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Amelioration of Ozone-Induced Oxidative Damage in Wheat Plants Grown under High Carbon Dioxide[1]

Role of Antioxidant Enzymes

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O_3-induced changes in growth, oxidative damage to protein, and specific activities of certain antioxidant enzymes were investigated in wheat plants (*Triticum aestivum* L. cv Roblin) grown under ambient or high CO_2. High CO_2 enhanced shoot biomass of wheat plants, whereas O_3 exposure decreased shoot biomass. The shoot biomass was relatively unaffected in plants grown under a combination of high CO_2 and O_3. O_3 exposure under ambient CO_2 decreased photosynthetic pigments, soluble proteins, and ribulose-1,5-bisphosphate carboxylase/oxygenase protein and enhanced oxidative damage to proteins, but these effects were not observed in plants exposed to O_3 under high CO_2. O_3 exposure initially enhanced the specific activities of superoxide dismutase, peroxidase, glutathione reductase, and ascorbate peroxidase irrespective of growth in ambient or high CO_2. However, the specific activities decreased in plants with prolonged exposure to O_3 under ambient CO_2 but not in plants exposed to O_3 under high CO_2. Native gels revealed preferential changes in the isoform composition of superoxide dismutase, peroxidases, and ascorbate peroxidase of plants grown under a combination of high CO_2 and O_3. Furthermore, growth under high CO_2 and O_3 led to the synthesis of one new isoform of glutathione reductase. This could explain why plants grown under a combination of high CO_2 and O_3 are capable of resisting O_3-induced damage to growth and proteins compared to plants exposed to O_3 under ambient CO_2.

Atmospheric levels of CO_2 are expected to double during the 21st century (Long et al., 1993). This has prompted the evaluation of many plant species for their responses to high CO_2 concentrations (Eamus and Jarvis, 1989; Sage et al., 1989; Yelle et al., 1989; Ziska et al., 1990, 1991). This topic has been extensively reviewed by Krupa and Kickert (1989) and Stitt (1991). High CO_2 influences plant growth primarily by increasing net photosynthetic rates (Long et al., 1993), and transpiration is usually reduced by partial stomatal closure (Allen, 1990). The combustion sources that produce CO_2 also emit a number of gaseous pollutants (and their precursors) into the atmosphere (Carlson and Bazzaz, 1985). Recent data concerning global climate predict increasing CO_2 levels in conjunction with similar increases in tropospheric gaseous pollutants, including ozone (O_3) (Krupa and Kickert, 1989), and other environmental stress factors, including increased temperature (Long, 1991), UV-B radiation, and drought (Krupa and Kickert, 1989; Rozema, 1993). Despite widespread recognition that the tropospheric concentrations of several gases will increase along with CO_2 in ambient air, very little information is available about how air pollutants such as O_3 will influence plant response to increasing CO_2 and vice versa (Barnes and Pfirrmann, 1992; Mulchi et al., 1992).

The effect of CO_2 and O_3 on plant growth and productivity has been determined separately for a large number of species, but very little work has focused on their interaction. Growth under a combination of high CO_2 and UV-B not only counteracted the negative impact of UV-B but also reduced the influence of high CO_2 in rice plants (Ziska and Teramura, 1992). It is unclear whether the stimulation of growth and photosynthesis provided by high CO_2 will persist despite increased tropospheric O_3. However, Rao and DeKok (1994) have recently reported the absence of SO_2 effects in plants grown under a combination of high CO_2 and SO_2. Similar observations were reported by Sandhu et al. (1992) for soybean exposed to high CO_2 and SO_2.

It is generally believed that high-CO_2-induced partial stomatal closure reduces the impact of air pollutants by regulating their flux (Allen, 1990). However, this prediction is based on intuition or speculation rather than on experimental evidence. Carlson (1983) and Kropff (1987) exposed plants to SO_2 at varying concentrations of CO_2 and concluded the high-CO_2-induced stomatal regulation of SO_2 flux to be minimal. Similarly, Rao and DeKok (1994) have observed that high-CO_2-induced regulation of SO_2 flux did not account for the complete absence of SO_2 effects in wheat (*Triticum aestivum* L.) plants grown under high CO_2. Independent studies by Barnes and Pfirrmann (1992) and Mulchi et al. (1992) have also suggested that high-CO_2-induced regulation of O_3 uptake may not be the only protecting mechanism and speculated on an efficient pro-

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Abbreviations: AP, ascorbate peroxidase; CSTR, continuous stirred tank reactor; GR, glutathione reductase; POD, peroxidase; ppb, parts per billion; ppm, parts per million; RLSU, Rubisco large subunit; RSSU, Rubisco small subunit; SOD, superoxide dismutase.

tecting mechanism(s) operating at the cellular level. However, O_3 detoxification has been reported to be independent of changes in free radical scavengers like polyamines in plants exposed to O_3 under high CO_2 (Kramer et al., 1991).

Although increasing CO_2 levels have long been considered beneficial to plants, O_3 is a widespread phytotoxic air pollutant (Runeckles and Chevone, 1992; Heath, 1994; Kangasjarvi et al., 1994). O_3, upon entry into plants, instantaneously dismutates and generates activated oxygen species. Unless efficiently metabolized, O_3-derived activated oxygen species may alter plant metabolism by structurally modifying proteins and enhancing their susceptibility to "proteolytic degradation" (Pell and Dann, 1991). Metabolism of O_3-derived activated oxygen species is largely dependent on the activities of certain antioxidant enzymes such as SODs, PODs, GR, and AP. O_3-induced alteration of antioxygenic activities and/or the gene expression of several antioxidant enzymes has been reported in various plants (Creissen et al., 1994; Van Camp et al., 1994a; Willekens et al., 1994).

Apart from enhanced dry matter production, growth under high CO_2 for prolonged periods has been shown to modify anatomical features and protein synthesis (Robertson and Leech, 1995) as well as the expression of various genes (Van Oosten et al., 1994). Under these conditions, it is not clear how plants grown under high CO_2 would cope with activated oxygen species induced by O_3. Since most of the environmental stresses anticipated in the near future, such as water deficits, cold, heat shock, UV-B, and O_3, act via generation of activated oxygen species, we believed it imperative to investigate how plants grown under high CO_2 would respond to O_3. We present a series of experiments in which we investigated the impact of O_3 on growth, oxidative damage to proteins, and responses of certain antioxidant enzymes in wheat plants grown under different CO_2 concentrations.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wheat (*Triticum aestivum* L. cv Roblin) was grown from seed in 6-L pots containing Promix-BX (Premier Brands Inc., Red Hill, PA). After the seed was sown, pots were transferred to CSTRs (Hale-Marie et al., 1991). In this experiment four CSTRs were used to provide ambient CO_2, high CO_2, O_3, or high CO_2 and O_3. Pressurized CO_2 diluted with nitrogen was mixed with air to obtain desired CO_2 concentrations. O_3 was generated by a high-voltage corona discharge generator (Aqua Air, model SL 4000; A.H. Simpson Industries Ltd., Toronto, Ontario, Canada). Both CO_2 and O_3 concentrations were controlled with a programmable gas-dispensing system (Hale-Marie et al., 1991).

