Comparative Effects of Paraquat on Antioxidant Components and Scavenging Enzymes in Kwangkyo and Hood Soybean

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The effect of paraquat on hydroxyl radical production, glutathione and ascorbate content, and glutathione reductase and superoxide dismutase activities was assayed in the tolerant "Kwangkyo" and the susceptible "Hood" cultivars of soybean [*Glycine max* (L.) Merr.]. Seedlings of both cultivars at the fully expanded first trifoliate leaf stage were treated with paraquat at 0, 1, 10, 100, 500, and 1000 μM. The *in vivo* generation of hydroxyl radicals was estimated by measuring methanesulfonic acid (MSA) produced in soybean leaves treated with dimethylsulfoxide (DMSO) and paraquat. The levels of paraquat-induced production of hydroxyl radicals were similar in Kwangkyo and Hood soybean. Ascorbate levels in the two cultivars were comparable and were reduced by paraquat treatment. Total glutathione levels were similar in both cultivars, but Kwangkyo had more glutathione in the reduced form than Hood. Treatment with low concentrations of paraquat enhanced oxidized glutathione levels in Hood. At high concentrations, paraquat decreased the levels of total and reduced glutathione in both cultivars. The endogenous activities of glutathione reductase and superoxide dismutase were similar in Kwangkyo and Hood soybean. Treatment with selected concentrations of paraquat enhanced the activity of these enzymes in trifoliates of the tolerant Kwangkyo. Overall, the results of this study do not suggest a strong involvement of components of the ascorbate-glutathione cycle in the observed tolerance of Kwangkyo soybean to the herbicide paraquat.

Introduction

It is well established since 1965, that the herbicide paraquat mediates the production of reactive oxygen species in the chloroplasts of treated plants by interacting with photosystem I in the vicinity of ferredoxin and diverting electrons from $NADP^+$ [1]. Although superoxide ion is the first toxic oxygen species produced in this process, hydroxyl radicals generated from superoxide are more likely to be the major toxic agent [2]. It is also possible that hydroxyl radicals could be generated from reduced paraquat and hydrogen peroxide. The produced toxic oxygen species are responsible for the cell damage and death of green plants treated with paraquat.

Chloroplasts and the cytosol of plant cells are well endowed with scavenging systems to cope with toxic species produced during the reduction of molecular oxygen. The ascorbate-glutathione cycle [3], also known as the Halliwell-Asada pathway [4], plays a major role in scavenging the active oxygen species produced under oxidative stress conditions induced by air pollutants like O_3 and herbicides such as paraquat and nitrodiphenylethers [4]. Higher constitutive levels of the components (substrates, cofactors, and enzymes) of the ascorbate-glutathione cycle have been correlated with resistance to oxidative stresses [4]. Changes in the levels of ascorbate-glutathione cycle components induced by oxidative stresses that are mediated by air pollutants and/or herbicides have also been reported [4].

Enhanced levels and/or activities of the components of the ascorbate-glutathione cycle may also contribute to the observed paraquat tolerance or resistance in several weed species. Such a mechanism has been implicated in the resistance of biotypes of perennial ryegrass (*Lolium perenne* L.) and hairy fleabane [*Conyza bonariensis* (L.) Cronq.], to the herbicide paraquat [4–8].

In the preceding paper [9] we showed that Kwangkyo and Hood soybean differ in their response to the herbicide paraquat. Kwangkyo is approximately 10 times more tolerant to paraquat than Hood soybean. The objectives of the present study were: a) to quantify the production of hydroxyl radicals in paraquat-treated and non-treat-

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ed leaf tissues of Kwangkyo and Hood soybean and b) to compare the effects of paraquat on the levels and/or activities of selected components of the ascorbate-glutathione cycle of Kwangkyo and Hood soybeans. Antioxidants and enzymes examined were ascorbate, glutathione (total, oxidized, reduced), glutathione reductase (GR; EC 1.6.4.2) and superoxide dismutase (SOD; EC 1.15.1.1).

