

Comparative Effectiveness and Mode of Action of Safeners for Chloroacetamide Herbicides in Maize Seedlings*

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In growth room bioassays, four herbicide safeners {BAS-145138: 1-dichloroacetyl-hexahydro-3,3,8a-trimethyl-pyrrolo(1,2a)pyrimidin-6(2H)-one, dichlormid: N,N-diallyl-2,2-dichloroacetamide, flurazole: phenylmethyl 2-chloro-4-(trifluoromethyl)-5-thiazole-carboxylate and MG-191: 2-dichloromethyl-2-methyl-1,3-dioxolane} were compared for their effectiveness in reducing metazachlor {N-(2,6-dimethylphenyl)-N-(1-pyrazolylmethyl)-chloroacetamide} injury to maize seedlings. Three of these compounds (BAS-145138, dichlormid and MG-191) and two different compounds (AD-67: N-dichloroacetyl-1-oxa-4-azaspiro-4,5-decane and DKA-24: N,N'-diallyl-N'-dichloroacetyl-glycineamide) were also evaluated as safeners for acetochlor {N-(2-ethyl-6-methylphenyl)-N-(ethoxymethyl)-chloroacetamide} in maize seedlings. Both BAS-145138 and dichlormid were highly effective as safeners for metazachlor and BAS-145138 and DKA-24 were equally good safeners for acetochlor.

Metazachlor and acetochlor uptake and metabolism by maize seedlings were enhanced significantly in response to treatment with every safener examined. Rates of metazachlor and acetochlor uptake and metabolism were greatest in seedlings treated with BAS-145138 – the most effective safener.

In the absence of any safener treatment, the non-enzymatic conjugation of the herbicides with glutathione either exceeded (metazachlor) or was similar (acetochlor) to enzymatic conjugation. However, the rate of enzymatic conjugation was generally increased by safener treatment. This was particularly true when the herbicide (¹⁴C-labeled) was employed as a conjugation substrate instead of the less specific substrate, CDNB (1-chloro-2,4-dinitrobenzene).

A significant linear correlation was observed between safener effectiveness and rates of metazachlor metabolism or glutathione S-transferase activity in maize seedlings. However, a similar correlation could not be established for the five compounds evaluated as safeners for acetochlor.

Introduction

Chloroacetamide herbicides [1] control germinating grasses and some broadleaved weeds. They can successfully be applied in broadleaved crops (e.g. beans, cotton, peanuts and rapeseed). However, highly effective chloroacetamides [2] such as acetochlor [3] and metazachlor [4] can seriously injure maize at rates over 1 kg/ha. Although the toxicity of thiocarbamate [5] herbicides to maize can be fully overcome by a number of different safeners [6] such as dichlormid and MG-191, most of the good thiocarbamate safeners produce insuffi-

cient protection against maize injury by acetochlor and metazachlor [7, 8].

AD-67 [9] was developed primarily as a safener for thiocarbamate herbicides in maize fields, but, an acetochlor – AD-67 EC product [10] has also been on the Eastern European market for almost ten years. BAS-145138 [11] was introduced as a metazachlor safener in maize. The most widely applied and studied compound has still been dichlormid [12], the first commercialized EPTC safener in the early seventies. Like AD-67, BAS-145138 and dichlormid, DKA-24 [13] is also a dichloroacetamide compound. Although several investigators have suggested that the dichloroacetamide group is responsible for the protective activity of herbicide safeners, flurazole [14], and MG-191 [15] are effective safeners but they are not dichloroacetamides. Seed treatment (1.25–2.5 g/kg seed) with flurazole allows the use of alachlor [N-(2,6-di-

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ethylphenyl)-N-(methoxymethyl)chloroacetamide] and acetochlor in grain sorghum [16]. MG-191 [17] is a highly effective thiocarbamate safener for maize and sorghum, and also for other crops such as rice (unpublished data).

