} in the OZ-376 treatment ($P \le 0.05$). The combined effect of elevated CO_2 and O_3 (OZ-730) was also significantly different from the control for root, stem, and leaf biomasses, which increased by 26 to 43% ($P \le 0.05$). Root biomass increased to 2.62 ± 0.23 g plant^{-1} in this treatment. Increases of about 35% in stem and leaf biomasses were observed in the NF-730 treatment compared with the control ($P \le 0.05$).

Leaf Nitrogen Concentration and $\delta^{15}N$ at Mid-Vegetative Growth

Leaf samples collected in July from the elevated CO_2 treatments had significantly lower N concentrations compared with the control (CF-376) (Table 3, Fig. 2A). Foliar N concentrations decreased with increased CO_2 concentrations, from 34 ± 1 g kg^{-1} (mean ± SE) in the control to 27 ± 1 and 23 ± 1 g kg^{-1} in the CF-550 and CF-730 treatments, respectively. In contrast, leaf N concentration was significantly greater by 13% in the OZ-376 treatment compared with the control, but otherwise O_3 treatment effects were minor. Treatment effects on foliar $\delta^{15}N$ values were not statistically significant (Table 3). The overall average foliar $\delta^{15}N$ value was 3.94 ± 0.90‰ (mean ± SD).

Stem, Leaf, and Seed Nitrogen at Harvest

Averaged across all treatments, the mean (± SE) N concentration in stem, leaf, and seed tissues at harvest was 9.7 ± 0.4, 22.7 ± 0.4 and 48.0 ± 0.4 g kg^{-1}, respectively ($P \le 0.001$). Main effects of the CO_2 and O_3 treatments on N concentrations in the stem, leaf, and seed tissue were not statistically significant (Table 4). However, one-way statistical comparisons revealed that a 20% decline in leaf N concentrations in the OZ-550 and OZ-730 treatments

was significantly different from the control ($P \leq 0.05$) (data not shown). There was a significant CO_2 effect on plant N concentration (Table 4, Fig. 2B), which was attributed to an 8% decline in plant N concentration in the high-level CO_2 treatment when averaged over the O_3 treatments ($P \leq 0.06$). Nitrogen concentration in the OZ-730 treatment was 14% less than that in the control ($P \leq 0.01$).

Although there was a tendency for high-level CO_2 to lower plant N concentration in the NF and OZ treatments, elevated CO_2 enhanced total N accumulation in each plant component via an increase in biomass (Table 4, Fig. 1, 3). When averaged across the O_3 treatments, N accumulation in stems, leaves, seeds, and plants was increased by as much as 74, 34, 28, and 35%, respectively, in the elevated CO_2 treatments compared with controls ($P \leq 0.05$). There was no statistically significant effect of the NF treatments on total N mass in stems, leaves, seeds, and plants (Fig. 3). Total N accumulation in leaves, seeds, and plants was decreased by 46, 36, and 40%, respectively, in the OZ-376 treatment compared with the control (Table 4, Fig. 3). The main effect of O_3 on stem N mass was not statistically significant (Table 4), but N accumulation in stems from the high O_3 treatment (OZ-376) was 55% less than in the control ($P \leq 0.05$) (Fig. 3). The $CO_2 \times O_3$ interactions for N concentration and mass were not statistically significant (Table 4).

Stem, Leaf, and Seed $\delta^{15}N$ at Harvest

Compared with the average leaf $\delta^{15}N$ value obtained at midgrowth, the control leaf $\delta^{15}N$ value at harvest (2.71 ± 0.39‰) was 31% lower ($P \leq 0.05$). Values of $\delta^{15}N$ for stems, leaves, seeds, and plants collected at harvest decreased by 20 to 41% with elevated CO_2 (Table 4, Fig. 4). There were no significant effects of elevated O_3 on $\delta^{15}N$ values in stems, leaves, and plants (Table 4). However, $\delta^{15}N$ values for seeds were 47 and 56% greater in the NF-376 and OZ-376 treatments, respectively, compared with the control ($P \leq 0.05$) (Fig. 4). As in the charcoal-filtered air treatments, $\delta^{15}N$ values for seeds in the elevated O_3 treatments declined with increased concentrations of CO_2 (Fig. 4). However, no significant $CO_2 \times O_3$ interactions were detected (Table 4).

