

Effect of Flurazole and Other Safeners for Chloroacetanilide Herbicides on Cysteine Synthase in Sorghum Shoots

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The effects of selected herbicide safeners on shoot growth inhibition and on cysteine synthase [CS; EC 4.2.99.8] activity of sorghum were examined. Seed treatment (1.25 g/kg seed) of flurazole [phenylmethyl 2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate] protected seedlings from growth inhibition by alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide]. Flurazole seed treatment (1.25 g/kg) increased the specific activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein) and total activity ($\mu\text{mol}/\text{min}/\text{g}$ fresh weight) of CS from 1.13- to 1.41-fold and from 1.40- to 1.75-fold at 48 and 72 h after planting, respectively. CS activity at 48 h after planting increased as the dosage of flurazole increased from 0.01 to 2.5 g/kg seed. Protection from growth inhibition by alachlor (100 μM) also increased as the flurazole dosage increased from 0.01 to 0.625 g/kg seed. Flurazole had little inhibitory effect on CS activity between 3 and 30 μM in the *in vitro* enzyme assay, suggesting that increased CS activity by this safener was not due to the activation of constitutive CS. Protection from growth inhibition by alachlor was also observed for the seed treatments of fluxofenim [*O*-(1,3-dioxolan-2-yl)-2,2,2-trifluoro-4'-chloroacetophenone-oxime; 0.1–0.4 g/kg seed], naphthalic anhydride [1*H*, 3*H*-naphtho(1,8-*cd*)-pyran-1,3-dione; 0.625–2.5 g/kg seed], benoxacor [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2*H*-1,4-benzoxazine; 0.625–2.5 g/kg seed], and dichlormid [2,2-dichloro-*N*'-di-2-propenylacetamide; 0.625–2.5 g/kg seed]. The safening efficacy of dichlormid was the lowest for the compounds tested. Fluxofenim (0.4 g/kg seed), naphthalic anhydride (2.5 g/kg seed), benoxacor (2.5 g/kg seed), and dichlormid (2.5 g/kg seed) increased CS activity ($\mu\text{mol}/\text{min}/\text{g}$ fresh weight) by 36, 38, 61, and 22%, respectively at 48 h after planting. However, the safening efficacy by these compounds and the increase in CS activity were not clearly correlated. These results suggest that safener treatments increase the extractable CS activity in sorghum shoots. © 2001 Academic Press

INTRODUCTION

Chloroacetanilide herbicides provide excellent weed control in sorghum (*Sorghum bicolor* L.) and corn (*Zea mays* L.), but they can cause crop injury (1–3). Safeners are compounds that protect these crops from injury by chloroacetanilide herbicides (4, 5). Flurazole, fluxofenim, and naphthalic anhydride are used as seed treatments, and benoxacor and dichlormid are usually applied to the soil. The whole mechanism of safening action by these compounds has not been clearly revealed, but many studies have suggested that safeners protect crops from chloroacetanilide herbicide injury by enhancing herbicide detoxification in the treated plants (6–12). In corn (6, 13) and sorghum (7, 10, 11, 14), chloroacetamide herbicides are detoxified by conjugation with glutathione (GSH).²

Although nonenzymatic conjugation of chloroacetanilides with GSH is known to occur *in vitro* (15), the safening activity of particular safeners against metolachlor injury in sorghum is related to the ability to enhance glutathione *S*-transferase (GST), which conjugates GSH with metolachlor (7), suggesting that safeners confer protection by increasing GST activity.

GSTs are dimeric multifunctional enzymes catalyzing conjugation of GSH to endogenous or exogenous substrates (16). These enzymes are found as isozymes in mammals (17), insects (18), and plants (19, 20). GSTs in plants detoxify herbicides such as EPTC (9) and atrazine (21).

If conjugation of chloroacetanilide herbicides with GSH, either by nonenzymatic or by GSTs,

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² Abbreviations used: CS(s), cysteine synthase(s); GSH, glutathione; GST(s), glutathione *S*-transferase(s); OAS, *O*-acetylserine.

is a major mechanism of detoxification, sufficient GSH must be supplied for the conjugation. It was reported that GSH content was increased in corn roots exposed to dichlormid (9). Dichlormid and flurazole increased GSH content of sorghum shoots (7). These findings suggest that biosynthesis of GSH is enhanced by safeners.

