SESSION 8C

MODE OF ACTION AND METABOLISM OF HERBICIDES

Session Organiser DR D J COLE
Rhône-Poulenc Agriculture Limited, Ongar, UK

Poster Papers 8C-1 to 8C-12

REGULATION OF MAIZE GLUTATHIONE TRANSFERASES DURING DEVELOPMENT AND THEIR INDUCTION BY XENOBIOTICS

D P DIXON, R EDWARDS
Dept. of Biological Sciences, University of Durham, South Road, Durham DH1 3LE, UK

D J COLE
Rhône-Poulenc Agriculture Ltd., Fyfield Road, Ongar, Essex CM5 0HW, UK

ABSTRACT

Maize glutathione transferases (GSTs) play a crucial role in the detoxification of a number of important classes of herbicide, including the chloro-*s*-triazines and chloroacetanilides, by catalysing the substitution of an electrophilic group on the herbicide with the tripeptide glutathione, rendering the herbicide non-phytotoxic and more water soluble. We have raised antibodies to the maize GST subunits *Zm* GST I, *Zm* GST II and *Zm* GST V present in safener-treated seedlings and used these antibodies to investigate the expression of GSTs in maize plants during development. *Zm* GSTs I and V were present in all plant organs, while *Zm* GST II was only detected in the roots of young seedlings. The antibodies were also used to study the inducibility of GST subunits in seedlings treated with a range of xenobiotics including herbicides, safeners and auxins, and showed that while *Zm* GST I was unaffected by any treatment, *Zm* GST II was inducible by a range of compounds including some herbicides and *Zm* GST V was specifically induced by the herbicide safener dichlormid.

INTRODUCTION

Glutathione transferases (GSTs, EC 2.5.1.18) are a group of widespread enzymes 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. GSTs exist as homo- or hetero-dimers with subunits of 23-30 kDa. The function of GSTs in plants seems mainly to be the detoxification of endogenous and exogenous chemicals, with the GSH conjugates usually being non-phytotoxic and more water-soluble than the unreacted molecules. A GSH conjugate-specific ATP-dependent pump in the tonoplast membrane then transports GSH conjugates into the vacuole (Martinoia *et al.*, 1993).

Maize GSTs play an important role in tolerance to herbicides, by catalysing the conjugation of atrazine and related chloro-*s*-triazines, and alachlor and other chloroacetanilides. The susceptibility of certain maize cultivars to these herbicides is inversely proportional to their GST activities (Shimabukuro *et al.*, 1970).

The increasing number of known maize GST subunits, and the numerous heterodimers known to be formed between these has made the existing maize GST nomenclature confusing. We have therefore proposed an updated nomenclature (Dixon *et al.*, in press), based on that used for mammalian GSTs (Jakoby *et al.*, 1984), where GST dimers are described by their subunit composition, with an additional species identifier to avoid confusion between similarly named

GSTs from different species. For example, maize GST I, a homodimer of 29 kDa subunits, is renamed *Zm* GST I-I (where *Zm* = *Zea mays*), while maize GST II, a heterodimer of 29 and 27 kDa subunits, will be called *Zm* GST I-II. Similarly, maize GST III becomes *Zm* GST III-III and GST IV becomes *Zm* GST II-II. This new nomenclature will be used throughout this paper.

A number of GST enzymes active in herbicide detoxification have been found in maize, some of which have been shown to be constitutively present while others are induced by the application of herbicide safeners. Safeners are compounds applied with, or before, herbicide application to differentially increase the crop plant's tolerance to a herbicide as compared to that of the target weeds. The *Zm* GST subunits I and III are expressed constitutively, while *Zm* GST II has been shown to be constitutively present in root tissue, but highly induced in roots and shoots in response to safeners. In addition, other safener-inducible subunits are known to exist but have not been characterised. We have raised antibodies to *Zm* GST I-II and *Zm* GST V-VI, a newly characterised GST similar to auxin-inducible GSTs found in dicots (Dixon *et al.*, in press), and used these antibodies to examine the expression of *Zm* GST subunits I, II and V during maize plant development. We have also used the antibodies (Dixon *et al.*, in press) to investigate the induction of GSTs by a wide range of chemicals, including herbicides, safeners and synthetic auxins.