From the numerous processes [18] that can be induced or enhanced by safener treatment, the role of the plant glutathione (GSH) and glutathione S-transferase (GST) enzyme system has been most thoroughly investigated. Several investigators [19–21] have shown that effective safeners significantly elevate both GSH content and GST activity in young seedlings. Because both thiocarbamates and chloroacetamides are detoxified *via* enzymatic conjugation with glutathione, safener treatment eventually leads to an accelerated herbicide detoxification. If this is the predominant pathway of safener action, a direct relationship can be found between safener effectiveness and plant GSH level, GST activity or herbicide metabolism rate as it was presented for metolachlor [21] and metazachlor [7] and several safeners. A number of chloroacetamides are so reactive that they can conjugate with GSH even in the absence of GST enzymes [2].

The objective of this study was to compare the effectiveness of different compounds as safeners for acetochlor and metazachlor as well as to thoroughly investigate the importance of the plant GSH/GST system in safener action and herbicide metabolism for these two weed killers.

Materials and Methods

Chemicals

Acetochlor and AD-67 were provided by Nitrokémia Chemical Works (Füzögyártelep, Hungary). [^{14}C]acetochlor (carbonyl-labeled, sp. act. 38.7 MBq/mmol) [22] and MG-191 [23] were synthesized by the Department of Pesticide Research, Central Research Institute for Chemistry, Hungarian Academy of Sciences (Budapest, Hungary). BAS-145138, metazachlor and [^{14}C]metazachlor (uniformly ring-labeled, sp. act. 198.69 MBq/mmol) were provided by BASF AG (Ludwigshafen, Germany). Dichlormid was provided by Stauffer Chemical Co. (Richmond, C.A., U.S.A.). DKA-24 and flurazole were received from the North Hungarian Chemical Works (Sajóbáony, Hungary) and from Monsanto Co. (St. Louis, M.O., U.S.A.), respectively. All active ingredients

used in the experiments were technical grade (purity > 95%). GSH, CDNB (1-chloro-2,4-dinitrobenzene), DTNB {5,5-dithiobis-(2-nitrobenzoic acid)} and PVPP (polyvinyl-polypyrrolidone) were obtained from Sigma Chemical Co. (St. Louis, M.O., U.S.A.). The Coomassie Blue G-250 based protein assay reagent was purchased from Reanal Fine Chemicals (Budapest, Hungary).

Plant growth

Maize (*Zea mays* L., var. Pioneer 3737) seeds (8/box) were planted in plastic boxes (12 × 12 cm, × 9 cm deep) in air-dried foundry sand (1500 g/box). Seeds were placed 2.5 cm deep and the sand was moistened with 250 ml water. Acetochlor and the safeners were dissolved in ethanol and then added to a 10 ml aqueous solution of TWEEN 20 surfactant (0.1%). The treatment solutions were sprayed on the sand surface (rates: acetochlor 2.0 kg/ha, safeners 200 g/ha) of three replicate boxes one day after planting. The conditions in the growth room were: 60–70% relative humidity, 16 h light period, light intensity of 10 lux, and temperatures of 23 °C and 16 °C during the light and dark periods, respectively. The plants were watered three times per week up to the weight of the boxes at the first watering (1750 g). Every third watering was with half-strength Hoagland's nutrient solution. Height and weight of the shoots were obtained two weeks after planting to facilitate a comparison of safener effectiveness against acetochlor injury.

Growth room studies with metazachlor ± safeners were carried out in a different way [7]. Maize (var. PAG SX-111) seeds were planted into vermiculite in foam cups (350 ml) with holes on the bottom. A 100 ml aqueous solution or emulsion containing either the herbicide (7.5 µM) or the herbicide and one of the safeners (1.0 µM) was poured onto the vermiculite surface. Growth room conditions were similar to the acetochlor ± safener experiments.

Herbicide uptake and metabolism

Maize seedlings were planted, treated and grown as described above. Seedlings which were either 4.0 or 2.5 days old were taken from the boxes or cups, washed and immersed into solutions (500 ml) of either [^{14}C]acetochlor (10 µM) or

[14 C]metazachlor (7.5 μ M) and one of the safeners (1 μ M), respectively. Five seedlings were taken from the solution at periodic intervals up to 2 h. The samples were frozen, pulverized in liquid nitrogen and the unchanged herbicide as well as its water soluble metabolites were extracted by a method published earlier [7]. The samples were analyzed by LSC (liquid scintillation counting).

