Influence of Oxime Ether Safeners on Glutathione Content and Glutathione-Related Enzyme Activity in Seeds and Seedlings of Grain Sorghum*,**

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Oxime Ether Safeners, Oxabetrinil, Metolachlor, Glutathione, *Sorghum bicolor* (L.) Moench.

The influence of the oxime ether safeners, oxabetrinil and CGA-133205, on glutathione content, glutathione reductase (EC 1.6.4.2), and glutathione-S-transferase (EC 2.5.1.18) activity in seeds and seedlings of grain sorghum (*Sorghum bicolor* (L.) Moench. var. Funk G-522-DR) was investigated. Plant material for these experiments was derived from seed that was either untreated, or treated with 1.25 or 0.4 g ai/kg seed of oxabetrinil or CGA-133205, respectively. Measurements were conducted at 0, 1, 2, 4, 8, 12, 24, 36, and 72 h after the initiation of germination. In safener-treated sorghum seeds, the levels of total and reduced glutathione decreased gradually after the initiation of germination reaching a low at 8 to 12 h and then increased continuously reaching a maximum at 48 h. The greatest increases in glutathione levels were observed in oxabetrinil-treated sorghum seedlings. Glutathione reductase activity was consistently higher in untreated seeds of grain sorghum for the first 24 h of imbibition, but at 36 to 72 h glutathione reductase activity increased in the safener-treated tissues. CGA-133205 appeared to have a greater stimulatory influence on the activity of glutathione reductase than did oxabetrinil. For the first 8 h after germination was initiated, glutathione-S-transferase activity in oxabetrinil- and CGA-133205-treated seeds remained enhanced compared to that of untreated seeds, but was at or below the levels of the activity of glutathione-S-transferase extracted from untreated seeds for the remainder of the experiment. Non-enzymatic conjugation of metolachlor with reduced glutathione increased as the pH of the reaction solution increased from 6.0 to 8.0. Oxabetrinil at 1 to 40 μM and CGA-133205 at 1 to 160 μM enhanced non-enzymatic conjugation of metolachlor with glutathione. At 80 and 160 μM, oxabetrinil reduced the amount of non-enzymatic conjugation of metolachlor. Oxabetrinil conjugated with reduced glutathione at low rates either enzymatically or non-enzymatically at pH 8.0 while CGA-133205 did not at any pH. In the presence of metolachlor, the amount of non-enzymatic conjugation of oxabetrinil was decreased, indicating that metolachlor is more reactive towards glutathione than is oxabetrinil. Overall, these data suggest that during the early stages of seed germination and seedling development of grain sorghum, oxime ether safeners can enhance the detoxication of the herbicide, metolachlor, through enzymatic or non-enzymatic conjugation to reduced glutathione by enhancing either the level of reduced glutathione and/or the activity of glutathione-related enzymes.

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Abbreviations: Alachlor, 2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide; CDNB, 1-chloro-2,4-dinitrobenzene; CGA-133205, O-[1,3-dioxolan-2-yl-methyl]-2,2,2-trifluoro-4′-chloroacetophenone-oxime; cyometrinil, (*Z*)-α[(cyanomethoxy)imino]benzeneacetonitrile; flurazole, phenylmethyl 2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione (glutathione disulfide); GST, glutathione-S-transferase; metolachlor, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide; oxabetrinil, α-(1,3-dioxolan-2-yl-methoxy)-imino-benzeneacetonitrile; *R*-29148, 3-(dichloroacetyl)-2,2,5-trimethyl-1,3-oxazolidine.

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Introduction

The oxime ether safeners, cyometrinil, oxabetrinil and CGA-133205, have been developed by CIBA-GEIGY Corporation (Basel, Switzerland) to protect grain sorghum against injury caused by the chloroacetanilide herbicide, metolachlor [1–3]. With a broadcast application, these oxime ether safeners will also protect some grass weed species of the *Sorghum* genus against metolachlor; therefore, to achieve crop selectivity, these safeners are applied directly to grain sorghum as seed dressings [3, 4]. This mode of application allows for the uptake of these safeners into the germinating seed and the young seedling of grain sorghum and confers protection to this crop against a subsequent application of the herbicide, metolachlor [3].

Our current understanding of how treatment with a seed-applied safener protects grass crops against chloroacetanilide herbicide injury is equivocal [5, 6]. Two possible theories have been proposed [5, 6]: (1) a safener-induced enhancement of herbicide detoxication in the safened plant, and (2) a competitive antagonism between the safener and the herbicide at a common site of action [5, 6]. At present, most of the accumulated evidence supports the enhanced degradation theory as a mode of action for herbicide safeners [5–7].

