Session 3D

Plant Selectivity and the Metabolism of Herbicides

Session Organiser	Mr D J Cole
Poster Papers	3D-1 to 3D-7

MECHANISMS OF SELECTIVE ACTION OF THE PEROXIDIZING HERBICIDE ET-751 ON WHEAT AND *GALIUM APARINE*

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ABSTRACT

ET-751 (ethyl 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetate) is a novel herbicide for cereals, highly effective against several important broad-leaved weeds, especially *Galium aparine*. To elucidate the mechanisms of its selective activity on wheat and *G. aparine*, the intrinsic activity on the target enzyme, foliar deposition and absorption, and metabolism of the compound were compared. Although protoporphyrinogen IX oxidase activities in both wheat and *G. aparine* chloroplasts were inhibited equally by ET-751, a foliar application of the compound caused much more accumulation of protoporphyrin IX in *G. aparine* than in wheat. Distinct differences were observed in foliar deposition and absorption, and in the rate of metabolic detoxification of the compound between both plants. The foliar deposition and absorption of the compound were much greater in *G. aparine* than in wheat, while metabolism to give non active or less active metabolites was much greater in wheat than in *G. aparine*. These differences would play an important role in the accumulation of protoporphyrin IX and the resulting selectivity of ET-751 between these plants.

INTRODUCTION

ET-751 (ethyl 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetate) is a new selective post-emergence herbicide for cereals. The compound is highly effective against several important broad-leaved weeds, especially *Galium aparine* (Miura *et al.*, 1993). To clarify mechanisms of selective action of ET-751 on wheat and *G. aparine*, differences of the intrinsic sensitivity of the target enzyme, of foliar deposition and absorption, and of metabolism of the compound were investigated in the two plant species.

RESULTS AND DISCUSSION

Mode of action and selectivity at the target enzyme level

The herbicidal mode of action of ET-751 was very similar to other peroxidizing herbicides (Miura *et al.*, 1993). We identified an accumulation of protoporphyrin IX (Proto IX) after a foliar application of ET-751 at 10 g a.i./ha (2.5% EC). After 24h of the treatment, high level of Proto IX accumulated in *G. aparine* (17.6 nmol/g fresh weight), while little accumulation took place in wheat (Table 1). This result would explain the selective action

of ET-751 on wheat and *G. aparine*, and suggest that the site of action of ET-751 was protoporphyrinogen IX oxidase (Protox) and therefore the same as other peroxidizing herbicides.

Table 1. Accumulation of protoporphyrin IX *In vivo* in wheat and *G. aparine* after 24 h of a foliar application of ET-751.

Species	No treatment	ET-751 treated
	nmol / g fresh weight	
Wheat	<0.20	0.37±0.09
Galium aparine	<0.20	17.58±4.76

ET-751 (2.5% EC; 10 g a.i./ha) was applied to wheat c.v. Mercia at the 3 leaf stage and *G. aparine* at the 2 whorl stage using a spray volume rate of 600 l/ha in a glasshouse. After 24 h, accumulated Proto IX in the shoots was extracted and measured with a HPLC equipped with a fluorescence detector. Results were presented as means and SD's of 3 replications.

To examine the inhibitory activity of ET-751 on Protox, chloroplasts were isolated from wheat and *G. aparine*. Protox activities in chloroplasts were determined by measuring accumulated Proto IX with a HPLC equipped with a fluorescence detector. The I_{50} values of ET-751 on Protox in wheat and *G. aparine* chloroplasts were 1.6 and 1.2 nM, respectively (Figure 1). The fact that Protox activity in wheat chloroplasts was as susceptible to ET-751 as in *G. aparine* suggested the intrinsic sensitivity of target enzyme was not important in the selectivity in whole plants. Therefore, other factor(s) such as foliar absorption and/or metabolism of the compound might play a more important role in the selectivity.

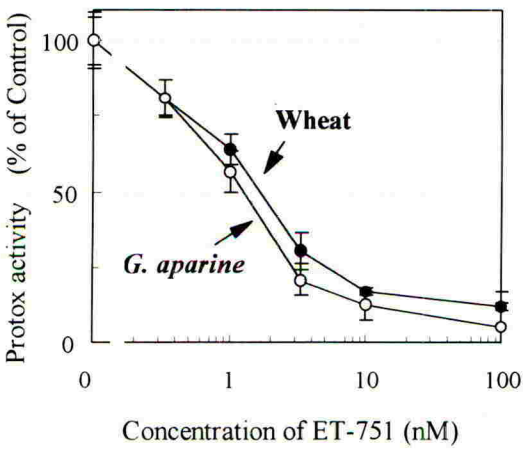

Figure 1. Effect of ET-751 on Protox activities in wheat and *G. aparine* chloroplasts.

Protox activities in untreated chloroplasts of wheat and *G. aparine* were 17.5 and 10.0 nmol Proto IX/mg chlorophyll/h, respectively. The I_{50} values of ET-751 on Protox in wheat and *G. aparine* chloroplasts were 1.6 and 1.2 nM, respectively. Results were presented as means and SD's of 3 replications.

Selectivity in foliar deposition and absorption

Foliar deposition of ET-751 (2.5% EC) was compared for wheat and *G. aparine* in a glasshouse. The application rates of the compound were 3, 10 and 30 g a.i./ha in a spray volume rate of 600 l/ha. As shown in Figure 2, the deposition of the compound on *G. aparine* was about 3 to 5 times higher than that on wheat at each application rate. Foliar absorption of the compound was compared for wheat and *G. aparine* in a laboratory using radiolabelled compound. After 1 h of the application, the amount of radioactivity absorbed by *G. aparine* was about 7 times as large as that absorbed by wheat (Figure 3). The amount of the compound absorbed by *G. aparine* increased in a time dependent manner, while that in wheat remained almost constant. Consequently, the amount of the compound absorbed by *G. aparine* was almost 13 times as large as that by wheat after 42 h. These results revealed that there were large differences between wheat and *G. aparine* in terms of the foliar deposition and absorption of the compound. These differences might be attributable to differences in the shape of the leaves, the structure of the leaf surface and the composition of the epicuticular wax of plants. Further study of these aspects would be needed to understand the differences in the foliar deposition and absorption of ET-751 between wheat and *G. aparine*.

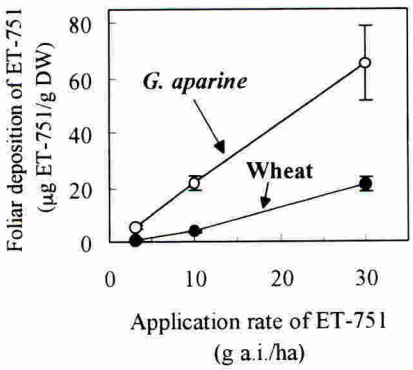

Figure 2. Foliar deposition of ET-751 on Wheat and *G. aparine*.

ET-751 (2.5% EC) was applied to wheat at the 3 leaf stage and *G. aparine* at the 2 whorl stage. The application rates of the compound were 3, 10 and 30 g a.i./ha in a spray volume rate of 600 l/ha. Immediately after the application, the compounds deposited on wheat and *G. aparine* were removed by rinsing with 30 ml of acetone for 15 s and measured with a HPLC equipped with a UV detector. Results were presented as means and SD's of 3 replications.

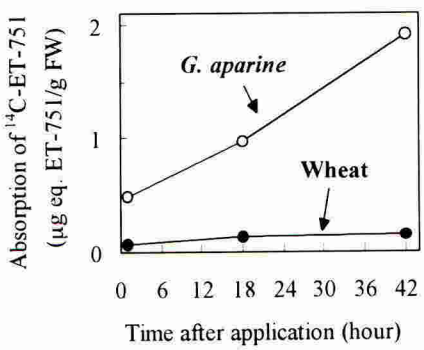

Figure 3. Foliar uptake of radiolabelled ET-751 by Wheat and *G. aparine*.

[*Pyrazole*-5-^{14}C] ET-751 (5.6 MBq/mg, 2.5% EC, 6 g a.i./ha) was applied to wheat at the 3 leaf stage and *G. aparine* at the 2 whorl stage in a spray volume rate of 600 l/ha. After 1, 18 and 42 h, shoots were weighed and the non-absorbed radioactivity was removed by rinsing with 30 ml of acetone for 15 s. the absorbed and non-absorbed radioactivity was measured by liquid scintillation counting. Results were presented as means of duplicates.

Selectivity in metabolic detoxification

Figure 4 shows the proposed metabolic pathway of ET-751 in plants. Major metabolites identified in leaves were metabolites I and II Although metabolite III was not characterized well, it appeared to be conjugate(s) produced from metabolite II. To elucidate the mechanisms of the selectivity of ET-751, the rate of the metabolism of the compound in the two species and the inhibitory effects of ET-751 and its metabolites on Protox activity were examined. Figure 5 shows the time course of metabolism of the compound when absorbed by wheat and *G. aparine* after a foliar application. To ensure a similar absorption of the radioactivity in both plants, the application rates of the compound to wheat and *G. aparine* were 6 and 0.6 g a.i./ha, respectively. Although the amount of the total radioactivity which could not be removed by rinsing with acetone increased in a time dependent manner, the amount of the parent compound remained almost constant for 42 h in both plants. In wheat, metabolite I was rapidly produced but then decreased as metabolites II and III increased. In *G. aparine*, however, metabolite I increased with time and the metabolites II and III were minor products only. These results indicated that the rate of ethyl ester hydrolysis of the compound was very rapid in both wheat and *G. aparine*, but N-demethylation and subsequent metabolism in wheat were much faster than that in *G. aparine*. It would be speculated that almost of the parent compound which could not be removed by rinsing with acetone was localized in the intercellular space of both wheat and *G. aparine* leaves and that there was little parent compound in the cells of both plants. Considering that the Protox was localized in chloroplasts and mitochondria, it would be suggested that not the parent compound but its metabolites might play an important role in the herbicidal activity in whole plants.
Inhibitory effects of ET-751 and its metabolites on Protox activity in wheat chloroplasts are shown in Figure 6. The I_{50} value for Protox in wheat chloroplasts by metabolite I was 0.36 nM, which was about one fifth of the parent compound, ET-751. The I_{50} value of metabolite II was 4.6 nM, which was about 3 times higher than that of ET-751. Metabolite III was inactive. These results suggested that ET-751 absorbed in both plants underwent a degree of bioactivation by hydrolysis of its ethyl ester, and this more active metabolite I was rapidly decomposed to the inactive metabolite III *via* metabolite II in wheat, but not in *G. aparine*. These differences in metabolism seemed to be important factors in the selective action as well as those of the foliar deposition and absorption.

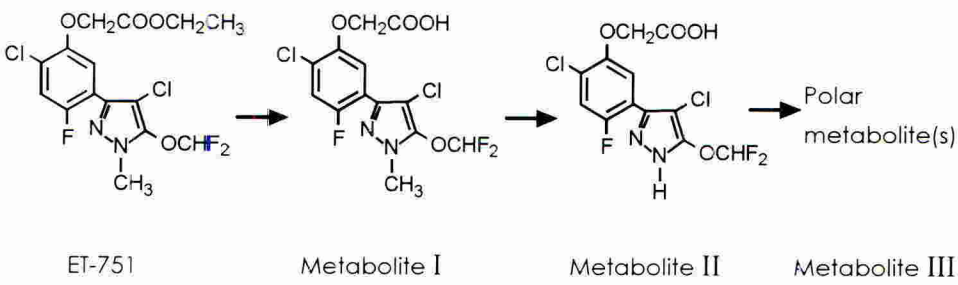

Figure 4. Proposed metabolic pathway of ET-751 in plants.

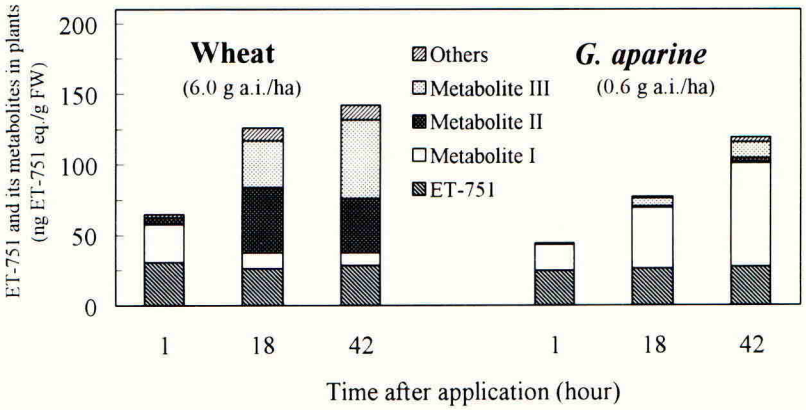

Figure 5. Metabolism of the absorbed ET-751 in wheat and *G. aparine*.

[*Pyrazole*-5-^{14}C] ET-751 (5.6 MBq/mg, 2.5% EC) was applied to wheat at the 3 leaf stage and *G. aparine* at the 2 whorl stage in a spray volume rate of 600 l/ha. The application rates of the compound to wheat and *G. aparine* were 6 and 0.6 g a.i./ha, respectively. After 1, 18 and 42 h of the application, shoots were weighed and the deposited radioactivity was removed by rinsing with 30 ml of acetone for 15 s. Absorbed radioactivity was extracted with acetone/methanol (1/1) and analyzed by TLC/auto radiography. Results were presented as means of duplicates.

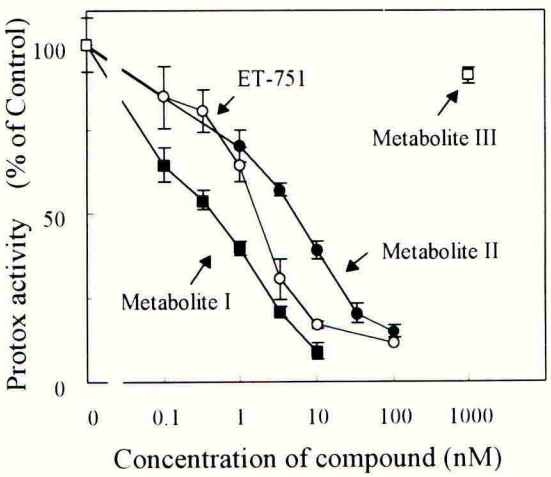

Figure 6. Effect of ET-751 and its metabolites on Protox activity in wheat chloroplasts.

Protox activity in untreated chloroplasts of wheat was 17.5 nmol Proto IX/mg chlorophyll /h. The I_{50} values of ET-751, metabolites I and II were 1.6, 0.36 and 4.6 nM, respectively. Results were presented as means and SD's of 3 replications.