CO_2 and O_3 concentrations in the air stream were continuously monitored with a computerized IRGA (Analytical Developmental Corp. model 225, Hoddesdon, UK) and a Dasibi model 1008-AH O_3 analyzer (Dasibi Environmental Corp., Glendale, CA), respectively. The mean (day and night) CO_2 concentration in the CSTRs was 390 (ambient) or 800 ppm (high), and the mean O_3 concentration was 120 ppb. O_3 concentration in the CSTRs assigned to ambient or high CO_2 averaged 4 ppb. Plants were exposed to O_3 for 8 h d^{-1} during the photoperiod for 5 weeks and irrigated with Hoagland nutrient solution on alternate days. Differential treatments were imposed immediately after sowing. To minimize spatial effects within a chamber, pots were randomly relocated on alternate days.

The environmental conditions in the chambers averaged 25/18°C temperature (day/night), 50 to 70% RH, and a PPFD of 500 μmol m^{-2} s^{-1} at the plant canopy height with a 14-h photoperiod. The desired PPFD was obtained by adjusting the height of either the pots or the lamp. A Sylvania 1000-W lamp was used as a light source. To dissipate heat energy, a tray containing a 6- to 7-cm water layer was placed between the lamp and chamber top. In total, five pots, each with five plants, were assigned to each chamber.

Growth Measurements

At indicated intervals, whole shoots were harvested for the determination of whole shoot fresh and dry weights. Whole shoots were dried at 80°C for 72 h for dry weight measurements.

Leaf Injury

Twenty-four hours after the termination of exposure (i.e. 36-d-old plants) total leaf area of six randomly selected plants was determined with a leaf area meter, and leaf area exhibiting chlorotic symptoms was scored. The percentage of leaf injury was calculated as

$$\text{Percentage of leaf injury} = \frac{\text{Leaf area damaged}}{\text{Total leaf area}} \times 100.$$

Photosynthetic Pigments

Recently developed and matured leaves (80–90% of total leaf area) were homogenized with pure methanol and centrifuged at 12,000*g* for 10 min. Chls and carotenoids were calculated with the absorption spectra provided by Lichtenthaler (1984).

Enzyme Assay

Extraction

Recently developed and matured leaves (80–90% of total leaf area) (1 g) exhibiting no visible injury symptoms were homogenized with 100 mM potassium phosphate (pH 7.5) containing 1 mM EDTA and 1% PVP-40. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000*g* for 20 min. The supernatant was collected and stored at −80°C for further analyses. For comparisons between treatments, the activities of different enzymes were determined with leaf extracts equivalent to 100 μg of protein. Foliar protein was determined according to the method of Bradford (1976) with BSA as a standard.

SOD

SOD activity was determined spectrophotometrically as described by Spychalla and Desborough (1990). The assay was performed at 25°C in a 3-mL cuvette containing 50 mM $Na_2CO_3/NaHCO_3$ buffer (pH 10.2), 0.1 mM EDTA, 0.015 mM ferricytochrome *c*, and 0.05 mM xanthine. One unit of SOD is defined as the amount of enzyme that inhibited the rate of ferricytochrome *c* reduction by 50%.

POD

Guaiacol POD activity was based on the determination of guaiacol oxidation (extinction coefficient 26.6 mM cm^{-1}) at 470 nm by H_2O_2. The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 16 mM guaiacol, and 10 μL of 10% H_2O_2 in a 3-mL volume. The reaction was initiated by adding plant extract and was followed for 10 min.

GR

GR activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM cm^{-1}) as described by Rao (1992). The 1-mL assay mixture contained 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM GSSG, and the leaf extract. The assays were initiated by the addition of NADPH at 25°C. Corrections were made for any GSSG oxidation in the absence of NADPH.

Extraction and Determination of AP

For measurement of AP activity, foliar samples were homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1% PVP-40, and 5 mM ascorbate as described above. AP activity was determined by following the decrease in A_{290} for 3 min (extinction coefficient 2.8 mM cm^{-1}). The 1-mL reaction volume contained 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM ascorbate, and 0.2 mM H_2O_2 at 25°C (Nakano and Asada, 1981). Corrections were made for oxidation of ascorbate in the absence of H_2O_2.

In Vivo Oxidative Damage

Oxidative damage to protein was estimated as the content of carbonyl groups (Levine et al., 1990). Recently developed and matured leaves (80–90% of total leaf area) (2 g) sampled from plants exposed to different concentrations of CO_2 and/or O_3 for 35 d were homogenized with 5 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA and 2.5 μg each of the protease inhibitors PMSF, leupeptin, and pepstatin A. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000*g* for 25 min at 4°C. The supernatant was treated with streptomycin to remove the contaminating nucleic acids, and the foliar carbonyl groups were determined by reaction with 2,4-dinitrophenylhydrazine (Levine et al., 1990).

SDS-PAGE

Foliar proteins were denatured by the addition of an equal volume of SDS sample buffer and heated at 95°C for 5 min. Equal amounts of protein (5 μg) from extracts of different treatments were subjected to electrophoresis. One-dimensional SDS-PAGE was carried out following the method of Laemmli (1970) except that 8 to 20% gradient polyacrylamide gels with a 4% stacking gel were used. Electrophoretic separation was performed at 4°C for 4 h with 35 mA of current per gel. Proteins were visualized by staining the gels with Silver Stain Plus Kit (Bio-Rad) following the manufacturer's protocol.

Native PAGE and Activity Staining

Equal amounts of protein from plants exposed to different treatments were subjected to discontinuous PAGE under nondenaturing, nonreducing conditions, essentially as described by Laemmli (1970) except that SDS was omitted and the gels were supported with 10% glycerol (Mittler and Zilinskas, 1993). Electrophoretic separation was performed at 4°C for 4 h with a constant current of 35 mA per gel. After completion of electrophoresis the gels were stained for the activities of SOD, POD, and GR following the procedures of Misra and Fridovich (1977), Castillo et al. (1984), and Madamanchi et al. (1992), respectively.

Native gels were stained for SOD activity by incubation in a solution containing 2.5 mM nitroblue tetrazolium for 25 min, followed by incubation in 50 mM potassium phosphate buffer (pH 7.8) containing 28 μM riboflavin and 28 mM tetramethyl ethylene diamine for 20 min in the dark. The gels were placed in distilled water and exposed on a light box for 10 to 15 min at room temperature. Visualization of Cu, Zn-, Mn-, and Fe-SOD was achieved by incubating gels in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM KCN or 5 mM H_2O_2 for 30 min before staining for SOD activity.

