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ScienceDirect

Environmental Pollution 150 (2007) 355–362

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Leaf extracellular ascorbate in relation to O_3 tolerance of two soybean cultivars

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Received 5 September 2006; received in revised form 22 January 2007; accepted 27 January 2007

Leaf extracellular metabolites other than ascorbic acid are a factor in the differential ozone tolerance of two soybean cultivars.

Abstract

Soybean [*Glycine max* (L.) Merr.] cultivars Essex and Forrest that exhibit differences in ozone (O_3) sensitivity were used in greenhouse experiments to investigate the role of leaf extracellular antioxidants in O_3 injury responses. Charcoal-filtered air and elevated O_3 conditions were used to assess genetic, leaf age, and O_3 effects. In both cultivars, the extracellular ascorbate pool consisted of 80–98% dehydroascorbic acid, the oxidized form of ascorbic acid (AA) that is not an antioxidant. For all combinations of genotype and O_3 treatments, extracellular AA levels were low (1–30 nmol g^{-1} FW) and represented 3–30% of the total antioxidant capacity. Total extracellular antioxidant capacity was twofold greater in Essex compared with Forrest, consistent with greater O_3 tolerance of Essex. The results suggest that extracellular antioxidant metabolites in addition to ascorbate contribute to detoxification of O_3 in soybean leaves and possibly affect plant sensitivity to O_3 injury.
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Keywords: Antioxidant capacity; Apoplast; Ascorbic acid; Dehydroascorbic acid; Ozone tolerance; Soybean

1. Introduction

During the twentieth century, rapid industrialization has contributed significant amounts of toxic gaseous pollutants to the atmosphere, which eventually become damaging to many ecosystems (Wellburn, 1994; Karnosky et al., 2003). Among the various air pollutants, ozone (O_3) causes the most damage to plants (Krupa and Manning, 1988; Heagle, 1989). Ozone was first described to have toxic effects on plants when it was associated with foliar injury and suppressed growth in grape (*Vitus* spp.) (Richards et al., 1958). Ensuing research indicated that ambient O_3 concentrations in many industrialized regions worldwide significantly reduce yields of susceptible crops, which is accompanied by substantial economic costs (Wang and Mauzerall, 2004; Fiscus et al., 2005; U.S. EPA, 2006).

Ozone initiates toxicity in plants mainly via uptake by the foliage (Runeckles, 1992). Once O_3 has entered a leaf through open stomata, it dissolves into the aqueous phase of the cell wall (Laisk et al., 1989) and can react with apoplastic and symplastic components of the cell (Pryor and Church, 1991; Runeckles and Chevone, 1992; Long and Naidu, 2002; Fuhrer and Booker, 2003; Fiscus et al., 2005). In the apoplast, O_3 likely reacts with water, ascorbic acid (AA), phenolics, transition metals, and thiols to form reactive oxygen species (ROS) (Long and Naidu, 2002). The ROS are thought to include superoxide, singlet oxygen, hydroxyl radicals, and hydrogen peroxide (Heath, 1987). Studies suggest that in addition to being toxic, O_3-derived ROS interact with signaling pathways that influence plant stress responses, including programmed cell death (Sandermann, 1998; Schraudner et al., 1998; Wohlgemuth et al., 2002; Overmyer et al., 2003; Kangasjarvi et al.,

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doi:10.1016/j.envpol.2007.01.022

2005). It is hypothesized that if the ROS generated directly from O_3 and indirectly by induced plant-derived oxidative bursts are not detoxified within the extracellular space, ROS can initiate reactions that lead to damage (Sandermann, 1998; Rao et al., 2000; Overmyer et al., 2003; Kangasjarvi et al., 2005). Ozone exposure is known to impair ion regulation, induce stress ethylene production, stimulate antioxidant and phenylpropanoid metabolism, and eventually suppress carboxylation activity and carbon assimilation (Heath, 1987; Runeckles and Chevone, 1992; Pell et al., 1997; Guidi et al., 2001; Fuhrer and Booker, 2003; Fiscus et al., 2005).

Antioxidant compounds localized in the apoplast are potential scavengers of ROS that could attenuate O_3 injury. To date, most attention has focused on AA (Plöchl et al., 2000; Conklin and Barth, 2004; Chen and Gallie, 2005). Ascorbic acid is synthesized in the cell and then transported into the leaf apoplast, where it plays a role in many cell wall processes (Smirnoff, 2000; Smirnoff et al., 2001). Ascorbic acid can protect against O_3 damage by reacting directly with O_3 (Chameides, 1989), by scavenging ROS, and by serving as a substrate in enzymatic reactions that scavenge ROS (Polle et al., 1990; Chen and Gallie, 2005; Fiscus et al., 2005). Ascorbate biosynthesis and transport has been implicated in cell wall biosynthesis and signaling processes as well (Conklin and Barth, 2004). Previous studies found that apoplastic AA is oxidized during O_3 exposures, resulting in the production of dehydroascorbic acid (DHA), which is then transported back into the cytoplasm where it is reduced again to AA by coupled reactions involving DHA reductase and reduced glutathione (Luwe et al., 1993; Noctor and Foyer, 1998; Horemans et al., 2000). Apoplastic AA concentration and redox status change in response to O_3 in some plants, suggesting that extracellular AA may be involved in O_3 detoxification processes (Castillo and Greppin, 1988; Luwe and Heber, 1995; Burkey and Eason, 2002; Conklin and Barth, 2004). Sensitivity to O_3 among genotypes of snap bean (*Phaseolus vulgaris* L.) (Burkey, 1999; Burkey and Eason, 2002; Burkey et al., 2003) and *Plantago major* (Zheng et al., 2000) is correlated with concentrations of extracellular AA. *Arabidopsis thaliana* mutants with low foliar concentrations of AA (*vtc*1) exhibit hypersensitivity to O_3 (Conklin and Barth, 2004). Transgenic tobacco (*Nicotiana tabacum* L.) plants with altered expression of DHA reductase exhibited changes in leaf AA concentrations that positively correlated with their tolerance to O_3 (Chen and Gallie, 2005). However, the efficacy of AA in protecting plants against O_3 injury has been questioned in some studies because apoplastic concentrations appeared to be insufficient for effective detoxification of ROS (Luwe et al., 1993; Turcsanyi et al., 2000). Also, the differential O_3 sensitivity of NC-S and NC-R clover (*Trifolium repens* L.) clones was not correlated with apoplastic AA concentrations (D'Haese et al., 2005).