After the last seedlings were harvested, 1.0 ml portions of the treatment solutions were analyzed in order to determine whether any herbicide metabolite(s) had leached from the tissue during the incubation period.

Glutathione levels

Nonprotein thiol (NP-SH) content of 4 days old maize seedlings was measured spectrophotometrically (412 nm) using DTNB color reagent at pH = 7.0–7.1 [7]. Since 80% of the NP-SH in maize seedlings is usually identified as GSH [24], we have referred to this component as glutathione or GSH in the remainder of this report.

Glutathione S-transferase activity

GST activity in maize seedlings was determined by two different methods, using CDNB [25] (spectrophotometric determination at 340 nm) and the [14 C]labeled herbicide [21] (LSC determination) as substrate. The protein content of the seedlings was determined spectrophotometrically [26] at 595 nm with bovine serum albumin as a standard.

Non-enzymatic conjugation with glutathione

The rate of non-enzymatic conjugation of both acetochlor and metazachlor was measured [8] by incubating a solution of [14 C]acetochlor or [14 C]metazachlor with a solution of GSH in phosphate buffer (pH = 7.2) at 25 °C for 1 h. The conjugation reaction was stopped by trichloroacetic acid (32.5%) and the rate of conjugation was measured by LSC.

GST ([14 C]herbicide) activity was calculated by subtracting the amount of [14 C]acetochlor and [14 C]metazachlor conjugated non-enzymatically with GSH from the total [14 C]herbicide conjugated in the presence of the enzyme extracts from maize seedlings.

Results

Herbicide and safener effects on maize growth

Acetochlor (2.0 kg/ha) and metazachlor (7.5 μ M) rates causing nearly 50% injury in maize shoot height were determined in preliminary experiments.

Significant differences (Fig. 1) were found between the effectiveness of some compounds studied as safeners for acetochlor in maize. Although none of the safeners produced a complete protection against the relatively high rate (2.0 kg/ha) of acetochlor in sand, both acetochlor + BAS-145138 and acetochlor + DKA-24 treated maize seedlings grew almost twice as high as did the seedlings receiving only acetochlor treatment. The influence of AD-67 and MG-191 was considerably weaker and comparable (about 10% protection). Dichlormid did not protect maize from acetochlor at all.

All safeners were more protective against maize injury caused by metazachlor (Fig. 1). BAS 145138 was the most effective safener. It provided nearly total prevention of the shoot height reduction caused by 7.5 μ M metazachlor. Dichlormid was the second best metazachlor safener (39% protection). MG-191 and flurazole were less effective but they also provided significant protection against metazachlor injury in maize (30 and 21% reduction in injury, respectively).

BAS-145138 was found to be the best safener for both acetochlor and metazachlor.

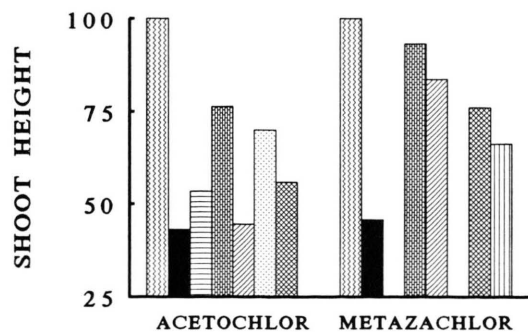


Fig. 1. Herbicide (acetochlor: 2.0 kg/ha; metazachlor: 7.5 μ M) and safener (200 g/ha and 1.0 μ M for acetochlor and metazachlor, respectively) effects on maize shoot height [% of untreated control]. ■, control; ■, herbicide (H); ▨, H + AD-67; ▩, H + BAS-145138; ▤, H + Dichlormid; ▥, H + DKA-24; ▦, H + Flurazole; ▧, H + MG-191. Differences greater than 6.7% are statistically significant ($P < 0.05$) according to the LSD.

Table I. Effect of safener pretreatment on the uptake* of [14 C]acetochlor and [14 C]metazachlor by 4 days old maize seedlings.