POD isozymes were stained by incubating gels in sodium acetate buffer (pH 4.5) containing 2 mM benzidine (dissolved in DMSO). The reaction was initiated by adding 3 mM H_2O_2 and was allowed to continue for 20 min. GR activity was detected by incubating gels in 50 mL of Tris-HCl (pH 7.5) containing 10 mg of 3-(4,5-dimethylthiazol-2–4)-2,5-diphenyl tetrazolium bromide, 10 mg of 2,6-dichlorophenolindophenol, 3.4 mM GSSG, and 0.5 mM NADPH. Duplicate gels were assayed for GR activity, one with and one without GSSG.

Electrophoresis and Detection of AP Activity

Samples were subjected to native PAGE (gels supported with 10% glycerol) as described above except that the carrier buffer contained 2 mM ascorbate. The gels were prerun for 30 min to allow ascorbate, present in the carrier buffer, to enter the gel prior to the application of samples following the method of Mittler and Zilinskas (1993). Following electrophoretic separation, gels were equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min. The gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 4

mM ascorbate and 2 mM H_2O_2 for 20 min. The gels were washed briefly with buffer for 1 min and submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM tetramethyl ethylene diamine and 2.45 mM nitroblue tetrazolium with gentle agitation. The reaction was continued for 10 to 15 min and stopped by a brief wash in water. Proteins for SDS-PAGE and native PAGE were extracted from recently developed, matured leaves (80–90% of total leaf area) of plants exposed to different concentrations of CO_2 and/or O_3 for 35 d.

The experiments described were repeated in their entirety and the mean values are averages of the two experiments (variation between two experiments is ≤10%). To eliminate the compounding effect of CSTRs, the second experiment was conducted by switching the CSTRs for treatments. Analysis of data on growth and enzyme activities utilized a commercial analysis of variance program (SAS Institute, Inc., Carey, NC). Mean separation was based on calculation of 95% confidence limits from the appropriate experiment error mean squares and table *t* value. For comparisons among treatments, gels were loaded with an equal amount of protein initially found to be in the linear activity range (data not shown). Activity staining was conducted at least twice with as many extracts from each experiment and only representative photographs are presented. The intensities of different isoforms of SOD, POD, GR, and AP were determined after scanning the negatives with a Bio-Rad computer-aided scanner (data not shown).

RESULTS

Whole shoots were harvested once a week during the 5-week exposure period for estimating growth responses. The data presented in Figure 1 indicate substantial increases in the shoot biomass of plants grown under high CO_2 compared to plants grown under ambient CO_2. In contrast, shoot biomass of plants grown under O_3 for 5 weeks decreased significantly by 34% compared to plants grown under ambient CO_2. The shoot biomass of plants grown under a combination of high CO_2 and O_3 was almost the same as that of plants grown under high CO_2 (Fig. 1). Our earlier studies have suggested that plants grown under high CO_2 for 5 weeks continue to respond to high CO_2 (i.e. there is no photosynthetic desensitization) (data not shown).

Exposure of plants to O_3 normally results in the appearance of visible injury symptoms (Heath, 1994). Under our exposure conditions, no foliar injury symptoms were visible during the first 3 weeks of O_3 exposure. However, prolongation of O_3 exposure for 5 weeks resulted in 36% of the total leaf area being damaged. Although growth under a combination of high CO_2 and O_3 did not affect shoot biomass, about 10% of the total leaf area exhibited chlorotic symptoms (Table I). Decreases in photosynthetic pigments have been widely used as an indicator of O_3 stress (Darrall, 1989). O_3 is believed to induce degradation of Chls and/or inhibit their biosynthesis (Darrall, 1989; Heath, 1994). O_3 exposure for 5 weeks under ambient CO_2 decreased both Chls and carotenoids by 34 and 30%, respectively, compared to those of plants grown under ambient CO_2 (Table I). However, both Chls and carotenoids were slightly higher in plants grown under a combination of high CO_2 and O_3 compared to those of plants grown under high CO_2 (Table I).

Increased free radical production in plants exposed to O_3 has been widely related to impaired plant metabolism (Bowler et al., 1992). Free radicals such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) enhance the susceptibility of proteins to "proteases" by causing structural modification (Pacifici and Davies, 1990). Growth under high CO_2 slightly decreased soluble proteins, whereas O_3 exposure decreased proteins by 30% ($P < 0.05$) compared to those of plants grown under ambient CO_2 (Fig. 2A). However, soluble protein content of plants grown under a combination of high CO_2 and O_3 was similar to that of plants grown under high CO_2 (Fig. 2A). Since O_3 is believed to induce oxidative damage to proteins, we have conducted experiments to estimate in vivo carbonyl groups. Foliar carbonyl groups serve as an indicator for oxidative damage to proteins (Pacifici and Davies, 1990; Stadtmann and Oliver, 1991). O_3 exposure enhanced carbonyl groups in vivo by 2.2-fold compared to those of plants grown under ambient CO_2, whereas growth under a combination of high CO_2 and O_3 enhanced carbonyl groups by 37% (Fig. 2B).

Because measurements of total soluble protein and carbonyl groups do not indicate qualitative changes in protein profiles, we subjected foliar extracts to SDS-PAGE. Protein profiles of plants grown under different concentrations of CO_2 and/or O_3 are presented in Figure 3A. A protein of 58 kD was absent from plants grown under high CO_2 but

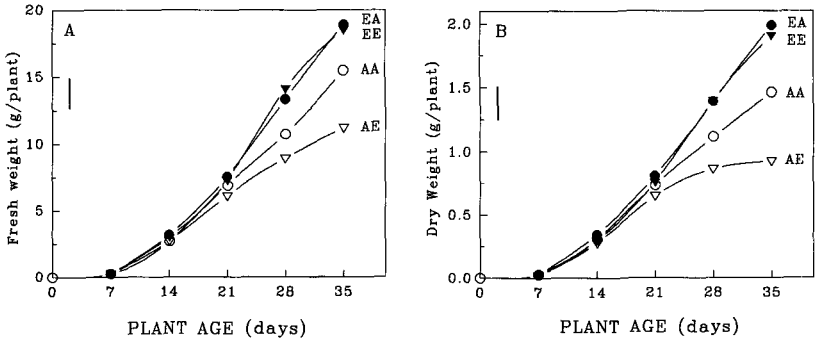

Figure 1. Growth responses of wheat plants grown under different concentrations of CO_2 and/or O_3. AA, Ambient CO_2 plus ambient O_3 (390 ppm of CO_2 plus 5 ppb of O_3); EA, high CO_2 plus ambient O_3 (800 ppm of CO_2 plus 4 ppb of O_3); AE, ambient CO_2 plus high O_3 (390 ppm of CO_2 plus 120 ppb of O_3); EE, high CO_2 plus high O_3 (800 ppm of CO_2 plus 120 ppb of O_3). Bars denote LSD ($n = 10$) ($P < 0.05$).