Therefore, the objective of this study was to determine whether the difference in O_3 sensitivity between two soybean (*Glycine max* (L.) Merr.) genotypes, Essex and Forrest, could be related to differences in leaf extracellular AA concentration and ascorbate redox status. Previous studies have found that Forrest was more sensitive than Essex to O_3 injury (Chernikova et al., 2000; Robinson and Britz, 2000). The hypothesis was tested by comparing apoplastic AA metabolites and total extracellular antioxidant capacity with visible O_3 injury in leaves of Essex and Forrest soybeans.

2. Materials and methods

2.1. Plant growth and treatment conditions

Essex and Forrest seeds were germinated in moist paper towels incubated at 29 °C for 3 days. Four seedlings per pot were transplanted into 6-l pots containing Metro-Mix 200 supplemented with slow release fertilizer (Osmocote Plus, Scotts-Sierra Horticultural Products, Marysville, OH, USA) and placed in a greenhouse supplied with charcoal-filtered (CF) air (<10 nmol O_3 mol^{-1}). Supplemental lighting was used to provide a day length of 20 h. After seedlings were established, plants were thinned to one per pot.

To determine antioxidant levels under clean-air conditions, plants were grown in CF air on three benches in a greenhouse for 4 weeks in April–May 2003. Two plants on each bench were sampled for metabolite assays. Sampling of tissues from the second, fourth, and sixth main stem leaves (counting acropetally) was conducted between 10:00 and 15:00 h on three consecutive days beginning at 28 days after planting (DAP).

To determine O_3 treatment effects on apoplastic antioxidants in the two genotypes, plants were grown for 3 weeks in CF air during October–November 2003 in a greenhouse and then placed in 1.2 m^3 continuous-stirred tank reactors (CSTRs) for 2 days before the 6-day O_3 treatment period began. One plant of each genotype was placed in each of 20 chambers. Plants were treated with CF air (1.4 ± 0.6 nmol O_3 mol^{-1}) or CF air plus O_3 (77 ± 5 nmol O_3 mol^{-1}) 7 h (09:00–16:00 h) daily. Relative humidity in the CSTRs was maintained at an average of 53% during the 7 h exposure period by adding steam to the chambers. Chamber temperature and photosynthetic photon flux density (PPFD) averaged 28.5 °C and 397 μmol m^{-2} s^{-1}, respectively, during O_3 exposure. Tissue samples for determination of AA metabolites were obtained from the second main stem leaf between 10:00 and 13:00 h on day 6 of the O_3 treatments.

2.2. Determination of visible injury

An assessment of visible O_3 injury to the first through the fifth main stem leaves was conducted one to 2 days after completion of the 6-d O_3 treatment. The level of foliar injury was determined by estimating the percentage of adaxial leaf surface area exhibiting stipple and necrotic or chlorotic lesions. Three observers assessed injury on each leaf and the results were averaged for use in statistical comparisons.

2.3. Determination of stomatal conductance

Leaf conductance of the two cultivars was measured using a steady-state porometer (Model 1600M, Li-Cor, Inc., Lincoln, Nebraska, USA) to ascertain whether differences in O_3 sensitivity between the genotypes might be related to O_3 uptake rates. Measurements were taken from 11:00 to 15:00 h on the abaxial and adaxial surfaces of the second leaf on the third and fourth day of O_3 treatment in the chambers. During the measurements, average relative humidity, PPFD, and leaf temperature were 50%, 312 μmol m^{-2} s^{-1}, and 25 °C, respectively. Leaf conductances were corrected for the standard boundary layer conductance imposed by the instrument (2.7 mol m^{-2} s^{-1}, Li-Cor, Inc., 1600M Instruction Manual, Revision 6, 1989) and reported as stomatal conductance (g_s). Statistical analyses were conducted using the average g_s value obtained for each plant over the 2-day period.