GSH is a tripeptide consisting of three amino acids, glutamate, cysteine, and glycine. GSH is formed by the two enzymes, γ -glutamylcysteine synthetase and GSH synthetase, from these three amino acids (22). In this case, the rate of GSH synthesis appears to depend on the availability of cysteine and glycine. Hence, a sufficient amount of cysteine and glycine must be available or supplied when plants are treated with safeners. The cysteine required for synthesis of GSH in response to safeners might be released from seed storage protein or synthesized *de novo* from sulfate (23). Indeed, dichlormid increased cysteine and decreased free sulfate content in corn roots, suggesting that the lowered sulfate level is due mostly to incorporation into GSH and cysteine synthesis (23).

In plants, sulfur is assimilated into cysteine through the cysteine biosynthesis pathway (24). Inorganic sulfate is reduced to sulfite and then to sulfide through the sulfate reduction pathway. Cysteine synthase [CS; EC 4.2.99.8] catalyzes the formation of cysteine from *O*-acetylserine (OAS) and sulfide. Dichlormid and benoxacor increased activity of two enzymes in sulfate assimilation, ATP-sulfurylase and adenosine 5'-phosphosulfate sulfotransferase, in corn (23, 25). One report stated that CS activity was unaffected by these safeners, although supporting data were not presented (25). Therefore, whether CS activity might be increased in safener-treated plants warrants further investigation.

In this study, we examined the effects of flurazole and other safeners for chloroacetanilide herbicides on CS activity in shoots of sorghum and their safening activity for alachlor injury.

MATERIALS AND METHODS

Chemicals

Alachlor (analytical grade, 99.9%) and flurazole (technical grade) were obtained from

Monsanto Co. Concep III 8 EC was used as fluxofenim (96%). Naphthalic anhydride (98%) was purchased from Chem Service Co. Benoxacor and dichlormid were both technical grade obtained from Syngenta Crop Protection, Inc. All chemicals used in the CS assay [OAS, Na₂S, dithiothreitol, and pyridoxal-5'-phosphate] were purchased from Sigma Chemical Co.

Safener and Herbicide Treatment

Flurazole (0.01, 0.039, 0.156, 0.625, 1.25, and 2.5 g/kg seed), fluxofenim (0.1 and 0.4 g/kg seed), naphthalic anhydride (0.625 and 2.5 kg/seed), benoxacor (0.625 and 2.5 kg/seed), and dichlormid (0.625 and 2.5 kg/seed) were dissolved in methanol and then added to sorghum (cv. Beef-Builder T; Asgrow) seeds at the rate of 50 ml/kg seed in a closed container. After shaking to distribute the safener solution over the seed, the methanol was evaporated. The control was also treated with the same volume of methanol without safener. The treated seeds were stored at 4°C until planting. Alachlor (3 to 300 μ M) was applied to the vermiculite surface in aqueous solutions containing 0.5% methanol (v/v) at the time of initial watering after planting as described below. The corresponding control was applied with deionized water containing 0.5% methanol (v/v).

Effect of Safeners on Alachlor Injury

Vermiculite (2 cm deep, coarse; Strong-Lite Products Corp.) was placed on the bottom of plastic trays (3 \times 4 \times 5 cm) and saturated with deionized water. Five sorghum seeds, either control or safener-treated, were placed on the vermiculite surface and covered with 2 cm deep vermiculite. At the initial watering, 16 ml of the deionized water for the safener-treated control or the aqueous solutions of alachlor were applied on the vermiculite surface by pipettes. Safener-treated control was prepared for each compound and for each dosage. The containers were placed in a growth chamber under 16 h photoperiod (150 μ E/s \cdot m²) at 25°C and watered (deionized water) as necessary. Shoot length was measured 7 days after planting, and percentage shoot

length was calculated compared to safener-treated control in each compound and in each dosage. Each treatment consisted of three replicates with three shoot length measurements per replicate, and the experiment was repeated three times.