The metabolic detoxication of metolachlor in grasses such as corn and grain sorghum proceeds primarily *via* the formation of a conjugate of metolachlor with reduced glutathione (GSH) [3]. This reaction can be either non-enzymatic or enzymatic with the enzymatic reaction being catalyzed by glutathione-S-transferase enzymes (GSTs) [5, 8]. Recent reports have indicated that at least three isozymes of GSTs exist in corn and these isozymes exhibit differential substrate specificity towards chloroacetanilide herbicides [9–11]. These isozymes have been designated GST I, GST II, and GST III and are distinguished by their specificity towards the chloroacetanilide herbicides and by their separation characteristics [11]. GST I is a constitutive isozyme that catalyzes the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) and of the herbicide, alachlor, with GSH. GST II appears to be induced by safener treatments, and also catalyzes the conjugation of CDNB and alachlor, but has different separation characteristics from GST I. GST III appears to be a constitutive enzyme with a higher specific activity for alachlor and metolachlor than GST I [11]. O'Connell [10] demonstrated that at least 80% of the enzymatic activity for the conjugation of metolachlor and alachlor with GSH resides in GST III with the remainder of the activity in GST I. The existence of GST isozymes in grain sorghum has been speculated [12], but detailed characterization of such isozymes is currently unavailable.

Gronwald [7] recently reported that the degree of protection provided by safeners to grass crops against injury from chloroacetanilide herbicides correlates rather strongly with the ability of safeners to enhance GST(s) activity in corn and grain sorghum. However, other questions regarding the influence of safeners on glutathione levels and the activity of other glutathione-related enzymes such as glutathione reductase (GR) (EC 1.6.4.2) need to be examined. Komives *et al.* [13] reported that treatment of 2.5-day old corn shoots with the dichloroacetamide safener, dichlormid, for 24 and 48 h resulted in a 1.78- and 2.5-fold increase in GR activity, respectively. Questions regarding the potential enzymatic and non-enzymatic conjugation of the oxime ether safeners with glutathione should also be addressed. Flurazole, a thiazole carboxylate safener, has been shown to conjugate with reduced glutathione in corn and sorghum shoots within 2 h after treatment [14]. Breaux *et al.* [14] postulated that the formation of the GS-flurazole conjugate may override the normal feedback inhibition of glutathione biosynthesis, resulting in the observed increases in glutathione levels. In addition, he noted that the molecules of most of the currently available safeners are reactive enough to conjugate with GSH and that the formation of GS-safener conjugates may be an important aspect involved in their protective nature.

All of the currently available information on the safener-induced enhancement of metolachlor conjugation with GSH has been generated using shoot tissues of corn or grain sorghum. The influence of seed-applied safeners on glutathione levels and the activity of glutathione-related enzymes during seed imbibition and early seedling establishment of grain sorghum have not been examined.

Therefore, the objectives of this research were to determine: a) the influence of seed-applied oxabetrinil and CGA-133205 on the levels of glutathione and the activity of GR and GST of grain sorghum

seeds during the imbibition phase of germination and early seedling establishment, b) the influence of oxabetrinil and CGA-133205 on the non-enzymatic conjugation of metolachlor with reduced glutathione, and c) the potential non-enzymatic conjugation of oxabetrinil and CGA-133205 with reduced glutathione as influenced by pH and metolachlor.

Material and Methods

Plant material

Since oxabetrinil and CGA-133205 are applied as seed-coat treatments and would be taken into the seed early in the germination process, seeds imbibed for 0 to 24 h and germinating seedlings (36 to 72 h) were used as plant material for these studies. For the 0 to 8 h period of imbibition phase of germination, 16 g of seed was placed into a scintillation vial with 8 ml of distilled water. At 0, 1, 2, 4, and 8 h after germination was initiated (beginning of imbibition), 2 g of seed was removed, frozen in liquid nitrogen, and ground in a mortar and pestle. For the 12 to 72 h time points, seeds were put into petri dishes containing filter paper and 5 ml of distilled water. At 12 and 24 h, seeds were removed, frozen, and ground as above. At 36, 48, and 72 h, the shoots and roots were removed from the seed tissue and both parts were frozen and ground separately. All seeds were incubated at 30 °C. Final concentrations of all reagents are given in parenthesis.

Glutathione extraction

A modified method of Tietze [15] was used for this study. Plant tissue (0.25 g) was ground in a mortar and pestle with 1.5 ml of trichloroacetic acid (5% w/v). The slurry was centrifuged for 15 min in a microcentrifuge (approximately 13,000 × *g*). A portion of the extract (0.4 ml) was diluted with 2.8 ml of Na_2HPO_4-KOH buffer (0.36 M, pH 7.5) for a 1:8 dilution. The shoot and root tissue was diluted 1:80. Diluted extracts were used in the glutathione assays as follows:

Glutathione assay

This assay [15] and the GR assay [16] described below are based on the conjugation of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with reduced glutathione to form a GS-TNB conjugate and 2-nitro-5-thiobenzoic acid (TNB). The formation of TNB was monitored spectrophotometrically at 412 nm. Reduced glutathione was formed by the reduction of oxidized glutathione (GSSG) catalyzed by NADPH-dependent GR. Commercial yeast GR (Sigma Chemical Co., St. Louis, MO) was used for the glutathione assays. For total glutathione determination, the reaction was conducted in a cuvette which contained; 400 µl of sample or standard; 400 µl of reagent I (110 mM Na_2HPO_4, 40 mM NaH_2PO_4, 15 mM EDTA, 0.04% BSA, and 0.3 mM DTNB; approximate pH is 6.9), 320 µl of reagent II (1 mM EDTA, 0.02% BSA, 50 mM imidazole, and 0.48 units of glutathione reductase; approximate pH is 7.1). The reaction was initiated by addition of 80 µl of NADPH (0.9 mM). The reaction is monitored at 412 nm for 2 to 4 min at 24 to 26 °C.