2.4. Leaf intercellular washing fluid (IWF) infiltration and extraction

Leaves were excised from plants, placed in plastic bags, and immediately processed for IWF infiltration and extraction. After the leaf mid-vein was removed and initial fresh weight (FW) was determined, tissue samples were

vacuum infiltrated and extracted using the procedure described by Luwe and Heber (1995), with modifications (Burkey, 1999). Briefly, leaf tissues were placed in 50 ml of 100 mM KCl in a 60 ml syringe barrel. A syringe plunger was inserted into the barrel, the chamber was cleared of air, and sealed. The tissues were infiltrated by several retractions and compressions of the plunger. Afterward, leaf tissues were blotted dry, weighed, rolled into a cylinder, and inserted into a 5 ml syringe barrel that was placed inside a 15 ml polypropylene centrifuge tube containing a 1.5 ml microfuge tube. The nested tubes were centrifuged at 556 *g* for 5 min at room temperature, and the IWF was recovered in the microfuge tube. IWF recovery efficiency (Essex: $42 \pm 1\%$; Forrest: $38 \pm 1\%$, mean $\pm$ SD) was calculated from measurements of leaf tissue mass before and after infiltration, and again following IWF extraction. IWF was recovered into a microfuge tube containing 0.05 ml of 6% (w/v) meta-phosphoric acid and 0.2 mM diethylenetriamine pentaacetic acid (DTPA) to prevent oxidation of ascorbate. The IWF extracts were placed on ice, centrifuged at 11 000 *g* for 2 min at 4 °C to pellet precipitated protein, and the supernatants were stored at −80 °C. Following IWF extraction, leaf tissue samples were frozen in liquid N_2 and stored at −80 °C for analysis of AA and DHA contents and guaiacol peroxidase (GuPOD) activity.

Glucose 6-phosphate (G6P) was used as a marker for cytoplasmic contamination of IWF in all IWF extracts. G6P was detected using commercial glucose 6-phosphate dehydrogenase (EC 1.1.1.49) as described previously (Burkey, 1999). Detection of G6P in an IWF sample served as a criterion for exclusion of the sample from the data.

2.5. Guaiacol peroxidase (GuPOD) assay

Frozen leaf tissue samples were ground in liquid N_2 using a mortar and pestle. Powdered tissue samples (0.5 g) were combined with 0.5 g insoluble polyvinyl-polypyrrolidone and extracted with 3.75 ml of ice-cold 66 mM KP_i, 100 mM KCl (pH 7.0) followed by centrifugation at 28 000 g for 10 min at 4 °C. Guaiacol peroxidase (EC 1.11.1.7) activity in the supernatant was determined by measuring the increase in absorbance at 436 nm at 25 °C with an extinction coefficient of 25 $mM^{-1} cm^{-1}$ (Polle et al., 1990). The 1-ml assay contained 100 mM KP_i buffer (pH 6.3), 10 mM H_2O_2, 40 mM guaiacol, and 20 µl of plant extract. Protein content of the extracts was measured using the Bradford method with bovine serum albumin as the standard (Bradford, 1976).

2.6. Ascorbate extraction and assay

Frozen leaf tissue samples were ground in liquid N_2 using a mortar and pestle. Powdered tissue samples (0.2 g) were extracted with 2 ml of cold extraction buffer [6% (w/v) meta-phosphoric acid, 0.2 mM DTPA]. The homogenate was centrifuged at 10 500 *g* for 10 min at 4 °C, and supernatants were recovered for analysis of AA and DHA.

AA concentration in IWF and total leaf extracts was determined by measuring the decrease in absorbance at 265 nm in the presence of ascorbate oxidase (AO) (Luwe and Heber, 1995). The assay solution contained 100 mM KP_i buffer (pH 7.0) and an aliquot of plant extract in a total volume of 1 ml. The absorbance at 265 nm was measured before and after addition of a 1 µl-aliquot of 1 U of AO (EC 1.10.3.3, Calbiochem-Novabiochem, La Jolla, CA, USA). DHA concentration in IWF and total leaf extracts was determined by measuring the increase in absorbance at 265 nm in the presence of dithiothreitol (Luwe and Heber, 1995). The reaction solution contained 100 mM KP_i buffer (pH 7.0) and an aliquot of extract in a total volume of 1 ml. The absorbance at 265 nm was measured before and after addition of a 1 µl-aliquot of 200 mM dithiothreitol. An extinction coefficient of 14.3 $mM^{-1} cm^{-1}$ at 265 nm was used for calculation of AA and DHA concentrations.

For calculations of apoplast AA and DHA concentration per leaf, the recovery efficiency for each sample was used to normalize the values to 100% recovery. Ascorbate redox status was expressed as [AA]/[AA + DHA].

2.7. Total antioxidant capacity assay

Total antioxidant capacity in leaf IWF extracts was determined using the ferric reducing ability of plasma (FRAP) assay based on the reduction of Fe^{+3} to Fe^{+2} by antioxidant compounds in a non-specific reaction (Benzie and Strain, 1996; Deighton et al., 2000). Briefly, an aliquot (typically 30 µl) of IWF was combined with 900 µL of FRAP reagent, and distilled water in a total volume of 1 ml. The FRAP reagent consisted of 25 ml of 300 mM acetate buffer (pH 3.6), 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine dissolved in 40 mM HCl, and 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$. Reactions were conducted in individual micro-centrifuge tubes, incubated at 37 °C for 5 min, and then centrifuged at 12 000 *g* for 2 min to remove a precipitate formed by the presence of KCl in the infiltration solution used to isolate the IWF. Absorbance at 593 nm of the supernatant was determined and compared with a standard curve (100–1000 µM Fe^{+2} from $FeSO_4$). Preliminary tests showed that precipitate formation did not interfere with color formation in the assay, but for protocol consistency KCL equivalent to the unknowns was included in the ferrous sulfate reactions used to construct the standard curve. The IWF recovery percentage for each leaf was used to calculate antioxidant capacity in ferrous ion equivalents so that the reported values are normalized to reflect 100% recovery.

