

Influence of the Safener Benoxacor on the Metabolism of Metolachlor in Corn*

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The herbicide safener benoxacor (CGA-154281) is effective in protecting corn from metolachlor injury. Glutathione-S-transferase (GST) activity of corn seedlings was stimulated by low concentrations of benoxacor as was the formation of a polar metabolite identified as the glutathione (GSH) conjugate of metolachlor. A similar degree of enhancement of metolachlor metabolism was observed in both a metolachlor-tolerant ('Cargill 7567') and a metolachlor-susceptible ('Northrup-King 9283') corn line. The total GSH content of shoots of the metolachlor-susceptible corn hybrid was not affected by benoxacor treatment, but an increase was noted for shoots of the tolerant corn hybrid. The two corn hybrids with differential tolerance to metolachlor also differ in their dose response to benoxacor with higher safener concentrations failing to induce or inhibit GST activity of the tolerant 'Cargill 7567' corn line. Stimulation of GST activity and a corresponding enhanced rate of metolachlor metabolism can account for the safening effect of benoxacor. These results are consistent with the proposed mechanism of action of dichloroacetamide safeners.

Introduction

Benoxacor [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine] is a recently developed safener synthesized by Ciba-Geigy Corporation for use in corn as a formulated mixture with the herbicide metolachlor [1]. The mechanism by which this safener confers its protective effect is believed to be by enhancing the detoxication of metolachlor in treated plants. The chloroacetanilide class of herbicides, to which metolachlor belongs, are detoxified in corn [2, 3] and sorghum [4, 5] by conjugation with the tripeptide glutathione (GSH). This conjugation has been shown to occur both as an enzymatic process [3, 6, 7] catalyzed by glutathione-S-transferase isozymes (GSTs) and nonenzymatically [8]. The relative contributions of these two processes is currently a matter of controversy [9]. There is a growing body of evidence suggesting that herbicide safeners confer their protection primarily by inducing GST isozymes specific for chloroacetanilide herbicides [4, 7, 10–12].

The objectives of this study were to determine the effects of benoxacor on metabolism of meto-

lachlor, GST induction, and glutathione content in seedlings of two hybrid corn lines. These hybrids, known to respond differentially to metolachlor [13], were utilized in order to determine if predisposition to herbicide injury (susceptibility or tolerance) is a factor in their response to the safener.

Materials and Methods

Chemicals

Analytical grade (> 95% purity) metolachlor, benoxacor, and radiolabeled metolachlor (carbon-14 labeled, sp. act. 59.5 $\mu\text{Ci}/\text{mmol}$) were provided by Ciba-Geigy Corp., Greensboro, N.C. All other chemicals were obtained from commercial sources.

Metolachlor metabolism study

For metabolism experiments seeds of both corn hybrids were germinated on filter paper saturated either with distilled H_2O or 1 μM benoxacor at 30 °C in a dark growth chamber. After 72 h, apical sections (20 mm) of the seedlings were excised and placed, six per vial, in 400 μl of incubation medium (1 mM CaCl_2 , 10 mM HEPES, pH 7.5) and 10 nCi [^{14}C]metolachlor for 1, 2, 4, and 8 h at 27 °C. After incubation the apices were removed, rinsed with 80% methanol, and the absorbed [^{14}C]metolachlor extracted by grinding in 1 ml of 80% methanol.

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The extract was clarified by centrifuging for 5 min in a microcentrifuge and the radioactivity contained in the extract was determined by liquid scintillation spectrometry. The amount of [^{14}C]metolachlor metabolized to the glutathione conjugate was determined by mixing a 60 μl aliquot of the extract with 60 μl of H_2O , and fractionating with 1 ml of methylene chloride.

Metolachlor metabolism by unsafened seedlings was also determined in the presence of varying concentrations of benoxacor. These experiments were conducted as above except for the inclusion of 0, 1, 10, and 50 μM benoxacor to the metabolism reaction mix.

Influence of benoxacor on GST activity

Seeds of the metolachlor-tolerant 'Cargill 7567' and the metolachlor-susceptible 'Northrup-King 9283' corn hybrids were germinated for 48 h on H_2O saturated filter paper at 30 °C in a dark growth chamber before being transferred to continuously aerated incubation medium containing 0, 0.2, 2, 5, 10, or 20 mg/l benoxacor. After 24 h the seedlings were removed from the liquid medium, rinsed, and frozen immediately in liquid nitrogen for GST analysis.