Materials and Methods

Chemicals

Formulated paraquat (GRAMOXONE®) and the surfactant X-77 were obtained from Chevron Chemical Company, Richmond, California. Analytical grade paraquat, yeast glutathione reductase and other reagents were purchased from Sigma Chemical Company, St. Louis, Missouri.

Plant material and paraquat treatment

Seeds of the two soybean cultivars were planted and grown in a 1:2:2 (v/v/v) mixture of peat moss: vermiculite: weblite, plus a slow release fertilizer, in a greenhouse with temperature of 25 ± 5 °C, 16 h photoperiod and photosynthetic photon flux density (PPFD) of 600 μE m^{-2} s^{-1}. When seedlings of both cultivars reached the stage of the fully expanded first trifoliate leaf, paraquat and the surfactant X-77 were applied as a foliar spray at 0, 1, 10, 100, 500, and 1000 μM with a hand atomizer until run-off. The treated soybean seedlings were placed in a continuously illuminated growth chamber with 27 °C and 600 μE m^{-2} s^{-1} PPFD. 24 h after paraquat treatment, soybean leaves were collected, frozen immediately in liquid nitrogen and stored at −70 °C until they were used for the following analyses.

Determination of hydroxyl radical ($OH^{\cdot}$) production

The *in vivo* production of $OH^{\cdot}$ was estimated according to the method of Babbs *et al.* [10], which uses dimethyl sulfoxide (DMSO) as a molecular probe to trap the reactive and unstable $OH^{\cdot}$. DMSO is oxidized by $OH^{\cdot}$ forming methanesulfinic acid (MSA), a stable non-radical product, which can be extracted from plant tissue and measured spectrophotometrically. Two grams of leaf tissue were harvested from Kwangkyo and Hood seedlings that had been treated with 5% DMSO (controls) or with 5% DMSO plus 1000 μM paraquat. The tissue was frozen with liquid nitrogen, pulverized with a mortar and pestle and then extracted with distilled water (15–20 ml). Plant debris was removed by centrifugation, and the aqueous supernatant was concentrated by lyophilization. Interfering substances were removed with the aid of Sep-Pak columns and the color reaction was initiated with the addition of fast blue BB dye as has been described previously [10]. The concentration of methanesulfinic acid was determined spectrophotometrically at 420 nm and was expressed as nmol MSA per g fresh weight.

Antioxidant assays

Leaf tissue (0.2 g) from control or paraquat-treated seedlings of both soybean cultivars was ground in 1.5 ml 2% *meta*-phosphoric acid containing 2 mM EDTA in a precooled mortar. The homogenate was centrifuged at $17{,}000 \times g$ for 10 min. The supernatant was used for the spectrophotometric determination of ascorbate and glutathione contents.

Ascorbate was assayed by derivatizing dehydroascorbate with 2,4-dinitrophenylhydrazine (2,4-DNPH) following the procedure of Hauslanden *et al.* [11]. 15 ml of a mixed solution of 5% *meta*-phosphoric acid and 10% acetic acid were added to 0.05 ml of the leaf extract. After the addition of about 5 mg of activated, acid-washed charcoal, the solution was stirred for 30 s and centrifuged at $17{,}000 \times g$ for 5 min. Then 0.125 ml of 2% 2,4-DNPH/4% thiourea in 9 N H_2SO_4 was added and the solution was incubated for 3 h at 37 °C. The resulting red precipitate was dissolved with ice-cold H_2SO_4 (85%) and the solution was incubated at room temperature for 30 min. Absorbance was read at 523 nm and ascorbate content was calculated from a standard curve.

Total and oxidized (GSSG) glutathione were assayed following the procedure of Griffith [12]. The pH of the leaf extract was brought to 5.5 by the addition of citrate. For GSSG determination, the extract was neutralized with 2-vinylpyridine and following stirring for 1 min, the solution was 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 di-

ethylether. The assay mixture contained 100 mM phosphate buffer (pH 7.5), 2 mM EDTA, 6 mM DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], 5 mM NADPH, 0.5 units of yeast glutathione reductase and the extract. Total glutathione content was determined by reference to a standard curve and expressed on the basis of GSH equivalents. Reduced glutathione (GSH) was calculated by subtracting oxidized from total glutathione.