CONCLUSIONS

Although Protox activities in both wheat and *G. aparine* chloroplasts were inhibited equally by ET-751, a foliar application of the compound caused much more accumulation of Proto IX in *G. aparine* than in wheat. The foliar deposition and absorption of the compound were much greater in *G. aparine* than in wheat, while metabolism to give non active or less active metabolites was much greater in wheat than in *G. aparine*. From these results, it was concluded that the intrinsic sensitivity of target enzyme, Protox was not important in the selectivity of ET-751 in whole plants and that differences in the foliar deposition and absorption and the metabolism of the compound would play an important role in the accumulation of Proto IX and the resulting selectivity of ET-751 between these plants.

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MODE OF SELECTIVE ACTION OF THE HERBICIDE HOE 095404

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ABSTRACT

The mode of selective action of the herbicide Hoe 095404 [3-(4,6-dimethoxypyrimidin-2-yl)-1-(2-ethoxyphenoxysulfonyl)-urea], proposed common name: ethoxysulfuron, was studied using radiolabelled compound in tolerant crops (rice, wheat, sugarcane) and in representative susceptible weed species. After foliar application of [^{14}C]Hoe 095404 the percentage of uptake by wheat was about half that of *Galium aparine* whereas the percentage of uptake by rice and the sedge weed *Cyperus serotinus* was about equal. In both cases translocation was less in the crop than in the corresponding weed. The concentration of [^{14}C]Hoe 095404 equivalents after flood water application was significantly lower in the shoot base of rice than in the shoot base of *C. serotinus*. The rate of degradation of [^{14}C]Hoe 095404 to hydrophilic products was markedly faster in rice than in *C. serotinus*, both after shoot or root treatment of whole plants.

An additional series of degradation studies was carried out with excised shoot material of rice and *Scirpus maritimus*, wheat and *Galium aparine*, and sugarcane and *Cyperus esculentus*. It showed consistently high rates of degradation of the ^{14}C-labelled parent compound to hydrophilic products in the crops, contrasting to slow degradation in the weeds. It is concluded that differential degradation of Hoe 095404 in tolerant crops and susceptible weeds is a key factor in the selective action of this herbicide, but differences in herbicide uptake and translocation may also contribute towards crop selectivity.

INTRODUCTION

Hoe 095404 [3-(4,6-dimethoxypyrimidin-2-yl)-1-(2-ethoxyphenoxysulfonyl)-urea], proposed common name: ethoxysulfuron, has been developed as a post-emergence herbicide for the control of annual and perennial sedges and dicotyledonous weeds. It is selective in transplanted and seeded rice, in wheat and barley, and in sugarcane. The compound is taken up via the shoot, but also via the root, and acts by inhibition of the enzyme acetohydroxyacid synthase (Bauer *et al.*, 1995).

Enzyme inhibition data from *in vitro* studies suggest that the selective action of Hoe 095404 is not based on differential susceptibility of the acetohydroxyacid synthase in tolerant and susceptible species. As an example, the inhibition value for tolerant rice (I_{50} = 1.7 x 10^{-8} M) was not significantly different from the inhibition value (I_{50} = 9.2 x 10^{-9} M) for the susceptible sedge weed *Cyperus serotinus* (Trinks, 1995). Therefore comparative studies on the uptake and particularly on the degradation of Hoe 095404 were carried out with tolerant and susceptible plant species, in order to elucidate the mode of selective action of this herbicide.

MATERIALS AND METHODS

[*Pyrimidyl*-2-^{14}C]Hoe 095404, specific activity 870 MBq/mmol, radiochemical purity 98-99% (tlc-analyis) was synthesised by the Radiochemical Laboratory of Hoechst AG. All other chemicals were obtained from commercial sources.

Plants were cultivated in the greenhouse in pots of loamy sand, 16 cm diam. (sugarcane) or 9 cm diam. (other species) and were transferred after herbicide treatment to a growth cabinet with 12 hours daily lighting by fluorescent lamps, 155 µE m^{-2} s^{-1}. Spring wheat (*Triticum aestivum*), cv. Ralle, and *Galium aparine* were kept at 18/12°C, sugarcane (*Saccharum officinarum*) and *Cyperus esculentus* at 23/18°C, and rice (*Oryza sativa*), cv. Binalien, *Scirpus maritimus* and *Cyperus serotinus* were maintained at 30/19°C and about 55/85% r.h. (day/night). Sugarcane and sedges were cultivated from vegetative parts, the other species from seeds. At the time of treatment with [^{14}C]Hoe 095404 plants were at the growth stage appropriate for practical herbicide application. A paddy rice field situation was simulated by growing rice and *Cyperus serotinus* in pots with a flood water level of 2 cm above the soil surface.

Application solutions for treatment of whole plants were prepared with [^{14}C]Hoe 095404 formulated as a 75% water-dispersible granulate (WDG). Fatty alcohol-diglycolether-sulphate wetting agent (final concentration 0.1% washing active substance) was added to treatment solutions being applied to the leaf surface. Foliar treatments were carried out by application of microdroplets (1 µl droplet volume) on the adaxial leaf surface by syringe, and uptake rates determined after two short leaf rinses with methylene chloride. Herbicide degradation in excised shoot material was studied after immersion of the cut basal end of the shoots in 1 mM potassium phosphate buffer solution, pH 6.8, with 7 ppm of non-formulated [^{14}C]Hoe 095404. Parent compound and degradation products were extracted from 5 g of homogenised plant tissue with 10 ml acetone + water (80 + 20). After centrifugation this procedure was repeated twice. Acetone was evaporated from the combined supernatants at 35°C under vacuum and the pH adjusted to 3.5 by addition of diluted sulphuric acid. After partitioning with methylene chloride radioactivity was determined in the methylene chloride and the water phase. Radioactivity was quantified by liquid scintillation counting in washing solutions and extracts directly, in plant tissue after combustion in a sample oxidiser (Fa. Zinsser). Concentrated extract phases were chromatographed with acetonitrile + water (55 + 45) on RP 18 tlc plates and the chromatograms evaluated on a Radio-tlc scanner (Fa. Berthold).

RESULTS

Uptake and translocation

The application of [^{14}C]Hoe 095404 to the flood water, simulating a paddy rice field situation, resulted in an initial concentration of 184 ng a.i./ ml flood water. Seven days after application the level of [^{14}C]Hoe 095404 equivalents in the shoot base (exposed to the flood water) was markedly higher in the susceptible weed species *Cyperus serotinus* than in rice. The concentration in the shoot base of *Cyperus serotinus* was higher than the initial flood water concentration, whereas the concentration in the shoot base of rice was equal to or lower than that in the flood water. In both species the concentration of radioactive material in the shoot above the flood water level remained much lower than in the shoot base and the

roots. In tendency, the results were similar for the two growth stages being tested in this experiment (Table 1).

When a solution of [^{14}C]Hoe 095404 (0.02 % a.i., 5 µl/plant) was applied to the 3rd leaf both of rice and *C. serotinus* at the 4-to 5-leaf stage, the percentage of uptake was similar in both species. Uptake 3 days after treatment amounted to about 52 % in rice, and 46.5% in *C. serotinus*. In rice, even after 3 days, very little (0.2 % of the applied ^{14}C) was translocated to the nontreated parts of the plants. In *C. serotinus* about 5 % had been translocated after the same period (Table 2).

Table 1. Concentration of [^{14}C]Hoe 095404 equivalents (ng/g fwt) in plant tissue 7 d after application to flood water

Plant part	Rice		*Cyperus serotinus*	
	3-leaf stage	4/5-leaf stage	2-leaf-stage	4-leaf stage
Shoot above water	45.5 ± 12.2	26.1 ± 3.2	18.5 ± 5.6	16.7 ± 6.8
Shoot base	178.3 ± 49.5	65.9 ± 21.7	477.6 ± 89.7	640.1 ± 269.8
Root	179.6 ± 48.6	332.6 ± 81.9	160.1 ± 41.0	120.7 ± 48.9
Tuber	-	-	65.3 ± 12.5	109.1 ± 46.9

(mean ± sd, n = 8)

After application of [^{14}C]Hoe 095404 to the 1st leaf of wheat or the 1st leaf whorl of *Galium aparine* (solution with 0.02 % a.i., 5 µl/plant) 41 % was taken up by *G. aparine*, and only 18 % by wheat 3 days after application. Accordingly, after the same period, the translocation rate was slightly higher in *G. aparine* than in wheat, though it did not exceed 1 % of the applied ^{14}C in both species (Table 2).

Table 2. Uptake and translocation (% applied radioactivity) after foliar application of [^{14}C] Hoe 095404

Species	Days	Uptake	Translocation		Recovery of ^{14}C
			shoot	root	
Rice	1	37.1 ± 4.2	0.2 ± 0.1	0.0	83.9 ± 4.1
	3	51.9 ± 4.0	0.2 ± 0.1	0.0	82.9 ± 5.5
C. serotinus	1	45.3 ± 6.5	0.3 ± 0.1	0.1	91.4 ± 7.3
	3	46.5 ±10.0	3.9 ± 1.4	1.1 ± 1.1	75.8 ± 9.0
Wheat	3	18.2 ± 4.7	0.4 ± 0.3	0.1	100.2 ± 7.9
G. aparine	3	41.2 ± 9.9	0.9 ±0.6	0.1	92.0 ± 8.1

(mean ± sd, n = 8)

Degradation

Degradation studies with whole plants were carried out with rice, 4/5-leaf stage, and *Cyperus serotinus*, 3/4-leaf stage, either after foliar application of a solution of formulated [^{14}C]Hoe 095404 (0.02 % a.i., 15 µl/plant) or after root exposure of plants to 7 ppm non-formulated [^{14}C]Hoe 095404 in hydroponic culture solution.

Extraction of surface-washed shoots 3 days after foliar application and subsequent phase partitioning of the extracts revealed that 47 % of the ^{14}C in the shoots of *C. serotinus* partitioned into the methylene chloride phase and 20% into the water phase. In contrast, only 28 % of the ^{14}C in the shoots of rice partitioned into the methylene chloride phase, but 45 % into the water phase. The percentage of ^{14}C in the nonextractable residue was very similar in both species (27 % in rice, 32 % in *C. serotinus*). Radio tlc analysis showed that the total amount of radioactive material in the methylene chloride phase from *C. serotinus* cochromatographed with the parent compound Hoe 095404, but only a fraction in the same extract phase from rice behaved in this way. Therefore 47 % of the total ^{14}C in the shoots of *C. serotinus* cochromatographed with the parent compound, but only 19 % in the shoots of rice (Table 3). Translocation of ^{14}C to the roots was very low in this experiment and did not allow a detailed analysis.

Table 3. Partitioning (%) of ^{14}C 3 days after application of [^{14}C]Hoe 095404 (A) to the foliage, (B) to the root

	Species	Methylene chloride phase	Water phase	Residue
A	Rice	28 (*19)	45	27
	C. serotinus	47 (*47)	20	33
B	Rice			
	root	31 (*22)	43	26
	shoot	32 (*8)	54	14
	C. serotinus			
	root	65 (*44)	25	10
	shoot	57 (*40)	30	13

(*percentage of parent compound)

When plants were analysed in the same way after they had absorbed the herbicide via the roots, more radioactive material from root and shoot extracts of *C. serotinus* partitioned into the methylene chloride phase than into the water phase, whereas the radioactive material extracted from rice partitioned predominantly into the water phase. Only 8 % of the ^{14}C in the shoots of rice cochromatographed with the parent compound Hoe 095404, in contrast to 40% in the shoots of *C. serotinus* (Table 3). Radiochromatograms of shoot extracts (methylene chloride phase), 3 days after foliar application to *C. serotinus* showed only one band which cochromatographed with the parent compound (R_f-value : 0.22), whereas after foliar application to rice additional bands were detected at R_f-values of 0.50, 0.66 and 0.72-0.78. After root application and analysis of the shoot extracts (methylene chloride phase) after 3 days degradation products were detected on the radiochromatogram from both species at R_f values of 0.50 and 0.72-0.78 (Table 4).

Table 4. Distribution of ^{14}C (%) in radiochromatograms of the methylene chloride phase from shoot extracts 3 days after (A) foliar, (B) root application of [^{14}C]Hoe 095404

Species		R_f-values				
		0.0	0.22*	0.50	0.66	0.72-0.78
A	Rice	-	67.4	11.6	10.7	10.3
	C. serotinus	-	100.0	-	-	-
B	Rice	5.2	24.8	16.5	-	53.5
	C. serotinus	-	69.4	16.0	-	14.6

*R_f-value of parent compound

An additional series of degradation experiments, which included the three tolerant crop species rice, sugarcane and wheat and characteristic weed species for comparison, was carried out with excised shoot material which was fed with [^{14}C]Hoe 095404 via the transpiration stream. The results obtained for rice and *Cyperus serotinus* were similar to those described for application to intact plants of these species (Table 5). Also extracts from shoots of the sedge weed *Scirpus maritimus*, after phase partitioning, had much less ^{14}C in the water phase than in the methylene chloride phase, while the opposite was determined in extracts of rice. A considerable amount of the ^{14}C in the methylene chloride phase of rice extracts was not identical with the parent compound, while most of the ^{14}C-labelled material in *C. serotinus* cochromatographed with Hoe 095404. The same tendencies were found, when *Cyperus esculentus* was compared with sugarcane, and *Galium aparine* with wheat. After a feeding period of one day, only 8% of the recovered ^{14}C cochromatographed with Hoe 095404 in wheat, but 61 % in *Galium aparine*; in sugarcane and *Cyperus esculentus*, after a feeding period of 3 days, it was 4 % and 36 %, respectively.

Table 5. Partitioning (%) of ^{14}C after feeding [^{14}C]Hoe 095404 to excised shoots

Experiment	Species	Feeding period (d)	Methylene chloride phase	Water phase	Residue
I	Rice	3	13 (*4)	57	30
	C. serotinus	3	27 (*19)	33	40
II	Rice	1	30 (*24)	49	21
		3	22 (*11)	40	38
	S. maritimus	1	67 (*59)	13	20
		3	50 (*45)	20	30
III	Sugarcane	3	18 (*4)	63	19
	C. esculentus	3	45 (*36)	41	14
IV	Wheat	1	28 (*8)	48	24
	G. aparine	1	61 (*61)	30	9

(*percentage of parent compound)

In wheat more ^{14}C was in the nonextractable residue than in *Galium aparine*, but this was not the case for the other crops and corresponding weeds (Table 5).

