Metabolic Bases for Differences in Sensitivity of Two Pea Cultivars to Sulfur Dioxide¹

Nageswara R. Madamanchi and Ruth G. Alscher*  
Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

ABSTRACT

An oxidative chain reaction of sulfite initiated by the superoxide ion produced in the Mehler reaction has been implicated in the damage of plants exposed to sulfur dioxide. The toxicity of SO₂ may be alleviated by free radical scavenging systems acting to terminate this chain reaction. Hence, the relative sensitivity of plants to SO₂ toxicity could depend on differences in the responses of the levels of antioxidant metabolites and enzymes. The effect of SO₂ exposure on glutathione and ascorbic acid contents, glutathione reductase, and superoxide dismutase activities was assayed in two cultivars (Progress, Nugget) of pea (Pisum sativum L.) in which apparent photosynthesis showed a differential sensitivity to 0.8 microliter per liter SO₂ (R. Alscher, J. L. Bower, W. Zipfel [1987] J Exp Bot 38:99–108). Total and reduced glutathione increased more rapidly and to a greater extent in the insensitive Progress than in the sensitive Nugget, as did glutathione reductase activities. Superoxide dismutase activities increased significantly in Progress, whereas no such change was observed in Nugget as a result of SO₂ exposure. This increase in superoxide dismutase activity was observed at 210 minutes after 0.8 microliter per liter SO₂ concentration had been reached, in marked contrast to the increases in reduced glutathione content and glutathione reductase activity, which were apparent at the 90 minute time point. These data suggest that one basis for the relative insensitivity of the apparent photosynthesis of the pea cultivar Progress to SO₂ is the enhanced response of glutathione reductase, superoxide dismutase activities, and glutathione content.

The resistance of plants to SO₂ is determined both by stomatal regulation of SO₂ absorption (avoidance) and by defense at the metabolic level against the toxicity of SO₂ absorbed. Once inside the leaf, SO₂ is hydrated to form HSO₃⁻, SO₃⁻, and H⁺ ions. The sulfite thus formed in the stroma in the light can affect carbon fixation, the activity of light-modulated enzymes, ribulose bisphosphate carboxylase, glycogen oxidase activity, cyclic and noncyclic photophosphorylation, and the phosphate translocator (2). Asada and Kiso (4) reported that the presence of sulfite or bisulfite leads to a free radical chain reaction generating increased superoxide (O₂⁻) levels in chloroplasts. The chain reaction is initiated by the interaction of sulfite with O₂⁻ produced as a result of the Mehler reaction. Superoxide ions and other reactive oxy-

gen species can cause oxidation of various cellular components with consequential damage to plants exposed to SO₂ (25).

The present investigation is a continuation of our studies aimed at understanding the metabolic bases for differences in sensitivity of two pea cv to SO₂ (2). Antioxidants function as metabolic protectants by removing oxygen radicals and associated species. Superoxide is scavenged by the enzyme SOD² (EC 1.15.1.1) and the reaction product hydrogen peroxide, a toxic molecule per se, is removed in subsequent reactions with ASC and glutathione (16). Glutathione exists in both a reduced form, GSH, and an oxidized form, GSSG. GSH, through its action as an antioxidant, helps to maintain proteins and other macromolecules in an active, reduced state (1). Glutathione is maintained in its reduced form through the activity of GR (EC 1.6.4.2), which in an NADPH-dependent reaction, catalyzes the reduction of GSSG to GSH. In our previous studies, GSH content increased in leaves of the insensitive pea cv during exposure to SO₂, whereas in the sensitive cv, no increase was observed until the postexposure period, when photosynthesis was recovering (2). If differential sensitivity to SO₂ is mediated by antioxidants and antioxidant enzymes, cv which differ in their relative sensitivity to the pollutant should exhibit differences in either the rate and/or magnitude of the responses of some or all of these enzymes or their substrates. Results obtained from the present investigation further implicate GSH, GR, and SOD in the resistance of peas to SO₂.