MATERIALS AND METHODS

Plant material

For chemical induction studies, maize (cv. Pioneer 3394) seeds were imbibed in tap water for an hour and grown in vermiculite at 25 °C with a 16 h photoperiod and light intensity of 150 $\mu mol/m^2/s$. Eleven day old seedlings were gently uprooted and washed to remove any vermiculite. For each treatment duplicate pairs of seedlings were placed with their roots in treatment solution. For heat-shock treatments, maize seedlings were exposed to 1% ethanol and were placed in an incubator at 41 °C for 3 h and then harvested immediately. For treatment with glutathione the thiol was made up to 5 mM after adjusting to pH 7, and 1% ethanol added. Treatment solutions of 1-chloro-2,4-dinitrobenzene (CDNB, 1 mM), 2,4-dichlorophenoxy-acetic acid (2,4-D, 0.1 mM) and α-naphthaleneacetic acid (NAA, 0.1 mM) were prepared by a 100-fold dilution of the respective stock solutions prepared in ethanol, while dichlormid (0.1 mM) fluorodifen (0.1 mM), alachlor (0.1 mM) and atrazine (0.1 mM) were diluted 200-fold from stock solutions prepared in acetone. Control solutions consisted either of 1% ethanol or 0.5% acetone. After incubation in the growth room for 24 h, the seedlings were separated into roots and shoots and each tissue was homogenised in 3 volumes of 100 mM Tris-HCl, pH 7.5, 2 mM sodium EDTA, 14 mM ß-mercaptoethanol in the presence of 5% m/V polyvinylpolypyrrolidone. After allowing to stand for ten minutes on ice, the extracts were centrifuged (12,000 *g*, 20 min, 4 °C) and the protein concentrations of the resultant supernatants determined using a dye-binding assay (Bio-Rad) and normalised to the concentration of the most dilute sample prior to SDS-PAGE (sodium dodecylsulphate - polyacrylamide gel electrophoresis) and Western blotting.

For dichlormid treatment studies, seeds were imbibed for 1 h in 10 µg/ml dichlormid in 1% acetone and sown in vermiculite. Seedlings were grown in the dark in laboratory conditions

and watered with 5 µg/ml dichlormid dissolved in 0.5% acetone. Control plants were grown under similar conditions but were imbibed in 1% acetone and watered with 0.5% acetone. Plants were harvested as seeds and at 2, 4, 6, 8 and 10 days after sowing by up-rooting the seedlings and washing them free of all traces of vermiculite. Protein was then extracted as detailed for the chemical-induction study and precipitated by adding ammonium sulphate to 80% saturation. After centrifugation (13,000 *g*, 20 min, 4 °C) the resulting protein pellets were dissolved in a minimal volume of 2 mM potassium phosphate buffer pH 6.8, 2 mM DTT, and dialysed for 16 h against the same buffer, prior to normalising the protein contents for analysis by SDS-PAGE and Western blotting. Extracts were also assayed for GST activity towards CDNB and the herbicides atrazine, metolachlor and fluorodifen as described previously (Dixon *et al.*, 1997).

For developmental studies, maize plants were grown in compost under greenhouse conditions and harvested at 7, 13, 17, 24, 32 and 38 days after sowing by separating into roots and shoots. Plants were also grown to maturity (11 weeks) and samples of stem, tassels (post-anthesis), silks (stigmas and styles) and immature kernels collected. Protein was extracted from each tissue as described for dichlormid-treated plants, and analysed by SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting

Protein samples were heated to 100 °C in an equal volume of 2× loading buffer for 5 min and allowed to cool. Samples were then loaded onto a 0.8 mm mini-gel (12.5% acrylamide, 0.33% N'N'-bis-methylene-acrylamide), and after electrophoresis at 150 V proteins were electroblotted from the gel onto Immobilon-P PVDF membrane (Millipore, Watford, UK) using a Bio-Rad tank electroblotter and supplied protocol. Membranes were used for Western blotting following published procedures (Harlow and Lane, 1988); primary antibody (anti-*Zm* GST I-II or anti-*Zm* GST V-VI) was used at a dilution of 1:5,000. Detection was performed using an alkaline phosphatase-linked secondary antibody and the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