DISCUSSION

It could clearly be shown in this study that intact plants or excised shoots of the tolerant crops rice, wheat and sugarcane can degrade Hoe 095404 much faster than the susceptible weed species. It is likely that the degradation products, found in the methylene chloride (lipophilic) extract phase, are at least in part intermediates in the degradation pathway leading to hydrophilic products. Corresponding R_f-values of the lipophilic intermediates in rice and *C. serotinus* suggest that the herbicide is degraded via a similar pathway, though with different rates, in the tolerant and the susceptible species.

The percentage of foliar uptake of Hoe 095404 and the subsequent translocation of ^{14}C was lower in wheat than in *G. aparine*. Rice and *C. serotinus* were not significantly different with respect to foliar uptake, but less ^{14}C was translocated in rice, and after addition of the herbicide to the flood water lower concentrations of ^{14}C were found in the shoot base of rice than in the shoot base of *C. serotinus*. It can be assumed that due to these differences in the uptake and translocation behaviour herbicide concentrations in the meristematic tissues were lower in rice and wheat than in the corresponding weed species.

It is concluded that differential degradation of Hoe 095404 in tolerant crops and susceptible weeds is a key factor in the selective action of this herbicide, but differences in herbicide uptake and translocation may also contribute towards crop selectivity.

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SPECTRUM OF HERBICIDE REACTIVE GLUTATHIONE TRANSFERASES IN MAIZE

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ABSTRACT

Glutathione transferases (GSTs) in maize catalyse the conjugation of glutathione to a broad range of exogenous electrophilic substrates. The GSTs with activity towards the chloroacetanilide herbicides alachlor and metolachlor have been well studied in both untreated and herbicide safener-treated plants. However the GSTs with activities towards other classes of substrate such as chloro-*s*-triazines and diphenylethers remain under-characterized. Here, we have compared constitutive and safener-inducible GSTs active towards a number of herbicides in maize using the enzymes isolated from plants. We also report on the comparative activity of GSTs purified from roots of herbicide safener-treated maize toward a range of substrate classes. In addition we have successfully used PCR to amplify the full coding sequence of maize GST I and have cloned this into a bacterial recombinant expression vector. We aim to use this and other recombinant maize GSTs to complement the substrate specificity work.

INTRODUCTION

Glutathione transferases (GSTs, EC 2.5.1.18) are enzymes found in a wide range of organisms, which catalyse the conjugation of the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) at the sulphydryl group of the cysteine residue with a wide range of electrophilic, often hydrophobic, substrates. Active GSTs exist as homo- or hetero-dimers with subunits of 23-30 kD. The function of GSTs in plants seems mainly to detoxify endogenous and exogenous chemicals; GSH conjugates of these are usually non-phytotoxic and more water-soluble than the unreacted molecules. GSH conjugation is thought to assist vacuolar sequestration following the action of an ATP-dependent pump in the tonoplast membrane which specifically transports GSH conjugates into the vacuole (Martinoia *et al.*, 1993).

Maize GSTs play an important role in tolerance to herbicides, since many herbicides are able to be detoxified by GSH conjugation. For example in maize, detoxification of the herbicides atrazine and related chloro-*s*-triazines, and alachlor and other chloroacetanilides is mainly due to conjugation with GSH. The susceptibility of certain maize cultivars to these herbicides is inversely proportional to their GST activities (Shimabukuro *et al.*, 1970).

A number of different GST isoforms have been found in maize with differing activities towards xenobiotic substrates. Some of these isoforms have been shown to be induced by the application of herbicide safeners, compounds applied with or before herbicide application to increase differentially a crop plant's tolerance to a herbicide as compared to that of the target weeds. To date four GSTs from maize have been purified and at least partially characterised. These have been named GSTs I to IV.

GST I is a homodimer of 29 kD subunits, with high activity towards CDNB and some activity towards chloroacetanilide herbicides such as alachlor (Mozer *et al.*, 1983). It is present constitutively in roots and shoots of etiolated maize seedlings, but is also slightly enhanced by the

application of herbicide safeners. The GST I sequence has been elucidated by Shah *et al.* (1986) and expressed in *E. coli* (Wiegand *et al.*, 1986). GST II exists as a heterodimer of 29 kD and 27 kD subunits, with the 29 kD subunit being identical to that found in GST I. GST II was first purified by Mozer *et al.* (1983) who could only show its presence in plants treated with herbicide safeners, indicating that this isoform is not present constitutively but is safener-inducible. GST II had about half the activity of GST I towards CDNB but had seven times its alachlor conjugating activity. GST III was first described by Moore *et al.* (1986) who identified the respective cDNA. This cDNA was expressed in *E. coli* and was found to encode a subunit of 26 kD which formed homodimers; this recombinant protein had higher activity than GST I towards alachlor. Purification of GST III by O'Connell *et al.* (1988) showed it to be expressed constitutively and responsible for more than 80% of chloroacetanilide herbicide conjugation. Alachlor conjugating activity was found to be 3-fold higher than metolachlor conjugating activity. GST III has also been sequenced by Grove *et al.* (1988). GST IV is a homodimer of 27 kD subunits, these subunits are identical to the 27 kD subunit found in GST II (Jepson *et al.*, 1994). Irzyk and Fuerst (1993) found GST IV to be induced by treatment with the safener benoxacor. GST IV has high activity towards metolachlor, low activity towards atrazine and no activity towards CDNB or *trans*-cinnamic acid. The 27 kD subunit found in GSTs II and IV is expressed constitutively at low levels in roots but not in shoots, and is highly induced in both tissues by safener treatment. (Holt *et al.*, 1995, Mozer *et al.*, 1983). The 27 kD subunit has been sequenced by Irzyk *et al.* (1995) and Jepson *et al.* (1994). Other GSTs are present in maize including presumptive atrazine and *trans*-cinnamic acid conjugating isoforms, but these have yet to be fully characterized.

MATERIALS AND METHODS

Plant material

Maize (cv. Pioneer 3394) seeds were imbibed in tap water for an hour and grown in vermiculite. The seeds of safener-treated plants were imbibed in 10mg/l dichlormid, and the seedlings subsequently watered with 5mg/l dichlormid as required. Light grown plants were grown at 25°C with a 16 hour photoperiod and light intensity of 150 $\mu mol/m^2/s$ while dark grown plants were grown in laboratory conditions. Plants were harvested, separated into roots and shoots and washed in distilled water to remove any vermiculite. All steps of the extraction were carried out at 4°C. Tissue was homogenised in 3 volumes of extraction buffer (100mM Tris-HCl, pH 7.5, 2mM sodium EDTA, 14mM 2-mercaptoethanol) with 5% m/V insoluble polyvinylpolypyrrolidone, then centrifuged at 17,000*g* for 30 minutes. Proteins were then precipitated from the supernatant by addition of ammonium sulphate to 80% saturation.

GST assays

Enzyme activity towards substrates able to be measured colorimetrically was determined using the methods described by Habig *et al.* (1974). Control experiments, omitting either enzyme, GSH or substrate, were also performed. For the routine analysis of GSTs 1-chloro-2,4-dinitrobenzene (CDNB) was used as the substrate.

A series of hplc based assays were developed to measure GST activity towards the following herbicides: the chloroacetanilides alachlor and metolachlor, the chloro-*s*-triazines atrazine, cyanazine and simazine, and chlorimuron ethyl, fluorodifen and fenoxaprop. GSH conjugates of the herbicides or their trimethylamino salts were synthesized by reacting the herbicides with GSH in a basic aqueous solution (Edwards and Owen, 1986).

Herbicide standards and the herbicide conjugates were used to calibrate the hplc. Analysis was performed using a Fisons Spherisorb ODS1 column (250mm × 4.6mm) equilibrated at 0.8ml/min with 5% acetonitrile in 1% phosphoric acid. Each sample (50µl) was injected onto the column and the following gradient was used to separate compounds: 5% to 10% acetonitrile in 1% phosphoric acid over 5 minutes followed by 10% to 100% acetonitrile in 1% phosphoric acid over 35 minutes.

The UV absorbance (at 264nm) of the column eluant was monitored and the peaks corresponding to the herbicide-GSH conjugates were quantified on the assumption that the conjugates had the same extinction coefficient at the analysed wavelength as the herbicide.

Enzyme assays contained the following:

	50mM glycine buffer, pH9.5 (for fluorodifen & fenoxaprop assays)	50µl
OR	100mM potassium phosphate buffer, pH6.8 (for remaining herbicide assays)	50µl
	Enzyme soln. in 2mM potassium phosphate buffer, pH6.8	120µl
	20mM herbicide in acetone (1mM final concentration)	10µl
	100mM GSH (10mM final concentration)	20µl

Assays were run in duplicate at 37°C for 1 hour and were terminated by the addition of 10µl of 0.6M HCl. Reaction mixtures were stored at -20°C until required and then the precipitated proteins were removed by centrifugation prior to analysis of 50µl of the supernatant by hplc. To correct for the non-enzymic conjugation rate protein was omitted from the assay.

Purification of CDNB-active GSTs

Hydrophobic interaction chromatography (HIC) was carried out using a phenyl sepharose CL-4B column (35ml total volume) to fractionate crude maize protein extracts. Samples were loaded in 0.5M ammonium sulphate in buffer A (10mM potassium phosphate buffer pH 7.4 containing 14mM 2-mercaptoethanol). The column was washed with the same buffer until no more protein (as measured by absorbance at 280nm) was eluted. The column was then washed with buffer A alone, again until no further protein was eluted. CDNB-active GSTs were eluted with 2mM GSH in 50% ethylene glycol and 50% buffer A. Fractions with CDNB conjugating activity were pooled and ethylene glycol and GSH were removed from the active fraction by loading onto a Q Sepharose FF (Fast Flow) column (6ml total volume). After washing with buffer B (20mM Tris-HCl pH 7.8, 14mM 2-mercaptoethanol) the GST activity was eluted with buffer B + 0.25M NaCl. Active fractions were pooled and dialysed against 10mM potassium phosphate buffer, pH 6.0 overnight, then loaded onto a dye affinity (Amicon orange A) column (6ml total volume). The initial eluate was reloaded to increase binding and the column was washed with 10mM potassium phosphate buffer, pH 6.0 until no more protein was eluted. The column was then similarly washed with buffer C (50mM potassium phosphate buffer, pH 7.0) and the CDNB-active fraction was then eluted with buffer C + 2mM GSH.

Analysis of CDNB-conjugating GST isoforms

Analysis of crude and partially purified GSTs was performed using ion exchange chromatography. Protein samples were loaded onto a Q Sepharose FF column (6ml total volume) in buffer B and eluted with a 50ml linear gradient of increasing NaCl concentration up to 0.25M in buffer B; fractions were assayed for activity towards CDNB.

SDS-PAGE (sodium dodecylsulphate - polyacrylamide gel electrophoresis)

Protein samples were heated to 100°C in an equal volume of 2× loading buffer for 5 minutes and allowed to cool. Samples were then loaded onto a 0.8mm mini-gel (12.5% acrylamide, 0.33% N'N'-bis-methylene-acrylamide), and after electrophoresis at 200V the gel was stained with Biorad silver stain as described by the manufacturer.

Cloning and expression of GST I cDNA

RNA was extracted from roots and shoots of light grown maize seedlings using TRIZOL reagent (Gibco BRL) and the accompanying protocol. This RNA was used as a template for cDNA synthesis using the reverse transcriptase M-MuLV. The primer used for this was either dT_{15} (to select for mRNAs) or a partial sequence from GST I (to selectively synthesise GST I cDNAs). *Taq* DNA polymerase and two oligonucleotide primers designed from the published sequence for GST I

(Grove *et al.*, 1988) was used to selectively amplify GST I cDNAs by polymerase chain reaction (PCR). One primer matched the 5' end of the coding sequence of GST I cDNA and the other primer matched the reverse complement of the 3' end of the coding sequence. Reaction products were separated on a 1% agarose gel, the dominant bands were excised and the DNA was recovered using sodium iodide treatment and silica fines. The identity of the amplified fragment was then confirmed by sequencing and the full length coding sequence ligated into the expression plasmid pKK233-2 and transformed into *E. coli* JM101.

To induce expression of GST I, 5ml of nutrient broth was inoculated with bacteria containing the expression vector and incubated at 37°C on a shaker for 8 hours. This was then used to inoculate 500ml of nutrient broth containing 1mM isopropyl-β-D-thiogalactoside (IPTG), which was then incubated at 37°C on a shaker for 16 hours. The cells were harvested by centrifugation, resuspended in a small volume of cold extraction buffer (10mM Tris-HCl pH7.5, 1mM EDTA, 250mM KCl, 10% v/v glycerol, 0.5mM dithiothreitol) and ground up in liquid nitrogen. The resulting powder was suspended in extraction buffer and sonicated. The extract was then centrifuged; proteins were precipitated from the supernatant by addition of ammonium sulphate to 80% saturation.

RESULTS AND DISCUSSION

GST activities in safener-induced maize

Table 1. GST activity towards herbicides in safener-treated and control maize seedlings.

	Activity (pmol/min/mg protein) ± SD			
Herbicide	Treated shoots	Control shoots	Treated roots	Control roots
Atrazine	216 ± 50	142 ± 11	315 ± 16	136 ± 25
Alachlor	351 ± 150	90 ± 88	1230 ± 60	920 ± 150
Metolachlor	390 ± 35	225 ± 22	1080 ± 90	720 ± 70
Fluorodifen	39 ± 8	19 ± 1	71 ± 6	31 ± 3

Control and dichlormid-treated maize were grown in the dark for 10 days and root and shoot extracts were then assayed with herbicide substrates; the results from this are summarised in Table 1. Previous studies have shown that treatment of maize with a number of safeners increases the GST complement in these plants, however most of these studies have concentrated on a single substrate and have not considered activities towards multiple substrates. These studies have also tended to concentrate on shoot GSTs.

Safener treatment increased significantly GST activity towards all the herbicides tested in both roots and shoots. In both tissues there was significant constitutive activity towards these herbicides without safener treatment; specific activities in the roots were generally higher than in shoots. No activity could be detected towards chlorimuron ethyl, fenoxaprop, simazine or cyanazine. Our studies have also shown that treatment with the safener dichlormid roughly doubled the GST activities in maize towards the colorimetric substrates CDNB, ethacrynic acid and *p*-nitrobenzyl chloride (data not shown).

Purification of maize GSTs

GSTs were purified from dichlormid-treated maize root extracts (Table 2). As determined by SDS-PAGE the active fraction from the orange A column contained two polypeptides with relative molecular masses of 29 kD and 27 kD. Further resolution of CDNB-conjugating GST isoforms using Q sepharose chromatography gave two CDNB-active fractions. These fractions were analysed by SDS-PAGE which showed that fraction 1 consisted of a single polypeptide with an

estimated molecular weight of 29 kD (GST I) while fraction 2 consisted of two polypeptides of 29 kD and 27 kD (GST II). A further minor GST isozyme, tentatively identified as GST III and consisting of 26 kD subunits eluted with the later fractions of GST I.