MATERIALS AND METHODS

Plant Material

Pea (Pisum sativum L.) cv Progress is insensitive and cv Nugget is sensitive to exposure to 0.8 µL L⁻¹ SO₂ (2). Seeds of Progress and Nugget were planted and grown in “containers” (Cone-Tainer Nursery, Canby, OR) containing Pro-Mix BX (Premier Brands Inc., Stamford, CT) in a growth chamber. The seedlings were grown under a 16 h photoperiod. Day and night temperatures were 25 to 27°C and 20°C, respectively. The seedlings were watered daily and fertilized once with Peter's Plant Soluble food (20–20–20 N:P:K) when they were 10 d old. Three-week-old plants were used in all experiments.

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² Abbreviations: SOD, superoxide dismutase; ASC, ascorbic acid; GR, glutathione reductase; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione.
Sulfur Dioxide Exposure Conditions

Seedlings were placed in continuously stirred tank reactors (CSTR) chambers 3 d before exposure to SO₂. A 16 h photoperiod (approximately 400 μE m⁻² s⁻¹ PAR at plant level) was supplied with a mercury vapor lamp, and temperature in the chambers was 20°C at night and was between 24 and 27°C during the day. Relative humidity varied between 50 and 80% and was supplemented by humidifiers whenever necessary during the day. SO₂ exposure began 7 h after the onset of illumination and lasted 210 min after the target concentration (0.8 μL L⁻¹⁻¹) was attained [0 (t) = time when target concentration was first reached, approximately 40 min from the start of exposure]. Control pea seedlings were placed in CSTR chambers in similar conditions without SO₂. SO₂ levels were measured by a fluorescent analyzer (model 8850, Monitor Labs Inc., San Diego, CA) and concentrations within the CSTR were controlled by a computerized system. Plant material collected for biochemical analyses was immediately frozen in liquid nitrogen and stored at -80°C until use.

Assay of Antioxidants

Preparation of Extracts

Leaf tissue (0.2 g) was ground in 1.5 mL 2% meta-phosphoric acid containing 2 mM EDTA in a precooled mortar. The homogenate was centrifuged at 17,000g for 10 min. The supernatant was used for assaying total and oxidized glutathione and ASC contents. All the spectrophotometric assays were performed using a Beckman DU-65 spectrophotometer.

Total and Oxidized Glutathione Assay

Total glutathione and GSSG were assayed following the procedure of Griffith (13). The leaf extract was brought to a pH of 5.5 by adding 0.6 mL 10% sodium citrate to 0.9 mL extract. For the determination of GSSG, 0.01 mL 2-vinylpyridine was added to 0.5 mL of neutralized extract. The solution was stirred for 1 min and incubated for 1 h at 25°C. Neutralized extraction medium was used as a blank. Excess 2-vinylpyridine was removed by extracting the solution twice with 1 mL of diethylether. The assay mixture contained 100 mM phosphate buffer (pH 7.5), 2 mM EDTA, 6 mM 5,5′-dithiobis-2-nitrobenzoic acid, 5 mM NADPH, 0.5 units of yeast GR, and the extract. Total glutathione content was determined by reference to a standard curve and expressed on the basis of GSH equivalents. Duplicates were run for each extract.

Ascorbic Acid Assay

ASC was analyzed by derivatizing dehydroascorbic acid with 2,4-dinitrophenylhydrazine following the procedure of Roe and Kuether (20) as outlined by Hausladen et al. (15). To 0.05 mL extract, 0.55 mL 5% meta-phosphoric acid/10% acetic acid solution was added. After adding about 5 mg activated, acid-washed charcoal, the solution was stirred for 30 s and centrifuged at 17,000g for 5 min. To 0.5 mL of supernatant, 0.125 mL of 2% 2,4-dinitrophenylhydrazine/4% thiourea in 9 N H₂SO₄ was added and the solution incubated for 3 h at 37°C. The resulting red precipitate was dissolved by addition of 85% H₂SO₄ on ice, and the solution was incubated at room temperature for 30 min. Absorbance was read at 523 nm against an extraction medium blank. ASC was calculated from a calibration curve. The absorbance was proportional to the ASC content between 2 and 200 nmol/assay mixture.

