

# Stimulation of Glutathione Synthesis in Photorespiring Plants by Catalase Inhibitors<sup>1</sup>

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## ABSTRACT

The effect of various herbicides on glutathione levels in barley (*Hordeum vulgare* L.), tobacco (*Nicotiana tabacum* L.), soybean (*Glycine max* [L.] Merr.), and corn (*Zea mays* L.) was examined. Illumination of excised barley, tobacco, and soybean plants for 8 hours in solution containing 2 millimolar aminotriazole (a catalase inhibitor) resulted in an increase in leaf glutathione from 250 to 400 nanomoles per gram fresh weight to 600 to 1800 nanomoles per gram fresh weight, depending on the species tested. All of this increase could be accounted for as oxidized glutathione. Between 25 and 50% of this oxidized glutathione was reduced when plants were darkened for 16 hours, but there was no significant decline in total glutathione. Another catalase inhibitor, thiosemicarbazide, was as effective as aminotriazole in elevating glutathione in soybean but was less effective in barley and tobacco. Glyphosate, an inhibitor of aromatic amino acid biosynthesis, had no significant effect on glutathione levels in any of the plants examined. Whereas methyl viologen (paraquat), which is a sink for photosystem I electrons, caused oxidation of leaf glutathione in all of the plants but did not increase the total amount of glutathione present.

Under photorespiratory conditions, a catalase-deficient mutant (RPr 79/4) of barley and wild-type barley treated with the catalase inhibitor aminotriazole, accumulated glutathione to 3-fold the level in nonphotorespiring plants (14, 15). Because a large fraction of this glutathione was oxidized, we proposed that H<sub>2</sub>O<sub>2</sub>, generated during photorespiration by the action of glycolate oxidase, directly or indirectly oxidized GSH to GSSG. A consequence of a lower GSH pool would be additional synthesis of glutathione, because GSH is a feedback inhibitor of glutathione biosynthetic enzymes (12).

We have two short-term objectives in the present study. First, to determine whether glutathione accumulation is a general phenomenon in photorespiring plants treated with catalase inhibitors. Second, to determine the extent to which plants can reduce GSSG in the dark. A long-term goal is to determine the stoichiometric relationship between photorespiratory H<sub>2</sub>O<sub>2</sub> production and GSSG formation, because the latter may be a more reliable measure of photorespiration rates than is currently available.

## MATERIALS AND METHODS

Barley (*Hordeum vulgare* L.), corn (*Zea mays* L.), soybean (*Glycine max* [L.] Merr.), and tobacco (*Nicotiana tabacum* L.) plants raised from seed were grown in a greenhouse for between

3 and 8 weeks. Usually, shoots were excised close to the ground, placed in 150 ml of solution in a 250 ml beaker, and the stem cut under liquid about 5.1 cm from the base to ensure adequate transpiration. Plants were exposed to 8 h of light, intensity 400  $\mu\text{E m}^{-2} \text{s}^{-1}$  at 27°C, unless specified otherwise. We previously showed that intact or excised shoots of barley behaved similarly