To analyze for oxidized glutathione, 40 µl of 2-vinylpyridine was added to 1 ml of the diluted extract and vigorously shaken every 15 min for 1 h. Reduced glutathione reacts with the 2-vinylpyridine and is effectively removed. This solution is then assayed as above to obtain the oxidized glutathione content of the sample. Reduced glutathione in the crude extract is obtained by subtracting the oxidized glutathione from the total glutathione. Glutathione concentration in the samples was calculated from standard curves using 0 to 2.0 µM of GSH and GSSG. These curves are linear over this concentration range and the GR is active for at least 15 min.

Glutathione reductase and GST extraction

Plant tissue (0.25 g) prepared as described above plus 0.13 g of polyvinylpolypyrrolidone were briefly ground with a mortar and pestle. Then 2.5 ml of extraction buffer (0.1 M K-phosphate buffer plus 0.5 M EDTA, pH 7.5) was added and the slurry was ground again. This slurry was filtered through two layers of Miracloth and then centrifuged for 20 min at 20,000 × *g*. The pellet was discarded and the supernatant was used as the crude extract for the following GR and GST assays. Protein was determined using the Coomassie Blue G-250 dye-binding assay procedure [17].

Glutathione reductase assay

GR was assayed according to the procedure of Smith *et al.* [16]. All reaction components except the crude extract were at room temperature and the reaction chamber temperature was maintained at 24 to 26 °C. The reaction mixture contained 1 ml of 0.2 M K-phosphate buffer (0.1 M) plus 1 mM EDTA (0.5 mM), pH 7.5; 0.5 ml of 3 mM DTNB (0.75 mM) in 0.01 M K-phosphate buffer; 0.25 ml distilled water; 0.1 ml of 2 mM NADPH (0.1 mM); 0.05 ml of crude extract; and the reaction was initiated by the addition of 0.1 ml of 5 mM GSSG (0.25 mM) to make a final volume of 2 ml. Formation of TNB was monitored at 412 nm for 2 to 4 min, but the reaction was linear for at least 15 min. The rate of TNB formation is proportional to the amount of GR activity. The extinction coefficient for the TNB is 11,500 moles cm^{-1} min^{-1} [18].

Glutathione-S-transferase assay

The procedures of Mozer *et al.* [19] and Ezra *et al.* [20] were used for assaying the activity of GST with slight modifications. The reaction mixture contained; 30 µl of 0.1 M K-phosphate buffer (pH 6.5); 10 µl of 60 mM reduced glutathione (10 mM); 10 µl of crude extract. The reaction was initiated by the addition of 10 µl of 6 mM of either carbonyl-labeled [^{14}C]metolachlor (1 mM; sp. act. 59.5 mCi/mmol), phenyl-labeled [^{14}C]oxabetrinil (1 mM; sp. act. 12.2 mCi/mmol), or phenyl-labeled [^{14}C]CGA-133205 (1 mM; sp. act. 9.8 mCi/mmol) in a final volume of 60 µl. The reaction vessel was centrifuged in a microcentrifuge for 30 sec for thorough mixing and then incubated at 35 °C for 60 min. After incubation, the reaction was terminated by adding 60 µl of distilled water and 1 ml of dichloromethane. The reaction vessel was shaken vigorously and then microcentrifuged for 3 min. 60 µl of the aqueous phase was counted using liquid scintillation counting to determine the amount of conjugate formed.

All extractions for glutathione determinations, GR, and glutathione-S-transferase, were twice repeated in time and all assays were run in duplicate for each extraction.

Non-enzymatic conjugation of metolachlor with GSH

To determine the influence of the safeners on non-enzymatic conjugation of metolachlor with glutathione, the methods of Mozer *et al.* [19] and Ezra *et al.* [20] for enzymatic conjugation were modified. Reaction mixtures contained 30 µl of 0.1 M K-phosphate buffer (pH 7.0), 10 µl of 6 mM reduced glutathione (1 mM), 10 µl of 0.3 mM [^{14}C]metolachlor (50 µM), and either 10 µl of oxabetrinil or CGA-133205 (0, 1, 10, 20, 40, 80, or 160 µM) in a final volume of 60 µl. The reaction vessel was microcentrifuged for 30 sec for thorough mixing and then incubated at 30 °C for 1 h. Addition of 60 µl of distilled water and 1 ml of dichloromethane terminated the reaction by partitioning any unreacted metolachlor and safener into the organic phase and any conjugate into the aqueous phase. The aqueous phase was subsampled (60 µl) for liquid scintillation spectrometry. This experiment contained three replicates and was repeated in time.