Enzyme Extraction

For assays of GR and SOD, 0.2 g leaf tissue was homogenized in 1.5 mL 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 17,000g for 10 min, and the supernatant was used for enzyme assays.

Glutathione Reductase Assay

GR activity was determined from the rate of NADPH oxidation as measured by the decrease in absorbance at 340 nm following the procedure of Foyer and Halliwell (11). The 1 mL assay mixture contained 0.1 M Tris buffer (pH 7.8), 2 mM EDTA, 50 μM NADPH, 0.5 mM GSSG, and the extract. The assays were initiated by the addition of NADPH and were carried out at 25°C. The initial velocity of the reaction was determined, and activity was expressed as nmol of NADPH oxidized min⁻¹ mg⁻¹ protein.

Superoxide Dismutase Assay

SOD activity was assayed based on the method of Beauchamp and Fridovich (5), as modified by Dhindsa et al. (9), by measuring the ability of the enzyme to inhibit the photodecomposition of nitro blue tetrazolium. The 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), containing 0.1 mM EDTA, 13 mM methionine, 75 μM nitro blue tetrazolium, 2 μM riboflavin, and the enzyme extract. Riboflavin was added last, the tubes were stirred, and the reaction initiated by placing the tubes under two 15 W fluorescent lamps. The reaction was terminated after 10 min by switching off the light. Nonilluminated tubes served as blanks. The tubes were stirred and the blue color was measured at 560 nm. The volume of enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit (5).

Protein Assay

Protein content of the enzyme extracts was measured according to the procedure of Bradford (6).

Statistical Analysis

The experiments were repeated twice and three plants were collected for each time period in each experiment. Two-way analysis of variance was performed to test for the main effects of SO₂ exposure, time, and their interactions.

RESULTS

Our previous studies have established that apparent photosynthesis in pea cv Progress was relatively insensitive and cv Nugget relatively sensitive to an 80 min exposure to 0.8 μL L⁻¹⁻¹ SO₂ (2). Apparent photosynthesis in both cv in this
investigation showed similar differential sensitivity during a 210 min exposure to 0.8 μL L⁻¹ SO₂ (data not shown).

Here we report on the contents of the antioxidants glutathione and ASC, and the antioxidant enzymes GR and SOD in pea cv Progress and Nugget during a 210 min exposure to 0.8 μL L⁻¹ SO₂. Mean total foliar glutathione content increased in SO₂-exposed plants compared with their respective controls in both Progress (Fig. 1A) and Nugget (Fig. 1B) (P < 0.001). However, the increase in total glutathione content over time in Progress exposed to SO₂ was greater than that of Nugget. The interaction between SO₂ treatment and time for total glutathione content in Progress was significant (P < 0.1). In Progress, the ratio of exposed/control total glutathione increased from 1.11 at 0 (t) min to 2.04 at the end of the 210 min exposure period. The interaction between SO₂ treatment and time with respect to total glutathione content was not statistically significant in Nugget (P > 0.1). In Nugget, the ratio of exposed/control total glutathione only increased from 1.42 at 0 (t) min to 1.69 at the end of 210 min exposure period.

Mean GSH content increased in SO₂-exposed Progress (Fig. 1C) and Nugget (Fig. 1D) compared with their respective controls (P < 0.001). As with total glutathione content, the response of GSH content to SO₂ exposure was greater in Progress than in Nugget. GSH content showed a significant change over time in Progress after exposure to SO₂ (P = 0.0067), whereas no significant change was observed in Nugget exposed to SO₂ (P = 0.262). In Progress, the ratio of exposed/control GSH increased from 1.11 at 0 (t) min to 1.93 at the end of the 210 min exposure period, whereas the exposed/control GSH ratio only increased from 1.37 at 0 (t) min to 1.59 at the end of 210 min exposure period in Nugget. The increases in GSH content were similar to the increases in total glutathione content, indicating that the major portion of increase in total glutathione was due to an increase in GSH.