DISCUSSION

Atmospheric CO_2 enrichment usually enhances plant growth and biomass production (Kimball et al., 1993; Nösberger et al., 2006; Prasad et al., 2005; Reddy and Hodges, 2000). In our experiment, elevated CO_2 increased seasonal *A* and total biomass at harvest (averaged over the O_3 treatments) (Table 2, Fig. 1). However, biomass production was less than what might be expected given the substantial stimulation of *A* with elevated CO_2. This suggests that competition for nutrients, space, light, and other resources may limit plant biomass responses to increased CO_2, as previously suggested (Poorter and Navas, 2003; Reid and Fiscus, 2008). Plants may also be unable to acquire sufficient quantities of nutrients utilizable at higher CO_2 concentrations because of changes in physiology and growth that reduce nutrient uptake efficiency, thus limiting plants in their ability to attain greater benefit from increasing atmospheric CO_2 concentrations (Pritchard and Rogers, 2000).

Table 3. Probabilities of elevated CO_2 and O_3 treatment effects on foliar N concentration and $\delta^{15}N$ values in tissue samples collected at mid-vegetative growth (July).

Source	N concentration	δ15N
CO_2	***	NS†
O_3	*	NS
$CO_2 \times O_3$	NS	NS

*Significant treatment effects and interactions $P \leq 0.05$.
***Significant treatment effects and interactions $P \leq 0.001$.
†NS, not statistically significant $P > 0.05$.

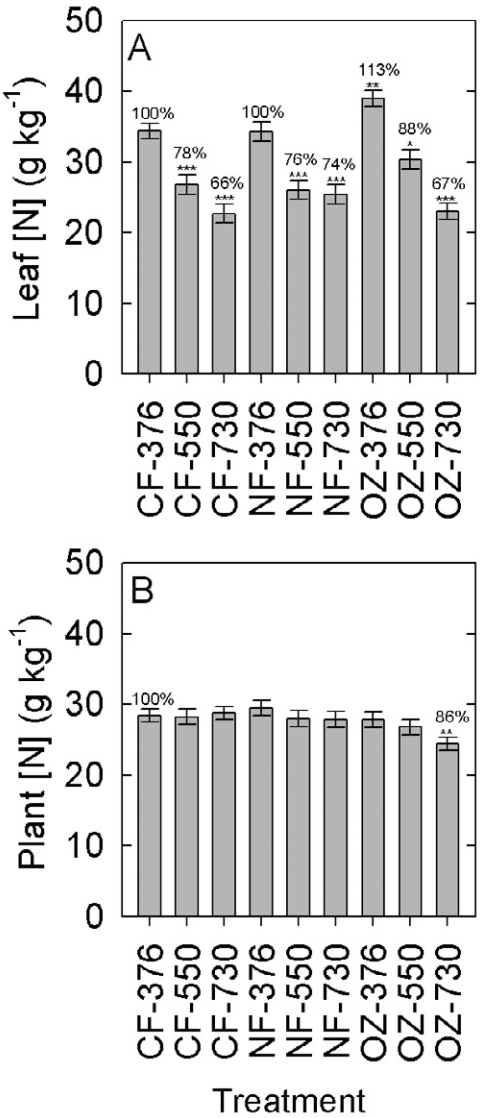

Figure 2. Mean (± SE) N concentrations (A) in leaves at mid-vegetative growth and (B) in plants at harvest of peanut exposed to mixtures of CO_2 and O_3 in open-top field chambers (see Table 1 for treatment descriptions). Values shown above the bars indicate percentage of the control treatment (CF-376), and asterisks indicate probabilities of statistical difference from the control. Statistical significance: $P \leq 0.05$ (*), $P \leq 0.001$ (***).

Table 4. Probabilities of elevated CO_2 and O_3 treatment effects on N concentration (conc.), N mass, and $\delta^{15}N$ values in peanut stem, leaf, seed, and composite plant tissues at final harvest.