Influence of pH on non-enzymatic conjugation

To determine the influence of pH on the conjugation of metolachlor, oxabetrinil, and CGA-133205 with glutathione, and to determine the influence of metolachlor and pH on the conjugation of safeners with glutathione, the above procedure was used with the following modifications. The reaction mixture contained 30 µl of 0.1 M K-phosphate buffer (pH 6.0, 7.0, or 8.0), 10 µl of 6 mM reduced glutathione (1 mM), and 10µl of 0.3 mM [^{14}C]metolachlor (50 µM, [^{14}C]oxabetrinil (1 mM), or [^{14}C]GCA-133205 (1 mM) depending on the conjugate that was being determined in a final volume of 60 µl. Reaction times and separation procedures were the same as above. This experiment contained three replicates and was repeated in time.

Results and Discussion

Influence of oxabetrinil and CGA-133205 on glutathione content of grain sorghum seeds and germinating seedlings

The levels of total and reduced glutathione in untreated seeds of grain sorghum decreased during the first 12 h after the initiation of seed imbibition,

Table I. Influence of seed-applied oxabetrinil and CGA-133205 on total and reduced glutathione levels of grain sorghum suring seed germination and early seedling establishment.

Imbibition time [h]	Seed treatment: Untreated Total	Untreated Reduced (A)	Oxabetrinil[a] Total	Oxabetrinil[a] Reduced (B)	CGA-133205[b] Total	CGA-133205[b] Reduced (C)	B/A ratio	C/A ratio
	Glutathione content (μmol/g tissue)[c]							
0	69.8^{D}	41.1^{C}	92.3^{D}	50.7^{D}	88.1^{D}	48.4^{D}	1.23	1.18
1	60.2^{D}	37.4^{C}	93.6^{D}	51.3^{D}	77.7^{D}	43.2^{D}	1.37	1.15
2	54.6^{D}	28.5^{C}	84.1^{D}	48.9^{D}	63.4^{D}	34.1^{D}	1.72	1.20
4	50.6^{D}	28.4^{C}	75.3^{D}	46.0^{D}	43.5^{D}	22.6^{D}	1.62	0.80
8	56.2^{D}	32.1^{C}	56.2^{D}	30.8^{D}	42.7^{D}	22.4^{D}	0.96	0.70
12	61.8^{D}	35.3^{C}	36.6^{D}	18.5^{D}	47.3^{D}	26.8^{D}	0.52	0.76
24	95.5^{D}	53.0^{C}	79.3^{D}	44.0^{D}	105.1^{D}	55.8^{D}	0.83	1.05
36[d]	89.9^{D}	51.2^{C}	90.9^{D}	56.8^{D}	119.2^{D}	70.3^{D}	1.11	1.37
36[e]	408.4^{C}	255.4^{B}	779.0^{C}	380.8^{C}	603.4^{C}	441.8^{C}	1.49	1.73
48	1558.5^{A}	761.3^{A}	3185.1^{A}	2012.8^{A}	1224.9^{A}	819.5^{A}	2.64	1.08
72	1044.5^{B}	690.6^{A}	1746.4^{B}	1006.2^{B}	950.9^{B}	561.9^{B}	1.46	0.81

[a] Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg of seed.
[b] Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg of seed.
[c] Columns followed by the same letter are not significantly different as determined by Fishers' protected $LSD_{(0.05)}$.
[d] Seed tissue only.
[e] Shoot and root tissue removed from seed.

reaching a minimum at 4 h (Table I). From 8 h to 72 h, the levels of total and reduced glutathione increased gradually in seeds as well as shoots and roots of germinating seedlings of grain sorghum reaching a maximum at 48 h (Table I). At 36 h after the initiation of seed germination, the majority of total and reduced glutathione was present in the shoots and roots of the emerging seedlings of grain sorghum rather than the seed tissue (Table I).

The levels of total and reduced glutathione in safener-treated seeds of grain sorghum during germination and seedling establishment followed a trend similar to that observed with untreated seeds (Table I). Glutathione levels decreased gradually following the initiation of germination, reaching a low at 8 to 12 h, and then increased continuously, reaching a maximum at 48 h (Table I). At most time periods following the initiation of seed germination, safener-treated seeds of grain sorghum contained higher levels of total and reduced glutathione than unsafened seeds (ratios of B/A and C/A in Table I). This was particularly true for oxabetrinil-treated seedlings which at 36 to 72 h contained 46 to 164% more reduced glutathione than untreated seeds (Table I). However, oxabetrinil-treated grain sorghum seeds at 8 to 24 h or CGA-133205-treated seed at 4 to 12 h had reduced glutathione levels when compared to the level found in untreated grain sorghum seeds (Table I).

