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

Fang-Yi Cheng [a], Kent O. Burkey [a,*], J. Michael Robinson [b], Fitzgerald L. Booker [a]

[a] *USDA-ARS Plant Science Research Unit and Department of Crop Science, North Carolina State University, 3127 Ligon Street, Raleigh, NC 27607, USA*
[b] *USDA-ARS Crop Systems and Global Change Laboratory, Beltsville, MD 20705, USA*

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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.

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.,

* Corresponding author. Tel.: +1 919 515 1620; fax: +1 919 856 4598.
E-mail address: koburkey@unity.ncsu.edu (K.O. Burkey).

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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