

## The influence of elevated CO<sub>2</sub> on the activities of antioxidative enzymes in two soybean genotypes

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**Abstract.** The effects of elevated compared to current atmospheric CO<sub>2</sub> concentration (720 and 365 µL L<sup>-1</sup>, respectively) on antioxidative enzymatic activities of two soybean (*Glycine max* (L.) Merr.) genotypes (R and S) grown in open-top field chambers were investigated. Enzymatic activities of leaves collected 40, 47, 54 and 61 d after planting were measured. Elevated CO<sub>2</sub> significantly decreased activities of superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APOD, EC 1.11.1.7), glutathione peroxidase (GPOD, EC 1.11.1.9) and glutathione reductase (GR, EC 1.6.4.2) in both genotypes. The activities of dehydroascorbate reductase (DAR, EC 1.8.5.1) and monodehydroascorbate reductase (MDAR, EC 1.1.5.4.) increased in genotype S, but decreased in genotype R under elevated CO<sub>2</sub>. Elevated CO<sub>2</sub> decreased rubisco activity and rubisco, chlorophyll, carotenoids and total soluble protein contents in both genotypes. Results indicate that constitutive antioxidative enzymatic activities may decrease in a high-CO<sub>2</sub> world. Significant CO<sub>2</sub> × genotype interactions, however, suggest that there may be key genotypic differences in response patterns, potentially conferring differential resistance to biotic and abiotic stress.

**Keywords:** antioxidative enzymes, elevated CO<sub>2</sub>, soybean genotypes.

### Introduction

The global atmospheric CO<sub>2</sub> concentration is rising, and may double by the end of the 21st century (Murray 1995). The influences of elevated CO<sub>2</sub> on plant structure (Pritchard *et al.* 1999), plant–herbivore interactions (Bezemer 1998), root growth (Rogers *et al.* 1997; Pritchard and Rogers 2000) and physiology (Bosac *et al.* 1995; Conroy *et al.* 1998) have been recently reviewed. However, although many reports dealing with the effects of CO<sub>2</sub> enrichment on plant growth and physiology have been published, effects of elevated CO<sub>2</sub> on antioxidative enzymes in plant cells have received little attention.

Production of reactive oxygen species (ROS), including superoxide (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the hydroxyl radical (•OH), is an inevitable consequence of life in an oxygen-rich environment (Polle *et al.* 1990). ROS

damage plant cells by oxidizing membrane lipids including the photosynthetic apparatus (Foyer *et al.* 1994), inhibit protoplast regeneration (Marco and Roubelakis-Angelakis 1996) and damage proteins, chlorophyll and nucleic acids (Foyer *et al.* 1994). ROS are formed within plant cells in several ways: (1) photochemical production of H<sub>2</sub>O<sub>2</sub> in the atmosphere from air pollution (Masuch *et al.* 1986); (2) donation of electrons directly to oxygen during photosynthesis, especially with high light or low chloroplastic CO<sub>2</sub> concentrations (Foyer *et al.* 1994); and (3) in response to stresses such as drought (Smirnoff and Colombe 1988), chilling (Hull *et al.* 1977), ozone (Sharma *et al.* 1996), salinity (Fadzilla *et al.* 1997), SO<sub>2</sub> (Asada and Kiso 1973), UV light and microbial attack (Low and Merida 1996).

In order to minimize cellular oxidative damage, plants produce antioxidative enzymes including superoxide

Abbreviations used: APOD, ascorbate peroxidase; BSA, bovine serum albumin; CAT, catalase; DAR, dehydroascorbate reductase; DAP, days after planting; GPOD, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDAR, monodehydroascorbate reductase; NADPH, nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; OTC, open-top chamber; POD, peroxidase; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase; UV light, ultraviolet light.

dismutase (SOD), catalases (CAT), peroxidases (POD), ascorbate peroxidase (APOD), dehydroascorbate reductase (DAR), monodehydroascorbate reductase (MDAR), glutathione peroxidase (GPOD) and glutathione reductase (GR). These molecules catalyse reactions that directly or indirectly detoxify ROS (Dalton *et al.* 1986; Schwanz *et al.* 1996b). These enzymes occur in both the chloroplast and cytoplasm (Polle *et al.* 1990). SOD catalyses the conversion of  $O_2^-$  to  $H_2O_2$  (and  $O_2$ ), and then  $H_2O_2$  is further detoxified by POD, CAT, APOD and GPOD. DAR, MDAR and GR function in the ascorbate–glutathione redox chain, utilizing nicotinamide adenine dinucleotide phosphate (NADPH) as a reductant in order to provide reduced forms of ascorbate and glutathione for APOD and GPOD (Polle *et al.* 1990).