Before the initiation of germination (0 h), seeds of grain sorghum treated with oxabetrinil (1.25 g ai/kg seed) and CGA-133205 (0.4 g ai/kg seed) contained total and reduced glutathione levels that were 20 to 30% higher than those of untreated seeds (Table I). Storage of safener-treated seeds of grain sorghum for periods greater than a year does not adversely influence seed germination or the protective activity of the oxime ether safeners [1]. Measurable effects of oxime ether safeners on the growth and respiration of grain sorghum during early stages of seed germination have been reported by Ketchersid and Merkle [21]. However, information on the potential influence of seed safeners on the metabolic activity of grain sorghum seeds during storage is currently unavailable. Therefore, the differences in glutathione content of untreated and safener-treated seeds of sorghum are difficult to explain at this time.

Reduced glutathione is an obligatory reactant for the enzymatic or non-enzymatic formation of the glutathione conjugate of metolachlor or other chloroacetanilide herbicide [8, 12]. The oxabetrinil-induced increases in the GSH content of grain sorghum seedlings at 36 to 72 h are significant and they may contribute to the mechanism of protective action of this safener. Data reported by Gronwald *et al.* [12] showed that oxabetrinil caused a slight (11%), but not significant increase in the lev-

els of total and reduced glutathione of excised shoots of etiolated 2-day old grain sorghum seedlings. The use of a different sorghum cultivar (G-623 GBR) as well as time-course differences may have accounted for the differential results obtained by Gronwald *et al.* [12] and the present study.

Relatively little is known about the content, synthesis, and metabolic fate of glutathione in mature or germinating seeds of higher plants. In a recent study on the thiol content of legume seeds, Klapheck [22] reported that the major thiol in Vicieae seeds was GSH while in Phaseoleae seeds the major thiol was homoglutathione (hGSH). Seeds from the Trifoleae tribe contained both GSH and hGSH [22]. The results of the present study show that the levels of GSH found in grain sorghum seeds are greater than the levels of GSH or hGSH found in the seeds of legume species [22]. The GSH reserves of grain sorghum seeds are depleted during early germination indicating that the biosynthesis of GSH in grain sorghum seeds may be initiated between 12 and 24 h following the initiation of seed imbibition.

Influence of safeners on glutathione reductase activity

The activity of GR extracted from seeds or seedlings of grain sorghum remained rather stable during the 72 h time-course of this study (Table II). Before the initiation of imbibition (0 h), the activity of GR extracted from safener-treated seeds of grain sorghum was 15 to 24% higher than that of untreated seeds (Table II). At the early stages of seed germination (1 to 12 h), the activity of GR from safener-treated seeds was lower than that of untreated seeds (B/A and C/A ratios in Table II). At 24 h or later time points following the initiation of seed imbibition, the activity of GR in safener-treated seeds or shoots and roots of sorghum seedlings was at or above the activity level of GR extracted from corresponding tissues of untreated grain sorghum (Table II). However, most of these safener influences did not appear to be significant.

Based on these data, it is safe to conclude that a safener-induced enhancement of GR activity does not appear to play a major role in the safening action of oxabetrinil. This statement is further sup-

Table II. Activity of glutathione reductase extracted from untreated, oxabetrinil-treated and CGA-133205-treated grain sorghum during seed germination and early seedling establishment.

Imbibition time [h]	Seed treatment Untreated (A)	Oxabetrinil[a] (B)	CGA-133205[b] (C)	B/A ratio	C/A ratio
		(μmol/min/mg protein)			
0	337.0^{BC}	387.3^{BAC}	417.2^{DC}	1.17	1.24
1	353.0^{BC}	330.3^{BC}	328.2^{DE}	0.94	0.93
2	483.3^{A}	371.4^{BAC}	333.2^{DE}	0.77	0.69
8	314.4^{BC}	247.4^{C}	343.0^{DE}	0.68	1.09
12	361.2^{BAC}	318.3^{BC}	250.8^{E}	0.88	0.69
24	264.3^{C}	470.9^{BA}	310.4^{DE}	1.78	1.17
36[d]	478.1^{A}	528.2^{A}	635.6^{A}	1.10	1.33
36[e]	476.1^{A}	488.7^{BA}	598.0^{BA}	1.03	1.26
48	401.1^{BA}	478.9^{BA}	493.2^{BC}	1.19	1.23
72	342.9^{BC}	435.5^{BA}	363.4^{DE}	1.27	1.06

[a] Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg of seed.
[b] Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg of seed.
[c] Columns followed by the same letter are not significantly different as determined by Fishers' $LSD_{(0.05)}$.
[d] Seed tissue only.
[e] Shoots and roots removed from seed.

Table III. Ratio of reduced (GSH) to oxidized (GSSG) glutathione extracted from untreated, oxabetrinil-treated and CGA-133205-treated grain sorghum during seed germination and early seedling establishment.