Table I. *Leaf injury (percentage of leaf area damaged), total Chl, and carotenoids (mg g^{-1} fresh weight) in plants grown under different concentrations of CO_2 and/or O_3*

Trait	Growth Conditions[a]			
	AA	EA	AE	EE
Leaf injury (%)	0	0	36	10
Total Chl	1.428a ± 0.101	1.216b ± 0.11	0.942c ± 0.082	1.388a,b ± 0.11
(mg g^{-1} fresh wt)	(100)	(85)	(66)	(97)
Carotenoids	0.448a ± 0.034	0.410a ± 0.033	0.312b ± 0.029	0.456a ± 0.039
(mg g^{-1} fresh wt)	(100)	(92)	(70)	(102)

[a] Growth conditions: AA, 390 ppm of CO_2 plus 4 ppb of O_3; EA, 800 ppm of CO_2 plus 4 ppb of O_3; AE, 390 ppm of CO_2 plus 120 ppb of O_3; EE, 800 ppm of CO_2 plus 120 ppb of O_3. Values in parentheses indicate relative values compared to AA plants. Shown are means ± SE (n = 6). Mean values followed by the same letter are not significantly different ($P > 0.05$).

appeared in plants grown under high CO_2 and O_3 (Fig. 3A, lanes b and d). Furthermore, some proteins of unknown identity (molecular mass ranging between 20 and 30 kD) disappeared or stained with decreased intensities in plants exposed to O_3 under ambient CO_2 compared to those of plants grown under a combination of high CO_2 and O_3 (Fig. 3A, lane c). The intensities of both RLSU and RSSU were slightly lower in plants grown under high CO_2 compared to those of plants grown under ambient CO_2 (Fig. 3B). Similarly, the staining intensities of both RLSU and RSSU were significantly lower ($P < 0.05$) in plants exposed to O_3 under ambient CO_2. However, no significant changes were observed in the intensity of either RLSU or RSSU in plants grown under a combination of high CO_2 and O_3 compared to that of plants grown under high CO_2 (Fig. 3B).

Metabolism of O_3-derived activated oxygen species is believed to determine plant tolerance to O_3 (Kangasjarvi et

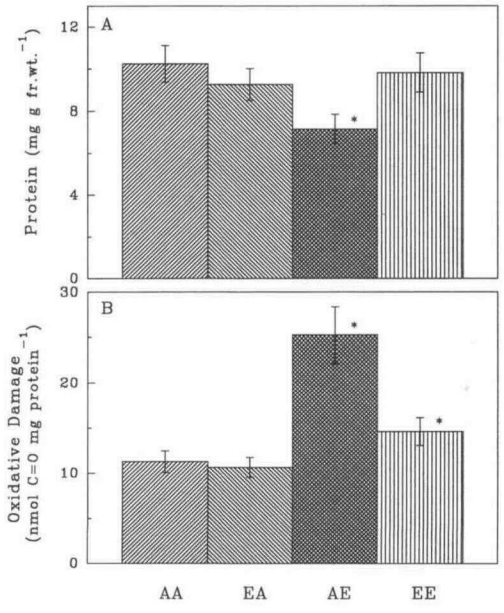

Figure 2. Total soluble protein (mg g^{-1} fresh weight [fr. wt.]) (A) and in vivo carbonyl groups (nmol C = O mg^{-1} protein) (B) of wheat plants grown under different concentrations of CO_2 and/or O_3. Growth conditions same as for Figure 1. An asterisk (*) indicates significantly different mean values compared to control plants. Error bars indicate SE (n = 6).

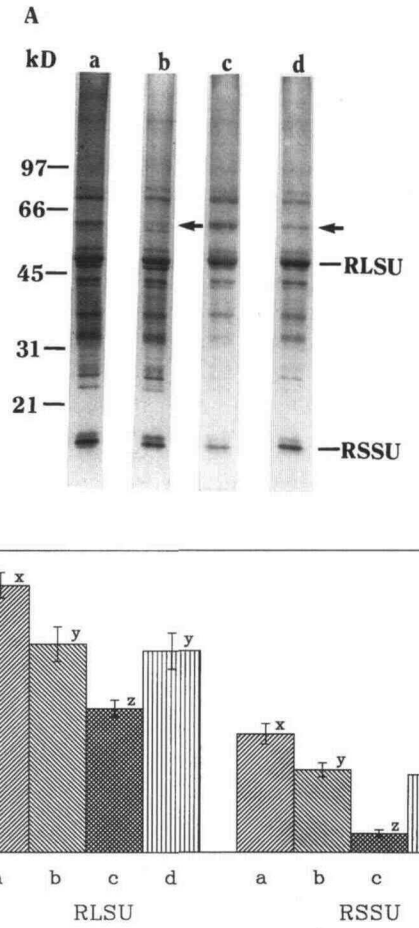

Figure 3. A, SDS-PAGE of soluble proteins (A) and staining intensities (B) (arbitrary units) of RLSU (○) and RSSU (●) of wheat plants grown under different concentrations of CO_2 and/or O_3. Lane a, Ambient CO_2 plus ambient O_3 (390 ppm of CO_2 plus 4 ppb of O_3); lane b, high CO_2 plus ambient O_3 (800 ppm of CO_2 plus 4 ppb of O_3); lane c, ambient CO_2 plus high O_3 (390 ppm of CO_2 plus 120 ppb of of O_3); lane d, high CO_2 plus high O_3 (800 ppm of CO_2 plus 120 ppb of O_3). Molecular mass (kD) is shown to the left. Arrows denote a protein of 58 kD that was absent in high-CO_2-grown plants but reappeared in plants grown under a combination of high CO_2 and O_3. Error bars (x, y, z) in B indicate SE (n = 4). Bars with same letter are not significantly different ($P > 0.05$). SDS-PAGE of proteins was repeated with at least two separate extracts from each experiment, and the data presented in B are the averages of four different gels.