Exposure to elevated  $CO_2$  significantly affects activity of antioxidative enzymes in plants. For example, it decreased SOD activities in spruce, pine and oak (Polle *et al.* 1993; Schwanz *et al.* 1996a, b) and reduced CAT activity in spruce and tobacco (Havir and McHale 1989; Polle *et al.* 1993). In orange, oak and pine leaves, however, elevated  $CO_2$  did not affect catalase activities (Schwanz *et al.* 1996a, b). Similarly, the  $CO_2$  treatment did not affect SOD activity in tobacco or wheat (Havir and McHale 1989; Rao *et al.* 1995). Badiani *et al.* (1993, 1997) reported reduced activities of Cu and Zn-SOD and APOD, but increased GR and Mn-SOD in soybeans grown in elevated  $CO_2$ . They warned, however, against drawing general conclusions from studies in which leaves were collected at a single sampling. Moreover, although the effects of elevated  $CO_2$  on plant growth and physiology are often reported to change over time, this has not been factored into most studies attempting to characterize the effect of  $CO_2$  enrichment on antioxidant activity. Furthermore, there may be significant inter- and intra-specific differences in response of plant antioxidative enzymes to elevated  $CO_2$  (Badiani *et al.* 1997).

In light of the important role of antioxidants in scavenging ROS, and the inevitability of rising global atmospheric  $CO_2$  concentrations, the effects of  $CO_2$  enrichment on constitutive antioxidant activity in plant cells must be better understood. Furthermore, the effects of elevated  $CO_2$  on antioxidative enzymes must be considered throughout the life cycle of a plant. Finally, different responses exhibited by different species (interspecific) or genotypes (intraspecific) must be elucidated. Therefore, the purpose of this study was to examine the effects of growth in elevated  $CO_2$  on antioxidant activity in two soybean (*Glycine max* (L.) Merr.) genotypes. These soybean genotypes exhibit differential resistance to the fungal pathogen, *Cercospora sojina*, causative agent of frogeye leaf spot disease. Characterization of constitutive antioxidant activity will provide baseline information necessary to understand subsequent studies on the effects of  $CO_2$  enrichment on plant–pathogen interactions.

## Materials and methods

### Plant materials

Soybean (*Glycine max* (L.) Merr.) seeds (five seeds per pot) were planted in 31 cm diameter, 31 cm deep round containers (17.2 L) in promix potting mixture. Immediately after planting, pots were transferred to open-top chambers (OTCs) and grown for 61 d under ambient ( $365 \mu\text{L L}^{-1}$ ) or elevated ( $720 \mu\text{L L}^{-1}$ )  $CO_2$ . Plants were watered daily and fertilized twice a week with full-strength Peters Professional Water Soluble Fertilizer (Peters Fertilizer Products, Fogelsville, PA USA). The chambers,  $CO_2$  supply and  $CO_2$  monitoring/dispensing systems were as described by Mitchell *et al.* (1995). The factors included two concentrations of  $CO_2$  ( $365$  and  $720 \mu\text{L L}^{-1}$ ) and two soybean genotypes (R and S). The R genotype exhibits resistance to the fungus *Cercospora sojina* and the S genotype is susceptible. These genotypes were derived from a single heterozygous  $F_5$  plant from the cross of Stonewall and Coker 6738. Three replicates per treatment were used, for a total of 60 plants in 12 pots. The youngest fully expanded trifoliolate leaf was collected from a different plant in each pot on growing days 40, 47, 54 and 61 in order to avoid injury induction of antioxidative enzymes. The experimental design was a completely randomized split plot design with  $CO_2$  levels as the main plots, genotype as the subplots and days after planting (DAP) the sub-sub plots. Three replications were used (six OTCs). ANOVA was performed using the 'proc Mixed' procedure in SAS (SAS Institute 1988).

### Preparation of total protein extracts

A single trifoliolate leaflet from the youngest fully expanded leaf was chosen from each plant. Leaves (1 g) were immediately frozen on site in liquid nitrogen, transported to the laboratory, lyophilized and homogenized in 2 mL of 0.1 mM potassium phosphate (pH 7.8). Homogenates were centrifuged at 12 000  $g$  for 15 min at  $4^\circ\text{C}$ . The supernatants were separated into 10 Eppendorf tubes (200  $\mu\text{L}$  of extract per tube) and stored at  $-20^\circ\text{C}$  until needed for determination of enzymatic activities. Total soluble protein content was determined by the method of Bradford (1976), using BSA Standard II as standard and Protein Assay Dye Reagent (Bio-Rad, California). Results are presented as  $\text{mg g}^{-1}$  fresh leaves.