GST and GSH assays

For GST activity determinations 6 seedlings were pulverized in liquid nitrogen and homogenized with a mortar and pestle in 3 ml of 0.1 M K-phosphate buffer, pH 6.9, and 0.1 g polyvinylpyrrolidone (PVPP). The resulting homogenate was centrifuged at $20,000 \times g$ for 20 min and the supernatant was used as enzyme source. GST activity was determined with [^{14}C]metolachlor as substrate. The enzyme reaction contained 30 μl of 0.1 M K-phosphate buffer, pH 6.9; 10 μl of 60 mM

GSH; 10 μl of 60 μM metolachlor (1.3 nCi/ μmol); and 10 μl of plant extract. Enzyme reactions were incubated for 1 h, at 30 °C. Assays were terminated by the addition of 60 μl of 5% trichloroacetic acid (TCA). Metolachlor conjugated to GSH was determined by fractionating with 1 ml of methylene chloride and determining the radioactivity remaining in the aqueous phase by liquid scintillation spectrometry.

The total glutathione content of 72 h old seedlings was determined by the method of Tietz [14] as described by Gronwald *et al.* [7].

Protein content was determined spectrophotometrically by the Coomassie blue G-250 dye-binding assay [15] using bovine serum albumin as a standard.

Results and Discussion

Apical sections from seedlings grown in the presence of 1 μM benoxacor were found to metabolize metolachlor to a greater extent than control sections in a 1 h incubation (Table I). A similar enhancement of metolachlor metabolism (65–70%) was observed for both corn varieties. This increase in herbicide degradation is consistent with the findings of other investigators for a number of chemicals safening chloroacetanilide herbicides [9, 16].

Various concentrations of benoxacor were tested for their ability to influence the metabolism of metolachlor by excised apical sections of 'Northrup-King 9283' corn (Table II). No effect was observed with any of the concentrations tested. This suggests that the safener must be present for some time before its action occurs, and that there is no direct interaction between the herbicide and the safener. This finding fits well with the concept of

Table I. Formation of the GS-metolachlor conjugate in unsafened and benoxacor-treated corn seedlings*.

Corn Hybrid	Unsafened % of extracted [^{14}C] as GS-metolachlor	Benoxacor**	Ratio cols 2:1
Cargill 7567	29.6 \pm 5.3	48.8 \pm 6.4	1.65
Northrup-King 9283	23.9 \pm 2.8	41.4 \pm 5.6	1.73

* Data presented represent the average and standard errors of two experiments with 3 replications.

** 1 μM benoxacor was included in the imbibing solution of treated seeds.

Table II. Formation of the GS-metolachlor conjugate by unsafened 'Northrup-King 9283' shoots when benoxacor is added to the reaction mixture*.

Benoxacor [μ M]	% of [14 C]metolachlor conjugated
0	36.0 ± 2.6
1	34.2 ± 3.8
10	38.2 ± 3.8
50	35.0 ± 5.7

* Data presented as percent of extracted [14 C]metolachlor partitioning into the aqueous phase after fractionation with methylene chloride. Data presented represent the average and standard errors of two experiments with 3 replications.

safener action occurring at the molecular level with enzyme induction being a prerequisite for enhanced herbicide metabolism [10, 17].

The effect of benoxacor concentration on GST activity is shown in Fig. 1. The lowest safener concentration tested (0.2 mg/l) resulted in the greatest stimulation of GST activity. This approximately 35% increase in GST activity was observed for both of the corn varieties tested. The level of induction reported here is substantially less than the

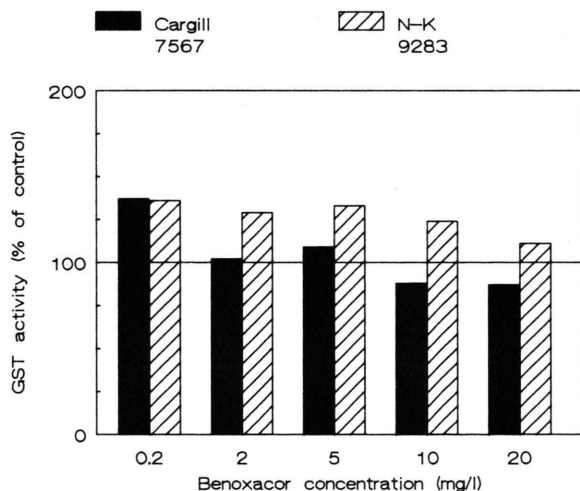


Fig. 1. Effect of benoxacor concentration on GST-metolachlor activity*.