Enzyme extraction and assay

Leaf tissue (0.5 g) was homogenized in 1.5 ml phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 17,000 × *g* for 10 min and the supernatant was used for assaying enzyme activity.

Glutathione reductase (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 [3]. The 1 ml assay mixture contained 0.1 mM 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 and GR activity was expressed as nmol NADPH oxidized per mg of protein per min. Protein content of crude enzyme extracts was measured according to the Bradford method [13] using bovine serum albumin (BSA) as a standard.

Superoxide dismutase (SOD) activity was assayed polarographically monitoring the ability of the enzyme to consume O_2 with an oxygen electrode [14]. The 2 ml reaction mixture contained 66 mM Tris-HCl buffer (pH 8.9) containing 9 mM TEMED, 0.1 mM EDTA, BSA (33 μg/ml), 0.1 mM nitro blue tetrazolium, 20 μM riboflavin and the enzyme extract. The reaction was initiated by exposing the oxygen electrode chamber to light of 600 $\mu E\ m^{-2}\ s^{-1}$ PPFD. The O_2 consumption rate was calculated based on the amount of O_2 air saturation (0.253 μmol O_2/ml H_2O at 25 °C).

Statistical analysis

All experiments were completely randomized designs and were repeated in time twice. All treatments within each experiment were replicated two or three times. Treatment means and their standard errors are presented graphically as histograms.

Results and Discussion

Production of hydroxyl radicals in Kwangkyo and Hood soybean

The generation of hydroxyl radicals in trifoliate leaves of Kwangkyo and Hood soybean treated with DMSO or DMSO plus paraquat was estimated by spectrophotometric measurements of methanesulfinic acid (MSA) (Fig. 1). Under normal photosynthetic conditions, the levels of MSA produced in DMSO-treated (control) leaves of the tolerant Kwangkyo soybean were considerably greater than those produced in leaves of the susceptible Hood soybean (Fig. 1). This result shows that Kwangkyo soybean may be able to tolerate greater levels of toxic oxygen species such as free radicals. Nevertheless, additional research utilizing more direct techniques (*e.g.* ESR spectroscopy) will be needed to validate such a postulation.

Treatment with 1000 μM of paraquat plus DMSO increased significantly the production of MSA in both soybean cultivars (Fig. 1). These results confirm indirectly that treatment with paraquat induces the production of hydroxyl radicals in the leaves of both soybean cultivars and are in agreement to those reported earlier by Babbs *et al.* with ryegrass and lemna plants [10]. Overall, it can be concluded that differential paraquat-induced production of hydroxyl radicals does not seem to explain the observed differential response of Kwangkyo and Hood soybean to this herbicide.

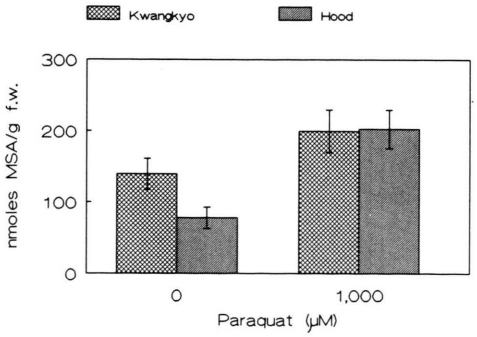

Fig. 1. Production of methanesulfinic acid (MSA) in Kwangkyo and Hood soybean leaves at 24 h after treatment with DMSO and paraquat. Values are means of four replicates ± standard error.

Antioxidant levels in Kwangkyo and Hood soybean

Finkh and Kunert [15] have proposed that ascorbate can contribute to plant resistance against oxidative damage, not only because it participates in the scavenging of H_2O_2, but also because of its role in the direct regeneration of α-tocopherol (vitamin E) during peroxidation. Endogenous levels of ascorbate were slightly higher in first trifoliates of Kwangkyo compared to those of Hood soybean (Fig. 2). Treatment with low concentrations of paraquat did not affect ascorbate levels in any of the two soybean cultivars. However, paraquat at concentrations of 100 μM or higher reduced considerably ascorbate levels in both cultivars (Fig. 2). These results agree in part with those of Gullner *et al.* [16], who reported that treatment with paraquat did not affect ascorbate levels in leaves of paraquat-resistant and susceptible tobacco cultivars.