### Enzymatic activity assays

Total SOD activity was determined spectrophotometrically by measuring the inhibition of blue diformazan formation in the presence of riboflavin/nitroblue tetrazolium (NBT) and light (Beauchamp and Fridovich 1971). The modified assay solution consisted of 1 mL of 50 mM sodium phosphate (pH 7.8) containing 0.1 mM EDTA, 0.3 mM riboflavin and 30  $\mu\text{L}$  of plant extract. After incubation for 5 min at room temperature, the solution was combined with NBT to give a final concentration of 0.03 mM NBT. The reaction mixture was then illuminated by a fluorescent lamp (75 W, 20 cm above the mixture) for 3 min and absorption determined at 560 nm. The control rate was obtained from the reaction mixture without extract. The absorption of NBT was negligible. Activity is presented as units  $\text{mg}^{-1}$  protein; one unit is defined as 50% inhibition of blue diformazan formation (Beauchamp and Fridovich 1971).

Catalase activity ( $\text{nmol min}^{-1} \text{mg}^{-1}$  protein) was determined by measuring  $H_2O_2$  consumption according to the method of Summermatter *et al.* (1995) using ABTS (Boehringer Mannheim, Mannheim, Germany) and peroxidase (Sigma, St Louis, MO USA).

Peroxidase activity was estimated by measuring the rate of increase of absorbance at 470 nm using *o*-dianisidine as substrate (Chance and Maehly 1964). The assay solution was 1 mL of 0.01 M sodium phosphate (pH 6.0) containing 1.3 mM  $H_2O_2$ , 1 mM *o*-dianisidine and 5  $\mu\text{L}$  of extract. Activity was expressed as  $\Delta\text{OD}_{470\text{nm}} \text{min}^{-1} \text{mg}^{-1}$  protein.

Ascorbate peroxidase activity was measured by a modified spectrophotometric procedure based on the rate of decrease in absorption of

ascorbate at 290 nm during ascorbate oxidation (Dalton *et al.* 1986). The assay was performed in a 1-mL plastic cuvette containing 0.20 mM ascorbate, 50 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 7.0), 0.5 mM EDTA, 1.5 mM H<sub>2</sub>O<sub>2</sub> and 15 µL of extract. Dehydroascorbate reductase activity was measured by the rate of increase in absorbance at 290 nm due to ascorbate formation (Dalton *et al.* 1986). The assay was performed in a 1 mL plastic cuvette containing 50 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 7.0), 0.15 mM dehydroascorbate, 0.5 mM EDTA, 2 mM GSH and 15 µL of extract. The control rate was obtained by using the same amount of double-distilled water instead of enzyme extract. Ascorbate peroxidase and dehydroascorbate reductase activities in the soluble fraction were analysed using an extinction coefficient of 2.8 mm<sup>-1</sup> cm<sup>-1</sup> for ascorbate at 290 nm, and recorded as nmol min<sup>-1</sup> mg<sup>-1</sup> protein.

Monodehydroascorbate reductase activity was measured at 340 nm by NADH oxidation (Polle *et al.* 1990). The modified assay included 1 mL of 100 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 7.8), containing 0.25 mM NADH, 1.5 mM sodium ascorbate, 15 µL of extract and 10 mg ascorbate oxidase (146 units mg<sup>-1</sup>, Sigma).

Glutathione reductase activity was determined from the change in NADPH absorbance at 340 nm (Foyer and Halliwell 1976). The modified reaction mixture contained 1 mL of 50 mM Tricine (pH 7.8) with 0.5 mM EDTA, 0.25 mM oxidized glutathione (GSSG) (Sigma) 0.15 mM NADPH and 30 µL of extract.

Glutathione peroxidase activity was measured by coupling to NADPH oxidation in the presence of excess glutathione reductase at 340 nm (Polle *et al.* 1990). The modified reaction mixture was 1 mL of 50 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 7.0) containing 0.5 mM EDTA, 2 units glutathione reductase (Fluka, Switzerland), 2 mM GSSG (Sigma), 0.15 mM NADPH, 100 mM H<sub>2</sub>O<sub>2</sub> and 30 µL of extract. Control rates were obtained in the absence of extract.

Activities of glutathione peroxidase, glutathione reductase and monodehydroascorbate reductase were calculated using an extinction coefficient of 6.22 mm<sup>-1</sup> cm<sup>-1</sup> for NADPH at 340 nm. Glutathione reductase and monodehydroascorbate reductase activities were presented as nmol min<sup>-1</sup> mg<sup>-1</sup> protein; glutathione peroxidase activity was presented as the ratio of oxidized/total NADPH.