* GST activity of safener treated seedlings as compared to untreated controls with [14 C]metolachlor as substrate. Control values were 41.2 and 28.0 nmol/min/mg protein for 'Cargill 7567' and 'Northrup-King 9283', respectively.

four to 5-fold increase reported by other investigators using different corn lines [18, 19]. There is however no reason to believe that GST activity need be elevated to such extremes to prevent the phytotoxic effects of metolachlor. When untreated 72 h old seedlings were compared the GST activity of the metolachlor-tolerant 'Cargill 7567' corn was found to be 35% greater than that of the susceptible 'Northrup-King 9283' corn [13]. Therefore, the increase in GST shown here for the susceptible variety would be sufficient to raise its GST activity to an equivalent level of the naturally tolerant corn variety. This is supported by our results on the significant enhancement of metolachlor metabolism by benoxacor on both corn lines shown in Table I. It has been shown that GST isozymes with greater affinity for chloroacetanilide herbicides can be induced by herbicide safeners [4, 7, 10–12]. It is expected that the induction of GST activity shown here involves such a mechanism.

Interestingly, the two corn hybrids respond differently to higher concentrations of the safener (Fig. 1). GST activity of the tolerant corn variety, 'Cargill 7567', was only induced slightly by 2 mg/l and 5 mg/l benoxacor (2% and 9% respectively) and was inhibited by higher concentrations of the chemical. GST activity of the susceptible corn hybrid, 'Northrup-King 9283', was induced to the same level at safener concentrations up to 5 mg/l, was only reduced slightly by higher concentrations of the safener, and was not inhibited by any of the concentrations tested. The reason for this differential response to the safener is unknown, but is believed to be related to the biochemical and physiological differences underlying the differential metolachlor tolerance observed for these two corn hybrids.

The effect of benoxacor on the total glutathione content of 72 h old corn shoots is shown in Table III. An increase in the glutathione content

Table III. Effect of benoxacor on glutathione content of corn shoots*.

Corn Hybrid	Control μ mol/g fresh weight	+ 1 μ M benoxacor
Cargill 7567	1.25 ± 0.05	1.50 ± 0.04
Northrup-King 9283	1.20 ± 0.07	1.30 ± 0.09

* Data presented are the average and standard error of at least 4 determinations.

of the metolachlor-tolerant corn hybrid, 'Cargill 7567', was observed but no change in glutathione content was observed for the susceptible hybrid. Previous studies have shown different herbicide safeners to have variable effects on glutathione content of protected crops. Lay and Cassida [20] found an increase in glutathione in the roots of corn treated with dichlormid, Gronwald *et al.* [7] found dichlormid and flurazole treatment increased glutathione levels in sorghum shoots but did not find increases in glutathione content with naphthallic anhydride or oxabetrinil treatments. Viger *et al.* [19] found benoxacor to have no effect on the glutathione content of 'Pioneer 3906' corn. In our study the glutathione content of untreated shoots (about 1.2 $\mu\text{mol/g}$ fresh weight) was similar for both varieties and is two-times the level of glutathione reported for sorghum shoots [7]. These findings suggest that glutathione level is not the limiting factor in determining the metolachlor tolerance of these two corn varieties, and also suggest that regulation of glutathione content is not essential to the mechanism of action of benoxacor.

Conclusion

The dichloroacetamide safener benoxacor is capable of stimulating GST activity with an asso-

ciated increase in the metabolism of metolachlor to the glutathione conjugate. The increase in GST activity observed in this study is believed to be sufficient to account for the safening effect of this compound. The failure of benoxacor to elevate glutathione levels in the metolachlor-susceptible corn variety and the relatively high endogenous glutathione content of both varieties tested suggest that regulation of glutathione content is not a primary mechanism of action of this safener. Further characterization of the GST isozymes from these corn varieties is underway to determine if the increase in GST activity reported here involves the stimulation of constitutive enzyme activity or the induction of newly synthesized GST isozymes. The variable effect of benoxacor on the two corn varieties tested in regard to the regulation of GST and glutathione content suggests fundamental differences in their physiology which may be useful in explaining the basis of differential herbicide tolerance.

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