2.8. Statistics

Statistical comparisons were conducted using analysis of variance (SAS for Windows, Version 8, SAS Institute Inc., Cary, NC). The experiment comparing soybean genotypes under clean-air conditions (CF air) was a split-plot, randomized complete block with genotype as the main plot factor and leaf position as the subplot factor. Greenhouse benches were treated as blocks in the analysis. There were four pots of each genotype on each of three benches. Because the volume of IWF extract per leaf was insufficient for the antioxidant assays, IWF extracts from leaves at the same position on two plants in the same experimental block were pooled. Data were checked for homogeneity of variance prior to statistical analysis. A ln transformation was applied to the AA content and redox status data prior to statistical analysis. To accommodate pooling of IWF samples, standard errors for comparisons among different leaf positions within the same genotype were calculated as $(2 \times \text{MSE}/n)^{1/2}$, and as $[2(2 \times \text{MSE} + \text{MS Rep} \times \text{Genotype})/3 \times n]^{1/2}$ for comparisons between genotypes at the same leaf position, where *n* is the number of replicates, MSE is the mean square error, and MS Rep × Genotype is the mean square for the interaction effect of replicate and genotype.

The O_3 treatment experiment was a split-plot, randomized complete block with O_3 treatment level as the main plot factor and genotype as the subplot factor. Twenty CSTRs were divided into 10 experimental blocks consisting of pairs of CF and O_3 treatments, with one plant of each genotype placed in each chamber. Ten blocks were used to assess canopy injury profiles. Eight blocks were used to assess stomatal conductance of the second trifoliate leaf. Six blocks were used to assess metabolite concentrations in the second trifoliate leaf. To obtain sufficient quantities of IWF extract for the assays, six experimental blocks were grouped into three based on similarity of O_3 injury in the O_3 treatment chambers. IWF extract from one plant in each of two CSTRs was pooled and assayed as one sample. Data were checked for homogeneity of variance prior to statistical analysis. A ln transformation was applied to apoplast and whole-leaf ascorbate content and redox status data. To account for pooling of IWF samples, the standard errors were calculated as $(2 \times \text{MSE}/n)^{1/2}$ for comparisons among different genotypes within the same O_3 treatment, and as $[2(2 \times \text{MSE} + \text{MS Rep} \times \text{Treatment})/3 \times n]^{1/2}$ for comparisons between O_3 treatments within the same genotype where MS Rep × Treatment is the mean square for the interaction effect of replicate and treatment.

3. Results

3.1. Leaf position and genotype effects on antioxidants under clean-air conditions

3.1.1. Apoplast ascorbate

AA concentration in IWF extracts was not statistically significantly different between genotypes or among leaf