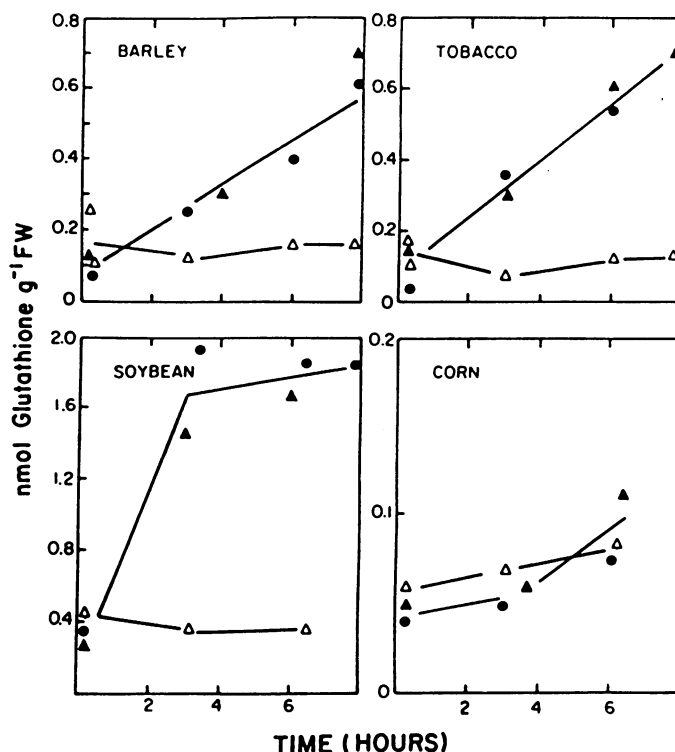


FIG. 1. Effect of aminotriazole on glutathione content of leaves. Plants were illuminated ( $400 \mu\text{E m}^{-2} \text{s}^{-1}$ ) in the presence or absence of 2 mM aminotriazole as described in "Materials and Methods." Each point is the average of two replicates. ( $\Delta$ ), Controls not treated with aminotriazole; ( $\bullet$ ,  $\blacktriangle$ ), treated plants in two separate experiments.

Table 1. Effect of Various Light Regimes on Glutathione Levels in Soybeans Treated with 2 mM Aminotriazole

Light Regime	GSH GSSG	
	nmol g <sup>-1</sup> fresh wt	
4 h light ( $400 \mu\text{E m}^{-2} \text{s}^{-1}$ )	[-83] <sup>a</sup>	1593 ± 178
4 h light + 20 h dark	1221 ± 10	453 ± 59
4 h light + 20 h dark + 4 h light	125 ± 65	2764 ± 286
28 h dark	486 ± 6	0

<sup>a</sup> Negative value means that oxidized glutathione was higher than total amount of glutathione and indicates experimental error.

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Table II. Effect of 2 mM Aminotriazole on the Glutathione Content of Leaves

Excised shoot systems were exposed to 8 h of light (400  $\mu\text{E m}^{-2} \text{s}^{-1}$ ), 8 h of light followed by 16 h of darkness or 24 h of darkness.

Plant	Light Regime <sup>a</sup>	+ Aminotriazole			- Aminotriazole		
		GSSG	GSH	Total	GSSG	GSH	Total
<i>nmol g<sup>-1</sup> fresh wt</i>							
Soybean	L	922 ± 274	87 ± 67	1008 ± 206	14 ± 6	243 ± 8	257 ± 14
Primary leaf	L + D	557 ± 51	403 ± 59	960 ± 110	17 ± 13	240 ± 110	257 ± 123
	D	18 ± 0	197 ± 0	215 ± 0	10 ± 10	239 ± 154	249 ± 164
Soybean	L	1567 ± 70	152 ± 47	1719 ± 237	44 ± 28	277 ± 74	321 ± 102
First trifoliolate	L + D	1121 ± 173	452 ± 112	1573 ± 285	34 ± 26	232 ± 90	266 ± 116
	D			467 ± 205	18 ± 10	342 ± 116	360 ± 126
Soybean	L	1585 ± 359	121 ± 97	1706 ± 335	51 ± 58	262 ± 71	313 ± 94
Second trifoliolate	L + D	1331 ± 136	763 ± 0	2097 ± 93	30 ± 0	283 ± 100	313 ± 100
	D			310 ± 72	24 ± 12	351 ± 63	375 ± 75
Barley	L	1037 ± 45	49 ± 12	1086 ± 33	18 ± 10	219 ± 114	237 ± 124
	L + D	749 ± 41	365 ± 183	1164 ± 92	42 ± 2	328 ± 25	370 ± 22
	D	49 ± 9	571 ± 93	620 ± 102	41 ± 20	310 ± 225	350 ± 245
Tobacco	L	529 ± 31	55 ± 31	583 ± 0	6 ± 6	195 ± 5	201 ± 10
	L + D	529 ± 116	298 ± 79	827 ± 196	5 ± 5	74 ± 25	79 ± 30
	D	2 ± 2	93 ± 28	96 ± 31	2 ± 2	138 ± 8	140 ± 10

<sup>a</sup> L = light, D = dark.