Imbibition time [h]	Seed treatment		
	Untreated	Oxabetrinil[a]	CGA-133205[b]
		(GSH/GSSG ratio)	
0	1.43	1.22	1.22
1	1.64	1.21	1.25
2	1.09	1.39	1.16
4	1.28	1.57	1.08
8	1.33	1.21	1.11
12	1.34	1.02	1.30
24	1.25	1.25	1.13
36[c]	1.32	1.66	1.43
36[d]	1.67	0.96	2.73
48	0.96	1.72	2.02
72	1.95	1.36	1.44
Average	1.39	1.32	1.44

[a] Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg seed.
[b] Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg seed.
[c] Seed tissue only.
[d] Shoot and root tissue removed from seed.

ported by data on the GSH/GSSG ratios calculated for untreated and oxabetrinil-treated sorghum tissues and presented in Table III, Averaged over time the GSH/GSSG ratios of control and oxabetrinil-treated sorghum tissues were 1.39 and 1.32, respectively (Table III). Thus, the increase in reduced GSH levels of grain sorghum seedlings caused by the safener oxabetrinil (Table I) can not be explained as a result of safener-induced influence of GR activity. Instead it may be the result of a direct influence of oxabetrinil on the biosynthesis of GSH in grain sorghum. Dichloroacetamide safeners such as dichlormid and R-29148 have been shown to directly influence the *de novo* synthesis of GSH from sulfate by enhancing the activity of the enzyme ATP sulfurylase in corn [23].

CGA-133205 caused an increase in the GSH/GSSG ratio in shoots and roots of germinating sorghum seedlings at 36 and 48 h after the initiation of seed imbibition (Table III). At the same time periods, CGA-133205 increased the activity of GR extracted from these tissues by 26 and 23%, respectively (Table II). Thus, it is likely that CGA-133205 might act by enhancing the activity of GR to maintain a high GSH/GSSG ratio in the cells of protected grain sorghum. A 2.5-fold increase in the activity of GR extracted from 2.5-day old corn treated with the safener dichlormid for 48 h, has been reported recently by Komives *et al.* [13]. Nevertheless, further research is needed to define more clearly the potential influences of CGA-133205 on GR activity.

The ratios of reduced to oxidized glutathione determined in grain sorghum seeds and seedlings (Table III) were low compared to ratios observed in photosynthetic tissue of other plant species [18]. Given the activity of GR determined in these experiments (Table II), it is difficult to explain these low GSH/GSSG ratios. Information on the levels of GSH and GSSG in germinating sorghum seeds is not available in the literature.

Influence of safeners on GST activity

The activity of crude extracts of glutathione-S-transferase (GST) obtained from untreated and safener-treated seeds or seedlings of grain sorghum was assayed by monitoring formation of GS-metolachlor conjugate with [^{14}C]metolachlor as substrate (Table IV). GST activity from untreated grain sorghum tissues remained somewhat stable during the first 8 h following the initiation of seed imbibition. However, from 12 to 72 h, GST activity increased constantly, reaching a maximum at 72 h. At time points equal to or greater than 36 h, most of the GST activity was associated with the shoots and roots of germinated sorghum seedlings rather than the seed (Table IV). GST activity obtained from untreated tissues of the Funk G 522-DR cultivar of grain sorghum used in the present study was significantly higher than that reported by Gronwald *et al.* [12] for the grain sorghum variety, Funk G-623 GBR.

Similar to the aforementioned studies on glutathione content and GR activity, at time 0 h, the activity of GST obtained from oxabetrinil- and CGA-133205-treated seeds of grain sorghum was 67 and 98% greater than that of untreated seeds (ratios of B/A and C/A in Table IV). Again this safener effect is difficult to explain based on current information available in the literature. However, it is evident that oxime ether safeners have an apparent influence on the metabolism of grain sorghum seeds during storage which needs to be examined more thoroughly in the future.

Following the initiation of seed imbibition, GST activity in oxabetrinil- and CGA-133205-treated seeds remained enhanced compared to that of un-

Table IV. Metolachlor-conjugating activity of glutathione-S-transferase extracted from untreated, oxabetrinil-treated and CGA-133205-treated grain sorghum during seed germination and early seedling establishment.

Time [h]	Seed treatment Untreated (A)	Oxabetrinil[a] (B)	CGA-133205[b] (C)	B/A ratio	C/A ratio
	(μmol/min/mg protein)[c]				
0	154.9^{C}	258.9^{A}	292.8^{BA}	1.67	1.89
1	161.7^{C}	190.6^{A}	272.4^{BA}	1.17	1.68
2	197.4^{C}	387.3^{A}	135.8^{B}	1.96	0.69
4	239.8^{C}	298.9^{A}	288.8^{BA}	1.25	1.20
8	198.0^{C}	205.8^{A}	215.6^{A}	1.39	1.46
12	288.8^{CB}	302.8^{A}	282.3^{BA}	1.04	0.93
24	318.5^{CB}	209.6^{A}	271.9^{BA}	0.66	0.85
36[d]	114.0^{C}	111.3^{A}	275.2^{BA}	0.98	0.66
36[e]	596.8^{CB}	325.3^{A}	228.0^{BA}	0.54	0.38
48	799.3^{B}	481.2^{A}	624.4^{A}	0.60	0.78
72	1825.0^{A}	541.2^{A}	355.2^{A}	0.29	0.19

[a] Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg of seed.
[b] Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg of seed.
[c] Columns followed by the same letter are not significantly different as determined by Fishers' $LSD_{(0.05)}$.
[d] Seed tissue only.
[e] Shoots and roots removed from seed.

treated seeds up to the time point of 8 h (B/A and C/A ratios, Table IV). From 12 to 72 h, GST activity in safener-treated seeds or germinating seedlings of grain sorghum was consistently lower than that of untreated seeds or seedlings (Table IV).