Exposure to SO₂ had no significant effect on GSSG content in either Progress (Fig. 2A) or Nugget (Fig. 2B) compared with their respective controls (P > 0.001). The interaction between SO₂ treatment and time was also not significant for GSSG content in either cv (P > 0.1).

Mean GR activity increased in Progress (Fig. 2C) and Nugget (Fig. 2D) compared with their respective controls due to exposure to SO₂ (P < 0.001). The interaction between SO₂ treatment and time with respect to GR activity showed a

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**Figure 1.** Effect of exposure to 0.8 μL L⁻¹ SO₂ on mean total glutathione and mean GSH contents in the leaves of pea cv Progress and Nugget. 0 (t) represents the time when the target concentration of 0.8 μL L⁻¹ SO₂ is attained, approximately 40 min from the start of exposure. Values represent the mean ± se of three replicates from each of two experiments.
significant trend in Progress (P = 0.065), whereas it was not significant in Nugget (P = 0.199). In Progress, mean GR activity increased 17.7 and 35.2% in SO₂-exposed plants over their respective controls at the 90 and 210 min exposure periods, respectively. In contrast, the mean GR activity in Nugget increased only 4.5 and 21.0% in SO₂-exposed plants over their respective controls at the 90 and 210 min exposure periods, respectively. The rapid and greater increase in GR activity in SO₂-exposed Progress corresponded with a similar increase in GSH content.

Mean SOD activity increased in Progress (Fig. 3A) exposed to SO₂ compared with the respective control (P < 0.001), whereas SO₂ exposure had no effect on the mean SOD activity in Nugget (P > 0.1) (Fig. 3B). The response of SOD activity to SO₂ exposure was much slower than that of GR. In Progress exposed to SO₂, SOD activity was similar to that of the controls up to 150 min of exposure, but increased greatly thereafter. The change in SOD activity over time was significant in Progress exposed to SO₂ compared with the control (P = 0.000). In Progress, the exposed/control SOD activity ratio increased from 0.87 at 0 (t) min to 3.38 at the end of the 210 min exposure period. Exposure to SO₂ had no significant effect on SOD activity over time (p = 0.556) in Nugget. Mean ASC content did not change significantly in either Progress (Fig. 3C) or Nugget (Fig. 3D) after exposure to SO₂ (P > 0.001). There was also no significant interaction between SO₂ treatment and time with respect to ASC content in either cv (P > 0.1).

**DISCUSSION**

Apparent photosynthesis has been shown to be differentially sensitive to SO₂ (0.8 μL L⁻¹) exposure in pea cv Progress and Nugget, with Nugget being more sensitive (2). Differences in the relative abilities of metabolic resistance mechanisms to respond to the oxidative stress imposed by SO₂ exposure may be responsible for this differential sensitivity. Alscher et al. (2) have reported that more sulfite accumulated in Nugget exposed to SO₂ than in Progress. This suggests that sulfite detoxification processes are important in differential resist-
Sulfite can undergo free radical chain oxidation mediated by photoproduced O$_2^-$ (Mehler reaction) in chloroplasts and can amplify production of O$_2^-$, the hydroxyl and bisulfite radicals (3), causing increased oxidative stress. This is a possible explanation for SO$_2$-mediated damage of plants in the light.

The mechanism by which SO$_2$ decreased apparent photosynthesis was not through an effect on stomatal conductance, as was shown earlier (2). Total glutathione increased over time in both the cv after exposure to SO$_2$ in the present investigation, but this increase was only statistically significant in Progress, indicating higher rates of glutathione biosynthesis in the insensitive cv after exposure to SO$_2$. Increases in GSH content over time after exposure to SO$_2$ corresponded with an increase in total glutathione content in both cv because GSSG is found in low concentrations (about 15% of the total glutathione content). Neither total glutathione nor GSH contents changed greatly in controls of either cv during the treatment period. Hence, the observed increase in total glutathione is truly a response to the stress due to exposure to SO$_2$. Increased levels of GSH on exposure to SO$_2$ and other forms of oxidative stress have been reported (2, 7, 14).