Rubisco content was measured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with a 12% separating gel and 5% stacking gel (Laemmli 1970) with 10 µL of each protein sample loaded into a well of a vertical slab gel; proteins were separated using a mini-protein system (Bio-Rad, Hercules, CA USA). Molecular weight standards (Bio-Rad) and a rubisco standard (from spinach, Sigma) were included. Separating gels were stained in 0.1% Coomassie blue R–250 (Kodak, New York, NY USA) for 10 min and then destained with 10% methanol and 10% acetic acid solution. Gels were scanned with a GS-700 Imaging Densitometer (Bio-Rad). Rubisco content was expressed as mg g<sup>-1</sup> fresh leaves.

Rubisco activity was measured spectrophotometrically by the methods described by Tissue *et al.* (1993). A 30-µL extract was incubated with 970 µL reaction mixture for 30 min. RuBP (Sigma) was then added, and the rate of NADH oxidation was measured at 340 nm. Rubisco activity was expressed as the amount of NADH oxidized min<sup>-1</sup> mg<sup>-1</sup> protein.

Chlorophyll and carotenoids contents (mg g<sup>-1</sup> fresh leaves) were determined by the method of Lichtenthaler and Wellburn (1983).

## Results

### *Response of basic parameters to elevated CO<sub>2</sub>*

Rubisco activity and content, chlorophyll content, carotenoid content, and total soluble protein were decreased 29%, 23%, 23%, 37% and 14% in elevated compared to ambient CO<sub>2</sub> respectively (Table 1). There were no signifi-

cant three-way interactions (CO<sub>2</sub> × genotype × DAP). However, significant genotype × CO<sub>2</sub> interactions were observed for both total chlorophyll ( $P = 0.002$ ) and total carotenoids ( $P = 0.002$ ) (Figs 1A and B). When grown in ambient CO<sub>2</sub>, genotype S had a higher chlorophyll content than genotype R; however, this was reversed when grown with CO<sub>2</sub> enrichment. Significant CO<sub>2</sub> × DAP interactions were noted for rubisco content ( $P = 0.002$ ) (Fig. 2A). Plant growth and biomass were obviously stimulated by elevated CO<sub>2</sub> (data not shown). There were no significant differences in rubisco or chlorophyll content between the two genotypes (Table 1).

### *Response of antioxidative enzymes to elevated CO<sub>2</sub>*

The activities of all antioxidative enzymes were significantly lower in leaves from plants grown in elevated compared to ambient CO<sub>2</sub>. Only the activity of DAR and MDAR of genotype S increased. The activities of SOD, CAT, POD, APOD, DAR, MDAR, GPOD and GR decreased by 15, 24, 54, 18, 32, 71, 3 and 35%, respectively, in leaves of genotype R grown at elevated CO<sub>2</sub>. Activity of CAT, POD, APOD, GPOD and GR of genotype S decreased 30, 54, 40, 2 and 55%, respectively. Activities of all enzymes differed between the two genotypes with the exception of GPOD and CAT (Table 1).

Significant three-way interactions were observed for POD ( $P = 0.0003$ ; Fig. 3A), GR ( $P = 0.006$ ; Fig. 3B), MDAR ( $P = 0.0001$ ; Fig. 3C), GPOD ( $P = 0.0003$ ; Fig. 3D), APOD ( $P = 0.003$ ; Fig. 3E) and SOD ( $P = 0.0001$ ; Fig. 3F) resulting from fluctuations in both rank and magnitude of response patterns over time (Fig. 3). A significant CO<sub>2</sub> × genotype interaction was observed for DAR ( $P = 0.0008$ ) (Fig. 1C). A similar response pattern was observed for MDAR (Fig. 3C). A significant CO<sub>2</sub> × DAP interaction ( $P = 0.0006$ ) was observed for catalase activity (Fig. 2B); CAT activity generally increased over time in plants growing in ambient CO<sub>2</sub>, while activity increased in elevated CO<sub>2</sub> until 54 DAP. CAT activity decreased from 54 to 61 DAP in plants growing in elevated CO<sub>2</sub>.

## Discussion

Soybean genotypes grown in elevated CO<sub>2</sub> produced greater total biomass than those grown in normal CO<sub>2</sub>. Growth in CO<sub>2</sub>-enriched environments typically enhances growth and photosynthesis by directly increasing the amount of carbon available for fixation, decreasing CO<sub>2</sub> lost to photorespiration, and reducing oxygenase activity of rubisco (Lawlor and Mitchell 1991). Increased plant growth and photosynthesis due to long-term exposure to CO<sub>2</sub> enrichment in our study is consistent with other studies on soybean (Sionit *et al.* 1987; Cure *et al.* 1988; Del Castillo *et al.* 1989). Rogers and Dahlman (1993) summarized several studies and found that CO<sub>2</sub> enrichment increased photosynthesis (42%), total biomass (39%) and yield (29%) in soybean. Although