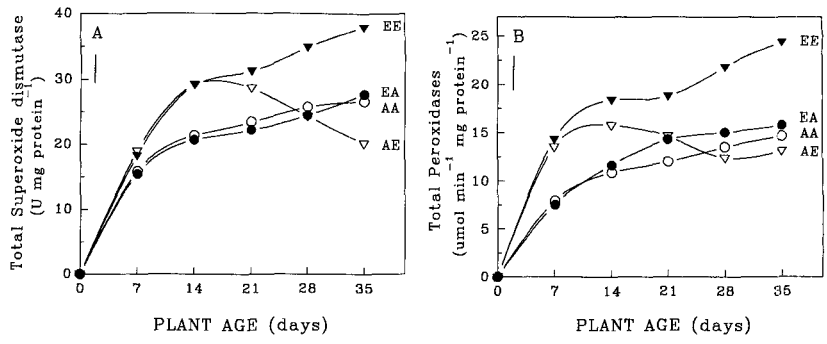

Figure 4. SOD (units mg^{-1} protein) (A) and total guaiacol-POD (μmol min^{-1} mg^{-1} protein) (B) of wheat plants grown under different concentrations of CO_2 and/or O_3. Abbreviations and symbols are the same as for Figure 1, except $n = 6$.

al., 1994). The ability of plants to metabolize activated oxygen species depends largely on the induction of the antioxygenic activity of SOD, PODs, GR, and AP (Foyer and Mullineaux, 1994; Foyer et al., 1994; Kangasjarvi et al., 1994). In an attempt to understand the role of antioxidant enzymes in plants grown under high CO_2 and O_3, we have estimated the activities of SOD, POD, GR, and AP spectrophotometrically. SODs (EC 1.15.1.1) are metal-containing enzymes that catalyze the dismutation reaction of superoxide into H_2O_2 and O_2 (Bowler et al., 1992). No major differences were observed in the total SOD activity of plants grown under ambient or high CO_2 (Fig. 4A). SOD activities were enhanced initially in plants exposed to O_3 under both ambient CO_2 and high CO_2. However, with prolonged O_3 exposure, SOD activities decreased significantly (30%, $P < 0.05$) in plants grown under O_3 and ambient CO_2, whereas the activities were significantly higher (33%, $P < 0.05$) in plants grown under high CO_2 and O_3 (Fig. 4A).

POD (EC 1.11.1.7) usually occurs as multiple molecular forms (isozymes) and has a number of potential roles in plant growth, development, and stress tolerance (Gaspar et al., 1991). No major changes were observed in the guaiacol-POD activity of plants grown under ambient or high CO_2 (Fig. 4B). O_3 exposure under ambient or high CO_2 initially enhanced POD activities. Prolonged O_3 exposure under ambient CO_2 decreased POD activity, and by 35 d of exposure, POD activity was similar to that of plants grown under ambient CO_2. However, POD activity remained enhanced in plants grown under a combination of high CO_2 and O_3. Growth under high CO_2 and O_3 in combination for 5 weeks enhanced POD activities by 55% compared to plants grown under high CO_2 (Fig. 4B).

GR (EC 1.6.4.2) and AP (EC 1.11.1.11) are two major enzymes that govern the ascorbate-glutathione cycle, or Halliwell-Asada pathway (Creissen et al., 1994). Although AP catalyzes the metabolism of H_2O_2, GR is essential to maintain the redox state of glutathione and ascorbate (Foyer et al., 1994). Growth under high CO_2 has no major effect on the activities of GR and AP compared to plants grown under ambient CO_2 (Fig. 5). O_3 exposure under ambient or high CO_2 enhanced GR and AP activities initially (Fig. 5). However, both GR and AP activities decreased with prolonged O_3 exposure under ambient CO_2, whereas GR and AP activities were enhanced by 39 and 56%, respectively, in plants grown under a combination of high CO_2 and O_3 (Fig. 5).

Plants are reported to possess different isoforms of SOD, POD, GR, and AP (Creissen et al., 1994; Van Camp et al., 1994a). It is strongly believed that oxidative stress-induced changes in the activities of antioxidant enzymes could be due to the synthesis of new isoforms (Creissen et al., 1994; Edwards et al., 1994). However, the changes in the antioxidant enzyme activities presented here do not reveal the possible changes in their isoform composition. To investigate whether the enhanced activities of SOD, POD, GR, and AP in plants grown under high CO_2 and O_3 are indeed due to changes in their isoform composition, we subjected foliar proteins to native PAGE and stained for their activities.

Wheat plants grown under ambient or high CO_2 have three isoforms of SOD (Fig. 6A, lanes a and b). Incubation of gels in either 2 mM KCN or 5 mM H_2O_2 before staining for SOD activity revealed no gel activity, suggesting that all three isoforms are of Cu,Zn (data not shown). SOD-1 and SOD-2 disappeared in plants exposed to O_3, and the staining intensity of SOD-3 decreased significantly compared to

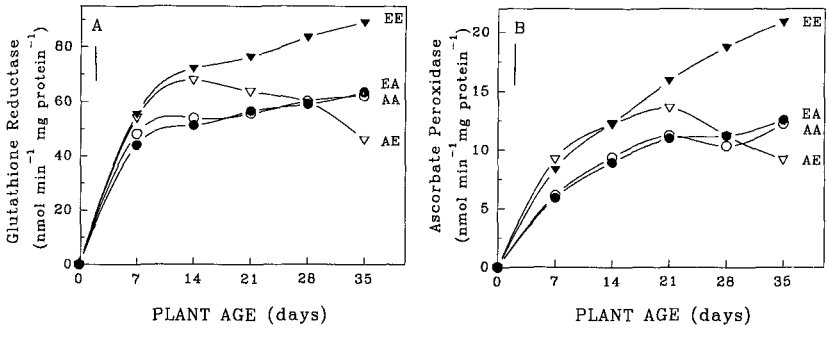

Figure 5. GR (nmol NADPH oxidized min^{-1} mg^{-1} protein) (A) and AP (nmol ascorbate oxidized min^{-1} mg^{-1} protein) (B) of wheat plants grown under different concentrations of CO_2 and/or O_3. Abbreviations and symbols are the same as for Figure 1, except $n = 6$.

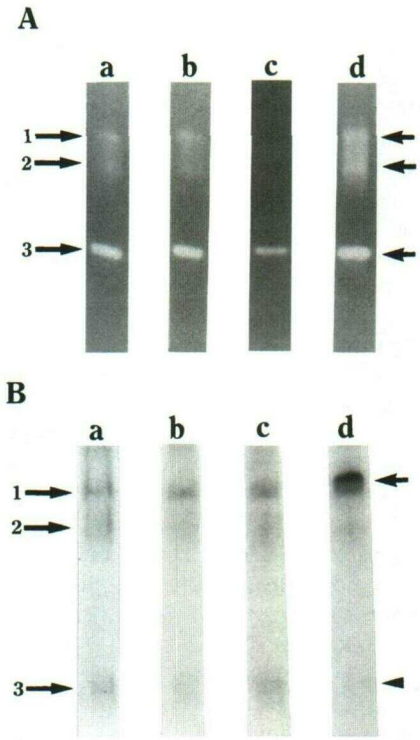

Figure 6. Native gels stained for SOD (A) and PODs (B) of wheat plants grown under different concentrations of CO_2 and/or O_3. Lane a, Ambient CO_2 plus ambient O_3 (390 ppm of CO_2 plus 4 ppb of O_3); lane b, high CO_2 plus ambient O_3 (800 ppm of CO_2 plus 4 ppb of O_3); lane c, ambient CO_2 plus high O_3 (390 ppm of CO_2 plus 120 ppb of O_3); lane d, high CO_2 plus high O_3 (800 ppm of CO_2 plus 120 ppb of O_3). Equal amounts of protein found to be in the linear activity range were loaded on the gel (150 μg for SOD and 200 μg for POD). Large arrows indicate different isoforms of SOD and POD in control plants. Incubation of gels in 3 mM KCN or 5 mM H_2O_2 before staining for SOD activity revealed no activity band, suggesting that all three isoforms are of Cu,Zn (data not shown). Small arrows indicate isoforms whose staining intensities were enhanced compared to control plants. The arrowhead in B indicates absence of the POD-3 isoform.