Table 2. Purification of GSTs from safener-treated maize roots.

Sample	Protein (mg)	CDNB activity (µmol/min/mg)	Purification (-fold)	Recovery (%)
Ammonium sulphate precipitate	280	1.51	1	100
Phenyl sepharose	45	8.8	5.8	93
Q sepharose	18	16.6	11.0	71
Orange A	2.2	85.3	56.6	44

Activities of purified GSTs I and II

Following complete purification of GSTs from dichlormid-treated roots GST I, GST II and a mixture of GST I and the unidentified GST were assayed with a range of substrates, giving the results shown in Table 3. These results show that GSTs I and II have broad substrate specificities. Compared to GST II, GST I has higher activity towards the colorimetrically assayed substrates and atrazine but lower activity towards the other herbicides. The unidentified GST seems to have high activity towards fluorodifen and metolachlor; activities towards the remaining substrates are difficult to assess due to contamination by GST I.

Table 3. Activities of purified GSTs.

	Specific activity (nmol/min/mg) ± SD		
Substrate	GST I	GST II	GST I & Unknown
Atrazine	5.1 ± 0.8	1.1 ± 0.4	1.0 ± 0.6
Alachlor	88 ± 13	192 ± 66	75 ± 30
Metolachlor	1.9 ± 4.8	101 ± 18	48 ± 12
Fluorodifen	0.33 ± 0.02	23.3 ± 0.7	9.5 ± 0.1
CDNB	101.6 ± 0.3*	67.5 ± 0.4*	74.4 ± 0.8*
1,2-Dichloro-4-nitrobenzene	66.0 ± 2.6	14.4 ± 2.5	10.8 ± 2.6
p-Nitrobenzyl chloride	3490 ± 140	1260 ± 400	1120 ± 200
1,2-Epoxy-3-(nitrophenoxy) propane	ND†	ND	ND
Ethacrynic acid	1620 ± 490	1500 ± 570	1180 ± 200
Nitrophenethyl bromide	34.0 ± 7.3	18.5 ± 8.7	16.5 ± 5.8

* µmol/min/mg † No detectable activity

Cloning and expression of GST I cDNA

Each RNA sample incubated with reverse transcriptase produced a similar sized cDNA fragment after PCR, with a size (about 680 bp) consistent with being GST I cDNA. Samples without reverse transcriptase showed no such fragment. Sequencing showed that the amplified cDNAs from root and shoot tissues were both GST I. The root sequence differed slightly from the published GST I sequence (Shah *et al.*, 1986), probably due to genetic differences or PCR errors but had the same deduced amino acid sequence. The shoot sequence also showed some differences from the published GST I sequence, including 3 amino acid differences. Minor differences between the coding sequence of root and shoot GST I are probably due to PCR errors, so it is likely that the same protein is found in roots and shoots.

GST I insert was ligated into the plasmid expression vector pKK233-2 and the resultant plasmid was transformed into *E. coli* JM101 for expression. Extracts from IPTG-induced *E. coli* JM101

containing the expression vector pKK233-2 with or without the GST I insert were assayed for CDNB activity; GST I expressing bacteria had approximately 20 times the conjugating activity of the control bacteria without the GST I insert, and a specific activity of 62 nmol/min/mg. Analysis by SDS-PAGE of proteins partially purified using orange A affinity chromatography showed that GST I expressing bacteria gave an extra band compared with the control, with an estimated molecular weight of 29 kD. Work is in progress to improve the expression of GST I in *E. coli*.

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THE BASIS FOR THE SYNERGIZING AND SAFENING ACTION OF FENCHLORAZOLE-ETHYL ON THE HERBICIDAL ACTIVITY OF FENOXAPROP-ETHYL: A REVIEW

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ABSTRACT

Laboratory studies show that the amount of glutathione (GSH) is higher in grass species that are moderately tolerant (wheat) and moderately susceptible (barley) to fenoxaprop-ethyl (FE) than in species that are very susceptible to the herbicide such as *Avena fatua*, *Echinochloa crus-galli*, *Digitaria ischaemum*, and *Setaria glauca*. FE was rapidly hydrolyzed to fenoxaprop (F) in wheat, barley, and all weed species. In wheat and barley, F underwent some metabolism to 6-chloro-2,3-dihydrobenzoxazol-2-one (HOE 054014) and rapid displacement of the phenyl group of F by GSH and /or cysteine, resulting in the production of a GSH conjugate, a cysteine conjugate, and 4-hydroxyphenoxypropanoic acid. There was considerably less metabolism of F in *A. fatua*, *E. crus-galli*, *D. ischaemum*, and *S. glauca*. The triazole, fenchlorazole-ethyl (FCE), acts as a safener against the phytotoxic action of FE in wheat and barley, and as a synergist for the herbicide in *E. crus-galli*, *D. ischaemum*, and *S. glauca*. However, FCE has no synergistic or safening effect on the toxicity of FE when applied to *A. fatua*. In addition, FCE was found to increase the quantity of GSH in barley and wheat but had no effect on GSH levels in *A. fatua*, *E. crus-galli*, *D. ischaemum*, and *S. glauca*. Consequently, in the presence of FCE, more F was non-enzymatically detoxified by conjugation with GSH and cysteine in wheat and barley than in the absence of FCE thereby safening both species against damage by the herbicide. In the presence of FCE, FE was rapidly converted to the more phytotoxic fenoxaprop (F) in the susceptible species with little subsequent metabolism. This resulted in synergism in *E. crus-galli*, *D. ischaemum*, and *S. glauca*. This does not happen in *A. fatua* since FCE is rapidly metabolized to water-soluble metabolites before it can mediate enhanced de-esterification of FE.

INTRODUCTION

The herbicide fenoxaprop-ethyl (ethyl(±)2-[4-[(6-chloro-2-benzoxazolyl)oxy]phenoxy]-propanoate), a member of the aryloxyphenoxypropionate chemical family, is an inhibitor of acetyl-CoA carboxylase (ACCase), and is active on many graminaceous weed species of the genera *Avena*, *Digitaria*, *Panicum*, *Setaria*, *Sorghum*, and *Echinochloa* (Lefsrud & Hall, 1989). Species from these genera are severe weed problems in cereal crops throughout North America. Wheat and rye crops are moderately tolerant to fenoxaprop-ethyl whereas barley

and oat, respectively, are moderately and extremely sensitive (Stephenson *et al.*, 1993). Unacceptable phytotoxicity and yield losses can occur in wheat and barley crops after application of this herbicide; however, these phytotoxic effects can be eliminated or reduced to acceptable commercial levels when the herbicide is applied in combination with the safener fenchlorazole-ethyl (ethyl-1-(2,4-dichlorophenyl)-5-trichloromethyl-1H-1,2,4-triazole-3-carboxylate) (Yaacoby *et al.*, 1991).

After application of fenoxaprop-ethyl there is cessation of growth and inhibition of lipid biosynthesis in susceptible grass species (Yaacoby *et al.*, 1991). These effects are followed by the destruction of the shoot meristem. After penetration into the leaf, the herbicide is rapidly hydrolyzed to the free acid, fenoxaprop. Studies with chloroplasts isolated from various grass species showed that the free acid is approximately 100 times more powerful than fenoxaprop-ethyl in inhibiting *de novo* fatty acid biosynthesis (Hoppe & Zacher, 1985, Kobek *et al.*, 1988, Kobek & Lichtenthaler, 1989). The I_{50} values for inhibition of ACCase were 0.1 and 10.0 μM for fenoxaprop and fenoxaprop-ethyl, respectively (Kobek *et al.*, 1988). In general, the selectivity of fenoxaprop-ethyl in tolerant and susceptible grass species is not based on differences in foliar uptake or translocation of the herbicide nor the degree of inhibition of ACCase activity (Hoppe & Zacher, 1985, Lefsrud & Hall, 1989). However, Lefsrud and Hall (1989) reported that there were qualitative and quantitative differences in the metabolism of fenoxaprop-ethyl in tolerant and sensitive grass species indicating that the primary selective action of fenoxaprop-ethyl in grasses may be due to differences in metabolism.

The triazole, fenchlorazole-ethyl, is used as a commercial safener against the phytotoxic action of fenoxaprop-ethyl in wheat (Romano *et al.*, 1991, Stephenson *et al.*, 1993, Yaacoby *et al.*, 1991). In combination with fenoxaprop-ethyl at application rates as high as 200 g ai/ha, fenchlorazole-ethyl at an application rate of 50 g ai/ha completely prevented injury to wheat without reducing the effectiveness of the herbicide for weed control (Stephenson *et al.*, 1993). In preliminary studies, Kocher *et al.* (1989) observed that fenchlorazole-ethyl did not reduce the foliar uptake or translocation of fenoxaprop-ethyl nor did it reduce the effects of the herbicide on ACCase activity at concentrations as high as 100 μM. However, preliminary experiments in wheat indicated that there were quantitative changes in the metabolism of fenoxaprop-ethyl in the presence of fenchlorazole-ethyl (Kocher *et al.*, 1989)

The objective of this paper is to provide a review of the research conducted in our laboratories over the past 7 years on the basis for the synergizing and safening action of fenchlorazole-ethyl on the herbicidal activity of fenoxaprop-ethyl. During this time we have investigated the dose-response relationships and metabolism of fenoxaprop-ethyl in the presence and absence of the safener in wheat, barley, *D. ischaemum*, and *E. crus-galli* (Lefsrud & Hall, 1989, Romano *et al.*, 1993, Romano *et al.*, 1991, Stephenson *et al.*, 1993, Tal *et al.*, 1993, Tal *et al.*, 1995, Yaacoby *et al.*, 1991). The two cereals are moderately tolerant and moderately susceptible, respectively, whereas the two weedy grasses are extremely susceptible to fenoxaprop-ethyl.

MATERIALS AND METHODS

All materials and methods have been described in detail by Lefsrud & Hall (1989), Romano *et al.* (1993), Romano *et al.* (1991), Stephenson *et al.* (1993), Tal *et al.* (1993), Tal *et al.* (1995), Yaacoby *et al.* (1991).

RESULTS AND DISCUSSION

Fenchlorazole-ethyl eliminated and reduced, respectively, the phytotoxicity of the herbicide fenoxaprop-ethyl to wheat and barley (Yaacoby *et al.*, 1991). In wheat, an ED_{50} value could not be determined because of its tolerance to the herbicide but there was a 30% reduction in dry weight after application of 600 g ai/ha (Figure 1a). In barley, the ED_{50} value for foliar-applied fenoxaprop-ethyl was 150 g ai/ha (Figure 1b). In both wheat and barley, no reduction in dry weight occurred when the same respective application rates of the herbicide were applied in combination with the safener (4:1 w/w, herbicide:safener). Conversely, addition of fenchlorazole-ethyl increased the toxicity of fenoxaprop-ethyl to *D. ischaemum* (Figure 1c).

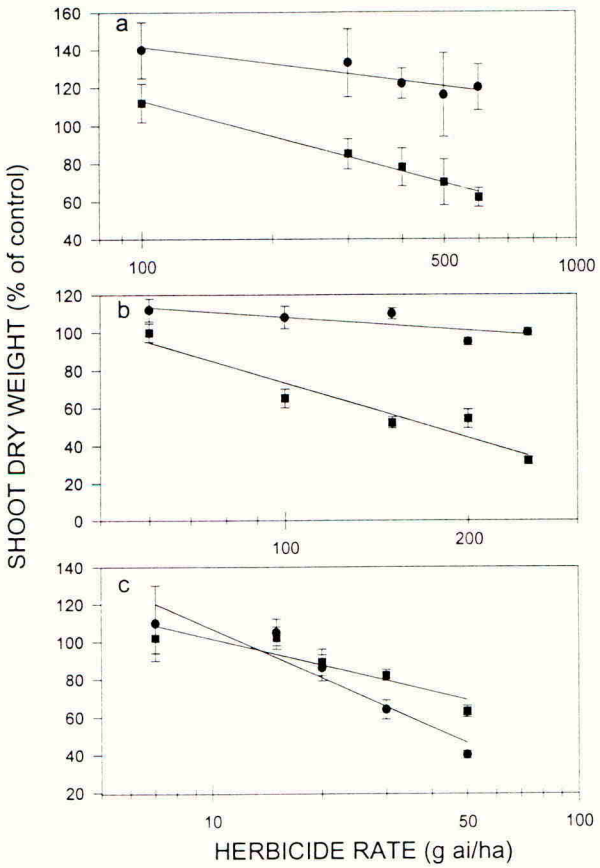

Figure 1. Effect of foliar application of fenoxaprop-ethyl in combination with (solid circles) or without (solid squares) fenchlorazole-ethyl on the shoot dry weights of wheat (a), barley (b), and *D. ischaemum* (c) (From Yaacoby *et al.*, 1991).

Preliminary metabolism studies on foliar-applied [^{14}C]fenoxaprop-ethyl indicated that the herbicide was deesterified to fenoxaprop in wheat, barley and *D. ischaemum* (Yaacoby *et al.*,

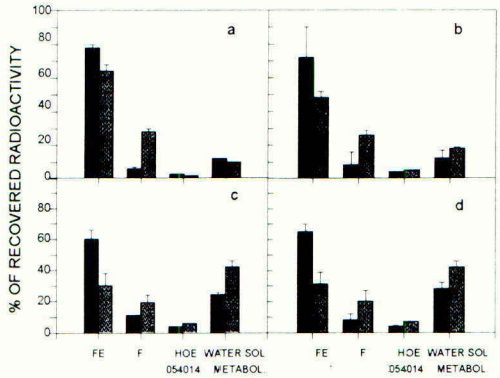

Figure 2. Influence of fenchlorazole-ethyl on the metabolism of [^{14}C]fenoxaprop-ethyl in wheat 16, 24, 36, and 48 hours (a-d, respectively) after application of herbicide (solid bars) or herbicide plus safener (hatched bars). Error bars represent standard errors of the means (From Yaacoby *et al.*, 1993).