**Table 1. Main effects of ambient (365  $\mu\text{mol mol}^{-1}$ ) or elevated  $\text{CO}_2$  (720  $\mu\text{mol mol}^{-1}$ ) treatments (grown in OTCs) and genotype (resistant or susceptible) on antioxidants and basic parameters of soybean**

Data are means of three replicates.  $P > F$  indicates the probability that the differences observed were due to chance. NS = not significant at  $P \leq 0.05$ . Units: rubisco, chlorophyll, carotenoid and total proteins are expressed as  $\text{mg g}^{-1}$  fresh leaves; superoxide dismutase,  $\text{units min}^{-1} \text{mg}^{-1}$ ; catalase,  $\text{nmol min}^{-1} \text{mg}^{-1}$  protein; peroxidase,  $\Delta\text{OD } 470 \text{ nm min}^{-1} \text{mg}^{-1}$ ; ascorbate peroxidase,  $\text{nmol min}^{-1} \text{mg}^{-1}$  protein; dehydroascorbate reductase,  $\text{nmol min}^{-1} \text{mg}^{-1}$  protein; monodehydroascorbate reductase,  $\text{nmol min}^{-1} \text{mg}^{-1}$  protein; glutathione peroxidase, % of control; glutathione reductase,  $\text{nmol min}^{-1} \text{mg}^{-1}$  protein

	$\text{CO}_2$ treatment			Genotype		
	Elevated	Ambient	$P > F$	Resistant	Susceptible	$P > F$
Rubisco activity	28.1	39.6	< 0.01	35.9	31.8	< 0.01
Rubisco content	4.34	5.66	< 0.01	4.87	5.12	< 0.06
Chlorophyll content	5.00	6.46	< 0.01	5.62	5.84	NS
Carotenoid content	0.67	1.00	< 0.01	0.92	0.78	< 0.01
Total proteins	13.9	16.1	< 0.01	15.5	14.4	< 0.01
Superoxide dismutase	23.1	27.1	< 0.01	24.4	25.8	< 0.01
Catalase	49.7	67.0	< 0.01	58.1	58.6	NS
Peroxidase	0.012	0.025	< 0.01	0.017	0.020	< 0.03
Ascorbate peroxidase	194.3	258.4	< 0.01	260.8	191.9	< 0.01
Dehydroascorbate reductase	52.3	48.7	NS	45.4	55.6	< 0.01
Monodehydroascorbate reductase	80.9	82.0	NS	58.6	104.2	< 0.01
Glutathione peroxidase	92.8	95.7	< 0.01	94.5	94.0	NS
Glutathione reductase	62.0	104.3	< 0.01	121.6	44.6	< 0.01

$\text{CO}_2$  enrichment stimulated growth, rubisco activity was significantly reduced, as has been reported by Vu *et al.* (1997) and Sims *et al.* (1998). Furthermore, chlorophyll *a* and *b* and carotenoids were significantly reduced, as has been reported for many plant species grown in  $\text{CO}_2$ -enriched atmospheres (Surano *et al.* 1986; Houpis *et al.* 1988; Polle *et al.* 1993). Decreased carotenoid content may predispose the photosynthetic apparatus to photodamage; carotenoids prevent destruction of chlorophyll in high light by dissipating excess excitation energy and may also stabilize and photo-protect the lipid phase of the thylakoid membranes (Havaux 1998).

Data obtained in the present study lend support to the hypothesis that plants growing in  $\text{CO}_2$ -enriched atmospheres will have lower antioxidative enzyme activity because of a decrease in cellular ROS production. Significant reductions in activity were observed for SOD, POD, CAT, APOD, GPOD and GR in plants grown in elevated  $\text{CO}_2$ , although results did fluctuate between collection dates. In general, it is thought that the status (up- or down-regulated) of plant antioxidant systems is controlled by the extent of oxidative stress (Polle *et al.* 1997). Therefore, it follows that the reductions in antioxidant activities observed here may reflect a reduction of oxidative stress resulting from growth in  $\text{CO}_2$ -enriched atmospheres. Similar reductions in SOD and catalase have been reported in the tree species spruce (Polle *et al.* 1993), oak and pine (Schwanz *et al.* 1996a, b), and also in tobacco (Havir and McHale 1989). A later study on spruce, however, showed that exposure to elevated  $\text{CO}_2$  did not significantly alter the activities of SOD, CAT or POD in October or January. In the only study to examine antioxidative enzymes in soybean, Badiani *et al.* (1993) reported

reductions in the activities of Cu, Zn-SOD, and APOD, but increases in GR and Mn-SOD in soybeans grown in elevated  $\text{CO}_2$  sampled on a single occasion.