The constitutive levels of total glutathione in leaves of both soybean cultivars appeared to be very similar (Fig. 3A). However, in Kwangkyo soybeans more glutathione was present in its reduced form (GSH), whereas Hood soybean contained more oxidized glutathione (GSSG) than Kwangkyo (Fig. 3B and 3C).

Treatment with low concentrations of paraquat did not affect the levels of total glutathione in the two soybean cultivars (Fig. 3A). At high concentrations, however, paraquat reduced the total glutathione content of both Kwangkyo and Hood. Treatment with high concentrations of paraquat reduced the GSH content in both soybean cultivars, but this effect of paraquat was more pronounced in leaves of Hood soybean (Fig. 3B). Paraquat did not affect the GSSG content of Kwangkyo soybean (Fig. 3C). However, at low concentrations, paraquat increased the GSSG content in leaves of Hood soybean (Fig. 3C).

The obtained results do not seem to make a strong case for the potential involvement of the antioxidants glutathione and ascorbate in the observed differential sensitivity of Kwangkyo and Hood soybean to oxidative stress induced by the herbicide paraquat.

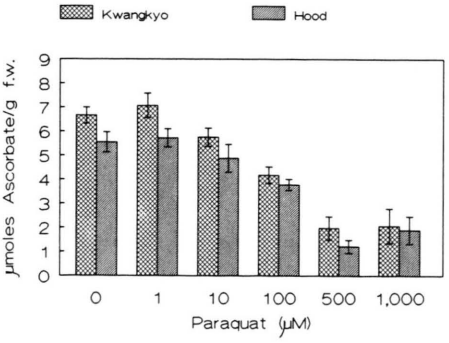

Fig. 2. The effect of paraquat on ascorbate content of the first trifoliate of Kwangkyo and Hood soybean at 24 h after treatment. Values are means of four replicates ± standard error.

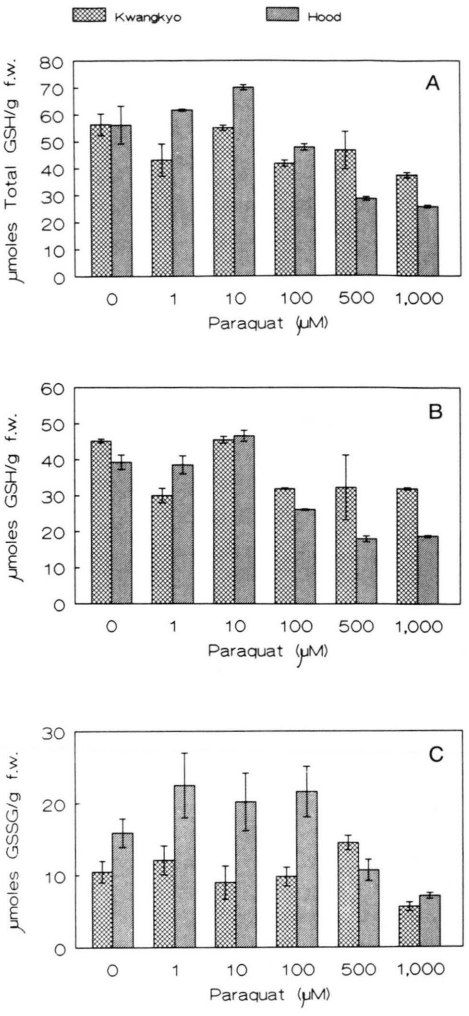

Fig. 3. The effect of paraquat on total (A), reduced (B), and oxidized (C) glutathione content of the first trifoliate of Kwangkyo and Hood Soybean at 24 h after treatment. Values are means of four replicates ± standard error.

Activities of scavenging enzymes

The constitutive activity of glutathione reductase was slightly higher in leaves of Kwangkyo than those of Hood soybean (Fig. 4). Treatment with low concentrations of paraquat induced GR activity in leaf tissues of both cultivars, but the induction was greater in Kwangkyo than in Hood soybean (Fig. 4).