Gronwald *et al.* [12] recently reported that oxabetrinil significantly enhanced GST activity extracted from excised apical sections of 48-h-old etiolated sorghum shoots. The oxabetrinil-induced enhancement of GST activity was relative and it decreased as metolachlor concentration in the assay medium increased [12]. Thus, while oxabetrinil enhanced GST activity of grain sorghum shoots by 21.8-fold when metolachlor was used at 0.5 μM, it caused only a 4.6-fold enhancement of GST activity when metolachlor was used at 0.5 mM. In the present study, the concentration of metolachlor used to assay GST activity was 1 mM and crude extracts were obtained from a sorghum cultivar different than that used by Gronwald *et al.* [12]. These two facts may explain the differences in the degree of safener-induced enhancement of GST activity between the present study and that conducted by Gronwald *et al.* [12].

Influences of safeners on non-enzymatic conjugation of metolachlor with GSH

Oxabetrinil used at concentrations ranging from 1 to 40 μM increased (41 to 126%) the rate of non-enzymatic conjugation of metolachlor with GSH at pH 7.0 (Table V). These data are in agreement with those reported by Zama and Hatzios [24] who also reported an oxabetrinil-induced enhancement of the non-enzymatic conjugation of metolachlor with GSH. Gronwald *et al.* [12] have reported that some non-enzymatic conjugation of metolachlor with GSH does occur *in vitro* at pH 7.4, and that the rate of this reaction increased with increasing concentrations of metolachlor.

At higher concentrations (80 and 160 μM), oxabetrinil significantly inhibited the non-enzymatic conjugation of metolachlor with GSH (Table V). CGA-133205 enhanced the non-enzymatic conjugation of metolachlor with GSH when used at concentrations of 1 to 160 μM (Table V). A 106% increase in the rate of the non-enzymatic conjugation of metolachlor with GSH was observed when CGA-133205 was used at 160 μM (Table V).

Table V. Influence of safener concentration on non-enzymatic conjugation of metolachlor with reduced glutathione[a].

Safener concentration [μM]	Oxabetrinil [nmol/h][b]	Percent of control [%]	CGA-133205 [nmol/h]	Percent of control [%]
0	37.2 ± 12.5	100	37.2 ± 12.5	100
1	75.3 ± 22.6	201	63.7 ± 13.1	170
10	84.8 ± 27.3	226	50.3 ± 12.7	134
20	52.9 ± 9.3	141	54.7 ± 8.6	146
40	68.9 ± 14.7	184	51.4 ± 18.8	137
80	13.4 ± 17.1	36	57.4 ± 21.9	153
160	0	0	77.4 ± 36.1	206

[a] Data represent the mean ± SE of 2 experiments with 3 replicates per experiment.
[b] nmol of metolachlor conjugated with GSH in 1 h at pH 7.0.

Comparison of the data presented in Tables IV and V shows that the rate of non-enzymatic conjugation of metolachlor with GSH is much slower than that of the enzymatic conjugation catalyzed by GST. The rate of non-enzymatic conjugation of metolachlor with GSH was found to be strongly dependent on the pH of the reaction solution with conjugation increasing with increasing pH (Table VI). Similar results have been reported by Leavitt and Penner [8] who showed that the non-enzymatic conjugation of the herbicide alachlor with GSH was dependent on the pH of the reaction solution and on the initial concentration of glutathione. The results of the present study show that the potential contribution of the non-enzymatic conjugation of metolachlor with GSH in the metabolic detoxication of this herbicide in grain sorghum and the protective action of the oxime ether safeners can not be ruled out.

Table VI. Influence of pH of the reaction solution on non-enzymatic conjugation of metolachlor, oxabetrinil and CGA-133205 with reduced glutathione[a].

pH	Metolachlor	Oxabetrinil [nmol/h][b]	CGA-133205
6.0	0.0	0.0	0.0
7.0	46.1 ± 25.5	0.4 ± 0.4	0.0
8.0	124.3 ± 9.9	2.6 ± 1.3	0.3 ± 0.1

[a] Data represent the mean ± SE of 2 experiments with 3 replicates per experiment.
[b] nmol of metolachlor, oxabetrinil or CGA-133205 conjugated to GSH in 1 h.

Conjugation of oxime ether safeners with GSH

Data presented in Table VI show that the safener oxabetrinil can conjugate with GSH non-enzymatically. The rate of this reaction is slow and is dependent on the pH of the reaction solution. CGA-133205 did not conjugate substantially with GSH at any pH (Table VI).