Table III. Effect of Various Herbicides on the Glutathione Content of Leaves Illuminated for 8 h

Additions	Barley		Tobacco		Soybean		Corn	
	Total	GSSG	Total	GSSG	Total	GSSG	Total	GSSG
<i>nmol g<sup>-1</sup> fresh wt</i>								
0	274 ± 114	14 ± 11	235 ± 75	5 ± 5	326 ± 84	25 ± 4	178 ± 60	5 ± 5
2 mM aminotriazole	965 ± 250	790 ± 324	558 ± 101	473 ± 165	1066 ± 100	1000 ± 90	150 ± 62	22 ± 21
4 mM thiosemicarbazide	451 ± 20	35 ± 30	274 ± 152	10 ± 4	1378 ± 200	197 ± 20	249 ± 41	4 ± 4
1 mM methyl viologen	194 ± 42	136 ± 56	241 ± 80	86 ± 20	554 ± 90	435 ± 85	120 ± 40	110 ± 30
20 mM glyphosate	187 ± 68	18 ± 4	111 ± 28	5 ± 2	205 ± 10	20 ± 5	267 ± 100	5 ± 2

with respect to glutathione synthesis (14).

Glutathione was assayed by a slight modification of the method of Griffith (5). One g fresh weight of leaves was homogenized in 5 ml 5% (w/v) sulfosalicylic acid with a small amount of sand, a further 5 ml sulfosalicylic acid was added and the brei centrifuged at 1000g for 10 min to sediment insoluble material. A 1 ml aliquot of the supernatant was neutralized with 1.5 ml 0.5 M K-phosphate buffer (pH 7.5); this sample was used for the assay of total glutathione. Another 1 ml aliquot of the supernatant was neutralized with 1.5 ml 0.5 M K-phosphate buffer, 0.2 ml 2-vinylpyridine added, the tube mixed until an emulsion formed and then incubated more than 1 h at 25°C; this sample was used for the assay of GSSG. Both tubes were extracted twice with 5 ml diethylether. The primary reason for doing this was to extract 2-vinylpyridine as a safety measure, although a secondary reason is a slight inhibition of the enzyme assay by this compound.

The standard incubation mixture contained: 0.5 ml 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, 0.2 ml 6 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.1 ml 2 mM NADPH, and 0.1 ml (1 unit) of yeast glutathione reductase type III (Sigma Chemical Co.). The reaction was initiated by the addition of 0.1 ml GSH standard (50–400 ng) or extract. The change in *A* at 412 nm was followed for 650 s at 25°C or until *A* reached 0.5.

## RESULTS AND DISCUSSION

### Effect of Aminotriazole on the Glutathione Content of Leaves.

Treatment of barley, tobacco, and soybean plants with 2 mM 3-

amino-1,2,4-triazole in the light induced an increase in total glutathione (Fig. 1). By contrast, aminotriazole had no effect on glutathione levels in plants kept in the dark, or barley in a high CO<sub>2</sub> atmosphere in light and only a small effect on glutathione levels in maize in the light (14). The increase in glutathione is a response to photorespiratory H<sub>2</sub>O<sub>2</sub> production which accumulates because of inhibition of catalase by aminotriazole. In barley and tobacco, an increase from <0.2  $\mu\text{mol g}^{-1}$  fresh weight to almost 0.7  $\mu\text{mol g}^{-1}$  fresh weight was observed in 8 h; continued exposure to light resulted in a further increase to 2  $\mu\text{mol g}^{-1}$  fresh weight which confirms earlier results with barley (14). In contrast, soybean synthesized 2  $\mu\text{mol g}^{-1}$  fresh weight within 3 h, after which time there was minimal increase. Many legumes, including soybean, contain homoglutathione ( $\gamma$ -glutamylcysteinyl- $\beta$ -alanine) rather than glutathione (3, 8, 11). We assume that homoglutathione would be active in our glutathione assay and that this is the compound being measured in soybean.