plants grown under ambient CO_2 (Fig. 6A, lane c). In contrast, staining intensities of all three isoforms were enhanced significantly in plants grown under a combination of high CO_2 and O_3 (Fig. 6A, lane d). Three different isoforms of POD could be distinguished in wheat plants grown under ambient or high CO_2 (Fig. 6B, lanes a and b). No major changes were observed in the staining intensities of any of the three isoforms of POD in plants exposed to O_3 under ambient CO_2 compared to those of control plants (Fig. 6B, lane c). However, growth under a combination of high CO_2 and O_3 preferentially enhanced the intensity of POD-1, whereas the intensity of POD-2 was unchanged. Furthermore, the POD-3 isoform was absent in plants grown under high CO_2 and O_3 (Fig. 6B, lane d).

Plants grown under ambient CO_2 or high CO_2 have two GSSG-specific and one GSSG-nonspecific GR isoform (Fig. 7A, lanes a and b). Growth under O_3 for 5 weeks decreased the staining intensity of both GR-1 and GR-2 isoforms (Fig. 7A, lane c). However, plants grown under a combination of high CO_2 and O_3 synthesized a new isoform, and the staining intensities of pre-existing isoforms were slightly enhanced (Fig. 7A, lane d). Three isoforms exhibiting AP activity were observed in wheat plants grown under ambient or high CO_2 (Fig. 7B, lanes a and b). AP-1 disappeared in plants grown under O_3, and the staining intensities of AP-2 and AP-3 decreased significantly (Fig. 7B, lane c). However, growth under a combination of high CO_2 and O_3 enhanced the staining intensities of AP-2 and AP-3, whereas there were no major changes in the intensity of AP-1 (Fig. 7B, lane d).

DISCUSSION

Growth under high CO_2 enhanced shoot biomass of wheat plants, whereas growth under O_3 decreased shoot biomass (Fig. 1). The observed changes in the shoot biomass of wheat plants were consistent with previous results obtained at high CO_2 (Du Cloux et al., 1987; Hocking and Meyer, 1991) and at high O_3 (Nie et al., 1993; Bender et al., 1994). The shoot biomass of plants grown under a combination of high CO_2 and O_3 was almost same as that of plants grown under high CO_2 and was significantly higher than that of plants grown under ambient CO_2 (Fig. 1).

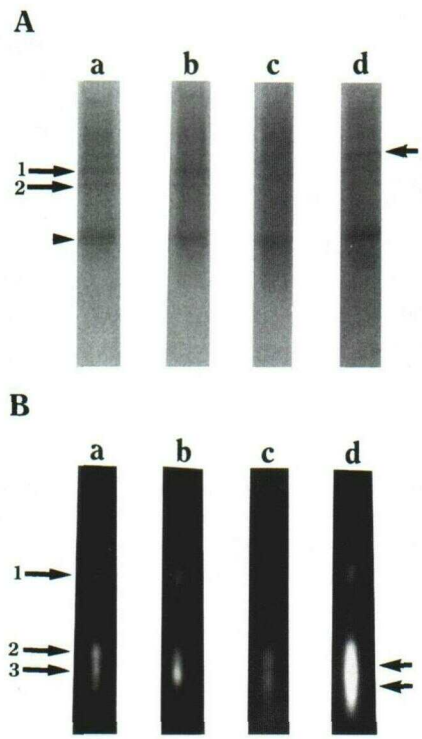

Figure 7. Native gels stained for GR (A) and AP (B) of wheat plants grown under different concentrations of CO_2 and/or O_3. Lane a, Ambient CO_2 plus ambient O_3 (390 ppm of CO_2 plus 4 ppb of O_3); lane b, high CO_2 plus ambient O_3 (800 ppm of CO_2 plus 4 ppb of O_3); lane c, ambient CO_2 plus high O_3 (390 ppm of CO_2 plus 120 ppb of O_3); lane d, high CO_2 plus high O_3 (800 ppm of CO_2 plus 120 ppb of O_3). Equal amounts of protein found to be in the linear activity range were loaded on the gel (100 μg for GR and 250 μg for AP). The arrowhead in A indicates the GSSG-nonspecific GR isoform. The short arrow in A indicates a new isoform of GR. The short arrows in B indicate preferential enhancement of AP-2 and AP-3 isoforms compared to control plants. Note the absence of AP-1 in lane c of B.

Amelioration of O_3-induced damage in high-CO_2-grown plants was consistent with the observations of Barnes and Pfirrmann (1992) and Mulchi et al. (1992). However, our results do not agree with their observations that O_3 exposure reduces the positive influence of high CO_2. In the present study, we did not observe an adverse impact of O_3 on the shoot biomass of wheat plants grown under high CO_2 (Fig. 1). Growth under high CO_2 has also been reported to counteract the toxic effects of SO_2 (Carlson and Bazzaz, 1982; Sandhu et al., 1992; Rao and DeKok, 1994) and UV-B radiation (Ziska and Teramura, 1992). The impact of O_3 on photosynthetic pigments was also absent in plants grown under a combination of high CO_2 and O_3 compared to that of plants exposed to O_3 under ambient CO_2 (Table I).

O_3 is considered to be a widespread phytotoxic air pollutant (Runeckles and Chevone, 1992). The ingress of O_3 seems to depend on the number and size of stomata and the size of their aperture (Heath, 1994). It has been shown that O_3 itself is not deleterious to plasma membranes (Grimes et al., 1983). As O_3 reacts, presumably instantaneously, with the components of cell walls and plasma membranes, reduced oxygen species such as O_2^-, OH^-, and H_2O_2 are formed (Mehlhorn et al., 1990). It is widely believed that these O_3-derived activated oxygen species affect different aspects of plant metabolism (Kangasjarvi et al., 1994).

One of the major roles of these activated oxygen species is to structurally modify cellular proteins (Pacifici and Davies, 1990) by altering their secondary and tertiary structure, thus enhancing their susceptibility to proteolysis (Pacifici and Davies, 1990; Pell and Dann, 1991; Stadtmann and Oliver, 1991). Growth under O_3 reduced total soluble protein, whereas growth in a combination of high CO_2 and O_3 had no major impact on total soluble protein compared to control plants (Fig. 2A). Foliar carbonyl groups were significantly lower in plants grown under a combination of high CO_2 and O_3 compared to those of plants exposed to O_3 in ambient CO_2. These results suggest that O_3-induced oxidative damage to proteins has been significantly reduced in plants grown in high CO_2 and O_3 compared to that of plants exposed to O_3 in ambient CO_2.