Several mechanisms have been advanced to explain how growth in elevated  $\text{CO}_2$  may reduce the amount of ROS formed within plant cells, thereby leading to reductions in constitutive antioxidant activity. First, increased  $\text{CO}_2/\text{O}_2$  ratios within chloroplasts may decrease electron leakage from photosystem I to  $\text{O}_2$ , thereby attenuating  $\text{O}_2^-$  formation (Schwanz *et al.* 1996b). Second, increased ratios of  $\text{CO}_2/\text{O}_2$  would decrease oxygenase activity of rubisco in favor of carboxylation, thus reducing photorespiration and resultant cellular  $\text{H}_2\text{O}_2$  production. Fixation of oxygen by rubisco (photorespiration) is greatly reduced in  $\text{C}_3$  plants grown in  $\text{CO}_2$ -enriched environments (Bowes 1991). Third, reductions in rubisco activity or content, as was observed for both soybean genotypes in the current study, may further decrease photorespiration by up to 50% (Polle 1996). Fourth, because antioxidant activities are stimulated by water stress (Smirnov 1993; Polle 1996; Schwanz *et al.* 1996a), and growth in elevated  $\text{CO}_2$  is often reported to alleviate water stress (Rogers *et al.* 1983), plants growing in  $\text{CO}_2$ -enriched atmospheres may exhibit lower antioxidant activities. Clearly, the relative contributions of these mechanisms to the observed reductions in antioxidative enzymes remain to be elucidated.

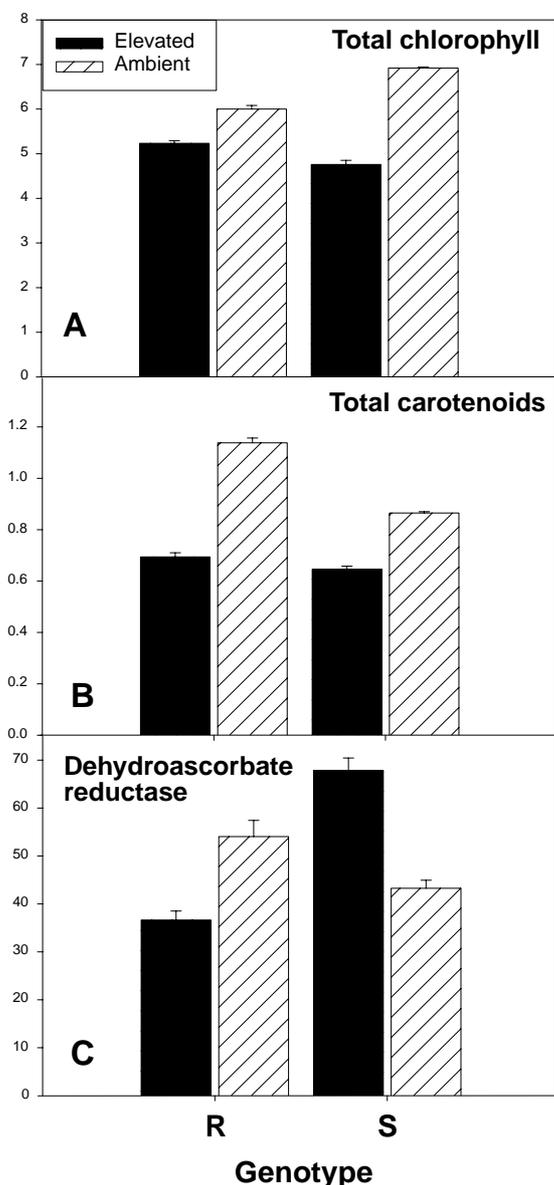
It is interesting to note the enhanced activities of DAR and MDAR in genotype S grown in elevated compared to ambient  $\text{CO}_2$ . These enzymes are both necessary for the regeneration of reduced forms of the antioxidative substrate ascorbate (Polle 1996). Badiani *et al.* (1993) also reported

that DAR and MDAR in soybean were either not affected or were enhanced in plants grown next to a natural CO<sub>2</sub> spring in Italy (at elevated [CO<sub>2</sub>]). Schwanz and Polle (1998) recently reported increased DAR and MDAR activities in *Quercus pubescens* growing in a CO<sub>2</sub>-enriched environment, and suggested that leaf ascorbate pools may have exhibited higher turnover rates. According to Foyer *et al.* (1994), there are two ways by which reduced forms of ascorbate are regenerated: directly by the electron transport chain, and by the ascorbate–glutathione cycle. Perhaps elevated CO<sub>2</sub>