Effect of Safeners on Extractable CS Activity

Approximately 30 seeds of sorghum, either safener-treated or control, were planted in plastic trays (6 × 8 × 5 cm) containing vermiculite as described above, and 16 ml of deionized water was applied on the vermiculite surface by pipettes. The trays were covered with aluminum foil to achieve dark conditions and incubated for 48, 72, and 96 h at 25°C, and shoots were collected for the CS assay.

CS Assay

Shoots of sorghum were weighed and homogenized with mortars and pestles on ice at approximately 4°C in 0.1 M phosphate buffer, pH 7.8, containing 1 mM dithiothreitol and 0.2% insoluble polyvinylpyrrolidone. The volume of the buffer was 40 ml per 1 g tissue. The homogenate was clarified by centrifugation at 15,000 g at 4°C for 30 min. The resulting supernatant was used for the CS assay. The CS assay (26–28) was performed in a final volume of 1 ml containing 50 mM phosphate buffer, pH 7.8, less than 0.02 mg protein, 5 mM OAS, 1 mM Na₂S, 1 mM dithiothreitol, 0.025 mM pyridoxal-5'-phosphate. The substrates were added to the enzyme to initiate the reaction, and assay test tubes were sealed with rubber caps. When the *in vitro* effect of flurazole on CS activity was examined, CS from untreated shoots was used. The safener dissolved in methanol (final concentration, 0.5%, v/v) was added to the reaction mixture. Controls contained the same concentration of methanol. After incubation at 30°C for 15 min, the reaction was stopped by the addition of 0.5 ml of 20% trichloroacetic acid (w/v), and precipitated protein was removed by centrifugation at 2000g for 10 min. An aliquot (1 ml) of the supernatant was collected and added to 1.5

ml of ninhydrin reagent (250 mg ninhydrin dissolved in 20 ml glacial acetic acid:concentrated HCl, 4:1 v/v). The mixture was heated in a boiling water bath for 6 min and then cooled. Cysteine was determined by measurement of the absorbance of the reaction mixture at 560 nm. The protein concentration was determined with the Bradford method (29) with bovine serum albumin as protein standard. All the enzyme experiments in this study were conducted with three to four replications and repeated two to three times.

RESULTS AND DISCUSSION

Effect of Flurazole on Sorghum Growth Inhibition by Alachlor

Alachlor reduced the shoot length of sorghum by 67–97% at 10 to 300 μM in the vermiculite assay system (Fig. 1). However, seed treatment of flurazole (1.25 g/kg seed) significantly reduced the growth inhibition by alachlor. This result is consistent with previous studies in which flurazole protected sorghum from metolachlor injury (11) and prevented yield losses in this crop due to chloroacetanilide herbicides (4) at 1.25 g/kg seed. The recommended rate of flurazole is also 1.25–2.5 g/kg seed to protect sorghum from injury by alachlor or acetochlor (30). Therefore, the effect of flurazole on CS

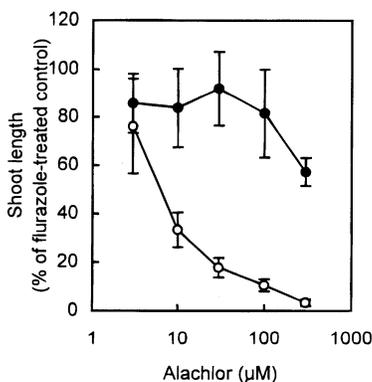


FIG. 1. Effect of flurazole seed treatment on growth inhibition by alachlor in sorghum. Vertical bars represent standard error. ●, Flurazole, 1.25 g/kg seed; ○, control.

activity in sorghum shoot was examined at 1.25 g/kg seed.

Effect of Flurazole on Extractable CS Activity

Flurazole increased the specific activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein) of CS 1.41- and 1.13-fold at 48 and 72 h after planting, respectively, but did not increase the activity 96 h after planting (Table 1). Flurazole also increased total activity ($\mu\text{mol}/\text{min}/\text{g}$ fresh weight) of CS 1.20- to 1.75-fold from 48 to 96 h after planting, but the difference between the safener treatment and the control in the activity at 96 h was not statistically significant. These results indicate that the increase in the activity is greater at early sorghum growth stages.