Changes in soluble protein content of plants grown in high CO_2 or in plants grown in high O_3 could be largely due to a decline in Rubisco protein (Fig. 3). Growth in high CO_2 has been reported to decrease Rubisco protein (Stitt, 1991; Long et al., 1993) whereas O_3 exposure has been shown to enhance proteolytic degradation of Rubisco protein (Pell and Dann, 1991). However, growth in a combination of high CO_2 and O_3 appeared to have no major effect on either RLSU or RSSU compared to plants grown in high CO_2 (Fig. 3). Exposure of plants to O_3 often causes accelerated foliar senescence characterized by premature leaf yellowing and abscission (Pell and Dann, 1991). O_3-induced acceleration of senescence is accompanied by a premature decrease in the activity and quantity of Rubisco protein. In this study, both RLSU and RSSU decreased significantly in plants exposed to O_3 and ambient CO_2 but not in plants exposed to O_3 and high CO_2 (Fig. 3). These results suggest that growth in a combination of high CO_2 and O_3 considerably reduces O_3-induced premature senescence, possibly by protecting the Rubisco protein from oxidative damage. In addition, the impact of O_3 on other foliar proteins was considerably reduced in plants grown in a combination of high CO_2 and O_3 compared to plants exposed to O_3 and ambient CO_2 (Fig. 3A).

These results clearly indicate that plants grown under high CO_2 have increased ability to tolerate O_3 toxicity. Considering the regulatory impact of high CO_2 on stomatal aperture, one may argue that growth under high CO_2 regulates O_3 flux and thereby offers protection. Since we have made no measurements of stomatal conductances, our data do not offer direct evidence for the role of stomata in regulating O_3 flux. However, high-CO_2-induced stomatal closure ranges between 10 and 20% and varies widely among plant species (Carlson and Bazzaz, 1985; Krupa and Kickert, 1989; Allen, 1990). The presumed small decrease in stomatal aperture is unlikely to account for the total absence of O_3 toxicity on growth and photosynthetic pigments (Carlson and Bazzaz, 1985; Barnes and Pfirrmann, 1992). Recently, Rao and DeKok (1994) reported a minimal role of high-CO_2-induced stomatal closure in regulating SO_2 flux in wheat plants. Accumulating literature suggests the protection to be minimal at the stomatal level and largely dependent on protective mechanisms operating at the cellular level (Rao, 1992; Rao and DeKok, 1994).

It has been established that plant tolerance of O_3 depends largely on the ability to detoxify O_3. Plants are endowed with an array of enzymes and small molecules such as ascorbate and glutathione to cope with free radicals. SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2, whereas PODs metabolize H_2O_2 (Foyer et al., 1994). In addition, plants are equipped with enzymes like GR and AP that are capable of metabolizing H_2O_2 by utilizing ascorbate and glutathione (Creissen et al., 1994; Foyer et al., 1994). The available literature, in general, relates plant tolerance of O_3 with enhanced antioxygenic activities and/or gene expression of various antioxidant enzymes (Kangasjarvi et al., 1994; Van Camp et al., 1994a; Willekens et al., 1994).

The data presented in Figures 4 and 5 indicate higher activities of SOD, POD, GR, and AP in plants exposed to O_3 and high CO_2 compared to those of plants exposed to O_3 at ambient CO_2. Although O_3 exposure at ambient CO_2 for 2 weeks enhanced the antioxygenic activities of SOD, POD, GR, and AP, prolonged exposure to O_3 decreased the activities of all antioxidant enzymes. However, plants grown under high CO_2 and O_3 maintained significantly higher activities of SOD, POD, GR, and AP. Oxidative damage to proteins occurs when the production of activated oxygen species overwhelms the antioxidant defense mechanisms. Since the oxidative damage to proteins was significantly greater in plants exposed to O_3 under ambient CO_2, we believe that higher activities of antioxidant enzymes of plants grown under a combination of high CO_2 and O_3 are indeed capable of protecting plant growth from O_3-induced oxidative damage.

Changes in the antioxygenic activities of SOD, POD, GR, and AP may be due to changes in their protein content

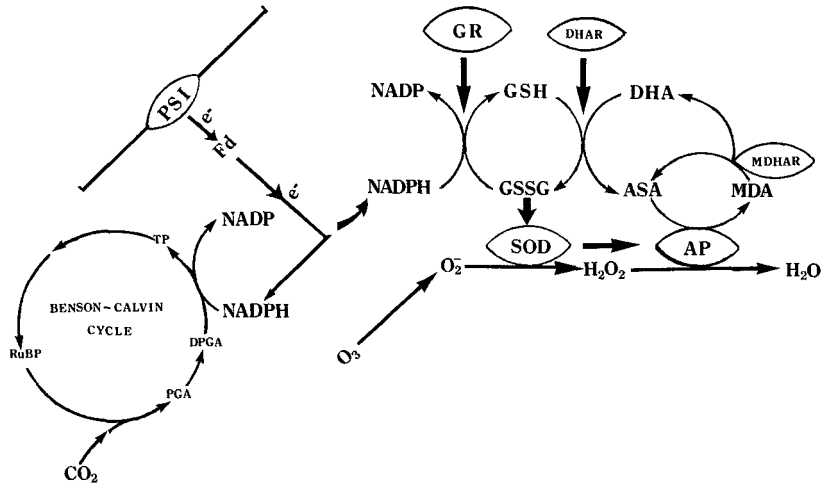

Figure 8. Schematic diagram of proposed scavenging system of O_3-derived activated oxygen species in plants grown under high CO_2 and O_3. O_3-derived O_2^-s are dismuted by SOD and the resulting H_2O_2 is metabolized by AP. Monodehydroascorbate radicals (MDA) produced by AP are converted to ascorbate (ASA) or dehydroascorbate (DHA) through a reaction catalyzed by monodehydroascorbate reductase (MDHAR). Dehydroascorbate is reduced to ascorbate by dehydroascorbate reductase (DHAR) utilizing glutathione. Reduced glutathione (GSH) is regenerated by GR in an NADPH-dependent reaction. NADPH generated by the photosynthetic electron transport pathway will be utilized by both Calvin and ascorbate- glutathione cycles. Plants grown in high CO_2 are believed to regulate the photosynthetic electron transport system and synthesize additional NADPH for enhanced carbohydrate synthesis. It has been proposed that plants grown in high CO_2 and O_3 are capable of diverting more NADPH to maintain higher GR activities and high redox states of ascorbate and glutathione (indicated by broad arrows) compared to plants exposed to O_3 in ambient CO_2. This, in turn, would have induced SOD and AP and scavenged O_3-derived activated oxygen species. Limited availability of NADPH in plants grown in ambient CO_2 could not have maintained high GR activities for longer durations, thus inactivating the antioxidant enzymes and antioxidant defense systems and enhancing oxidative damage to proteins. DPGA, Diphosphoglycerate; TP, triose phosphate; RuBP, ribulose-1,5-bisphosphate; PGA, glycerate-3-phosphate; e^-, electron.

and/or the synthesis of new isoforms. Recently, Edwards et al. (1994) reported that O_3 exposure induced two isoforms of GR in pea plants with no significant changes either in the GR protein or in the mRNA transcripts encoding GR. It has been suggested that the synthesis of new isoforms of antioxidant enzymes with altered kinetics properties may be more beneficial for the metabolism of free radicals than is the enhancement of the activities of existing antioxidant enzymes (Edwards et al., 1994). Since we observed higher antioxygenic activities of SOD, POD, GR, and AP in plants grown in high CO_2 and O_3, we have attempted to determine whether the changes in antioxidant enzymes represent changes in their isoform composition.