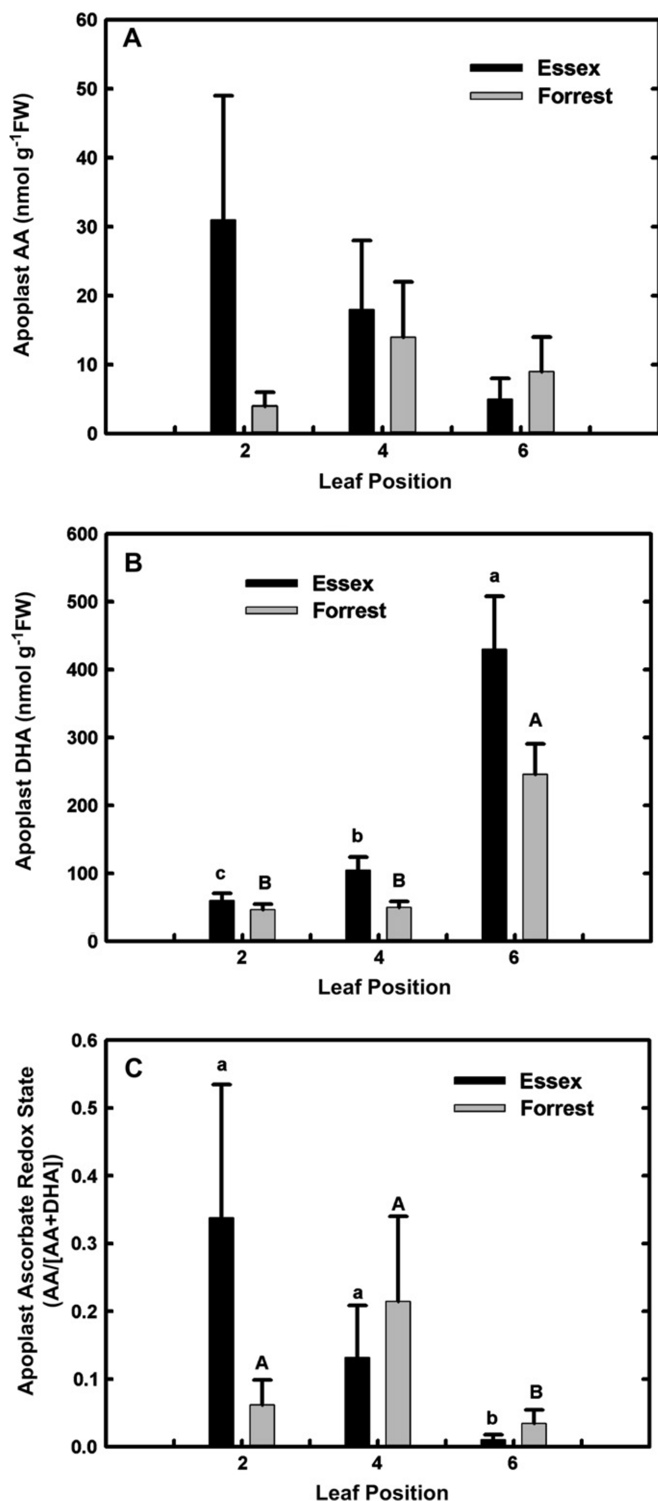

Fig. 1. Canopy profile of apoplast AA (A), DHA (B) and ascorbate redox status (C) in soybean leaves. Values are least square means ± SE ($n = 3$). Values marked with the different letters are statistically significantly different responses among leaf positions for the same genotype ($P \leq 0.05$). There were no significant genotype effects.

positions for plants grown in CF air (Fig. 1A). Youngest leaves (leaf position 6) of both genotypes had the highest DHA level (Fig. 1B) and lowest ascorbate redox ratio (Fig. 1C).

3.1.2. Apoplast total antioxidant capacity

Apoplast total antioxidant capacity, as assessed by the reduction of Fe^{+3} to Fe^{+2} in the FRAP assay, was not significantly different among leaf positions for either genotype (Fig. 2). However, apoplast total antioxidant capacity was 2.5-fold and 1.4-fold higher in the second and fourth leaves, respectively, of Essex (O_3-tolerant) compared with Forrest (O_3-sensitive).

3.1.3. Leaf tissue ascorbate concentrations

In whole-leaf tissue samples, there were no statistically significant differences in AA and DHA concentrations between genotypes at any leaf position. However, older leaves (leaf position 2) in both genotypes had significantly lower AA (Fig. 3A) and DHA (Fig. 3B) concentrations than younger leaves (leaf positions 4 and 6), although ascorbate redox state was similar in all leaves (Fig. 3C).

3.2. O_3 treatment effects

3.2.1. Leaf age and genotype effects on O_3 injury responses

In both genotypes, foliar injury due to O_3 was significantly less in younger leaves in the upper canopy (e.g. leaf positions 4 and 5) compared with older leaves in the lower canopy (leaf positions 1 and 2) (Fig. 4). In older leaves, Essex exhibited significantly less O_3 injury than Forrest. Foliar injury in Essex averaged 12% less than in Forrest for leaf positions 1 through 3 (Fig. 4). Ozone exposure stimulated GuPOD activity in extracts from leaf 2 (Table 1), but activities were 25% lower in Essex compared with Forrest plants and corresponded with lower foliar injury in Essex.

3.2.2. Stomatal conductance

The O_3 treatment caused a significant decline in g_s compared with the CF controls in leaf 2 for both Essex and Forrest, but no significant genotype differences in g_s were found (Table 1).

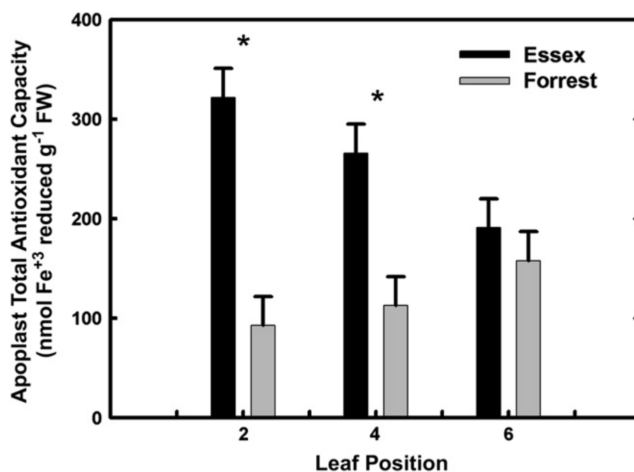

Fig. 2. Canopy profile of apoplast total antioxidant capacity in soybean leaves. Values are least square means ± SE ($n = 3$). Values marked with asterisk are statistically significantly different responses between genotypes for the same leaf position ($P \leq 0.05$). There were no significant leaf position differences.