CS total activity decreased in both control and safener-treated plants, depending on time after planting (Table 1). Generally, the activity of sulfur assimilation enzymes are dependent on the developmental stage (22). These enzymes may be active in tissues where there is a high demand for cysteine and methionine for protein synthesis.

Effect of Flurazole Dosage on Extractable CS Activity and on Sorghum Growth Inhibition by Alachlor

A dose response between flurazole and CS activity was found (Fig. 2). Specific activity was enhanced as flurazole dosage increased from 0.01 to 1.25 g/kg seed. The maximum increase in the activity was 1.44-fold at 1.25 g/kg seed. Total activity also increased up to 2.11-fold, where flurazole dosage was 2.5 g/kg seed.

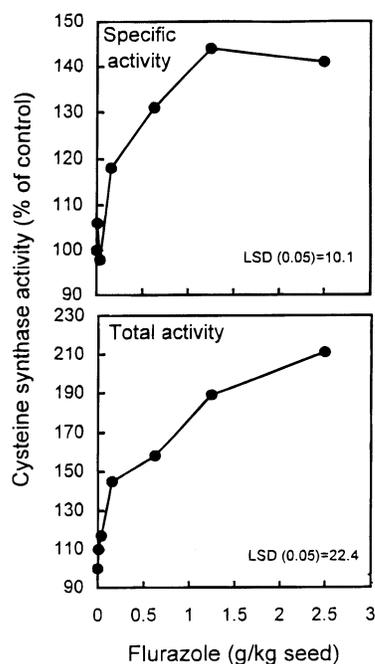


FIG. 2. Effect of flurazole dosage on extractable cysteine synthase activity in sorghum shoots. Cysteine synthase activity was measured 48 h after planting.

To examine the relationship between increase in CS activity and safening activity of flurazole, the effect of flurazole dosage on sorghum injury by 100 μM alachlor was tested. Although flurazole failed to reverse the growth inhibition by alachlor at 0.01 g/kg seed, the degree of recovery increased with the flurazole dosage between 0.01 and 0.625 g/kg seed (Fig. 3). The maximum recovery by flurazole was found between 0.625 and 2.5 g/kg seed. The relationship between the

TABLE 1
Effect of Flurazole on Extractable Cysteine Synthase Activity in Shoots of Sorghum

Time after planting (h)	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein)			Total activity ($\mu\text{mol}/\text{min}/\text{g}$ fresh weight)		
	Flurazole ^a	Control	Ratio (flurazole/control)	Flurazole ^a	Control	Ratio (flurazole/control)
48	4.07	2.89	1.41	96.6	55.2	1.75
72	5.85	5.20	1.13	62.0	44.4	1.40
96	6.05	5.79	1.04	40.0	33.4	1.20
LSD (0.05)	—0.43—			—8.13—		

^a Sorghum seeds were treated with flurazole (1.25 g/kg seed) dissolved in methanol before planting.

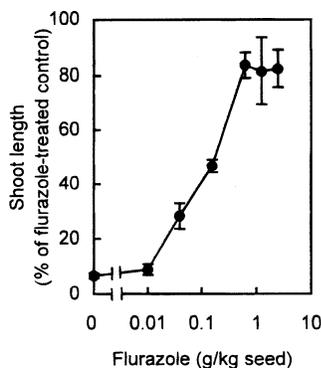


FIG. 3. Effect of flurazole dosage on growth inhibition by alachlor in sorghum. Alachlor ($100 \mu\text{M}$) was applied in all the treatments in this figure. Flurazole was treated on sorghum seeds. Vertical bars represent standard error.

total activity of CS and the safening activity of flurazole was examined (Fig. 4). Alachlor injury was not completely restored when the increase in the CS activity was less than 58%. The plant growth recovered up to 80% of the control when the CS activity was increased by 58%. However, the growth did not recover any further, although CS activity increased by 111%. This result suggests that increase in CS activity is not clearly correlated to the plant growth recovery.