Pretreatment	[14 C]acetochlor [mol/g fresh weight]		[14 C]metazachlor	
	30 min	120 min	30 min	120 min
No pretreatment	10.9	23.9	3.41	5.50
Herbicide (H)	17.6	32.4	4.83	8.50
H + AD-67	23.7	60.8	—	—
H + BAS-145138	27.3	72.3	6.05	16.3
H + Dichlormid	20.8	46.4	5.83	14.11
H + DKA-24	16.1	38.3	—	—
H + MG-191	22.7	51.4	6.33	11.75
H + Flurazole	—	—	5.17	10.0

* Differences greater than 4.6 and 4.3% for acetochlor and metazachlor, respectively, are statistically significant ($P < 0.05$) according to the LSD.

Safener influence on herbicide uptake

Both herbicide and herbicide + safener pretreatments significantly enhanced the uptake of [14 C]acetochlor and [14 C]metazachlor by 4-day-old maize seedlings (Table I).

Acetochlor (A) and A + DKA-24 pretreatments enhanced [14 C]acetochlor uptake (Table I) by 50–60% in the first half hour of the incubation period. Pretreatments with A + AD-67, A + dichlormid and A + MG-191 doubled uptake, while A + BAS-145138 pretreatment caused a 150% increase in herbicide absorption. In 2 h, the differences became even higher. Seedlings pretreated with A + BAS-145138, the most effective acetochlor safener and A + AD-67, a poor acetochlor safener absorbed the most [14 C]acetochlor.

Similar effects (Table I) were found in the metazachlor (M) experiments. Pretreatments with M +

BAS-145138 and M + dichlormid, the two best metazachlor safeners, tripled [14 C]metazachlor uptake in 2 h. M + flurazole and M + MG-191 pretreatments about doubled the absorption of [14 C]metazachlor. Although the concentration of [14 C]acetochlor (10 μ M) and [14 C]metazachlor (7.5 μ M) were nearly equal in the incubation medium, 3–4.5 times more acetochlor than metazachlor was absorbed by maize. One explanation for this significant difference can be the difference in the absorption ability of the two varieties used for the acetochlor (Pioneer 3737) and for the metazachlor (PAG S-111) experiments.

Safener influence on herbicide metabolism

About 50% of the absorbed [14 C]acetochlor was metabolized to a water soluble GSH conjugate by control maize seedlings within 50 min (Table II).

Table II. Effect of safener pretreatment on the metabolism* of [14 C]acetochlor and [14 C]metazachlor in 4 days old maize seedlings.

Pretreatment	[14 C]acetochlor [% of total DPM as water sol. metabolites]		[14 C]metazachlor	
	10 min	50 min	10 min	50 min
No pretreatment	31.9	52.7	21.1	37.6
Herbicide (H)	44.5	60.1	22.9	43.1
H + AD-67	60.7	79.3	—	—
H + BAS-145138	67.2	82.0	42.7	72.5
H + Dichlormid	60.4	79.7	36.7	66.1
H + DKA-24	48.3	66.4	—	—
H + MG-191	56.0	71.6	29.4	55.0
H + Flurazole	—	—	24.8	49.5

* Differences greater than 5.7 and 5.1% for acetochlor and metazachlor, respectively, are statistically significant ($P < 0.05$) according to the LSD.

The rate of conjugation was significantly increased even by acetochlor pretreatment (almost 50% in 10 min) and was even faster when pretreated with a safener as well. With the exception of AD-67 and dichlormid, safener effects were most different at the 10 min sampling and the differences decreased in time. BAS-145138 treated seedlings metabolized almost 70% of the absorbed [14 C]acetochlor in 10 min and more than 80% in 50 min. After these same incubation times, conjugation in DKA-24 treated seedlings was 48 and 66%, respectively. The effect of AD-67 and dichlormid were practically the same and near to BAS-145138. The influence of MG-191 was similar to DKA-24.