The endogenous activity of superoxide dismutase was slightly higher in Hood than in Kwangkyo soybean (Fig. 5). Treatment with paraquat had little effect on the SOD activity of Hood soybean. Treatment with most concentrations of paraquat enhanced slightly SOD activity in Kwangkyo soybean (Fig. 5).

The observed paraquat-induced enhancement of the activity of GR and SOD enzymes in Kwangkyo soybean shows that the ascorbate-glutathione cycle may be involved in the differential response of Kwangkyo and Hood soybean to paraquat. The lack of a good correlation between the enhanced activity of GR and the observed levels of GSH and GSSG in paraquat-treated Kwangkyo soybean might be due to the fact that while GR is mainly localized in the plastid, glutathione and its oxidized/reduced forms are distributed throughout the leaf cell, with large pools occurring in the cytosol as well as in the plastid. Thus, detailed studies comparing the antioxidant levels and activities of scavenging enzymes in specific cell compartments such as the chloroplast and cytosol rather than whole leaf tissues will be needed to provide a more conclusive answer as to the contribution of the free

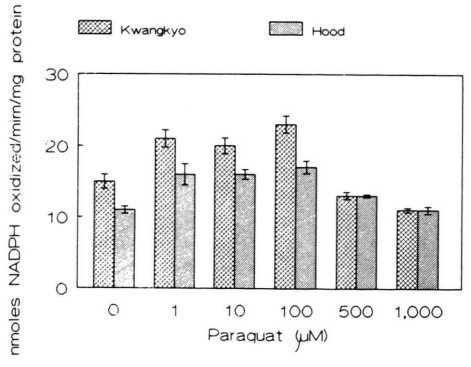

Fig. 4. The effect of paraquat on glutathione reductase activity in the first trifoliate of Kwangkyo and Hood soybean at 24 h after treatment. Values are means of four replicates ± standard error.

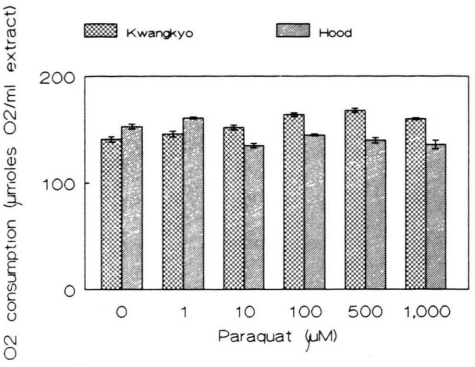

Fig. 5. The effect of paraquat on superoxide dismutase activity in the first trifoliate of Kwangkyo and Hood soybean at 24 h after treatment. Values are means of four replicates ± standard error.

radical detoxifying system in the tolerance of Kwangkyo soybean to paraquat.

The results of studies attempting to correlate the levels or activities of enzymes detoxifying free radicals and tolerance or resistance to oxidative stresses induced by paraquat or air pollutants are often contradictory. In a detailed survey of 15 lines of *Lolium perenne*, Harper and Harvey [5] found that the activity of the scavenging enzymes SOD, catalase, and peroxidase was slightly enhanced in tolerant lines. However, studies with SOD extracted from chloroplasts of these tolerant lines of *Lolium* failed to show any correlation between enhanced SOD activity and tolerance to paraquat [5]. Similarly, several investigators have failed to demonstrate enhanced activities of scavenging enzymes in paraquat-resistant biotypes of *Conyza bonariensis* [17–19].

Apart from the aforementioned problem of the compartmentation of scavenging enzymes in plant cells, such contradictory results may also result from differences in the activity of these enzymes caused by leaf age and treatment conditions. Gillham and Dodge [20] showed that the levels of ascorbate, ascorbate peroxidase, and glutathione reductase in pea leaves were variable during the course of the year and that illumination of peas with strong light enhanced the levels of these components. Variability in SOD activity of maize leaves throughout their life has also been demonstrated [21].

As it was pointed out in the previous paper [9], additional processes such as reduced mobility or

sequestration may be more important that the components of the ascorbate-glutathione cycle in contributing to the relative tolerance of Kwangkyo soybean to paraquat.

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