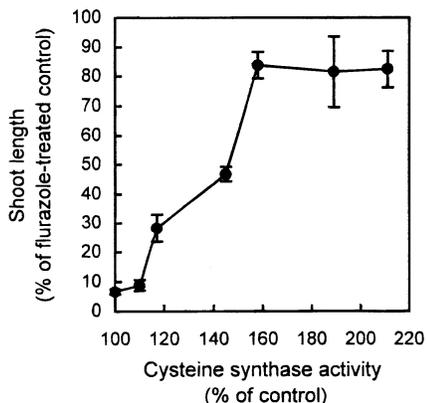


FIG. 4. Relationship between increase in extractable CS activity and growth recovery in sorghum. Shoot length in Fig. 3 was plotted against total activity of CS in Fig. 2. Vertical bars represent standard error.

In Vitro Effect of Flurazole on CS

Increased CS activity by flurazole seed treatment in sorghum shoot as shown above might be ascribed to the activation of constitutive CS or to the induction of *de novo* synthesis of this enzyme. When flurazole was added to the CS assay medium to examine the *in vitro* effect of this compound on CS activity, this compound had no or only a slight inhibitory effect on CS at 3 to $30 \mu\text{M}$ (Table 2). This result indicates that the increased extractable CS activity by flurazole was not due to the activation of constitutive CS.

Effect of Other Safeners on Growth Inhibition by Alachlor and on Extractable CS Activity

Fluxofenim, naphthalic anhydride, and benoxacor also protected sorghum from the growth inhibition caused by $30 \mu\text{M}$ alachlor at 0.1 to 0.4, 0.625 to 2.5, and 0.625 to 2.5 g/kg seed, respectively (Table 3). Dichlormid, however, had a poor safening activity on sorghum at 0.625 and 2.5 g/kg seed. It is reported that the safening activity of dichlormid in sorghum is lower than those of other safeners such as flurazole and naphthalic anhydride (7, 11). Fluxofenim is used as a seed treatment for sorghum with the recommended application rate of 0.4 g/kg seed (31). Naphthalic anhydride is also applied on seeds at 2.5 g/kg seed (11). Benoxacor and dichlormid are used to protect corn from injury by chloroacetanilide herbicides and is usually applied to soil (32, 33). In this study, however, these two safeners were applied on seeds to make it easier to compare the performance of these compounds. It was revealed that benoxacor had an ability to reverse the growth

TABLE 2
Effect of Flurazole on Cysteine Synthase Activity in Assay Medium (*in vitro*)

Flurazole (μM)	Activity (% of control)
3	96.4
30	91.8
Control	100
LSD (0.05)	4.4

TABLE 3
Effect of Safeners on Shoot Length of Alachlor-Treated^a Sorghum

Compound	Dosage (g/kg seed)	Shoot length (% of safener-treated control) ^b
Fluxofenim	0.1	73.2 ± 5.3
Fluxofenim	0.4	84.0 ± 6.8
Naphthalic anhydride	0.625	83.4 ± 7.4
Naphthalic anhydride	2.5	86.2 ± 7.6
Benoxacor	0.625	78.8 ± 4.2
Benoxacor	2.5	92.2 ± 2.7
Dichlormid	0.625	37.9 ± 6.9
Dichlormid	2.5	33.3 ± 4.8
Control (alachlor only)	0	16.8 ± 4.7

^a Alachlor was applied as 30 μ M aqueous solution containing 0.5% methanol (v/v) on the vermiculite surface at the time of initial watering in all the treatments shown in this table.

^b Values are means \pm standard error.

inhibition by alachlor with seed treatment. When dichlormid was applied to vermiculite surface as an aqueous solution, the safening efficacy of this compound was low (data not shown).