Comparison of the data presented in Table VI shows that the rate of the non-enzymatic conjugation of oxabetrinil with GSH is much slower than that of the non-enzymatic conjugation of metolachlor with GSH. In addition, the initial ratio of glutathione to metolachlor (33:1) is lower than the ratio of glutathione to oxabetrinil (20:1). These two facts indicate that metolachlor is much more reactive than oxabetrinil or CGA-133205 in terms of conjugating with GSH under non-enzymatic conditions *in vitro*. This is most likely due to the high reactivity of the electrophilic chlorine ion of metolachlor which is not present on the molecules of the oxime ether safeners.

At pH 8.0, 2.6 nmol of oxabetrinil conjugated with GSH in one hour (Table VI). However, when metolachlor at 50 μM was added to the reaction solution, only 0.2 nmol of oxabetrinil conjugated with GSH in 1 h at the same pH (8.0). Thus, the presence of metolachlor reduced the rate of non-enzymatic conjugation of oxabetrinil with GSH by more than 90% indicating again that metolachlor is more reactive than oxabetrinil in terms of non-enzymatic conjugation with GSH.

Influences of oxime ether safeners on GST activity using the safeners as substrate

The possibility that the conjugation of the safener oxabetrinil with GSH may proceed at a greater rate under enzymatic conditions was also examined. Crude extracts of GST obtained from untreated and oxabetrinil-treated tissues of grain sorghum catalyzed the conjugation of oxabetrinil with GSH (Table VII). However, GST activity from grain sorghum tissues utilizing oxabetrinil as a substrate was significantly lower than that utilizing metolachlor as a substrate (Tables IV and VII). Pretreatment of sorghum seeds with the safener, oxabetrinil, did not appear to enhance GST activity when this safener was used as a substrate (Table VII). Crude extracts of GST obtained from untreated or CGA-133205-treated tissues of grain sorghum did not catalyze the conjugation of the safener CGA-133205 with GSH (data not shown). These results illustrate, again, the poor reactivity of this safener with GSH.

Similar to studies conducted with the safener flurazole [14], the aforementioned evidence for the enzymatic and non-enzymatic conjugation of the safener oxabetrinil with GSH needs to be complimented by further analytical work (*i.e.* mass spectrometry or NMR spectroscopy) to conclusively demonstrate the formation of a GS-oxabetrinil conjugate in plant tissues. The biological significance of the conjugates of oxabetrinil with GSH also awaits further experimentation. Recent reports [14] have postulated that GS-conjugates of herbicide safeners such as flurazole could enhance GSH levels by deregulating the feedback control of GSH synthesis in tissues of the protected plants.

In summary, the results of the present study demonstrated that glutathione and glutathione-related enzymes in seeds and seedlings of grain sorghum play a key role in the mechanism of action of the oxime ether safeners. However, despite their chemical similarity, the oxime ether derivatives, oxabetrinil and CGA-133205, appear to behave differently when used as safeners of grain sorghum against injury from the chloroacetanilide herbicide, metolachlor. Oxabetrinil caused a significant enhancement of the levels of total and reduced glutathione in tissues of grain sorghum during seed germination and early seedling establishment with the levels increasing dramatically after 12 h following initiation of germination. This influence of oxabetrinil appeared to be a direct effect on GSH synthesis rather than an indirect one resulting from an oxabetrinil-induced stimulation of the activity of GR. Deregulation of the feedback control of GSH synthesis by oxabetrinil is a possible mechanism of action since this safener was reactive enough to form a conjugate with GSH. In contrast, CGA-133205 appeared to enhance slightly the activity of GR causing high GSH/GSSG ratios in tissues of grain sorghum during the early phases of seed germination. CGA-133205 did not conju-

Table VII. Oxabetrinil-conjugating activity of glutathione-S-transferase extracted from untreated and oxabetrinil-treated grain sorghum during seed germination and early seedling establishment.

Imbibition time [h]	Tissue examined	Seed treatment: Untreated (A)	Oxabetrinil[a] (B)	B/A ratio
		GST activity (μmol/mg protein/min)[b]		
24	seed	6.0^{C}	5.3^{B}	0.88
36[c]	seed	5.3^{C}	6.2^{B}	1.17
36[d]	shoot and root	28.1^{C}	14.6	0.52
48	shoot and root	107.9^{B}	107.5^{A}	1.00
72	shoot and root	160.4^{BA}	45.6^{B}	0.28

[a] Sorghum seed was coated with oxabetrinil at 1.25 g ai/kg of seed.
[b] Columns followed by the same letter are not significantly different as determined by Fishers' protected LSC $_{(0.05)}$.
[c] Seed tissue with shoot and root removed.
[d] Shoot and root tissue with seed tissue removed.

gate with GSH either enzymatically or non-enzymatically. Both safeners enhanced grain sorghum GST activity for conjugating metolachlor with GSH very early in the germination process (0–8 h following seed imbibition), but reduced activity at 24 to 72 h. In addition, both safeners increased the rate of the non-enzymatic conjugation of metolachlor with GSH in a concentration- and pH-dependent fashion.

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