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Effects of Paraquat on the Oxygen Free Radical Biology of Soybean Root Nodules

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Paraquat (methyl viologen) is a powerful herbicide that is readily taken up by chloroplasts where it leads to the formation of superoxide and hydrogen peroxide with subsequent peroxidation of membrane lipids and disruption of chloroplast structure (Farrington et al. 1973). Although paraquat effects are most pronounced in light, it can also kill plants in the dark and can be highly toxic in mammalian systems, presumably through free radical formation associated with mitochondrial electron transport (Dodge 1971). Paraquat is used worldwide in agriculture and field residues as high as 1,000 ppm have been reported (Calderbank et al. 1968).

Oxygen relations in nitrogen-fixing root nodules are important not only because of the direct oxygen sensitivity of nitrogenase, but also because of the presence of activated species of oxygen such as hydrogen peroxide and superoxide. Nitrogen-fixing organisms have high levels of superoxide dismutase (SOD), a ubiquitous enzyme in aerobic organisms that has an essential role in the protection of the nitrogen fixation process (Puppo and Rigaud 1986). In addition to SOD, nitrogen-fixing root nodules also contain an enzymic system to scavenge and remove H_2O_2 (Dalton et al. 1986). The initial reaction by which H_2O_2 is removed is catalyzed by ascorbate peroxidase. Peroxidation of ascorbate results in the formation of monodehydroascorbate (MDHA) - a free radical that has two possible fates. MDHA may spontaneously disproportionate to dehydroascorbate and ascorbate, in which case the dehydroascorbate is recycled back to ascorbate by dehydroascorbate reductase in a reaction utilizing reduced glutathione (GSH). GSH is in turn regenerated by a NADPH-dependent reaction catalyzed by glutathione reductase. Alternatively, MDHA may be directly reduced to ascorbate in a NADH-dependent reaction catalyzed by MDHA reductase. The enzymes of the ascorbate-glutathione pathway are also found in chloroplasts (Groden and Beck 1979) and cyanobacteria (Tel-Or et al. 1986).

This report examines the effects of paraquat on a number of nodule parameters relating to nodule free radical biology including the specific activities of defense enzymes and the concentrations of two key antioxidants - ascorbate and glutathione.

MATERIALS AND METHODS

Soybean seeds (*Glycine max* (L.) Merr. cv. Williams 82) were inoculated with *Bradyrhizobium japonicum* 122 DES. Plants were provided daily with nutrient solution and grown in Perlite as described elsewhere (Dalton et al. 1986). At 25 to 28 d after planting, the plants were divided into separate treatment groups. A control group continued to receive nutrient solution daily and experimental groups

received nutrient solution daily containing either 0.1 mM or 1.0 mM paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride, Sigma).

Reduced glutathione (GSH), oxidized glutathione (GSSG), total protein, and activities of enzymes of the ascorbate-glutathione cycle were measured using crude extracts of nodules as described by Dalton et al. 1991. SOD activity was determined by the xanthine oxidase - cytochrome c assay (Flohe and Otting 1984). Nitrogenase activity was assayed by measuring acetylene reduction of 0.2 to 0.4 g fresh weight of excised nodules incubated for 10 min in 24 ml sealed glass serum vials containing 10 kPa of C_2H_2. Ethylene was analyzed with a Varian model 3300 gas chromatograph equipped with a 0.318 cm x 1.83 m stainless steel column of Poropak N and a flame ionization detector.

The H_2O_2 content of nodules was determined by colorimetric reaction of 4-(2-pyridylazo)resorcinol (Sigma) and titanium potassium oxalate (Becana et al. 1986). Lipid peroxidation was analyzed based on the formation of substances that react with thiobarbituric acid (primarily malondialdehyde, Heath and Packer 1968). Nodule extract was prepared by grinding nodules (0.5 g fresh weight) with a mortar and pestle containing 5 ml of ice-cold 0.1 % trichloroacetic acid. The resulting macerate was centrifuged at 10,000 x g for 5 min. A 1 ml aliquot of the supernatant was added to 4 ml of a solution containing 20 % trichloroacetic acid plus 0.5 % (w/v) thiobarbituric acid (TBA). This was heated to 95°C for 30 min, rapidly cooled in an ice bath and then centrifuged at 10,000 x g for 10 min. The A_{532} was recorded against a blank that contained all reagents without nodule extract. Ascorbate content of nodules was determined by a modification of the procedure of Hewitt and Dickes (1961). 0.5 g of fresh weight of nodules was ground with mortar and pestle in 1.5 ml of 2% (w/v) metaphosphoric acid plus 1 mM EDTA. The extract was then centrifuged at 12,000 x g for 5 min. A 0.5 ml aliquot of the supernatant was removed and added to 0.25 ml of 10% (w/v) of sodium citrate. After mixing, 0.1 ml was placed in a 1 ml quartz cuvette along with 0.875 ml of 0.2 M K-PO_4 buffer (pH 7.0) plus 1 mM EDTA. After recording the A_{265}, 0.25 units of ascorbate oxidase (Sigma) were added. The A_{265} was monitored until no further decrease was observed and the ascorbate content was calculated based on the ε_{265} of 14.1 $mM^{-1} \cdot cm^{-1}$.