Fluxofenim (0.4 g/kg seed), naphthalic anhydride (2.5 g/kg seed), benoxacor (2.5 g/kg seed), and dichlormid (2.5 g/kg seed) increased the total activity of CS in sorghum shoots by 36, 38, 61, and 22%, respectively (Table 4). Benoxacor and dichlormid increased the specific activity of CS by 47 and 35%, respectively, at 2.5 g/kg seed, but fluxofenim and naphthalic anhydride did not significantly increase the specific activity. The increase in extractable CS activity by safeners is consistent with previous reports

which showed that safeners for chloroacetanilide herbicides increased the contents of cysteine and GSH (7, 23). However, dichlormid and benoxacor had no effect on CS in corn when the seeds were treated by soaking in nutrient solution containing these safeners for 24 h in the dark at 25°C (25). The data were not shown in that report. Differences in application methods, plant species, growth conditions, and measurement time after treatment could account for the difference in results.

With respect to the ability to increase total activity of CS in sorghum shoots, flurazole and benoxacor were most effective, fluxofenim and naphthalic anhydride were somewhat less effective, and dichlormid was least effective. Contrarily, the ability of fluxofenim and naphthalic anhydride to increase CS specific activity was lower than that of other compounds. The safening efficacy of flurazole, benoxacor, fluxofenim, and naphthalic anhydride was almost the same, so it cannot be asserted that the increase in CS activity was clearly correlated to the safening efficacy of these compounds. This is reasonable because the safening mechanism might be complicated, including various kinds of biochemical reactions. Conjugation of chloroacetanilide herbicides with GSH may be a direct mechanism of detoxification (6–14); therefore, increases in GST activity and in GSH (7, 23) are most important for the selectivity. Indeed, the degree of protection from metolachlor injury conferred by safeners was strongly correlated with their ability to enhance GST activity (7). In addition, sorghum contains a relatively large amount of

TABLE 4
Effect of Safeners on Extractable Cysteine Synthase Activity in Shoots of Sorghum

Compound	Dosage (g/kg seed)	Specific activity (μ mol/min/mg protein)	Total activity (μ mol/min/g fresh weight)
Fluxofenim	0.4	3.47 (107)	73.4 (136)
Naphthalic anhydride	2.5	3.55 (110)	74.4 (138)
Benoxacor	2.5	4.75 (147)	87.2 (161)
Dichlormid	2.5	4.36 (135)	65.8 (122)
Control	0	3.24 (100)	54.0 (100)
LSD (0.05)		0.33	9.6

Note. Cysteine synthase activity was measured 48 h after planting. Values in parentheses represent percentage of control.

GSH compared with the weeds susceptible to chloroacetanilide herbicides (34), suggesting a possibility that the levels of GSH in sorghum are not the limiting factors for detoxification of the herbicides. This might be the other reason that the increase in CS activity was not clearly correlated with the safening action of these safeners.

Research on the effect of these safeners on biosynthesis of GSH and its components, glutamate, cysteine, and glycine, is also important to clarify the whole mechanism of safening action. The enzymes responsible for sulfate assimilation, ATP-sulfurylase and adenosine 5'-phosphosulfate sulfotransferase, were affected by safeners in corn (23, 25). Safeners may interfere with the normal feedback inhibition of glutamylcysteine synthetase and thus increase GSH production (35). Flurazole was found to form a GSH conjugate in corn and sorghum, and this conjugate may also bind to glutamylcysteine synthetase, thereby circumventing the feedback regulation of the GSH biosynthesis pathway (36). Roots of corn pretreated with dichlormid showed no significant change in glutathione synthetase activity compared to that of the untreated control, but *in vitro*, the enzyme activity increased with the addition of the safener, suggesting a possible allosteric modification of the enzyme which increases the rate of glutathione synthesis (37).

The mechanism of the increase in CS activity in sorghum by the safeners is unknown. Because the correlation between the safening ability of these compounds and their effects to increase extractable CS activity was weak, increased CS activity is not involved in the direct action of the safeners. In this case, the increase might be a secondary effect. For example, increased GSH biosynthesis by safeners, which may lead to a depletion of cysteine, might cause a feedback up-regulation of CS.

In conclusion, although the mechanism is not revealed, the seed treatments of safeners for chloroacetanilide herbicides can increase extractable CS activity in sorghum shoots.

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