The cessation of net glutathione synthesis in soybean was investigated further, because I assume that photorespiratory H<sub>2</sub>O<sub>2</sub> production, and thus the need to synthesize additional glutathione, continues beyond 3 h. Excised shoot systems, with their bases in 2 mM Na<sub>2</sub>SO<sub>4</sub> and 2 mM aminotriazole, were exposed to 4 h of light and then placed in the dark for 20 h before a second exposure to 4 h of light. Glutathione was synthesized relatively rapidly during the second exposure to light (Table I), suggesting that the decline in glutathione synthesis after 4 h exposure to light is due primarily to limited substrates

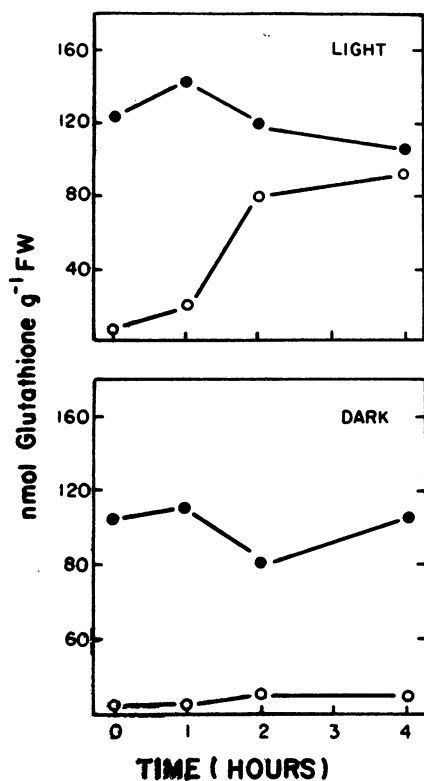


FIG. 2. Effect of methyl viologen on oxidized and reduced glutathione in corn leaves. Excised shoot systems were placed in 1 mM methyl viologen and either illuminated ( $400 \mu\text{E m}^{-2} \text{s}^{-1}$ ) or placed in the dark. (●), Total glutathione; (○), GSSG.

rather than end product inhibition of biosynthetic enzymes. Similar results were obtained in aminotriazole-treated barley, where the synthesis of glutathione accounted for more sulfur than that present in the sulfate entering the leaves (15).

Approximately 90% of the total leaf glutathione was reduced in control plants, whereas more than 90% was oxidized after treating plants with 2 mM aminotriazole for 8 h in the light (Table II). In soybean, the total accumulation was not markedly affected by the developmental stage of the leaf. The second trifoliate leaf (middle leaflet 30–40 mm long) and the first trifoliate leaf (55 mm long) accumulated similar amounts, and the lower levels of glutathione in the primary leaves is probably due to lower rates of photorespiration resulting from senescence. The pool of glutathione did not decline rapidly when plants were placed in the dark, but significant reduction occurred. In soybean, dark reduction elevated the GSH pool to twice the level in untreated plants. Experiments are in progress to measure the rate of GSSG reduction in the light by transferring aminotriazole-treated plants to high  $\text{CO}_2$  (2.5%), conditions which suppress photorespiration but allow light generation of reductant for glutathione reductase.