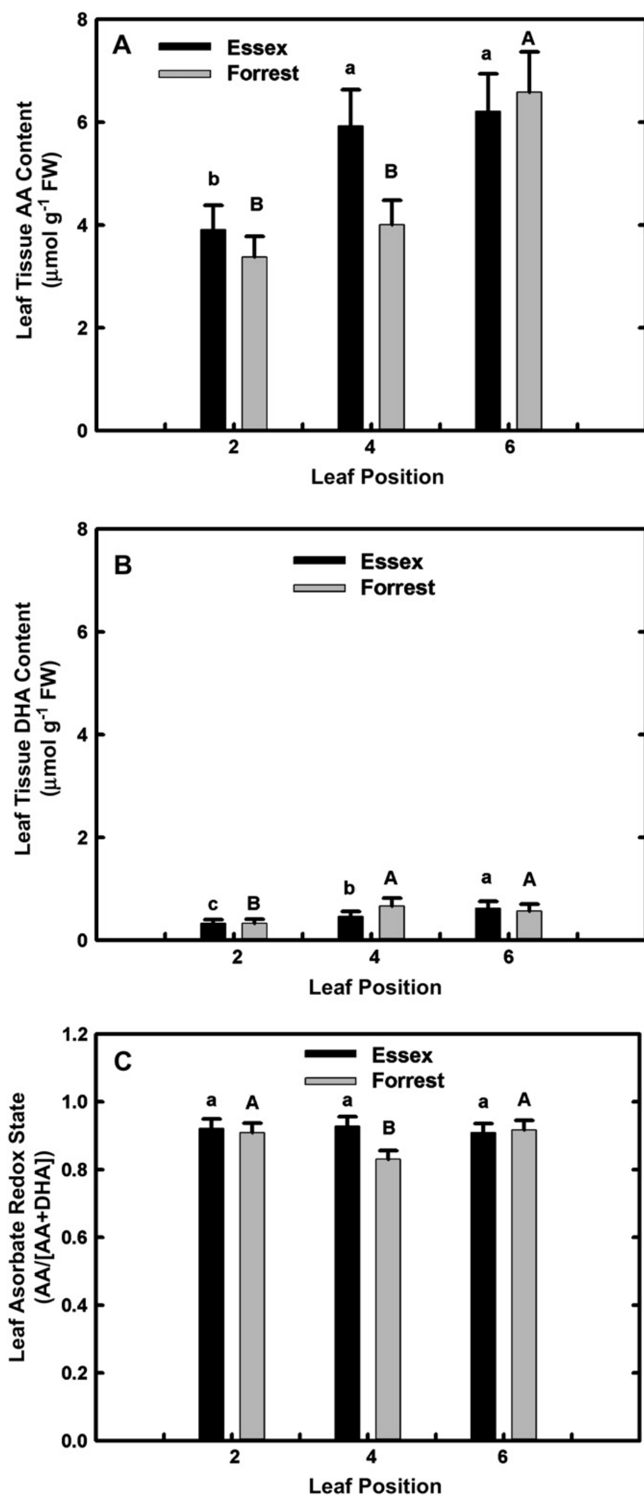

Fig. 3. Canopy profile of soybean leaf tissue AA (A) and DHA (B) content and ascorbate redox status (C). Values are least square means ± SE ($n = 3$). Values marked with the different letters are statistically significantly different responses among leaf positions for the same genotype ($P \leq 0.05$). There were no significant genotype differences.

3.2.3. Apoplast AA concentrations

Apoplast AA content of leaf 2 of both Essex and Forrest was significantly higher in O_3-treated plants compared with control plants (Table 2). However, in the elevated O_3 treatment, apoplast AA was 78% higher in Essex (O_3-tolerant) compared with Forrest (O_3-sensitive). No significant genotype or treatment effects were found for apoplast DHA concentrations (Table 2). In addition, no significant effect on ascorbate redox state was observed. However, ascorbate redox ratios were only 0.02–0.12 for all combinations of genotype and gas treatments (Table 2).

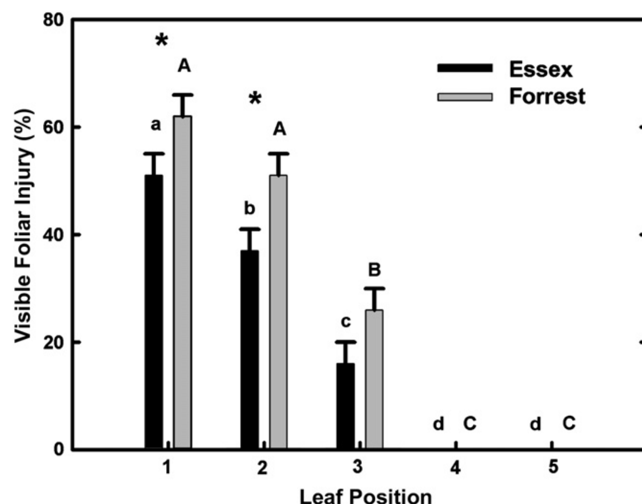

Fig. 4. Visible foliar injury on soybean leaves in O_3-treated plants. Values are least square means ± SE ($n = 10$). Values marked with asterisk are statistically significantly different responses between genotypes for the same leaf position ($P \leq 0.05$). Values marked with the different letters are significantly different responses among leaf positions for the same genotype ($P \leq 0.05$).

3.2.4. Apoplast total antioxidant capacity

Likewise to the clean-air experiment, apoplast total antioxidant capacity of leaves at position 2 was significantly greater in the more O_3-tolerant Essex cultivar than in Forrest (Table 2). The twofold difference in antioxidant capacity between genotypes was observed in both control and O_3-treated plants with no evidence that O_3 affected this parameter in either genotype.

3.2.5. Leaf ascorbate

Whole-leaf tissue samples from Essex and Forrest contained similar levels of AA in leaf 2 (Table 3). Leaf DHA levels were greater in Essex relative to Forrest in the CF treatment, but this was not observed in O_3-treated plants. Small differences in AA and DHA concentrations did not significantly affect the overall ascorbate redox state of the leaf tissue as the redox ratio remained above 0.8 for all combinations of genotype and gas treatments (Table 3).