RESULTS AND DISCUSSION

Chemical induction of maize GSTs

Antibodies which were specific to two major classes of maize GSTs were used to study of differential regulation of *Zm* GST subunits I, II and V in maize seedlings exposed to heat-shock or a variety of chemical treatments. Following SDS-PAGE, the resolved polypeptides were analysed by Western blotting using either the antibody raised to *Zm* GST I-II, or to *Zm* GST V-VI. The experiments were carried out in duplicate, but in view of the similar results, Figure 1 shows the results of only one of the replicates. The antibody raised to *Zm* GST V-VI identified the *Zm* GST V subunit as being constitutively expressed in control shoots and roots exposed to water containing the solvents used to dissolve the treatments. Relative to controls, *Zm* GST V expression in the roots was unaffected by all treatments except NAA and dichlormid, which caused a minor increase in immunodetectable polypeptide. In the shoots, dichlormid treatment resulted in a major increase in *Zm* GST V. CDNB and heat shock also gave a slight enhancement of expression.

Using the anti-*Zm* GST I-II serum, as reported in previous studies both *Zm* GST I and *Zm* GST II subunits were observed in control roots, while only the *Zm* GST I subunit was observed in control shoots. Expression of *Zm* GST I in the roots was unaffected by heat shock or any of the chemical treatments, while *Zm* GST II expression was clearly enhanced in response to treatment with NAA, and a slight enhancement was also seen with alachlor, fluorodifen, dichlormid and glutathione. In the shoots, the *Zm* GST II subunit was clearly visible following treatment with 2,4-D, dichlormid, NAA and CDNB.

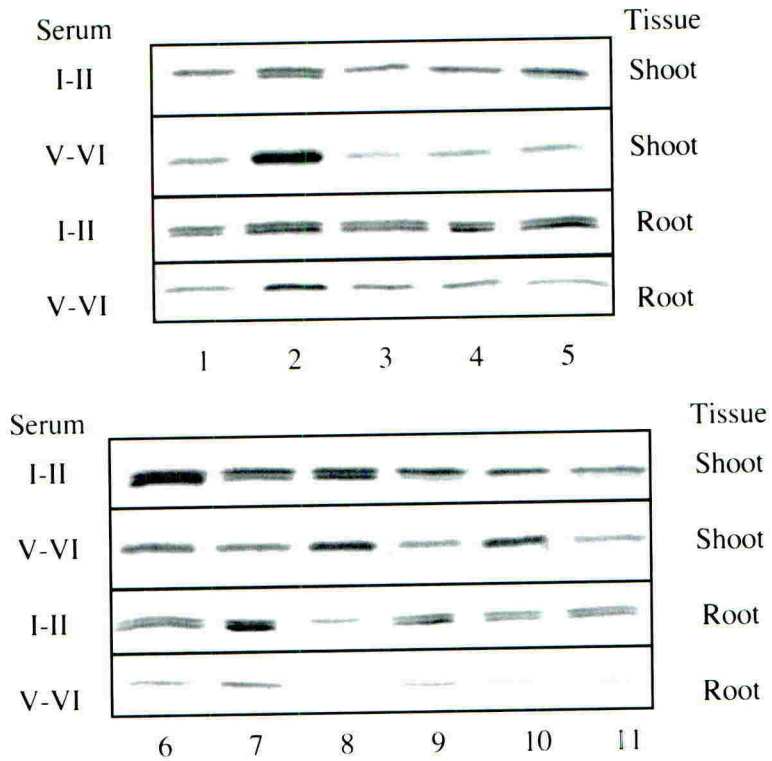

Figure 1. Composite digitised image showing Western blots of polypeptides resolved by SDS-PAGE of identical amounts of crude protein isolated from the roots and shoots of maize seedlings exposed for 24 h to: 1 = 0.5% acetone (control), 2 = 0.1 mM dichlormid, 3 = 0.1 mM atrazine, 4 = 0.1 mM alachlor, 5 = 0.1mM fluorodifen, 6 = 0.1 mM 2,4-D, 7 = 0.1 mM NAA, 8 = 1 mM CDNB, 9 = 5 mM glutathione, 10 = heat shock, 11 = 1% ethanol (control). For simplicity, only the bands corresponding to immunodetectable GSTs are shown, with neither antiserum recognising any other polypeptides.