RESULTS AND DISCUSSION

Treatment with 1 mM paraquat for 4 days led to significant increases in the activities of MDHA reductase, ascorbate peroxidase, and SOD (Table 1). Activities of these same enzymes in extracts of nodules treated with 0.1 mM paraquat were not statistically different from those of the control (no paraquat) plants. Nitrogenase activity, as measured by acetylene reduction, was markedly lower in those nodules that had received the 1 mM paraquat treatment (Table 1). In contrast, activity of DHA reductase was not affected by paraquat treatment.

There were no consistent differences in the amount of total protein per g of nodule fresh weight when control and paraquat-treated plants were compared. Expression of enzyme activities in terms of activity units per g of nodule fresh weight did not substantially affect the relationships that have been discussed based on activity units per mg total protein.

Additional experiments were performed to determine the timing of paraquat-related increases in enzyme activity Enzyme activities in nodule crude extracts were

determined at 1-day increments after the beginning of treatment with 1 mM paraquat. After 1 day, the specific activity of MDHA reductase in extracts from paraquat-treated nodules was 222 nmol NADH per min per mg protein compared to 159 nmol NADH per min per mg protein in extracts of the untreated control nodules. This increase was significant at the 0.05 level as were similar differences which were observed after 2 and 3 days.

Table 1. Effects of paraquat on the specific activity of oxygen defense enzymes and nitrogenase in soybean root nodules.

	Concentration of paraquat (mM)		
Enzyme	0	0.1	1
		(activity)	
Monodehydroascorbate reductase[a]	210 ± 10	199 ± 11	482 ± 22[e]
Ascorbate peroxidase[b]	230 ± 15	285 ± 18	398 ± 65[e]
SOD[c]	6.74 ± 1.0	7.71 ± 0.6	18.9 ± 4.2[e]
Dehydroascorbate reductase[b]	29.3 ± 3.6	32.5 ± 8.0	26.1 ± 4.4
Nitrogenase[d]	23.5 ± 5.6	18.7 ± 7.1	1.48 ± 0.4[e]

Roots were treated daily with nutrient solution containing 0, 0.1 or 1.0 mM paraquat for a total of 4 days. Each value is the mean of 4 replicates ± 1 SEM.

[a] nmol NADH · min^{-1} · mg $protein^{-1}$

[b] nmol ASC · min^{-1} · mg $protein^{-1}$

[c] units · mg $protein^{-1}$

[d] μmol C_2H_4 · g^{-1} · h^{-1}

[e] Significance level < 0.01 vs. no paraquat treatment as determined by t test.

The specific activity of ascorbate peroxidase did not show a significant increase until day 2 at which time the specific activity in extracts from paraquat-treated nodules was 519 nmol ASC per min per mg protein compared to 397 nmol ASC per min per mg protein in the extracts from the control group ($p < 0.01$). By day number 4, the specific activities were 534 nmol ASC per min per mg protein in the paraquat-treated group and 346 nmol ASC per min per mg protein in the control. Higher activity of SOD was observed only after 4 days of treatment with 1 mM paraquat. SOD activity was 29.2 units per mg protein in the paraquat-treated plants compared to 11.4 units per mg protein in the control group ($p < 0.02$, $n=6$). There were no differences between the specific activities of dehydroascorbate reductase and glutathione reductase in extracts of control and paraquat-treated plants during the 4 days of these experiments. The specific activity of DHA reductase ranged between 45.3 and 119 nmol ASC per min per mg protein. The specific activity of glutathione reductase ranged between 13.8 and 35 nmol NADPH per min per mg protein.

An increase in the specific activities of ascorbate peroxidase and MDHA reductase was observed in soybean plants treated with 1 mM paraquat and maintained in darkness for 48 hours (Table 2). In agreement with previous experiments, there was also an increase in the specific activities of these enzymes in nodule extracts from plants treated with paraquat and kept in the normal light regime of 16 h light and 8 h dark. Nitrogenase activity was markedly decreased by treatments involving paraquat (light or dark) or by darkness alone without paraquat (Table 2).

Table 2. Effects of light and paraquat on activities of oxygen defense enzymes and nitrogenase in soybean root nodules.