Source	Stem			Leaf			Seed			Plant		
	N conc.	N mass	$\delta^{15}N$	N conc.	N mass	$\delta^{15}N$	N conc.	N mass	$\delta^{15}N$	N conc.	N mass	$\delta^{15}N$
CO_2	NS†	**	*	NS	*	*	NS	***	***	*	***	***
O_3	NS	NS	NS	NS	*	NS	NS	**	*	NS	**	NS
CO_2 x O_3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

*Significant treatment effects and interactions $P \leq 0.05$.

**Significant treatment effects and interactions $P \leq 0.01$.

***Significant treatment effects and interactions $P \leq 0.001$.

†NS, not statistically significant $P > 0.05$.

Increased biomass production with elevated CO_2 often includes increased carbon allocation belowground via a larger root biomass with greater root length and more branching (Rogers et al., 1992). However, changes in root architecture with elevated CO_2, such as increased production of shallow, lateral roots, may render root systems less efficient at nutrient uptake (Pritchard and Rogers, 2000). For this and other reasons, root alterations at elevated CO_2 may be unable to provide proportionally more nutrients, and nutrient uptake efficiency and concentration in plant tissues decline (Rogers et al., 1999; Pritchard and Rogers, 2000; Taub and Wang, 2008). Results from our experiment showed that elevated CO_2 significantly reduced leaf N concentrations during mid-vegetative growth but did not affect $\delta^{15}N$ values. Lower leaf N concentrations with elevated CO_2 have been attributed in part to reduced nutrient uptake resulting from decreased transpiration rates and to a decrease in the N requirement for growth and thus demand for N (Cotrufo et al., 1998; Rogers et al., 1999; Taub and Wang, 2008). In our study, midday stomatal conductance declined by 42% at twice-ambient CO_2 (Booker et al., 2007). Decreased plant tissue N concentration with elevated CO_2 can also be related to dilution effects from increased biosynthesis of structural and nonstructural carbohydrates, as found in our study for leaf mass per area and starch levels in upper canopy leaves (Booker et al., 2007). Regression analysis of seasonal N and starch concentrations indicated that 7% of the decline in leaf N concentration was associated with increases in starch concentration. Lastly, the possibility exists that increased loss of N through root exudation with elevated CO_2 contributed to lower leaf tissue N concentrations as well (Taub and Wang, 2008).