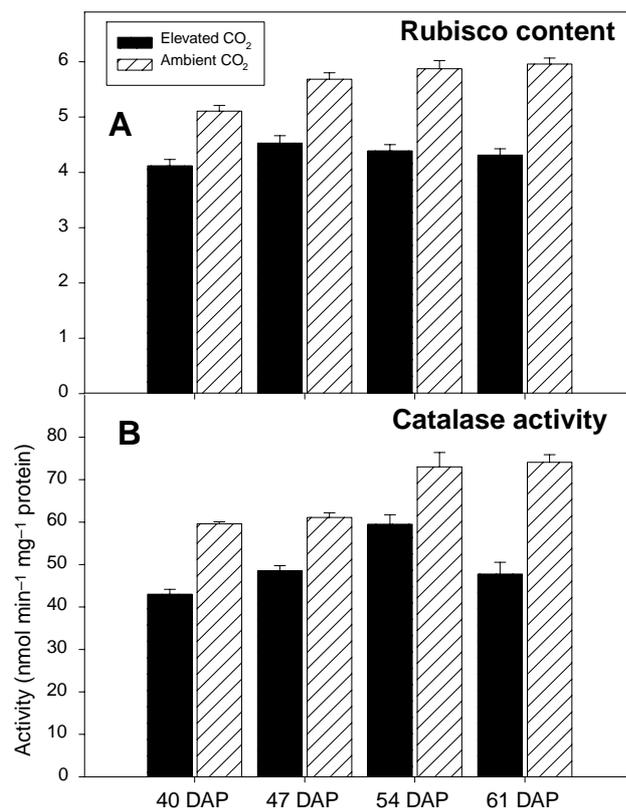
weakened electron transport from monodehydroascorbate to ascorbate, requiring up-regulation of the ascorbate–glutathione cycle in order to regenerate reduced ascorbate (see review paper by Foyer *et al.* 1994). Further work focusing specifically on the influence of elevated CO<sub>2</sub> on the ascorbate–glutathione pathway will be required to identify the mechanism driving enhanced DAR and MDAR activities reported here and in other studies, and also to determine what significance this phenomenon has for plant function.

Regardless of the mechanisms involved, it appears that constitutive antioxidant activity in both soybean genotypes examined here was down-regulated by growth in elevated CO<sub>2</sub>. Although this may reflect decreased basal oxidative stress, down-regulation of antioxidant systems suggests that growth in elevated CO<sub>2</sub> may compromise the ability of plants to cope with sudden stress events. More work needs to be done to determine if the inducibility of antioxidative enzymes is also impacted by growth in CO<sub>2</sub> enriched atmospheres.

The two soybean genotypes used in this experiment exhibit a differential response when challenged with the fungal pathogen *Cercospora sojina*, the causative agent of



**Fig. 1.** Significant CO<sub>2</sub> × genotype interactions for two soybean genotypes (R = resistant, S = susceptible) grown in elevated (720) or ambient (365 μmol mol<sup>-1</sup>) CO<sub>2</sub>. (A) total chlorophyll, *P* = 0.002; (B) total carotenoids, *P* = 0.002; and (C) dehydroascorbate reductase, *P* = 0.0008. Bars represent s.e. of mean.