Enzyme	Treatment			
	No paraquat		Paraquat (1 mM)	
	Dark	Light/dark	Dark	Light/dark
	(specific activity)			
Monodehydroascorbate reductase	120 ± 7	101 ± 4	148 ± 12[a]	170 ± 11[a]
Ascorbate peroxidase	306 ± 28	298 ± 7	372 ± 22[a]	339 ± 8[a]
Nitrogenase	0.79 ± 0.09[a]	8.74 ± 0.89	0.67 ± 0.11[a]	0.55 ±0.02[a]

Roots of plants were treated with nutrient solution or nutrient solution plus 1 mM paraquat at time zero and again after 24 hours. Plants were maintained in either complete darkness for 48 hours or in two cycles of 16 hours light followed by 8 hours dark. Each value is the mean of 5 replicate extractions ± 1 SEM.

[a]Significance level ≤ 0.02 vs. no paraquat and light/dark treatment (data column 2) as determined by t test.

The experiments in which paraquat-treated plants were kept in complete darkness were an attempt to determine to what extent the nodule responses to paraquat might have been caused by the interruption of photosynthate supply. The increase in specific activity of ascorbate peroxidase and MDHA reductase in the dark plus paraquat-treated plants (Table 2) suggest light-independent paraquat action in the nodules. However, since nitrogenase activity declined in dark-treated plants regardless of whether or not paraquat was present (Table 2), it is not possible to determine whether this decline was due to the interruption of photosynthate supply or to the direct action in the nodules by paraquat-generated free radicals.

Treatment of plants with 1 mM paraquat had no statistically significant effect on the level of TBA-reactive substances in nodule extracts. After 4 days, the content was measured at 58.7 nmol malondialdehyde per g for the paraquat-treated nodules and 67.1 nmol per g for the untreated nodules. Similarly, paraquat treatment had no effect on the H_2O_2 content of nodule extracts. After 4 days, the 1 mM paraquat-treated nodules had a content of 770 nmol H_2O_2 per g fresh weight. Analysis by t test showed this value was not significantly different from that of the untreated nodules (690 nmol per g fresh weight).

Plants treated with 1 mM paraquat displayed toxicity signs beginning after about 30 to 40 h. These symptoms included severe leaf necrosis beginning at the mid-vein and then proceeding towards the leaf margins along the primary lateral veins. By day 4, many leaves as well as the young terminal shoots were totally necrotic. No visible changes in root or nodule appearance were evident.

Treatment with paraquat led to substantial increases in the glutathione content of nodules (Table 3). The values reported for glutathione concentration also include homoglutathione, since the analytical procedures used do not distinquish between these two compounds. The glutathione content was 2.1-fold higher in the 1 mM paraquat-treated nodules when compared to untreated nodules. In contrast, the ascorbate content declined in response to paraquat treatment (Table 3). The ascorbate content of 1 mM paraquat-treated nodules was only 36% of the level of non-treated nodules.

The increases in enzyme activity reported here are similar to reported responses in several biological systems that have been challenged by free radical-generating

Table 3. Content of glutathione and ascorbate in soybean nodules treated with various concentrations of paraquat. Each value is the mean of 6 replicates ± 1 SEM.

Day no.	Paraquat treatment (mM)	GSH equivalents (nmol per g FW)	Ascorbate (nmol per g FW)
0	0	1330 ± 93	398 ± 19
1	0	1092 ± 82	364 ± 19
1	0.1	995 ± 137	321 ± 17
1	1.0	1256 ± 82	252 ± 16[a]
2	0	1386 ± 125	374 ± 6
2	0.1	1360 ± 90	361 ± 11
2	1.0	2027 ± 219[a]	291 ± 22[a]
3	0	1364 ± 64	365 ± 17
3	0.1	1521 ± 55	394 ± 11
3	1.0	2099 ± 53[a]	309 ± 14[a]
4	0	1340 ± 20	367 ± 26
4	0.1	1678 ± 72[a]	374 ± 20
4	1.0	2797 ± 102[a]	133 ± 20[a]

[a]Significance level of <0.05 by t test compared to the 0 paraquat treatment.

chemicals. Quinoline, a toxic soil pollutant that acts through generation of activated forms of oxygen, can lead to elevated levels of ascorbate peroxidase and dehydroascorbate reductase in legume root nodules (Wetzel and Werner 1990). Similar increases have been reported for ascorbate peroxidase in *Pisum* leaves treated with paraquat (Gillham and Dodge 1984) and in *Pisum* and *Spinacia* leaves treated with free radical-generating air pollutants such as ozone (Mehlhorn et al. 1987; Tanaka et al. 1985). Ozone treatment also results in an increase in the specific activity of MDHA reductase in leaves (Tanaka et al. 1985).

Oxidative stress may result in various changes in levels of antioxidants in plants. For example, treatment of illuminated chloroplasts with paraquat leads to decreases in ascorbate and GSH within a few minutes (Law et al. 1983). There are numerous examples in which longer exposure to xenobiotics and environmental stress have led to increases in glutathione and ascorbate content (Schmidt and Kunert 1986, Nieto-Sotelo and Ho 1990, Mehlhorn et al. 1986).

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