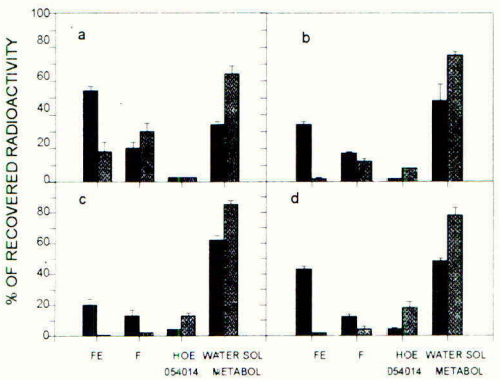

Figure 3. Influence of fenchlorazole-ethyl on the metabolism of [^{14}C]fenoxaprop-ethyl in barley 16, 24, 36, and 48 hours (a-d, respectively) after application of herbicide (solid bars) or herbicide plus safener (hatched bars). Error bars represent standard errors of the means (From Yaacoby *et al.*, 1993).

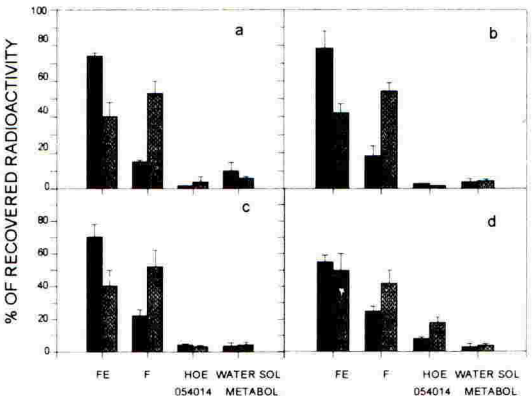

Figure 4. Influence of fenchlorazole-ethyl on the metabolism of [^{14}C]fenoxaprop-ethyl in *D. ischaemum* 16, 24, 36, and 48 hours (a-d, respectively) after application of herbicide (solid bars) or herbicide plus safener (hatched bars). Error bars represent standard errors of the means (From Yaacoby *et al.*, 1993).

1991). Regardless of whether the safener was present or not, three major metabolites, 6-chloro-2,3-dihydro-benzoxazol-2-one (HOE 054014), and two unidentified water-soluble metabolites, were found in all three species 48 hr after treatment (Figures 2, 3, and 4). When [^{14}C]fenoxaprop-ethyl was applied alone, approximately 29%, 63%, and 9% of the applied radioactivity was metabolized to products other than fenoxaprop 48 hr after treatment, whereas 48%, 96%, and 8% was metabolized when the safener also was applied to wheat, barley, and *D. ischaemum*, respectively. These results indicate that fenchlorazole-ethyl may protect wheat (Figure 2) and barley (Figure 3) from the phytotoxic action of fenoxaprop-ethyl by increasing both the rate of deesterification of fenoxaprop-ethyl to fenoxaprop and the subsequent rate of metabolism of fenoxaprop to other metabolites. In *D. ischaemum* (Figure 4), deesterification of fenoxaprop-ethyl was increased by the safener but the subsequent detoxification of fenoxaprop, the toxophore, was not increased, thereby explaining the observed synergism in *D. ischaemum* (Yaacoby *et al.*, 1991).

Based on the results from the dose-response and metabolism experiments (Figures 1-4), experiments were conducted to determine whether fenchlorazole-ethyl would act not only as a safener in wheat and barley but also as a synergist in susceptible weeds such as *D. ischaemum* (Stephenson *et al.*, 1993). In growth room experiments, fenoxaprop-ethyl was highly toxic to *E. crus-galli* (Figure 5) and *D. ischaemum* when applied postemergent at the two- to three-leaf stage of development. At this stage, shoot growth was generally so severely limited by fenoxaprop-ethyl at 50 g ai/ha it could not be determined whether the addition of fenchlorazole-ethyl had a synergistic effect on the herbicide. Conversely, there was little or no injury with 50 g ai/ha when *D. ischaemum* and *E. crus-galli* (Figure 5) were treated at the four- to five-leaf stage of development. However, combined treatments of fenchlorazole-ethyl

plus fenoxaprop-ethyl applied to *E. crus-galli* (Figure 5) and *D. ischaemum* at the later growth stage were significantly more phytotoxic than their appropriate fenoxaprop-ethyl controls. In the response surface (Figure 5) analyses this was reflected in large significant and negative cross product terms. These results (Stephenson *et al.*, 1993) indicate that fenoxaprop-ethyl and fenchlorazole-ethyl combinations are synergistic which confirms our earlier findings.

Detailed metabolism studies (Figure 6) were conducted by Tal *et al.* (1993) on wheat, barley, oat, and *D. ischaemum* indicating that [^{14}C]fenoxaprop-ethyl was rapidly hydrolyzed to fenoxaprop in all four species. However, in oat and *D. ischaemum* the radioactivity remained mainly in the form of fenoxaprop, while in wheat and barley, fenoxaprop In wheat and barley, F underwent some metabolism to 6-chloro-2,3-dihydrobenzoxazol-2-one (HOE 054014) and rapid displacement of the phenyl group of fenoxaprop by GSH and /or cysteine, resulting in the production of a GSH conjugate, a cysteine conjugate, and 4-hydroxyphenoxypropanoic acid. The GSH conjugate also may be catabolized to the cysteine conjugate which was subsequently metabolized to an unidentified metabolite (Metabolite I) which was speculated to be the N-glucoside of the cysteine conjugate. 4-Hydroxyphenoxypropanoic acid was further metabolized to yield a glucoside conjugate which upon hydrolysis with acid or β-glucosidase yielded 4-hydroxyphenoxypropanoic acid. Fenchlorazole-ethyl had no qualitative effect on fenoxaprop-ethyl metabolism. However, it enhanced the rate of fenoxaprop metabolism in wheat and barley but not in oat and *D. ischaemum*.

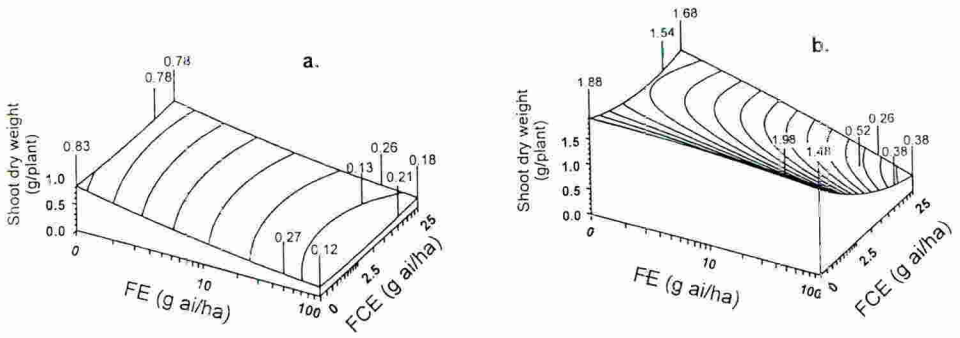

FIGURE 5. Response surfaces for the joint effect of fenoxaprop-ethyl and fenchlorazole-ethyl on shoot growth of *E. crus-galli*. a. Treated at the 2- to 3-leaf stage. b. Treated at the 4- to 5-leaf stage. Mean dry weights of treatment combinations are indicated on the surface. Contour lines on the response surfaces represent 0.1 g dry weight intervals (From Stephenson *et al.*, 1993).

In other studies, Tal *et al.* (1995) found that the amount of GSH is higher in grass species that are moderately tolerant such as wheat and moderately susceptible such as barley to fenoxaprop-ethyl than in species that are very susceptible to the herbicide such as *A. fatua*, *E. crus-galli*, *D. ischaemum*, and *S. glauca* (Table 1). Furthermore, fenchlorazole-ethyl was found to increase the quantity of glutathione in barley and wheat but had no effect on susceptible species. Consequently, in the presence of fenchlorazole-ethyl, more fenoxaprop was detoxified by conjugation with GSH in wheat and barley than in the absence of fenchlorazole-ethyl thereby safening both species against damage by the herbicide (Tal *et al.*,

1995). GSH conjugation is believed to be a non-enzymatic process because: 1) *in vitro* conjugation of fenoxaprop to GSH occurs equally well in boiled and non-boiled enzyme preparation, and 2) *in vitro* non-enzymatic conjugation occurs at physiological pH in the presence of only GSH and fenoxaprop.

Table 1. Concentrations of glutathione and cysteine in shoots of several grass species (Tal *et al.*, 1995).

Species	Glutathione	Cysteine
	----------(nmol/g fresh weight)----------	
Wheat	119 ± 15	80 ± 4
Barley	126 ± 21	72 ± 10
Triticale	121 ± 12	71 ± 7
S. glauca	26 ± 3	12 ± 12
Oat	5 ± 2	ND
A. fatua	3 ± 2	ND
E. crus-galli	trace	ND
D. ischaemum	trace	ND

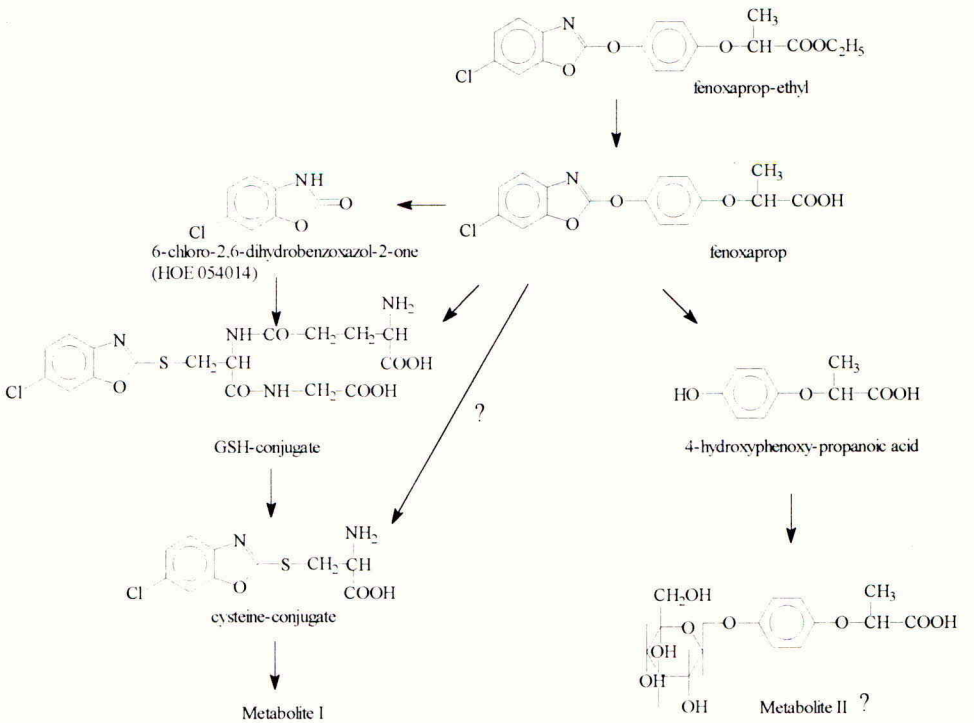

FIGURE 6. Pathway for the metabolism of fenoxaprop-ethyl in grass species (From Tal *et al.*, 1993).

SUMMARY

In the presence or absence of fenchlorazole-ethyl, fenoxaprop-ethyl was rapidly converted to the toxophore, fenoxaprop, in the susceptible species with little subsequent metabolism. Synergism in *E. crus-galli* and *D. ischaemum* resulted from enhanced deesterification of fenoxaprop-ethyl to phytotoxic fenoxaprop as a result of fenchlorazole-ethyl treatment. This does not happen in oat or *A. fatua* since fenchlorazole-ethyl is rapidly metabolized to water-soluble metabolites before it can mediate enhanced de-esterification of fenoxaprop-ethyl.

There are numerous chemical synergists and chemical safeners that have been developed to decrease or increase the selectivity of herbicides. However, fenchlorazole-ethyl is unique. It may be the first chemical that is known to be an effective safener for a particular herbicide (fenoxaprop-ethyl) on certain crops (wheat and barley) and at the same time act as an effective synergist for fenoxaprop-ethyl on some important weeds.

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STUDIES ON THE METABOLISM OF LINURON IN SOME UMBELLIFEROUS PLANTS

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ABSTRACT

Previous studies on the behavior of linuron 3-(3,4-dichlorophenyl)-1-methoxy-1- methylurea in 10 umbelliferous plants showed differences in tolerance of such plants to this herbicide. Carrot (tolerant) and dill (susceptible) were used as examples in the present investigations to further understand these differences. It was shown that the percentage of linuron translocated to the roots of the carrot was higher than that in dill. In carrot, the major proportion of linuron was found in the roots. In dill plants, linuron residues were shown to be higher in the shoots than in roots.
Degradation of linuron was greater in tolerant (carrot) than in susceptible (dill) plants. Four metabolites 3-(3,4-dichlorophenyl) methoxy urea (DCPMOU) , 3-(3,4-dichlorophenyl)-1- methyl urea (DCPMU), 3,4-dichloroaniline (DCA) and 3-(3,4 dichlorophenyl)urea (DCPU) of linuron were identified and showed to be present in both carrot and dill plants at varying levels. 3-(3,4-dichlorophenyl) methoxy urea (DCPMOU) and 3-(3,4-dichlorophenyl)-1- methyl urea (DCPMU) showed to be phytotoxic to chlorophyll.

INTRODUCTION

Several different crops of the Umbelliferae family are grown in Egypt as vegetables or medicinal plants. Linuron (3-(3,4-dichlorophenyl)-1- methoxy-1- methylurea) is recommended for weed control in carrots but can be less useful in some other plants species of the same family (Zaki *et al*, 1993). While carrot (*Daucus carota cv. Chantenay)* is tolerant to linuron, dill (*Anthum graveolens*) is susceptible to this herbicide.

MATERIALS AND METHODS

The present study was carried out in the Experimental Farm, Faculty of Agriculture, at Giza, Egypt, and the Institut Fur Okologische chemie und Abfallanalytik, der Technishe Universitat, Braunschweig, Germany. Plants were sprayed with linuron when they were about 15 cm high and untreated plants (check) were sprayed with water. Samples were randomly collected at various periods up to 42 days after treatment. Samples were then divided into shoots and roots. Four replicates used for each sample and these were analyzed immediately after collection. HPLC analysis was used for the UV determination of linuron and its metabolites, 3,4-dichloroaniline (DCA) and 3-(3,4

dichlorophenyl)urea (DCPU) according to the method of Miliadis *et al* (1990) with some modifications. Thin layer chromatography was also used for the determination of linuron and its metabolites, using chloroform: methanol: pyridine as a solvent system (100:5:1, v/v/v) (Katz, 1967). The phytotoxicity of linuron metabolites was also examined by using TLC technique (Lawrence, 1980).

RESULTS AND DISCUSSION

Persistence, distribution and metabolism of linuron in carrot and dill plants

Previous studies (Zaki *et al* 1993) on the behaviour of linuron in 10 umbelliferous plants showed differences in tolerance of such plants to this herbicide. Carrot (tolerant) and dill (susceptible) were used as examples in the present investigations to further understand these differences.