Since the maize safener dichlormid caused a significant accumulation of both *Zm* GST subunits II and V, a more detailed induction study was carried out with dichlormid in dark-grown seedlings. Whole seedlings (roots + shoots) were extracted and quantitatively analysed for changes in GST activities toward CDNB and the herbicides atrazine, fluorodifen and metolachlor, together with changes in immunodetectable polypeptides (Table 1). Using *Zm* GST I-II antibody, the *Zm* GST I subunit was found to be expressed at similar levels at all the

time points, and in extracts from whole seedlings, did not accumulate following dichlormid treatment. The *Zm* GST II subunit was not determined in seeds, but was detected in extracts of whole seedlings, with dichlormid treatment increasing its expression, particularly in the 4 to 6-day old plants. Probing with *Zm* GST V-VI antibody showed that the *Zm* GST V subunit was present in untreated tissue, and was significantly induced by application of dichlormid, particularly after 8 to 10 days. When changes in GST activities were related to changes in immunodetectable GST subunits (Table 1), it was apparent that the weak enhancement of GST activity towards CDNB in the dichlormid-treated seedlings was due to the major enhancement of *Zm* GST subunits II and V, both of which show limited activity toward CDNB. *Zm* GST I-I is responsible for the majority of CDNB-conjugating activity in maize and this enzyme was unaffected by safener treatment. GST activity toward metolachlor increased six-fold over the induction treatment and three-fold in untreated plants relative to the activities determined in the seeds, and these increases correlated well initially with the increase in the *Zm* GST II subunit and later with the increase in *Zm* GST V, both enzymes being highly efficient in detoxifying this herbicide. Following dichlormid treatment, GST activity toward fluorodifen doubled within 4 days and this could be accounted for by the rapid enhancement of both *Zm* GST II and *Zm* GST V subunits, both of which are associated with high fluorodifen activity. Activity towards atrazine, which is due partly to *Zm* GST I subunits, but mainly due to other, unidentified GSTs, did not change significantly over time, and was unaffected by dichlormid treatment.

Table 1. Effect of dichlormid treatment on GSTs in 0 to 10-day seedlings, as measured by enzyme activity towards CDNB and the herbicides atrazine (Atraz), metolachlor (Met) and fluorodifen (Flu), and by Western blotting using anti-*Zm* GST I-II and anti-*Zm* GST V-VI sera. [a] Integrated density of respective Western blot band, expressed as % of relative value obtained for the respective GST subunit in 4-day old untreated tissue.

Day	Dichlormid	GST activities (pkat/mg protein)				Immunodetectable GSTs[a]		
	(-/+)	CDNB	Atraz	Met	Flu	*Zm* I	*Zm* II	*Zm* V
0		5470	1.37	3.27	1.04	104	0	24
4	-	5640	1.29	5.22	1.40	100	100	100
4	+	5840	1.62	7.93	2.54	85	250	151
6	-	4840	1.15	5.41	0.98	101	198	118
6	+	7000	1.51	14.37	2.76	108	358	176
8	-	3580	1.23	7.38	0.61	73	167	120
8	+	7210	1.58	19.12	2.70	71	248	215
10	-	5200	1.58	9.16	0.90	106	238	132
10	+	7490	1.79	16.45	2.58	83	220	322

Changes in GST expression in response to plant age and development.

Changes in the relative expression of the *Zm* GST I, *Zm* GST II and *Zm* GST V subunits during the growth and development of maize were also monitored by Western blotting following SDS-PAGE using the two antibodies (data not shown). Using the anti-*Zm* GST I-II serum, 29.5 kDa *Zm* GST I and 29.0 kDa *Zm* GST II subunits were determined, together with a 27 kDa polypeptide degradation product of *Zm* GST I. For comparative purposes, *Zm* GST I and the 27 kDa polypeptide were considered to be a single entity. Expression of *Zm* GST I

decreased slightly in both root and shoot with age. At all time points, on the basis of % extracted protein, *Zm* GST I was five times more abundant in root tissue compared with shoot tissue. In mature plants *Zm* GST I was also present in male and female floral tissues, immature kernels and mature stems. *Zm* GST II could only be detected in young roots, while *Zm* GST III, which reacted poorly with the antibody used, could only be detected in 13-day roots. Immature kernel tissue also contained a larger protein (about 32 kDa molecular mass) which cross-reacted with the antibody; this protein was not observed in other tissues. Using the anti-*Zm* GST V-VI serum, expression of *Zm* GST V remained constant throughout development in roots and shoots. Again on the basis of % of extracted protein *Zm* GST V was five-fold more abundant in root tissue than shoot tissue. *Zm* GST V was barely detectable in male floral tissue and stem tissue but was present in higher amounts in female floral tissue and immature kernels.