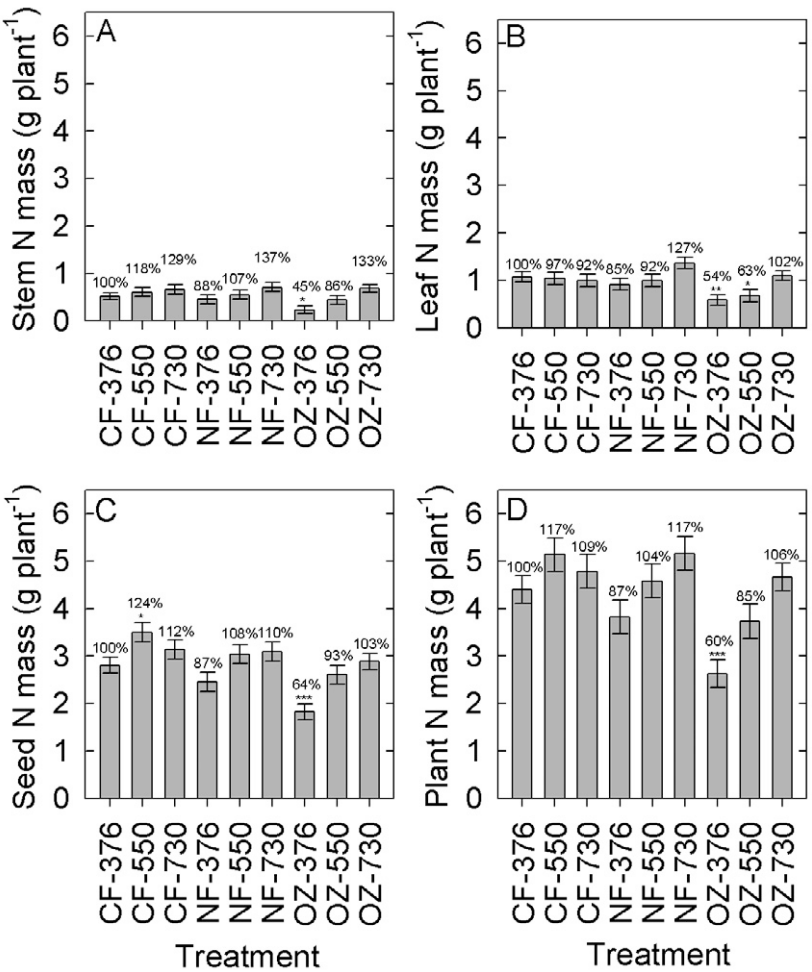

Figure 3. Mean (± SE) N mass in peanut stem, leaf, seed, and composite (stem + leaf + seed) plant samples at harvest following treatment with mixtures of CO_2 and O_3: (A) stems, (B) leaves, (C) seeds, and (D) plant composite (see Table 1 for treatment descriptions). Values above the bars indicate percent of the control treatment (CF-376) and asterisks indicate probabilities of statistical difference from the control. Statistical significance: $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

Once CO_2–enhanced root systems deplete readily available nutrients, plants need to acquire nutrients from alternative sources to sustain their responses to CO_2 enrichment. In some plants, CO_2 enrichment enhances arbuscular mycorrhizal growth and plant root colonization, which improve N acquisition from the soil (Hu et al., 2005; Treseder, 2004). Symbiotic N_2 fixation in N_2–fixing plants can also be enhanced during the growing season due to both reduced soil N availability (Zanetti et al., 1996) and increased nodule weight and number (Khan and Yoshida, 1994). Schiffmann and Löbel (1973) demonstrated that soil mineral N supply was important for leaf N in the early period of peanut growth while N_2

fixation played an increasingly important role in maintaining N levels as the growing season progressed. Evidence for this transition was found in our study. At harvest, the leaf $\delta^{15}N$ value in the control (CF-376) treatment was lower than at midseason. Lower $\delta^{15}N$ values indicated that the contribution by N_2 fixation to N acquisition increased during the latter part of the growing season. Furthermore, decreasing $\delta^{15}N$ values with elevated CO_2 indicated that symbiotic N_2 fixation increasingly contributed to maintaining N levels in peanut tissues.

In some legumes, elevated CO_2 enhances symbiotic N_2 fixation (Finn and Brun, 1982; Phillips et al., 1976; Rogers et al., 2006; West et al., 2005; Zanetti et al., 1996). This is likely related to increased availability of photosynthate from increased *A* that stimulates growth and demand for N along with increased C allocation belowground (Finn and Brun, 1982; Hu et al., 2006; Rogers et al., 2006). Increased C availability with elevated CO_2 could be advantageous for symbiotic N_2 fixation because it can be more carbohydrate costly compared to nitrate mineral nutrition (Chapin et al., 2002; Pate et al., 1979; Ryle et al., 1979). However, species conversion efficiency of C into dry matter depends on several factors, including root development, symbiotic N_2 fixation efficiency and the root contribution to nitrate assimilation (Atkins et al., 1980; Pate et al., 1979). It has been shown that with elevated CO_2, stimulated nitrogenase activity in legumes was directly correlated with enhanced C supply to nodules, as measured by ^{14}C specific activity in nodules (Finn and Brun, 1982; Tissue et al., 1997). It was generally concluded that CO_2 enrichment promotes symbiotic N_2 fixation by increasing root nodule development per plant rather than by increasing specific activity per nodule (Finn and Brun, 1982; Phillips et al., 1976), although the opposite effect was found in a tropical N_2–fixing woody legume (Tissue et al., 1997). Rogers et al. (2006) examined dynamics of foliar ureide, amino acid, and N concentrations in soybean grown at elevated CO_2 concentrations in free-air conditions and found that CO_2 enrichment ameliorated the early season decline in plant N concentration, indicating improved N assimilation and probably N_2 fixation during reproductive growth. In our study, small differences in plant N concentration among treatments at harvest (Fig. 2) and decreased $\delta^{15}N$ values with elevated CO_2 (Fig. 4) in physiologically mature plant tissues showed that CO_2 enhanced N acquisition from the soil and through symbiotic N_2 fixation in peanut. Both of these processes were modified by O_3.