In carrot, the major proportion of linuron residues was found in the roots 24 hr after foliar application (88.4 %), while only 11.6 % remained in the shoots (Figure 1). In comparison, the initial amounts of linuron residues in susceptible dill shoots and roots at this time were 35.9% and 64.1% respectively. The linuron concentration in dill plant shoots increased to a maximum after 14 days from initial treatment. Linuron was metabolized in both plants (Figures 2 & 3), the metabolism proceeding to the 3,4-dichloroaniline (DCA). The degradation products 3,4-dichloroaniline (DCA) and 3-(3,4 dichlorophenyl)urea (DCPU) were first observed in carrot shoots 4 days after foliar application, while in dill plants DCPU and DCA were first appeared only after 14 days. Greater quantities of metaboites were found in carrot shoots than in dill shoots. Four known degradation products of linuron 3,4-dichloroaniline (DCA), 3-(3,4-dichlorophenyl) methoxy urea (DCPMOU) , 3-(3,4-dichlorophenyl)-1- methyl urea (DCPMU), and 3-(3,4 dichlorophenyl)urea (DCPU) were detected in both carrot and dill plants, with the R_f values 0.84, 0.75, 0.53 and 0.24 respectively. Hogue and Warren (1968) reported that in resistant parsnip seedlings the major proportion of the parent linuron substance was retained in the fibrous roots after soil application. Sensitive tomato seedlings under the same by contrast, translocated most of the herbicide to the shoots. Walker and Featherstone (1973) also demonstrated that a high proportion of linuron absorbed by carrot and parsnip seedlings was retained in the root systems, whereas in lettuce and turnip (susceptible) over 60% of the herbicide was translocated to the shoot.

The Hil reaction inhibition spray technique descriped by Lawrance (1980) was useful for the detection of linuron and its metabolites which still retained photosynthesis inhibiting properties. It was shown in the present work that linuron and 2 of these metabolites were inhibitory to isloated chloroplasts. These two metabolites were 3-(3,4-dichlorophenyl) methoxy urea (DCPMOU) and 3-(3,4-dichlorophenyl)-1- methyl urea (DCPMU). The other two metabolites 3,4-dichloroaniline (DCA) and 3-(3,4 dichlorophenyl)urea (DCPU) showed to be inactive.

The results reported in the present investigations showed that linuron was readily translocated from shoots to roots and was retained in the roots to different degrees in carrot and dill plants. This root immobilistion in combination with metabolism in the shoots with higher rates of metabolites and less quantities of the original herbicide in

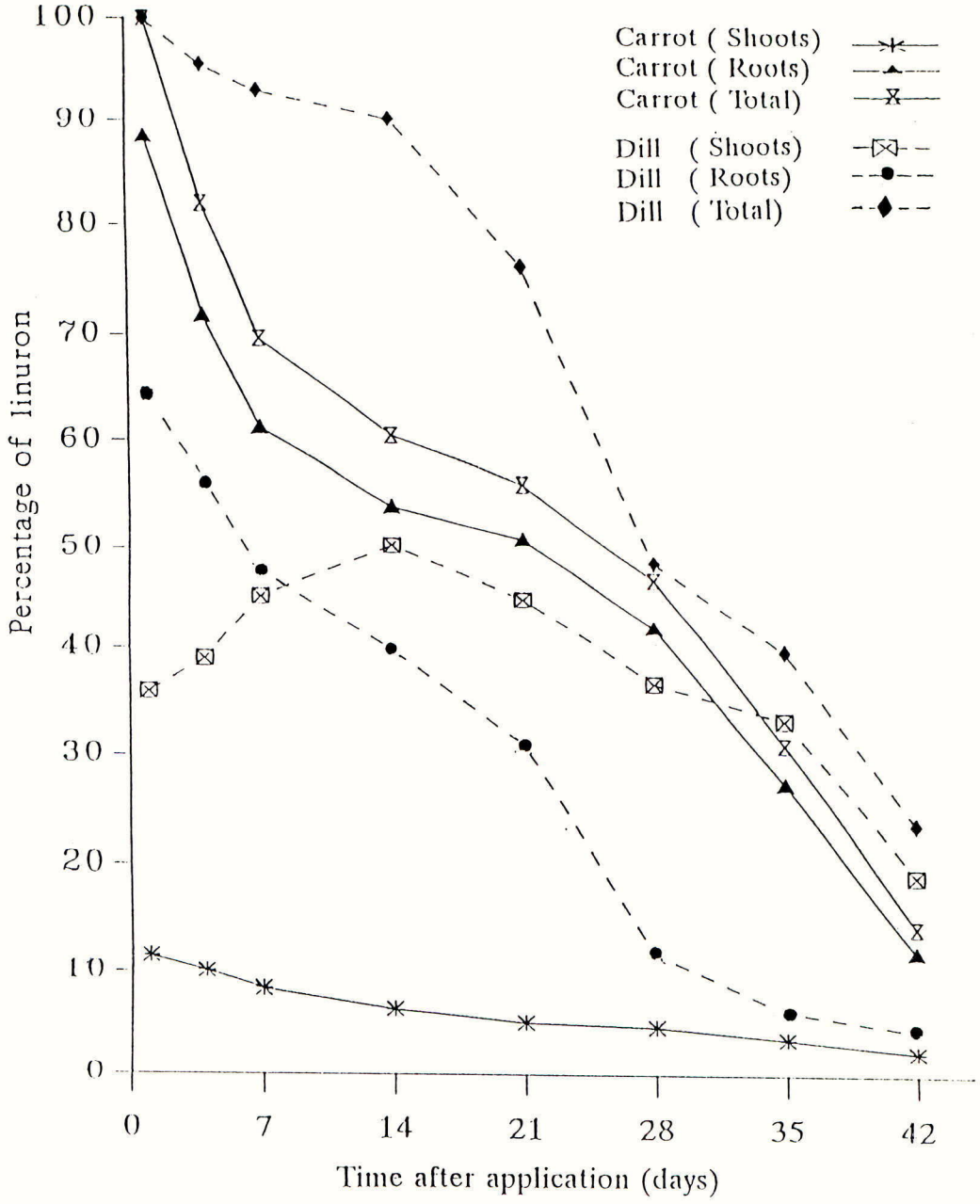

Figure (1) : Persistence and distribution of linuron in carrot and dill plants.

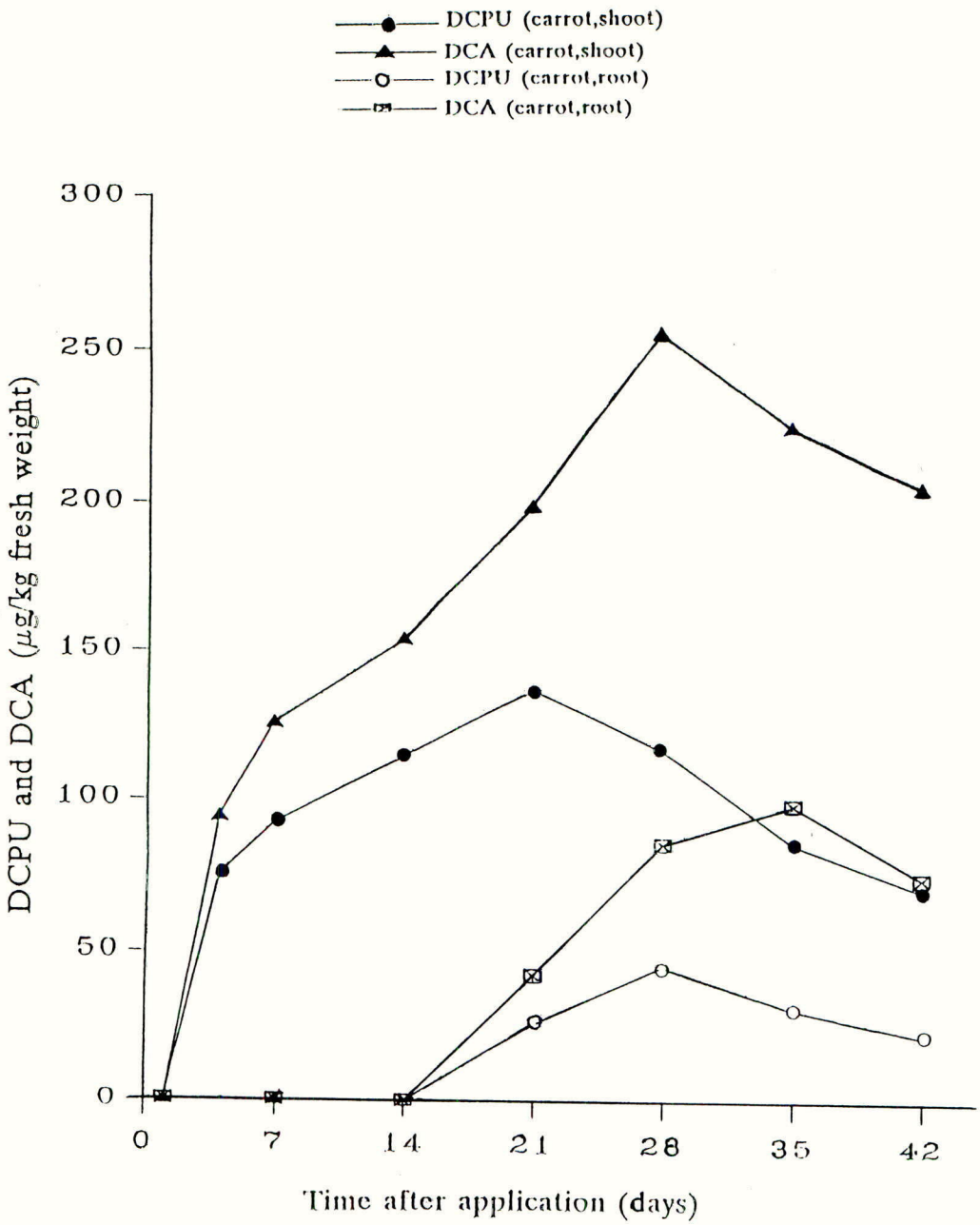

Figure (2) : Distribution of linuron metabolites (DCPU and DCA) in carrot plants.

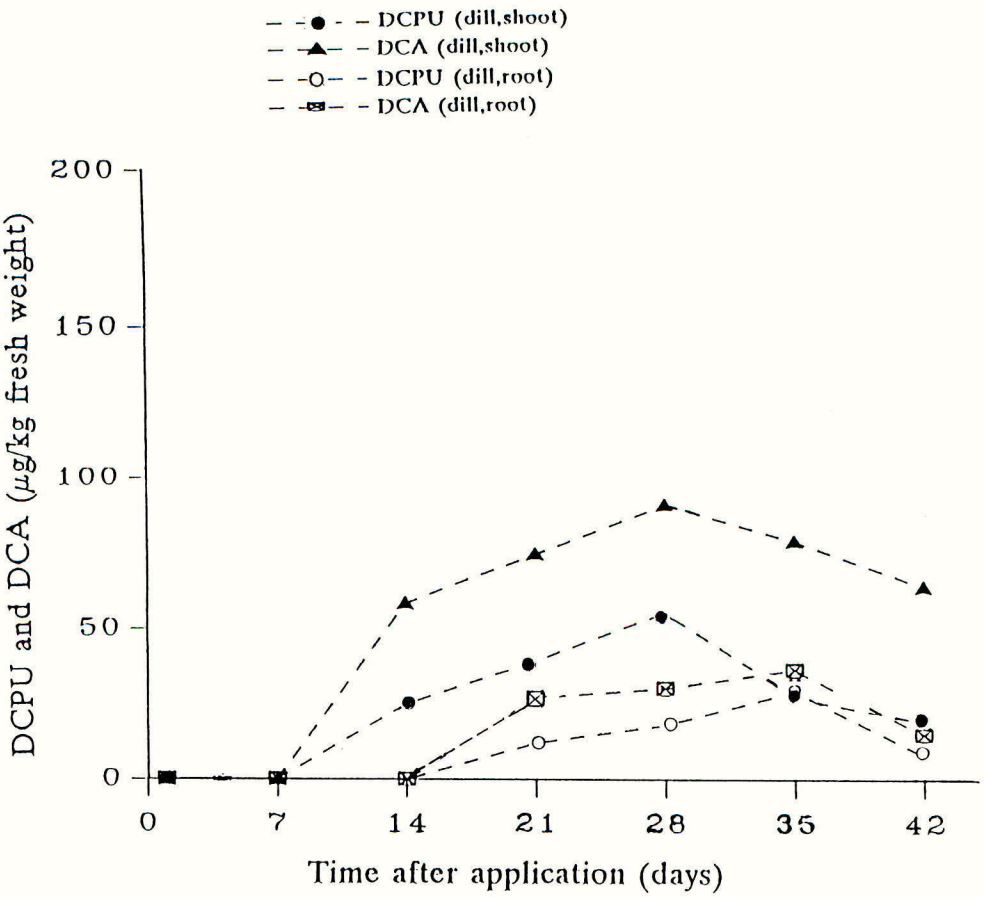

Figure (3) : Distribution of linuron metabolites (DCPU and DCA) in dill plants.

tolerant plants and more of the original compound for longer periods in susceptible plants were recorded, in addition to previous findings of significant reductions in protein, carbohydrate and sugar contents in susceptible plants (Zaki *et al* , 1993) and the effect on photosynthetic reactions, may explain these responses of different plant species of Umbelliferae family.

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MECHANISMS INVOLVED IN THE SAFENING OF IMIDAZOLINONE ACTIVITY IN MAIZE BY NAPHTHALIC ANHYDRIDE AND BAS 145138.

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ABSTRACT

In whole plant studies, the herbicide safener, naphthalic anhydride (NA), enhanced the tolerance of maize to the imidazolinone herbicides, imazethapyr, AC 263222 and, to a lesser extent, imazapyr. In addition, the safener BAS 145138 significantly reduced AC 263222 inhibition of maize shoot growth, while the safener MG 191 failed to elicit a protective effect. Results from *in vivo* metabolic studies indicated that enhanced oxidative herbicide metabolism was the predominant mechanism of NA action and evidence is discussed which suggests that this involves stimulation of a microsomal cytochrome P_{450} system. However, the ability of NA to reduce imazapyr injury, which does not undergo oxidative metabolism in maize, suggests that other selectivity mechanisms may also be induced by safener treatments. In this study, the contrasting effects of NA, BAS 145138 and MG 191 on the uptake, distribution and metabolism of AC 263222 are used to deduce the relative contribution of changes in these processes to safener action.