A variety of herbicides and other compounds increase glutathione levels in plants (12). The results obtained with the compounds we tested are presented in Table III. Thiosemicarbazide, which is as effective an inhibitor of catalase as aminotriazole (9), increased total glutathione in barley, corn and soybean but had little effect in tobacco. The most noticeable difference between plants treated with aminotriazole and thiosemicarbazide was the presence of primarily GSSG in the former and GSH in the latter. We have no explanation for this difference. Glyphosate, whose primary site of action is thought to be inhibition of aromatic amino acid biosynthesis, did not increase glutathione in any of the photorespiring plants tested (13). Methyl viologen, which stimulates  $\text{H}_2\text{O}_2$  production in the chloroplast (4, 7), caused

oxidation of GSH to GSSG but without a major increase in total glutathione.

In corn, the increase in GSSG was measured within 1 h of exposure to 1 mM methyl viologen and almost complete oxidation of leaf glutathione was observed in 4 h (Fig. 2). Similar results were obtained with isolated chloroplasts from spinach, where methyl viologen acts as an acceptor of PSI electrons thus preventing generation of the NADPH required by glutathione reductase (7). Since approximately 50% of the leaf glutathione is in the cytoplasm (15), the oxidation of 90% of the leaf GSH means that methyl viologen also caused oxidation of some cytoplasmic GSH.

## CONCLUSIONS

There are two pathways of peroxide production in illuminated plants. First,  $\text{H}_2\text{O}_2$  is generated when superoxide dismutase disproportionates the superoxide radicals which are produced by reduction of molecular oxygen in PSI (4, 6, 7). Second, in photorespiring plants,  $\text{H}_2\text{O}_2$  is formed in the peroxisome through the action of glycolate oxidase (17).

$\text{H}_2\text{O}_2$  is reduced in the chloroplast by a pathway, utilizing electrons generated by PSI and having ferredoxin, NADPH<sub>2</sub>, GSH, and ascorbate as intermediates (1, 2, 4, 6, 7). Light generated reductant is required and the system is inactivated when chloroplasts are exposed to  $\text{H}_2\text{O}_2$  in the dark (2, 6). Accumulation of toxic levels of  $\text{H}_2\text{O}_2$  occurs when plants are treated with methyl viologen. The latter has two effects, it stimulates  $\text{H}_2\text{O}_2$  production by generating superoxide radicals and, more importantly, acts as a sink for PSI electrons thus removing the source of reductant for the  $\text{H}_2\text{O}_2$ -scavenging system (4, 6, 7). In the present experiments, methyl viologen caused a rapid oxidation of glutathione in illuminated plants (Fig. 2). The fact that more than 90% of the GSH in the leaves is oxidized suggests that  $\text{H}_2\text{O}_2$  is released from the chloroplast, because only 50% of the glutathione pool is in chloroplasts (15).

Usually,  $\text{H}_2\text{O}_2$  generated in the peroxisome by photorespiring plants is degraded by catalase (17), when catalase is inhibited by aminotriazole one result is the oxidation of most of the leaf glutathione to GSSG. In animals,  $\text{H}_2\text{O}_2$  oxidizes GSH directly in a reaction catalyzed by GSH peroxidase. Although the presence of this enzyme in plants has been questioned (16), I think that the presence of a GSH peroxidase in leaves is the simplest explanation of my results and that the recent report of GSH peroxidases in plants supports this conclusion (10).

Generally, the inhibition of catalase caused an increase in the total glutathione present in the leaves and this increase could be accounted for as GSSG. The major exception was soybean, treated with thiosemicarbazide, where most of the increase was due to the presence of GSH (Table III). Differences were observed in the time course of glutathione accumulation in barley, tobacco, and soybean. For instance, glutathione synthesis was 4-fold higher in soybean but ceased after 3 to 4 h. This cessation may be due to a limited supply of substrates at the biosynthetic sites, because additional glutathione did accumulate after a dark period (Table I). The large pool of glutathione, established by illuminating plants in the presence of aminotriazole, did not decline when plants were transferred to the dark for 16 h, indicating relatively slow rates of turnover. However, the GSH content of this pool increased significantly, presumably due to the activity of a cytoplasmic or mitochondrial glutathione reductase.

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