4. Discussion

Our finding of greater O_3 sensitivity in soybean cultivar Forrest than in Essex was consistent with results reported previously (Chernikova et al., 2000; Robinson and Britz, 2000). The O_3 treatment employed in this study induced visible leaf injury on both genotypes, but Forrest developed significantly more injury than Essex on older leaves (Fig. 4, Table 1). The greater

Table 1
Visible O_3 injury, guaiacol peroxidase activity (GuPOD), and stomatal conductance (g_s) in the second main stem leaf following treatment with CF air or O_3 (77 nmol mol^{-1}) 7 h daily for 6 d

Genotype	Visible Injury (%)		GuPOD (μmol mg^{-1} protein min^{-1})		g_s (mmol H_2O m^{-2} s^{-1})	
	CF	O_3	CF	O_3	CF	O_3
Essex	0	41 ± 3^{b}*	0.30 ± 0.21	2.36 ± 0.21^{b}*	498 ± 33	361 ± 33*
Forrest	0	60 ± 3^{a}*	0.30 ± 0.21	3.14 ± 0.21^{a}*	551 ± 33	410 ± 33*

Values for visible injury and GuPOD specific activity are least square means ± SE ($n = 6$). Values of g_s are least square means ± SE ($n = 8$). Values with an asterisk indicate statistically significant O_3 treatment effects for the same genotype ($P \leq 0.05$). Values with different letters indicate significant differences between genotypes for the same gas treatment ($P \leq 0.05$).

visible injury in Forrest compared with Essex was accompanied by higher GuPOD specific activity (Table 1), an enzyme previously used as an indicator of O_3 stress in snap bean (Burkey et al., 2000; Burkey and Eason, 2002) and soybean (Chernikova et al., 2000). In both cultivars, foliar injury was greater for leaves in the lower canopy than for leaves in the upper canopy (Fig. 4), a result consistent with the general consensus that older leaves are more sensitive to O_3 than younger leaves (Lee et al., 1984; Heath, 1994).

Genotype differences in O_3 sensitivity of older leaves could be related to differences in O_3 uptake, detoxification mechanisms after O_3 has entered the leaf, or other, as yet unidentified, factors. In our study, possible variation in O_3 uptake was investigated by measuring g_s of the second main stem leaf that exhibited foliar injury when exposed to O_3. Stomatal conductance was similar in Essex and Forrest under CF control conditions where O_3 injury was not a factor (Table 1). In the same leaves of O_3-treated plants, foliar injury was observed and g_s declined in both Essex and Forrest, but there was no significant difference between the two genotypes (Table 1). These results suggested that the difference in O_3 sensitivity between the two genotypes was not related to differences in O_3 uptake.

Detoxification of O_3 and related ROS in the leaf apoplast by extracellular AA has been identified as a potential O_3 tolerance mechanism (Chameides, 1989), although its concentration varies among species and O_3 exposure conditions (Luwe et al., 1993; Luwe and Heber, 1995; Plöchl et al., 2000; Turcsanyi et al., 2000). Recent studies have found higher levels of AA in leaf IWF extracts of O_3-tolerant genotypes of snap bean (Burkey et al., 2003) and *Plantago major* (Zheng et al., 2000) compared with sensitive lines, suggesting that AA concentration in the leaf apoplast is important. In Essex and Forrest soybean, however, average leaf extracellular AA levels were low (1–30 nmol g^{-1} FW) in both CF control (Fig. 1A, Table 2) and O_3-injured (Table 2) leaves compared with concentrations found in broad bean (*Vicia faba* L.) and snap bean leaves (Turcsanyi et al., 2000; Burkey et al., 2003). Only in the case of O_3-treated plants did Essex have a statistically significant higher level of apoplast AA than Forrest, but it is questionable whether a difference of 7 nmol g^{-1} FW (Table 2) is biologically meaningful given that differences in apoplast AA concentration between tolerant and sensitive snap bean genotypes were on the order of 100 nmol g^{-1} FW (Burkey et al., 2003). In our study, the extracellular ascorbate pool in both CF control and O_3-injured leaves was primarily DHA (Fig. 1B, Table 2), an oxidized form of ascorbate that is not an antioxidant. The resulting extracellular ascorbate redox state was typically less than 0.2 (Fig. 1C, Table 2), and thus much lower than the 0.8 reported for spinach (Luwe et al., 1993) and 0.5–0.9 reported for O_3-tolerant snap beans (Burkey and Eason, 2002; Burkey et al., 2003). Thus, the difference in leaf extracellular AA concentration appeared unlikely to be a major factor contributing to the difference in O_3 response between Essex and Forrest. A similar observation was made for NC-S and NC-R clover (*Trifolium repens* L.) clones where differences in O_3 sensitivity were not correlated with apoplastic AA concentrations (D'Haese et al., 2005). These findings suggest that ascorbate-based mechanisms of O_3 detoxification in the leaf extracellular space may be a significant factor in determining differential O_3 sensitivity only in certain species and not generally applicable to all plants.