Increased concentrations of O_3 decreased N mass in leaves, seeds, and plants while the $\delta^{15}N$ value in seeds and plants increased, suggesting that symbiotic N_2 fixation was inhibited at elevated O_3. These effects were counteracted by elevated CO_2, although $CO_2 \times O_3$ interactions were not statistically significant. This suggests only partial amelioration of toxic O_3 effects at elevated CO_2 concentrations, particularly in the high-level O_3 treatment. The impact of elevated O_3 on N metabolism in legumes has not been fully explained (Flagler et al., 1987; Pausch et al., 1996). In our study, elevated O_3 had little effect on foliar $\delta^{15}N$ values at mid-vegetative growth, suggesting that elevated O_3 did not directly affect the N source for peanut during the early growing season. However, greater $\delta^{15}N$ values in seeds from the elevated O_3 treatments implied that plants obtained proportionately more N from the soil, possibly indicating O_3 inhibition of symbiotic N_2 fixation. Elevated O_3 concentrations typically inhibit *A* by decreasing Rubisco activity and reducing leaf longevity (Fiscus et al., 2005; Pell et al., 1997). These modifications inhibit C assimilation, plant biomass production, and C allocation belowground (Andersen,

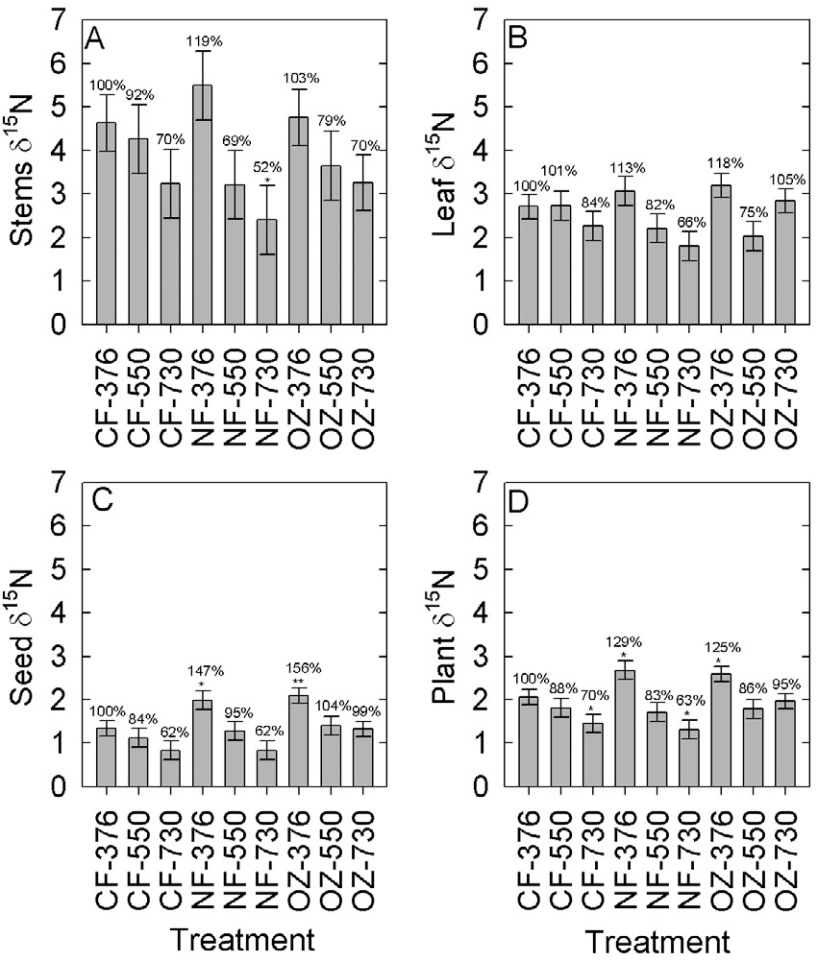

Figure 4. Mean (± SE) $\delta^{15}N$ values of peanut stem, leaf, seed, and composite (stem + leaf + seed) plant samples at harvest following treatment with mixtures of CO_2 and O_3: (A) stems, (B) leaves, (C) seeds, and (D) composite plant (see Table 1 for treatment descriptions). Values above the bars indicate percent of the control treatment (CF-376) and asterisks indicate probabilities of statistical difference from the control. Statistical significance: $P \leq 0.05$ (*); $P \leq 0.01$ (**), $P \leq 0.001$ (***).