Although [14 C]metazachlor was metabolized considerably slower than [14 C]acetochlor in the first 30 min, 80% of the metazachlor was converted to a GSH-conjugate within 2 h by BAS-145138 or dichlormid-pretreated maize seedlings. Metazachlor pretreatment alone did not significantly increase the detoxification ability of the control seedlings within the 50 min incubation period (Table II). The influence of flurazole and MG-191 was significant, but, not as great as was the influence of BAS-145138 or dichlormid.

Enzymatic vs. non-enzymatic conjugation

Almost 60% of [14 C]acetochlor and only 10% of [14 C]metazachlor conjugated enzymatically in control maize seedlings (Fig. 2). This ratio was in-

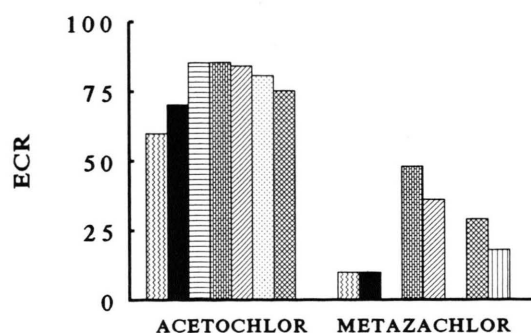


Fig. 2. Enzymatic conjugation rate ECR [% of total conjugation of the herbicide with glutathione] in herbicide \pm safener treated 4 days old maize seedlings. [Control; ■, herbicide (H); ▨, H + AD-67; ▩, H + BAS-145138; ▪, H + Dichlormid; ▫, H + DKA-24; ▬, H + Flurazole; ▮, H + MG-191]. Differences greater than 6.5% are statistically significant ($P < 0.05$) according to the LSD.

creased by acetochlor pretreatment and further increased by additional safener pretreatment. All safeners had a similar influence on acetochlor-GSH conjugation. However, significant differences were found between the effect of safeners on the conjugation of metazachlor with GSH. BAS-145138 and dichlormid pretreatments increased the original 10% enzymatic ratio to 48 and 36%, respectively. Flurazole and MG-191 had smaller, but also significant effects (18 and 29% enzymatic conjugation).

Glutathione *S*-transferase (GST) activity

Both acetochlor and all A + safener pretreatments significantly increased GST(CDNB) activity of the maize seedlings (Fig. 3). AD-67 had the greatest (162% increase) and BAS-145138 had the second greatest (118% increase) effect. Dichlormid, DKA-24 and MG-191 pretreatments produced similar increases (74–85%).

Metazachlor pretreatment had a smaller increasing effect on GST(CDNB) activity than acetochlor (Fig. 3). M + BAS-145138 and M + dichlormid pretreatments resulted in increases that were nearly as high as the A + BAS-145138 and A + dichlormid pretreatments. The influence of flurazole and MG-191 were intermediate (37 and 34% increase, respectively).

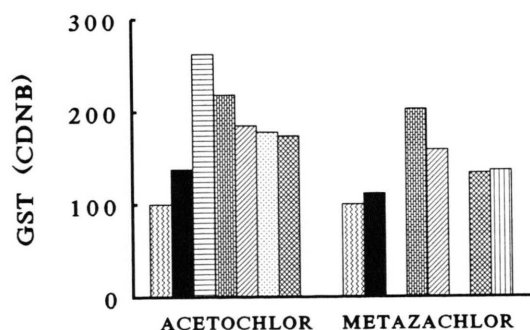


Fig. 3. Effect of acetochlor (2.0 kg/ha), metazachlor (7.5 μ M) and safeners (200 g/ha and 1.0 μ M for acetochlor and metazachlor, respectively) on glutathione *S*-transferase activity [GST, % of untreated control: acetochlor 352 ± 12 , metazachlor 388 ± 7 nmol mg prot $^{-1}$ min $^{-1}$] of 4 days old maize seedlings. Substrate: 1-chloro-2,4-dinitrobenzene (CDNB). [Control; ■, herbicide (H); ▨, H + AD-67; ▩, H + BAS-145138; ▪, H + Dichlormid; ▫, H + DKA-24; ▬, H + Flurazole; ▮, H + MG-191]. Differences greater than 3.8% are statistically significant ($P < 0.05$) according to the LSD. GST activity of the controls: 352 (acetochlor) and 388 (metazachlor) nmol mg prot $^{-1}$ min $^{-1}$.