Apoplastic antioxidant capacity was also assessed in our study using the FRAP assay, which is based on the capacity of a sample to reduce Fe^{+3} to Fe^{+2}. This assay indicated that the total antioxidant capacity of the leaf apoplast of both Essex and Forrest was much greater than could be accounted for by AA levels alone. For example, for all combinations of genotype and O_3 treatment, average extracellular AA

Table 2
Reduced ascorbate (AA), dehydroascorbate (DHA), ascorbate redox state (AA/[AA + DHA]), and total antioxidant capacity in apoplast extracts from the second main stem leaf after treatment with CF air or O_3 (77 nmol mol^{-1}) 7 h daily for 6 d

Genotype	AA (nmol g^{-1} FW)		DHA (nmol g^{-1} FW)		Ascorbate redox state AA/[AA + DHA]		Total antioxidant capacity (nmol Fe^{+3} reduced g^{-1} FW)	
	CF	O_3	CF	O_3	CF	O_3	CF	O_3
Essex	2.4 ± 0.3	15 ± 2^{a}*	92 ± 8	111 ± 10	0.022 ± 0.003	0.117 ± 0.010	134 ± 15^{a}	131 ± 15^{a}
Forrest	1.2 ± 0.3	8 ± 1^{b}*	86 ± 7	133 ± 12	0.025 ± 0.004	0.059 ± 0.005	60 ± 15^{b}	56 ± 15^{b}

Antioxidant capacity is assessed as the capacity to reduced Fe^{+3} to Fe^{+2} in the FRAP assay. Values are least square means ± SE ($n = 3$). Values with an asterisk indicate statistically significant O_3 treatment effects for the same genotype ($P \leq 0.05$). Values with different letters indicate statistically significant differences between genotypes for the same gas treatment ($P \leq 0.05$).

Table 3
Reduced ascorbate (AA), dehydroascorbate (DHA), and ascorbate redox state in whole-leaf tissues from the second main stem leaf after treatment with CF air or O_3 (77 nmol mol^{-1}) 7 h daily for 6 d

Genotype	AA (μmol g^{-1} FW)		DHA (μmol g^{-1} FW)		Ascorbate redox state AA/[AA + DHA]	
	CF	O_3	CF	O_3	CF	O_3
Essex	3.39 ± 0.21	4.09 ± 0.25	0.55 ± 0.09^a	0.32 ± 0.05	0.840 ± 0.022	0.926 ± 0.024
Forrest	3.33 ± 0.20	4.26 ± 0.19*	0.32 ± 0.05^b	0.22 ± 0.03	0.909 ± 0.023	0.949 ± 0.025

Values are least square means ± SE (n = 6). Values with an asterisk indicate significant O_3 treatment effects for the same genotype ($P \leq 0.05$). Values with different letters indicate significant differences between genotypes for the same gas treatment ($P \leq 0.05$).

levels were relatively low (1−30 nmol g^{-1} FW) compared with the FRAP results (56−332 nmol Fe^{+2} reduced g^{-1}FW). Given that each AA molecule can reduce two Fe^{+3} ions to Fe^{+2} in total antioxidant capacity equivalents, apoplast AA was calculated to represent only 3−30% of the total antioxidant capacity in the leaf apoplast. Clearly, antioxidant compounds other than AA are present in the leaf apoplast. More importantly, apoplast total antioxidant capacity was twofold greater in older leaves of Essex compared with Forrest (Fig. 2, Table 2), which corresponded with greater O_3 tolerance of Essex leaves. Apoplast antioxidant capacity was not affected by the O_3 treatment in either cultivar (Table 2), suggesting that constitutive steady-state capacity was not altered in response to O_3 stress. Taken together, the results suggest that unidentified antioxidants present in the apoplast of soybean leaves have the potential to detoxify ROS associated with O_3 stress and thus alter the O_3 response of the plant. Identification of these compounds and characterization of their potential reactions with ROS are important topics for future research on O_3 tolerance mechanisms. Possible compounds include phenolics, polyamines, and protein −SH groups (Plöchl et al., 2000). These compounds represent potentially new chemistries for detoxification of ROS in the leaf apoplast and thus may expand our current understanding of extracellular detoxification mechanisms.

Other aspects of antioxidant metabolism may also contribute to the observed differences in O_3 tolerance between Essex and Forrest. A decline in leaf sulfhydryl group content following O_3 exposure was greater in Forrest relative to Essex (Chernikova et al., 2000), but it remains unclear whether this reflected differences in protein −SH groups or possible elevation of glutathione content in Essex. In addition, antioxidant enzymes including superoxide dismutase, ascorbate peroxidase, and glutathione reductase are elevated 10−30% in Essex relative to Forrest (Chernikova et al., 2000). Thus, the differences in O_3 tolerance between Essex and Forrest may result from a combination of extracellular and cytosolic mechanisms.

5. Conclusions

The leaf extracellular ascorbate pool in soybean was predominately dehydroascorbate, an oxidized form of ascorbic acid that is not an antioxidant. It is unlikely that minor differences in the amount of reduced ascorbate present in the leaf apoplast were a significant factor in the differential O_3 tolerance of two soybean cultivars, Essex and Forrest. However, total antioxidant capacity in the leaf apoplast was twofold greater in the more O_3-tolerant Essex cultivar compared with Forrest, suggesting that antioxidant metabolites other than ascorbic acid play a role in detoxification of O_3 in soybean leaves and possibly affect plant sensitivity to O_3 injury.

Acknowledgements

Cooperative investigations of the United States Department of Agriculture, Agricultural Research Service, and the North Carolina Agricultural Research Service, Raleigh, NC 27695-7643. The authors thank Dr. Marsha Gumpertz for advice on statistical model development. The authors also thank Jeff Barton for greenhouse operations, and Renee Tucker and Mike Durham for excellent technical assistance.

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