2003; Fuhrer and Booker, 2003). The resulting decreases in root growth as well as suppressed C and energy availability for rhizobial bacteria may therefore inhibit symbiotic N_2 fixation. Several studies with elevated O_3 have documented lower number and weight of nodules on legumes (Blum and Tingey, 1977; Letchworth and Blum, 1977; Manning et al., 1973), and reduced total reductase activity rather than specific activity, suggesting that N_2 fixation was indirectly suppressed by O_3 through effects on C assimilation and availability (Flagler et al., 1987; Pausch et al., 1996).

A number of experiments have shown that elevated CO_2 can offset the adverse effects of O_3 on crop biomass production and yield (Fiscus et al., 2005; Fuhrer, 2003; Olszyk et al., 2000). The protective effect of elevated CO_2 against O_3 injury has been observed in a number of C_3 plant species, including cotton, peanut, rice, soybean, and wheat, due in large part to a reduction in O_3 uptake from reduced stomatal conductance and possibly from increases in photoassimilation rates and antioxidant metabolism (Booker and Fiscus, 2005; Booker et al., 2007; Fiscus et al., 2005; McKee et al., 2000). If the effects of elevated CO_2 and O_3 on N acquisition are related to their impacts on C and energy availability to N-fixing bacteria, it is likely that CO_2 stimulation of *A* would help ameliorate the O_3 indirect inhibitory effects on N_2 fixation.

Data from our experiment showed that the $\delta^{15}N$ signature in different plant components was in order of stems > leaves > seeds, suggesting that symbiotic N_2 fixation is important for maintaining seed N concentrations and that CO_2 enhancement of symbiotic N_2 fixation may compensate for low soil N availability. Plant tissue $\delta^{15}N$ signatures are known to vary, depending on the N source, assimilation preferences, partitioning patterns, and N_2 fixation activity (Evans, 2001; Wanek and Arndt, 2002). Bell and Wright (1994) showed that symbiotic N_2 fixation contributed 61 to 84% of pod N in peanut and that most of the remobilized N from the vegetative parts to the pods was exclusively N derived originally from N_2 fixation. It seems likely that newly fixed N_2 will become even more important in maintaining seed yield and protein accumulation under elevated CO_2 as CO_2 enhancement of plant vegetative growth will likely deplete soil N pool faster than at ambient CO_2 concentrations (Hu et al., 2006; Reich et al., 2006).

CONCLUSIONS

In our study, elevated atmospheric CO_2 affected peanut N acquisition differentially depending on O_3 concentrations although the effects were likely mediated in part by changes in C assimilation. Results suggested that atmospheric CO_2 enrichment enhanced plant N acquisition through symbiotic N_2 fixation during reproductive growth and increasing O_3 concentrations partially inhibited it, while elevated CO_2 counteracted this effect. Leaf N concentrations were up to 44% lower with elevated CO_2 at midseason, but plant N concentrations only differed by 8% among CO_2 treatments at harvest while N_2 fixation was increased (as evidenced by declining $\delta^{15}N$ values). Ozone suppressed biomass production, which limited N mass per plant and strongly inhibited N_2 fixation (as evidenced by higher $\delta^{15}N$ values). Elevated CO_2 attenuated O_3 effects on biomass and N mass per plant, including an increase in N_2 fixation (as evidenced by declining $\delta^{15}N$ values with increasing CO_2 levels in the NF and OZ treatments). Although the effects of elevated O_3 on N accumulation and $\delta^{15}N$ values were ameliorated with elevated CO_2, the $CO_2 \times O_3$ interactions were not statistically significant, which indicated that detrimental O_3 effects were not fully abated with elevated CO_2. These results suggest that the high O_3 concentrations used in this experiment, which occur or are predicted to occur in some major peanut producing regions worldwide (Booker et al., 2009; Vingarzan, 2004; Wang and Mauzerall, 2004), can critically modify plant responses to rising atmospheric CO_2 concentrations, in part by suppressing plant N acquisition.

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