Greater differences were measured when the [14 C]labeled herbicide was used as the substrate in the GST assays (Fig. 4). Even acetochlor pretreatment caused a 58% increase in GST activity. AD-67, BAS-145138 and dichlormid had similar effects (250–290% increase). DKA-24 almost tripled, MG-191 doubled GST([14 C]-acetochlor) activity.

The M + BAS-145138 pretreatment had the greatest influence (1000% increase) on GST([14 C]metazachlor) activity (Fig. 4). All safeners caused significant different increases in GST activity but there were significant differences in their effects on this enzyme (dichlormid 603%, MG-191 418%, flurazole 215% increase).

Glutathione (GSH) content

Like GST activity, maize GSH content was increased significantly by all herbicide and/or safener pretreatments (Fig. 5). BAS-145138 caused high increases in GSH levels when applied with either acetochlor or metazachlor. Dichlormid was at least as effective as BAS-145138 when applied with acetochlor but it had a significantly reduced effect on GSH levels when applied with metazachlor.

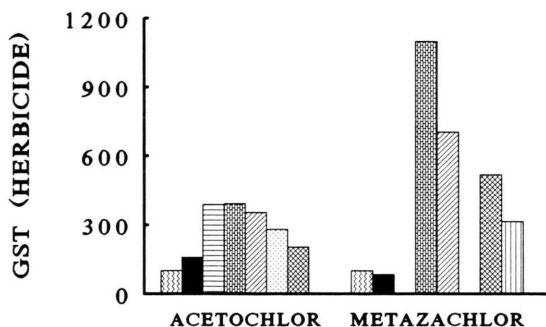


Fig. 4. Effect of acetochlor (2.0 kg/ha), metazachlor (7.5 μ M) and safeners (200 g/ha and 1.0 μ M for acetochlor and metazachlor, respectively) on glutathione S-transferase activity [GST, % of untreated control: acetochlor 99 ± 7 , metazachlor 80 ± 11 pmol mg prot $^{-1}$ h $^{-1}$] of 4 days old maize seedlings, substrate: [14 C]herbicide. [Control; H, herbicide (H); H + AD-67; H + BAS-145138; H + Dichlormid; H + DKA-24; H + Flurazole; H + MG-191. Differences greater than 5.2% are statistically significant ($P < 0.05$) according to the LSD. GST activity of the controls: 99 (acetochlor) and 80 (metazachlor) pmol mg prot $^{-1}$ h $^{-1}$.

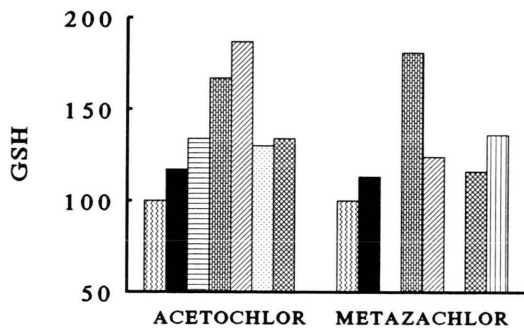


Fig. 5. Effect of acetochlor (2.0 kg/ha), metazachlor (7.5 μ M) and safeners (200 g/ha and 1.0 μ M for acetochlor and metazachlor, respectively) on the glutathione content [GSH, % of untreated control: acetochlor 495 ± 23 , metazachlor 570 ± 41 nmol/g f.w.] of 4 days old maize seedlings. [Control; H, herbicide (H); H + AD-67; H + BAS-145138; H + Dichlormid; H + DKA-24; H + Flurazole; H + MG-191. Differences greater than 5.3% are statistically significant ($P < 0.05$) according to the LSD.

Discussion

The major goal of these studies was to examine the relationship (Fig. 6–7) found between safener effectiveness (reduction of herbicide injury), herbicide metabolism rates, and levels of GSH and GST activity in maize seedlings.