INTRODUCTION

Herbicide safeners selectively protect crop plants from herbicide damage by reducing the ability of herbicides to reach and inhibit their target sites. This may be achieved through safener-induced modification of herbicide target enzymes, reductions in herbicide uptake and translocation, or safener-enhanced metabolism of herbicides to less active or immobile metabolites. On investigation, safeners have been found to affect all of these processes although evidence has now accumulated which advocates enhanced metabolism as the predominant consequence of safener treatments. For example, Thalacker *et al.* (1994) demonstrated stimulation of a wheat cytochrome P_{450} mono-oxygenase catalysing triasulfuron hydroxylation by naphthalic anhydride (NA). Isolation of the genes encoding such safener-inducible enzymes may facilitate the development of herbicide resistant crops with enhanced metabolic capacity. However, before embarking on such research, the relative contribution of other safener effects to their protective activity should be verified. Indeed, it is unlikely that a single mechanism can account for all reported herbicide-safener interactions or even the activity of one safener in a variety of herbicide-crop combinations.

In this paper, the contrasting effects of the safeners, NA, BAS 145138 and MG 191, on the activity, uptake, distribution and metabolism of AC 263222 will be used to deduce the relative contribution of changes in these processes to safener action. Furthermore, the ability of NA to protect maize from three imidazolinones subject to differing metabolic pathways, will be used to identify those pathways susceptible to stimulation by safener treatments.

METHODS

Herbicide tolerance studies

Maize seeds, cv. Monarque, were dressed with 0.5% w/w NA and sown at a depth of 3cm in a sandy loam soil amended with 30% grit and Vitax Q4 fertilizer (3.3 g/l). Commercially formulated imazethapyr, AC 263222 (2-[4,-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]-5-methylnicotinic acid) and imazapyr were prepared in deionized water and applied 2 days after sowing (DAS), using a track-sprayer fitted with a Teejet 8001 nozzle and delivering 200 l/ha. Pots were returned to glasshouse conditions with mean day/night temperatures of 20/15°C, 70% r.h. and watering by capillary action. When untreated plants reached Zadoks GS 14, shoots were harvested for fresh weight measurements.

Safener mode of action studies

NA-treated or untreated seed was sown on damp filter paper and germinated at 26°C and 80% r.h. in the dark. After 2 days, seedlings were suspended over plastic vials containing 5 ml treatment solution, such that seedling roots penetrated the solution to a depth of 1-2 cm. Treatment solutions contained AC 263222 (98%) or 18.5 kBq ^{14}C-AC 263222 (225.7 kBq μmol^{-1}) prepared in half-strength Hewitt's nutrient medium with 0.1 v/v acetone. Where required, this solution was supplemented with 400 μM BAS 145138 (1-dichloroacetyl-hexahydro-3,3,8-α-trimethylpyrrolo-[1,2α]-pyrimidin-6(2*H*)-one) or 600 μM MG 191 (2-dichloromethyl-2-methyl-1,3-dioxolane). Vials were then returned to the growth cabinet and maintained with a 14 h photoperiod of light intensity 59 W/m^2. After 24 h, seedlings were transferred to polystyrene seed trays floating on 500 ml nutrient solution. In experiments monitoring whole plant responses, this nutrient was renewed at 3-day intervals until 8 DAS when roots and shoots were harvested for fresh weight determinations. In separate experiments investigating the effect of the timing of safener applications on their ability to induce protective effects, 250 μM NA added in 0.4% DMF, was applied to maize seedlings 24 h before, during or after treatment with 5 μM AC 263222 at 3 DAS. Plants treated with [^{14}C]-AC 263222 were harvested 4 DAS and extracted for liquid scintillation counting and TLC as described by Davies *et al* (1993).

Experimental design and statistical analysis

The effects of safeners on herbicide activities were investigated in factorial experiments incorporating 6 herbicide application rates applied with and without safener. Glasshouse experiments included 3 replicate pots, each containing 2 plants, while hydroponic experiments included 5 replicate plants per treatment. Experiments investigating the effects of safeners on selectivity processes incorporated 4 replicates of 2 plants for root extractions and 2 replicates of 4 plants for shoot extractions. Data was subject to ANOVA using GENSTAT and significant herbicide dose responses were described by a logistic curve. The effects of safeners on parameters of this curve were assessed as described by Davies (1994).

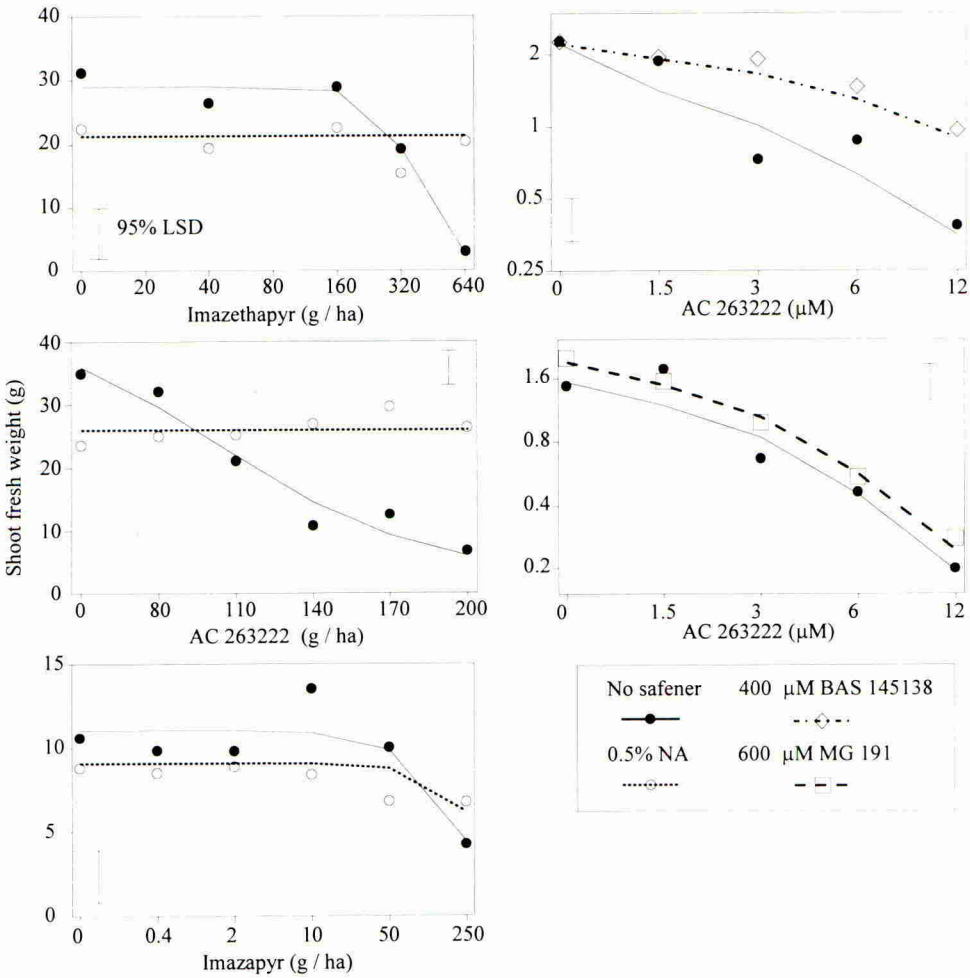
RESULTS

Effect of safeners on herbicide activity

In glasshouse experiments, shoot growth of maize, cv. Monarque, was susceptible to inhibition by imazethapyr, AC 263222 and imazapyr with GID_{50}s (dose causing 50%

inhibition of growth) of 358, 125 and 196 g/ha, respectively. Despite having a significant inhibitory effect itself, the herbicide safener NA enhanced the tolerance of maize to all of these herbicides. For example, imazethapyr and AC 263222 damage was completely eliminated over the dose ranges applied following seed treatment with 0.5% NA (Figure 1). NA also reduced imazapyr inhibition of maize shoot growth, increasing the GID_{50} to 400 g/ha. Although this increase was significant, the occurrence of a dose response in the presence of NA indicated that imazapyr injury was not completely eliminated. This contrasts sharply with the level of protection provided against imazethapyr and AC 263222.

Figure 1. Effect of safener treatments on herbicide activities in maize.



In hydroponic experiments, root treatment with 250 μM NA significantly enhanced the tolerance of maize to AC 263222, irrespective of application timing. However, maximum activity was achieved with NA pre-treatments which halved the level of inhibition caused by herbicide treatments alone. In contrast, concurrent and post-herbicide NA applications were less effective causing 35% and 19% reductions in activity, respectively, (Table 1). AC 263222 phytotoxicity was also significantly reduced by root applications of 400 μM

BAS 145138 applied simultaneously with herbicide treatments for a 24 hour period, 2 DAS (Figure 1). This was apparent as an increase in the GID_{50} for AC 263222 from 2.5 to 8.3 μM. Although BAS 145138 did not inhibit maize growth, the occurrence of a significant dose response in BAS 145138-treated plants indicated that this safener was less effective against AC 263222 injury than NA. In contrast, MG 191 root treatments applied concurrently with AC 263222 at 2 DAS did not significantly change the dose response of hydroponically-grown maize to AC 263222, (Figure 1). Increases in MG 191 dose and extension of the exposure period also failed to elicit a protective effect.

Table 1. Effect of NA application timing on its ability to protect maize from damage by AC 263222.

AC 263222 (μM)	Percent growth inhibition				95% LSD
	0 μM NA	250 μM NA			
		Before[1]	During	After	
0	0	17.7	33.6	40.9	12.4
5	79.5	40.8	51.1	64.1	

[1]Timing of NA application relative to AC 263222 treatment

Effect of safeners on the uptake, distribution and metabolism of AC 263222 in maize.

All safeners significantly reduced root uptake of [^{14}C]-AC 263222 from nutrient solution, (Table 2). While BAS 145138 and MG 191 did not affect the subsequent translocation of radiolabel to the shoot system, seed-treatment with 0.5% NA significantly reduced this process such that a greater proportion of radiolabel was retained in the root system. For example, only 6% of total absorbed radiolabel was translocated to the shoots of NA-treated plants while 13% was translocated in untreated plants (Table 2).

TLC analysis of maize extracts indicated that AC 263222 was metabolized to a single product which was identified by co-chromatography with an authentic standard as AC 263222 hydroxylated at the methyl group of the pyridinyl ring. Although treatment with BAS 145138 or MG 191 did not significantly affect this process in root tissue, the rate of hydroxylation was approximately doubled following seed-treatment with NA (Table 2). However, as hydroxylated AC 263222 is also a potent ALS inhibitor, enhanced metabolism alone cannot account for the ability of NA to reduce herbicide injury. Data on the mobility of the imidazolinones indicates that increased polarity and, therefore, reduced lipophilicity of AC 263222 following hydroxylation, would restrict its mobility relative to the parent molecule (Little and Shaner, 1991). Consequently, NA-induced reductions in translocation may be a consequence of enhanced metabolism. Further studies indicated that enhanced metabolism may be attributed to stimulation of a cytochrome P_{450} mono-oxygenase with the capacity for AC 263222 hydroxylation. Evidence for this proposal includes demonstration of NADPH-dependent hydroxylation of AC 263222 by a microsomal fraction extracted from maize shoots. This activity was promoted by NA seed treatment and inhibited by the cytochrome P_{450} inhibitor ABT (Davies *et al,* in preparation).

Table 2. Effect of safener treatments on the uptake, distribution and metabolism of [^{14}C]-AC 263222.

Safener	Dose	Uptake (dpm absorbed/mg fresh weight)	^{14}C in shoots (% of total absorbed ^{14}C)	Hydroxylated metabolite in roots (dpm/mg fresh weight)
NA	0	205.2	13.9	110.1
	0.5% w/w	166.9	6.45	221.4
BAS 145138	0	100.4	12.75	106.4
	400 μM	56.9	9.2	93.3
MG 191	0	100.4	12.75	106.4
	600 μM	66.4	11.3	105.4
95% LSD		16.02	3.9	33.5

DISCUSSION

These results suggest that NA protection of maize from AC 263222 injury results from enhancement of AC 263222 hydroxylation by a cytochrome P_{450} mono-oxygenase and concomitant reductions in herbicide translocation. As such, these results support those of Shaner (1991) who proposed a similar mode of action to account for the ability of NA to reduce activity of the closely related imidazolinone, imazethapyr. However, this mechanism of action cannot account for all herbicide-safener interactions reported here. In particular, enhanced oxidative metabolism would not appear to be responsible for the safening activity of BAS 145138 or the ability of NA to provide protection against imazapyr, which undergoes hydrolysis rather than oxidative metabolism (Lee *et al*, 1991). Consequently, other selectivity mechanisms may also contribute to safener activity.

Indeed, both BAS 145138 and NA were found to reduce herbicide uptake. Safeners may compete with herbicide molecules for sites of uptake, influence transpiration rates or disrupt the maintenance of pH gradients essential for the accumulation of weakly acidic molecules such as the imidazolinones. However, in this study, similar reductions in uptake were induced by MG 191 which failed to elicit a protective effect. This suggests that reductions of the magnitude observed are insufficient to reduce internal herbicide concentrations to a non-phytotoxic level and, thus, reduced uptake is not a predominant mechanism of safener action. The importance of reduced uptake is further undermined by the observation that protective effects can be achieved with post-herbicide NA applications. Under these circumstances, opportunities for safener interference with herbicide uptake processes is effectively eliminated. However, as this protective effect was significantly less than that induced by NA treatments applied with or after AC 263222, the possibility that inhibition of uptake contributes to activity cannot be eliminated. For example, reduced uptake may account for the limited protection provided by NA against imazapyr damage.

As metabolic rates are dependent on substrate concentrations, reductions in [^{14}C]-AC 263222 uptake following safener treatment may restrict metabolism in safener-treated tissue and, thus, obscure the effects of safener applications on AC 263222 hydroxylation. Consequently, the possibility that BAS 145138 and MG 191 may increase oxidative metabolism cannot be completely eliminated. Confirmation of their effects may require re-investigation using a biological system which circumvents the mechanisms responsible for reduced root-uptake.

This may be possible *in vivo* using an excised shoot system or *in vitro* by direct measurement of microsomal hydroxylation of AC 263222.

Nevertheless, the observation that BAS 145138 and MG 191 have identical effects on the uptake, distribution and metabolism of AC 263222 while only BAS 145138 elicits a protective effect, suggests that activity involves exploitation of selectivity mechanisms other than those we measured. For example, safeners may enhance other metabolic reactions. Although unlikely to explain safening of AC 263222, which did not undergo further metabolism within the duration of these experiments, enhanced hydrolytic activity may account for the ability of NA to reduce imazapyr injury. Alternatively, enhanced tolerance to the imidazolinones could result from modifications in the activity or sensitivity of the herbicide target enzyme, ALS. Although such effects are acknowledged, their relevance is usually dismissed on the grounds that such a mechanism cannot account for the ability of one safener to provide protection against herbicides with differing modes of action. Nevertheless, stimulation of ALS may account for the limited safening of imazapyr activity seen here.