It is assumed that synthesis of glutathione in plants occurs in a two-step process, as in animals and bacteria. In the first step, the dipeptide r-L-glutamyl L-cysteine synthesis is catalyzed by r-glutamylcysteine synthetase in an ATP-dependent reaction requiring L-glutamate and L-cysteine (19). De Kok et al. (8) have reported higher levels of cysteine in spinach leaves exposed to SO$_2$, which suggests that SO$_2$ exposure brings about an increased availability of substrate for glutathione biosynthesis. The synthesis of glutathione from its constituent amino acids was shown to be partially inhibited by GSH, suggesting that r-glutamylcysteine synthetase is sensitive to feedback inhibition by GSH (19). Hydrogen peroxide, a product of the action of SOD, may cause oxidation of GSH to GSSG and thus alleviate feedback inhibition, allowing additional GSH synthesis as suggested by Smith et al. (23). Both in Progress and Nugget, total glutathione content increased significantly after exposure to SO$_2$. However, GSSG content did not increase significantly in either cv during the exposure to SO$_2$, suggesting that release of feedback inhibition of GSH synthesis by GSSG may not be the primary mechanism responsible for enhanced GSH synthesis during oxidative stress. Perhaps increased substrate availability also contributed to the increased glutathione content in the pea cv exposed to SO$_2$.

Glutathione reductase activity increased in both the cv

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Figure 3. Effect of exposure to 0.8 µL L$^{-1}$ SO$_2$ on mean SOD activities and mean ASC contents in the leaves of pea cv Progress and Nugget. 0 t) represents the time when the target concentration of 0.8 µL L$^{-1}$ SO$_2$ is attained, approximately 40 min from the start of the exposure. Values represent the mean ± se of three replicates from each of two experiments.
exposed to SO$_2$ compared with their respective controls, but the increase was more rapid and greater in Progress than in Nugget. Onset of an appreciable and rapid increase in GR activity corresponded to a similar increase in GSH content in Progress exposed to SO$_2$. Increases in GR activity have been reported in various plants subjected to oxidative stress (10, 12, 21, 24) and the enzyme is thought to play a key role in protection against oxidative stress (11, 24). Our results suggest that SO$_2$ enhances both glutathione biosynthesis and GR activity in the pea cv, with the insensitive cv Progress showing greater responses. The increase in enzyme activity observed may be an expression of enzyme activation and/or enhanced transcription or translation. It has been demonstrated that ozone exposure causes de novo synthesis of GR in spinach leaves (24). Work is in progress to determine whether one or both of these mechanisms occur in peas exposed to SO$_2$. 

SO$_2$ exposure had no effect on ASC content in leaves of either pea cv. Although increased levels of ASC were reported in bean leaves exposed to ozone (18), no such increase was observed in spruce exposed to ozone (15).

SOD inhibits the chain oxidation of sulfite by scavenging O$_2^-$, thus SOD is one possible tolerance mechanism of leaf cells against SO$_2$ (3). Increased SOD activity affords increased protection against oxidative stress (25). We observed, also, that in the insensitive cv Progress, SOD activity increased 90% over the control in 180 min in response to SO$_2$ exposure (Fig. 3A), whereas no increase was observed in the sensitive cv Nugget.

The response of SOD activity to SO$_2$ exposure was slower than that of GR activity and total glutathione content in Progress. Similar results have been observed with poplars exposed to ozone (22). A plausible explanation for the slower response of SOD activity is that de novo synthesis of SOD occurs as more oxidant is produced than existing levels of enzyme could accommodate. However, in the sensitive cv, no increase in SOD was observed under conditions in which oxidant levels would be higher. Alternatively, a chain of events involving glutathione metabolism in the insensitive cv may be involved in the increased activity of SOD.

SOD occurs in three forms, depending on the prosthetic metals: Cu, Zn-SOD, Mn-SOD, and Fe-SOD (17). The identity of SOD isoforms involved in the response of pea to SO$_2$ is not yet known, nor is the mechanism(s) by which their activity is increased. Experiments are under way to determine the mechanisms regulating SOD in peas exposed to SO$_2$.

LITERATURE CITED