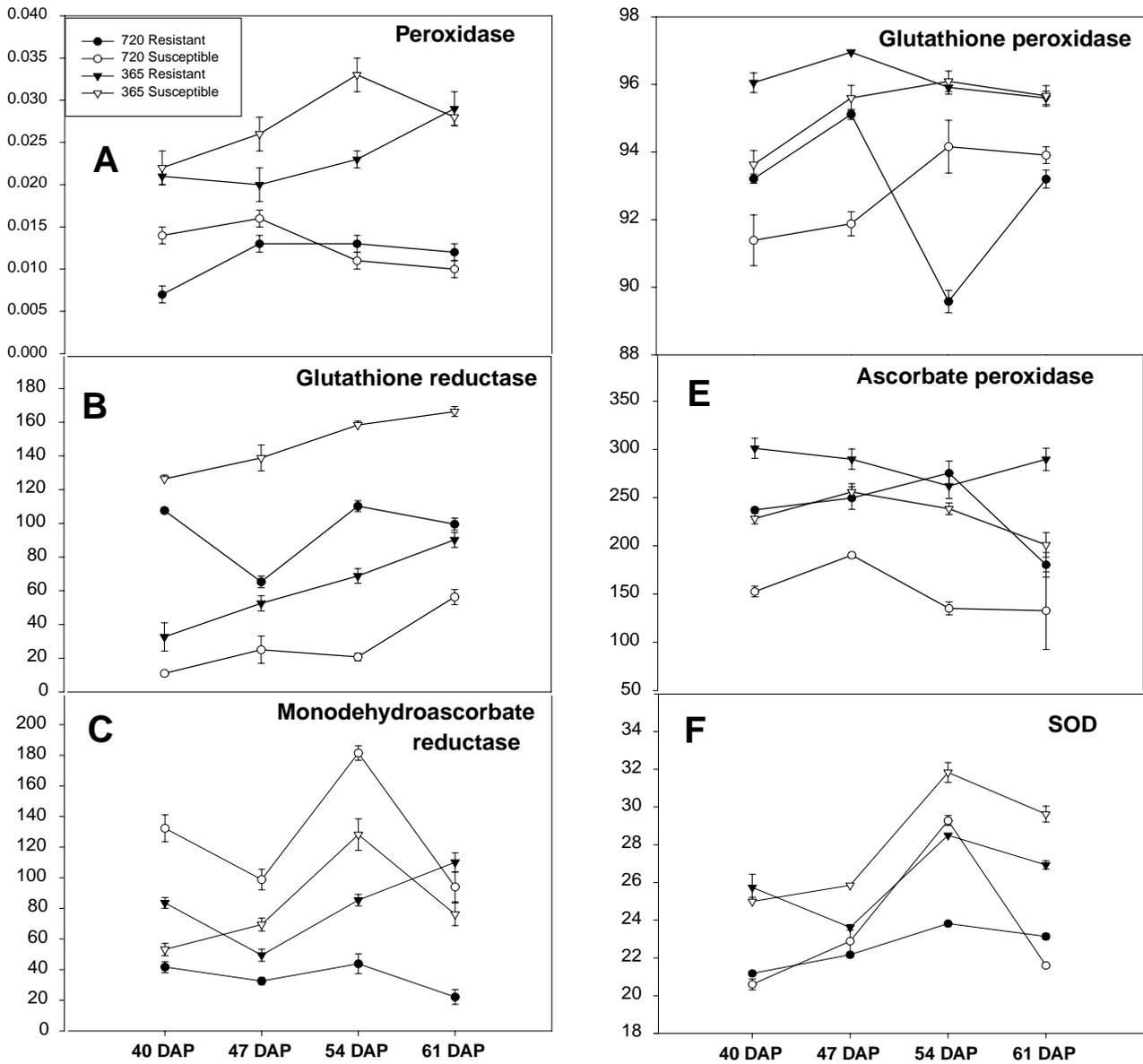


**Fig. 2.** Significant CO<sub>2</sub> × DAP interactions for soybeans grown at either elevated (720) or ambient (365 μmol mol<sup>-1</sup>) CO<sub>2</sub> sampled 40, 47, 54 and 61 days after planting (DAP). Values are averaged for R and S genotypes. (A) Rubisco content, *P* = 0.002; (B) catalase activity, *P* = 0.0006. Bars represent s.e. of mean.

frogeye leaf spot disease (Pace *et al.* 1993); genotype R exhibits resistance while genotype S is susceptible. Because the accumulation of ROS within plant cells is often associated with plant disease resistance (Meddy 1994), the shifts in antioxidative enzymes observed here suggest that the dynamics of interactions of *Cercospora* with the two soybean genotypes may be altered. Furthermore, significant CO<sub>2</sub> × genotype interactions observed here also suggest that antioxidant systems of different soybean genotypes may respond differently to rising CO<sub>2</sub>. These differences may change the

relative resistance/susceptibility of different genotypes to pathogen infection.

The rapid induction of ROS accumulation is often associated with the onset of pathogen infection, and is thought to contribute to the hypersensitive response that inhibits further spreading of the pathogen. So, with respect to pathogen infections, there is clearly a balance of ROS and antioxidative enzymes that will allow formation of the hypersensitive response without excessive oxidative damage to surrounding plant tissues. Further work is currently being



**Fig. 3.** Significant CO<sub>2</sub> × genotype × date interactions for two soybean genotypes (R = resistant, S = susceptible) grown in either elevated (720) or ambient (365 μmol mol<sup>-1</sup>) CO<sub>2</sub> sampled 40, 47, 54 and 61 days after planting. (A) Peroxidase, *P* = 0.0003; (B) glutathione reductase, *P* = 0.006; (C) monodehydroascorbate reductase, *P* = 0.0001; (D) glutathione peroxidase, *P* = 0.0003; (E) ascorbate peroxidase, *P* = 0.003; (F) superoxide dismutase, *P* = 0.0001. Bars represent s.e. of mean.

conducted to determine what effects growth in elevated CO<sub>2</sub> will have on interactions of pathogens with their plant hosts, and also what effect a down-regulation in antioxidant activity will have on sudden exposure to abiotic stress.

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