Results presented here suggest that the ability of NA and BAS 145138 to enhance the tolerance of maize to the imidazolinones results from modification of two or more selectivity processes. Although enhancement of oxidative metabolism and concomitant reduction in herbicide mobility, is the predominant mechanism, reduced uptake and/or modifications of ALS activitiy may also contribute to safener activity.

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GLUTATHIONE TRANSFERASES RESPONSIBLE FOR HERBICIDE METABOLISM IN *SETARIA FABERI.*

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ABSTRACT

Glutathione transferases (GSTs) catalyse the conjugation of electrophilic herbicides with the tripeptide glutathione (GSH). These enzymes have an important role in herbicide selectivity and have been studied extensively in crop plants. We are studying GSTs in the grass weed *Setaria faberi* Herrm active towards a range of herbicide substrates (alachlor, metolachlor, atrazine and fluorodifen) and the general substrate 1-chloro-2,4-dinitrobenzene (CDNB). GST activities in extracts from leaves and roots of light grown 10 day old seedlings and in extracts from etiolated shoots and cell suspension cultures have been compared. All extracts had GST activity towards the substrates tested with the highest activities being found in cell suspension cultures. In contrast to cell cultures of maize, GST activity was observed with atrazine as substrate. As determined by anion-exchange chromatography green leaves of *S.faberi* contained at least three GST isoenzymes with differential activities toward CDNB and herbicide substrates.

INTRODUCTION

Glutathione transferases (GSTs, EC 2.5.1.18) are a group of enzymes that catalyse the conjugation of electrophilic herbicides with the tripeptide glutathione (GSH). These enzymes have a role in determining selectivity of certain herbicides (Cole 1994), with more tolerant plants such as maize rapidly detoxifying these herbicide substrates by conjugation (Lamoureux and Rusness 1993). For this reason GSTs have been studied extensively in maize and up to four different isoenzymes composed variously of 26, 27 and 29 kD subunits have been identified (Timmermann 1989, Irzyk and Fuerst 1993, Holt *et al.*, 1993). In contrast, relatively less is known about the GSTs in competing weed species. The identification of GST isoenzymes which are unique to crops but absent in weeds would aid the design and optimisation of new herbicides for the selective control of important weeds.

Setaria faberi Herrm is a problem weed in maize crops in the USA and it has a wide distribution over most of the central and eastern states where it can be difficult to control by conventional chemical means (Wang and Dekker 1995). We are currently characterising the

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GSTs in *S.faberi* and comparing them with the corresponding isoenzymes in maize. We now report on the GST activities present in seedlings and suspension-cultured cells of *S.faberi* and the initial purification of GSTs from the foliage of *S.faberi*.

MATERIALS AND METHODS

Plant material

Setaria faberi seeds (Herbiseed Ltd, Wokingham, UK) were sown in Levington multi-purpose potting compost in a growth room at 25 °C with a 16 hr photoperiod of light intensity 150 $\mu mol\ m^{-2}\ s^{-1}$. Plants were watered daily. At 10 days after sowing the seedlings were harvested and the roots and shoots were extracted for GST activity. Etiolated plant material was obtained by sowing *S.faberi* seeds onto filter paper and placing in a dark room at 25°C, 90% r.h. The etiolated shoots were harvested 7 days after sowing.

Initiation and maintenance of cell suspension cultures of *S.faberi*

Mature seed of *S.faberi* were surface sterilised by immersing in 50 ml of 2% sodium hypochlorite containing a drop of Teepol surfactant and agitating on an orbital shaker for 10 minutes. The seeds were then washed in several changes of sterile distilled water and placed onto Murashige and Skoog basal medium supplemented with 20 g/litre sucrose, 2 mg/ litre 2,4-dichlorophenoxy acetic acid and 0.8% agar, pH6.5 (Murashige and Skoog 1962). Plates were incubated in darkness at 27 °C. Small calli formed after 2-3 weeks which were slow growing, dark in colour and mucilaginous. These calli were transferred to fresh medium at three weekly intervals, sub-culturing where possible. After two months areas of dense white callus appeared from the mucilaginous callus which was of a faster growing type. This distinct callus was isolated, plated further and readily formed a finely dividing suspension culture when transferred to liquid media. Suspension cultures were grown in the same nutrient medium (50 ml) and were sub-cultured at 7 day intervals using a 8 ml inoculum.

GST extraction

Frozen plant tissue was ground to a powder with a pestle and mortar using liquid N_2. The powder was then thawed in 3 v/w 0.1 M Tris:HCl (pH7.5) containing 1 mM EDTA, 14 mM 2-mercaptoethanol and polyvinylpolypyrrolidone (7.5% w/w). After filtering through muslin the homogenate was centrifuged (15,000g, 15 min, 4°C). Ammonium sulphate was added to the supernatant to 80% saturation and the protein pellet collected by centrifugation as above and stored at -20 °C until required.

Frozen cell tissue was thawed in 3 v/w 0.1 M Tris:HCl (pH7.5) containing 1 mM EDTA, 14 mM 2-mercaptoethanol and polyvinylpolypyrrolidone (7.5% w/w). The suspension was placed into a glass bead beater kept at 4 °C and pulsed twice for 30 secs with a 2 minute interval between. The homogenate was centrifuged (15,000g, 15 min, 4°C). Ammonium sulphate was added to the supernatant to 80% saturation and the protein pellet collected by centrifugation as above and stored at -20 °C until required.

Determination of GST activities

Protein pellets were taken up and desalted in 2mM potassium phosphate buffer (pH 6.8) on Sephadex G-25 columns (Pharmacia PD-10). The protein content was determined using the Bio Rad dye binding reagent as recommended by the manufacturer. After adjusting to 10 mg protein/ml this preparation was used for all enzyme assays. GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined spectrophotometrically using a standard procedure (Edwards and Owen 1986). GST activities toward the herbicide substrates were determined by adding the enzyme extract (120 µl) to the herbicide dissolved in acetone (10mM; 10 µl), 10 mM glutathione (20 µl adjusted to pH 7.0) and either 0.1M potassium phosphate buffer (pH 6.8; 50 µl) for the assay of triazine and chloroacetanilide substrates or 50mM glycine-NaOH buffer (pH 9.5; 50 µl) for the assay with fluorodifen. The mixtures were incubated at 37 °C for 60 minutes and the reactions terminated by the addition of 0.6M hydrochloric acid (10 µl). After freezing at -20 °C and then thawing the precipitated protein was removed by centrifugation (12,000g, 5 min) and the supernatant (50 µl) injected onto a Spherisorb octadecyl hplc column (250 x 4.6 mm, 5 µm particle size, Fisons Chromatography, Loughborough, Leicestershire UK). The column was eluted with an acetonitrile , phosphoric acid gradient and the eluant monitored for UV absorbance at 264 nm (Hatton *et al.*, 1995). Glutathione conjugates were identified by cochromatography with the synthesised reference standards and quantified by calibration of the system with known amounts of the corresponding herbicides. The GST-dependant formation of the conjugates was determined after correcting for the non-enzymic rate and activity was expressed as pmols of product formed per s/mg protein (pkats/mg)(Hatton *et al.*, 1995)

Analysis of GST isoenzymes

Desalted extracts of crude GST were applied onto a column of Fast Flow Q-Sepharose (Pharmacia, UK) in 20 mM Tris:HCl pH 7.8. The column was then washed with a NaCl gradient of 0-0.25M using a Pharmacia Gradi-Frac system at 1 ml/min. Fractions (1ml) were collected and tested for activity with CDNB, atrazine and fluorodifen as substrates.

RESULTS AND DISCUSSION.

GST activities in various tissues of *S.faberi*

GST activities towards CDNB and the herbicide substrates atrazine, alachlor, metolachlor and fluorodifen were determined in the roots and leaves of 10 day old light grown seedlings, the leaves of 7 day old etiolated seedlings and cell suspension cultures 3 days after subculture. GST activity was observed to all substrates in the different tissues tested (Table 1). With atrazine and the chloroacetanilides as substrates the activities were significantly higher in the roots than the aerial parts of the plant. Activity toward atrazine was significantly lower in etiolated shoots as compared to the light grown shoots. In contrast the specific activities with fluorodifen as substrate were similar in all tissues. These results suggest that distinct GSTs exist which are distributed differently in plant tissues. GST activities towards all substrates tested were highest in extracts from cell cultures of *S.faberi*. For some substrates GST

activities were higher in cells than in shoots or roots and this differential amplification suggests the presence of a number of distinct GSTs for these substrates. For example, cell cultures contained GST activity toward atrazine at a specific activity 8-fold greater than in leaves but GST activity toward the chloroacetanilides and fluorodifen was 30-fold or more than the corresponding activities from leaves. It is also interesting to note that in contrast to cultured cells of maize, GST activity was observed towards atrazine as substrate (Edwards and Owen 1986). Suspension cultured cells of *S.faberi* may therefore be a good source of GSTs for future purification work.

Table 1. A comparison of mean GST activities in different tissue extracts of *S.faberi* towards a range of substrates with standard deviations shown in brackets.

Extract		substrate	conjugated	pkats/mg	protein
	CDNB	Atrazine	Metolachlor	Alachlor	Fluorodifen
Green leaves	149 (50)	0.35 (0.15)	0.27(0.14)	0.19 (0.1)	0.22 (0.06)
Etiolated leaves	149 (9)	0.09 (0.02)	0.14 (0.04)	0.35 (0.07)	0.25 (0)
Roots	2367 (167)	0.50 (0.30)	0.46 (0.02)	1.53 (0.50)	0.28 (0.02)
Cells	5730 (500)	2.94 (0.2)	7.8 (1.0)	12.76 (0.85)	8.16 (0.08)

Effect of growth on GST activities in suspension cultured cells of *S.faberi*

GST activity in suspension cultured cells of *S.faberi* was determined at various periods following subculturing. Cells entered the rapid growth phase 2 days after subculture and reached stationary phase by 7 days. Desalted extracts prepared from *S.faberi* cells were assayed for GST activity towards CDNB and the herbicide substrates at 0, 3, 5 and 7 days growth after subculture. GST activity was observed towards all substrates with activity being highest at 3 days after subculture when cell growth was most rapid (Figure 1). The increase in specific activity with alachlor and metolachlor as substrates was 8 fold between day 0 and day 3. In contrast the activity toward atrazine only increased by 2 fold. This differential regulation suggests that the GSTs with activities toward chloro-*s*-triazines and chloroacetanilides are catalysed by distinct isoenzymes as has been shown in maize (Timmermann 1989).

Purification of GSTs from *S.faberi*

Initial purification studies of GST activity from *S.faberi* using hydrophobic interaction chromatography and anion exchange have yielded up to 70 fold purification of the crude protein whilst maintaining 70% of the initial GST activity (Table 2).The crude preparation of protein from *S.faberi* leaves was loaded onto an anion exchange Q-Sepharose column in 20 mM Tris:HCl pH 7.8 and was eluted with a gradient of 0-0.25M NaCl. This resulted in the separation of three peaks of GST activity. Peak 1 (fractions 24-30) had activity towards CDNB and atrazine. Peak 2 (fractions 33-37) had activity towards atrazine and fluorodifen. A third peak which had activity towards fluorodifen ran just before peak 1. Peak 2 also showed activity toward metolachlor (data not shown). Further work using alternative affinity chromatography matrices is in progress to complete the purification of GST isoenzymes.

Figure 1. GST activities towards a range of substrates in extracts of suspension cultures cells of *S.faberi*.

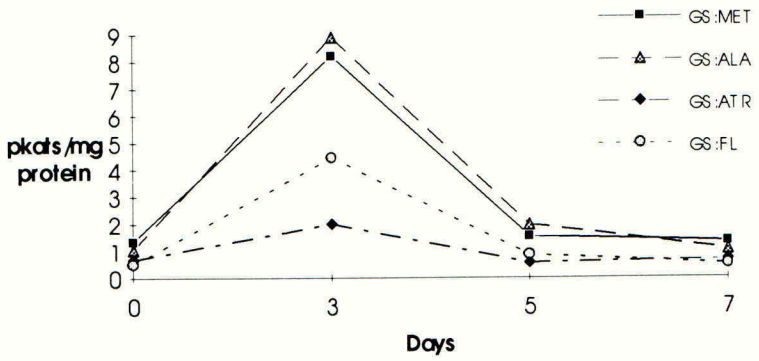
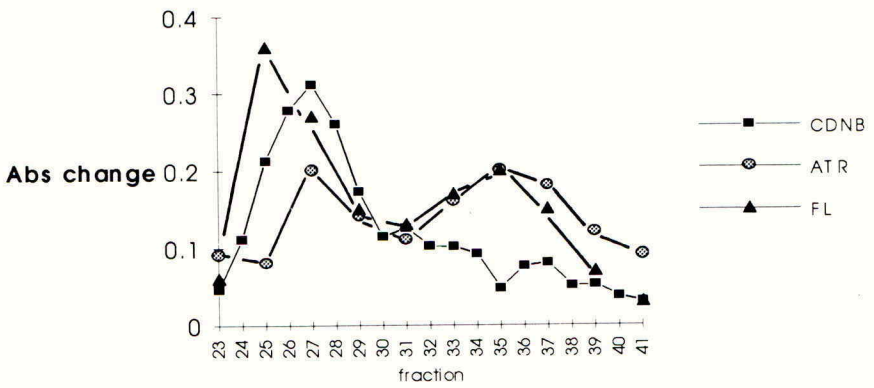


Table 2. Purification of GSTs from the leaves of 10 day old light grown *S.faberi* seedlings.

	Total protein (mg)	Specific activity (nkats/mg)	Total activity (nkats)	% Recovery	Fold Purification
80% Ammonium sulphate	1700	0.21	357	100	1
HIC	33.53	8.54	285.5	79.97	40.6
Q-Sepharose	15.36	16.4	251.9	70.6	78.1

Figure 2. Leaf GST activity in different fractions eluted off a Q-Sepharose column with a NaCl gradient.



CONCLUSIONS

These results show that GST activities towards CDNB and the herbicide substrates atrazine, alachlor, metolachlor and fluorodifen are catalysed by multiple isoenzymes in *S.faberi* plants and suspension cultured cells. Initial studies suggest that these multiple forms of GST can be partially resolved and their final purification is currently in progress.

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