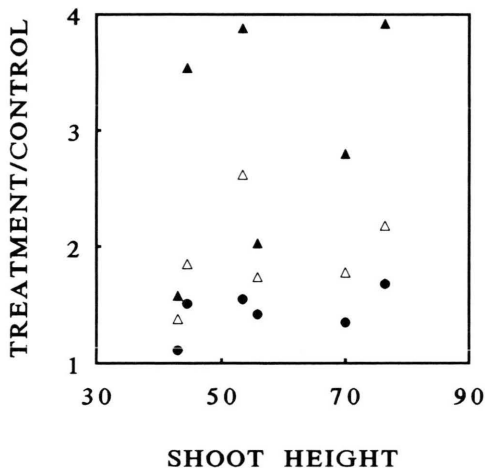


Fig. 6. Safener effectiveness [shoot height, % of control] versus GST(CDNB) activity [Δ , % of control], GST([14 C]acetochlor) activity [\blacktriangle , % of control] and the rate of acetochlor metabolism [\bullet , % herbicide conjugated with GSH in 70 min] in 4 days old maize seedlings pretreated with acetochlor (2.0 kg/ha) \pm safeners (200 g/ha).

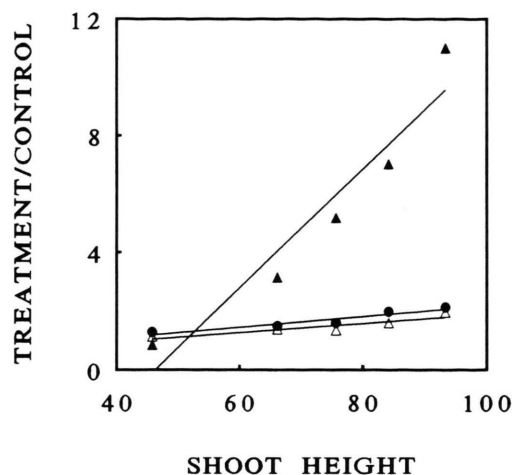


Fig. 7. Safener effectiveness (shoot height, % of control) versus GST(CDNB) activity [△, % of control], GST([¹⁴C]metazachlor) activity [▲, % of control] activity and the rate of metazachlor metabolism [●, % herbicide conjugated with GSH in 70 min] in 4 days old maize seedlings pretreated with metazachlor (7.5 μ M) \pm safeners (1.0 μ M).

In the metazachlor \pm safener experiments, there was a very clear and direct relationship between these processes. The rank of safener effectiveness for reducing metazachlor injury was found as follows: BAS-145138 > Dichlormid > MG-191 > Flurazole. Furthermore, these four safeners had the same order of effectiveness on metazachlor metabolism, GSH levels and GST activity (Fig. 7).

The results from the acetochlor \pm safener experiments did not show such a clear relationship

(Fig. 6). BAS-145138 and DKA-24 were almost equally effective as safeners for acetochlor. BAS-145138 also had the highest or second highest effect on acetochlor metabolism. However, DKA-24 had nearly the lowest activity for enhancing acetochlor metabolism or maize GSH activity. AD-67, a poor acetochlor safener increased both herbicide metabolism and GST activity to the same level as did BAS-145138. One explanation for the lack of a direct correlation in the acetochlor study may relate to the methods of safener treatment. Because both MG-191 and dichlormid are highly volatile, the preemergent spray application could have resulted in the loss of too much safener before the seedlings were able to absorb an adequate quantity. In contrast, in an earlier study, the safeners were applied as preplant incorporated treatments, and all five of them were similarly effective for reducing acetochlor injury [8].

These studies indicate that the enzymatic and non-enzymatic conjugation with non-protein thiols (GSH) is one of the primary mechanisms for chloroacetamide herbicide detoxication in maize seedlings. This detoxication system is inducible by the chloroacetamide herbicides themselves but the substrates (GSH) and enzymes (GST) are more markedly elevated by various less toxic herbicide safeners. In these studies, safener effectiveness was highly correlated with effects of the safeners on metazachlor metabolism. The lack of correlation between safener effectiveness and acetochlor metabolism may have been due to variations in the volatility of the safeners when applied as pre-emergence, soil surface treatments.

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