There were no major differences in the staining intensity of any isoforms of SOD, POD, GR, and AP between plants grown in ambient and high CO_2. However, exposure to O_3 and ambient or high CO_2 had differential effects on the isozyme composition of antioxidant enzymes. SOD-1, SOD-2, and AP-1 disappeared in plants exposed to O_3, whereas the intensities of SOD-3, GR-1, GR-2, AP-2, and AP-3 decreased significantly compared to plants grown at ambient CO_2 (Figs. 6 and 7). In contrast, growth under a combination of high CO_2 and O_3 enhanced the intensities of all three isoforms of SOD, whereas the POD-3 isoform was completely lost (Fig. 6). However, growth in a combination of high CO_2 and O_3 appeared to have preferentially enhanced the staining intensities of POD-1, AP-2, and AP-3 (Figs. 6 and 7). In addition, plants grown in a combination of high CO_2 and O_3 synthesized a new isoform of GR, and the staining intensities of existing GR isoforms was slightly enhanced (Fig. 7A). These results suggest that plants exposed to O_3 under high CO_2 are capable of differentially altering the isoform composition of SOD, POD, GR, and AP.

In the present study we have provided evidence for enhanced detoxification of O_3-derived activated oxygen species in plants grown under high CO_2 compared to that in plants grown under ambient CO_2. Growth under high CO_2 alone had no major effect on the specific activities of various antioxidant enzymes. Furthermore, O_3-enhanced specific activities of antioxidant enzymes were similar during initial stages of O_3 exposure irrespective of growth in ambient or high CO_2. However, prolonged O_3 exposure in ambient CO_2 inactivated antioxidant enzymes, whereas the activities were maintained in plants exposed to O_3 in high CO_2. Recently, Badiani et al. (1993) reported significant changes in the antioxidant metabolites and antioxidant enzymes of soybean grown under high CO_2; our results contradict their observations. It has been suggested that high CO_2 induces stromal acidification and decreases the activities of antioxidant enzymes that are relatively active at neutral-subalkaline pH. This condition has been suggested to predispose plants to oxidative stress (Schreiber and Neubauer, 1990). However, this did not happen in our experiments. Moreover, plants exposed to O_3 and high CO_2 for long durations are indeed capable of maintaining en-

hanced activities of several antioxidant enzymes compared to plants exposed to O_3 in ambient CO_2.

How does high CO_2 maintain O_3-enhanced specific activities of antioxidant enzymes? High-CO_2-induced photosynthesis requires additional NADPH for enhanced synthesis of carbohydrates (Long et al., 1993). Plants grown in high CO_2 are believed to regulate the photosynthetic electron transport system and synthesize additional NADPH to utilize CO_2 available in excess (Harbinson et al., 1990; Stitt, 1991; Long et al., 1993). Metabolism of activated oxygen species has been related to the high redox state of ascorbate and glutathione (Rao and DeKok, 1994) (Fig. 8). Regeneration of ascorbate is a critical component of the activated-oxygen-species scavenging system. The antioxidant enzyme GR, which utilizes NADPH, is primarily responsible for maintaining high redox states of ascorbate and glutathione (Foyer et al., 1994). Changes in the steady-state NADPH/NADP pool would not only affect the Calvin cycle but also the efficiency of the ascorbate-glutathione cycle (Foyer and Harbinson, 1994).

Excess availability of NADPH in plants grown in high CO_2 and O_3 would have maintained higher GR activities and thus, high redox states of ascorbate and glutathione. A high redox state of glutathione has been reported to induce Cu/Zn-SOD (Herouart et al., 1993). Enhanced expression of Cu/Zn-SOD would have enhanced AP because the mechanisms that control the expression of Cu/Zn-SOD and AP are believed to be similar (Sen Gupta et al., 1993b). This would have enabled plants grown in high CO_2 and O_3 to efficiently scavenge O_3-derived activated oxygen species compared to plants exposed to O_3 in ambient CO_2. Limited availability of NADPH in plants exposed to O_3 in ambient CO_2 could not have maintained high GR activities for long durations, resulting in enhanced oxidative damage to proteins. This would explain why plants grown in high CO_2 and O_3 are capable of detoxifying O_3-derived free radicals compared to plants grown in high O_3 and ambient CO_2. A schematic diagram indicating the possible mechanism is presented in Figure 8. No significant changes were observed in the antioxidant enzymes or in the redox state of ascorbate and glutathione of plants grown in high CO_2. These results suggest that the diversion of NADPH occurs only when required and such diversion appears to be regulated. Thus, additional, detailed studies are required to elucidate the role of high CO_2 in modifying the expression of various genes. Recently, high-CO_2-enhanced carbohydrates have been shown to modify the expression of various photosynthetic nuclear-encoded genes (Van Oosten et al., 1994).

In recent years efforts have been initiated to enhance plant tolerance to oxidative stress by modifying the plant antioxidant defense system through molecular biological techniques (Foyer et al., 1994; Allen, 1995). The importance of antioxidant enzymes was indicated by the enhanced tolerance to oxidative stress of plants overexpressing SOD or GR (Aono et al., 1993; Sen Gupta et al., 1993a; Van Camp et al., 1994b). In the present investigation, we provide evidence for the role of high CO_2 in maintaining an efficient antioxidant defense system upon receiving oxidative stress. However, it remains to be established whether these changes in specific activities represent similar increases in the protein content and/or gene expression of the respective antioxidant enzymes. Since we have shown that plants grown under high CO_2 are capable of resisting O_3-induced oxidative damage, it may also be possible that plants grown under high CO_2 would better overcome the adverse impact of other environmental stress factors (including heat shock, water deficit, UV radiation, and pathogens) that act via generation of activated oxygen species. This high-CO_2-induced tolerance to activated oxygen species should be considered when predicting the crop response to multiple stresses as well as while developing crop plants tolerant of changes in the environment.

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