CONCLUSIONS

The multiple GST isoenzymes of maize which are involved in herbicide detoxification are subject to complex and differential regulation in response to development, plant age and exposure to xenobiotics. Both *Zm* GST I and the newly reported *Zm* GST V are constitutively expressed in all plant parts, but *Zm* GST V differs from *Zm* GST I in being specifically induced by the safener dichlormid. In contrast, *Zm* GST II is responsive to a wide range of chemical treatments in addition to dichlormid. The differences in sensitivities of *Zm* GST I and *Zm* GST V to chemical treatments suggest that safeners like dichlormid must activate a signal-transduction pathway which is different from that activated by other xenobiotics.

ACKNOWLEDGEMENTS

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CALCIUM ION DYNAMICS IN AUXINIC-HERBICIDE RESISTANT AND SUSCEPTIBLE BIOTYPES OF *SINAPIS ARVENSIS*

Y WANG, S DESHPANDE, J C HALL
Department of Environmental Biology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada (jchall@evbhort.uoguelph.ca)

ABSTRACT

The effects of the auxinic herbicides dicamba and mecoprop (MCPP) were studied on the seedling growth of auxinic-herbicide-resistant (R) and -susceptible (S) biotypes of wild mustard (*Sinapis arvensis*). Dose-response studies in the laboratory confirmed that the S seedlings were more sensitive to the herbicides than R seedlings. Mecoprop (MCPP), which is more efficacious than dicamba in the field, was found to induce differential response between the biotypes at much lower concentrations than dicamba. The IC_{50} values for S and R seedlings were 1.0 and 50 μM for dicamba, and, 0.1 and 0.5 μM for mecoprop, respectively. The effects of the auxinic herbicides were ameliorated in both biotypes when the seedlings were pre-treated with calcium in the presence of the calcium ionophore A23187 at 1 μM. Conversely, the addition of the calcium channel blocker verapamil in the presence of either herbicide increased the sensitivity of R seedlings to that herbicide. These results agree with our previous results on the effects of dicamba and mecoprop on protoplasts from R and S plants using light scattering spectroscopy, thereby suggesting that calcium has a primary role in auxinic herbicide resistance.

INTRODUCTION

An auxinic herbicide resistant biotype of wild mustard (*Sinapis arvensis*) was found by Heap and Morrison (1992) in a field in west-central Manitoba, Canada which had been treated with mixtures of 2,4-D/mecoprop/dicamba for more than 10 years. This biotype was highly resistant to picloram and dicamba, moderately resistant to 2,4-D and MCPA, and only slightly resistant to mecoprop and 2,4-DP (Deshpande & Hall, 1996, Heap & Morrison, 1992). In subsequent research, we found no difference between the two biotypes with regard to absorption, translocation, or metabolism of foliar-applied 2,4-D, dicamba or picloram (Peniuk *et al.*, 1992). Hall *et al.* (1993) reported that the S biotype produced more ethylene than the R biotype after treatment with picloram. Characteristics of [^{3}H]IAA binding to auxin-binding proteins (ABP) in R and S biotypes were also investigated (Webb & Hall, 1995). When picloram, dicamba, and 2,4-D were used to inhibit [^{3}H]IAA binding, the S biotype was more sensitive to inhibition than the R biotype ABP preparations; however, no difference in ABP binding was observed between the biotypes following mecoprop treatment. The ABP binding results follow the same trends as do the results on the relative susceptibility of seedlings of the two biotypes to the auxinic herbicides (Webb & Hall, 1995). Furthermore, morphological studies showed that the R biotype was shorter, more branched, darker green, and higher in chlorophyll and cytokinin content than the S biotype (Hall & Romano, 1995).

Light-scattering spectroscopy, which can be used to discern rapid morphological changes in cells non-invasively, was used to compare the ATP-dependent flash-induced changes in dark-adapted protoplast suspensions isolated from S and R biotypes in the presence of picloram (Deshpande & Hall, 1995). Profiles of the scattering signals from R and S biotypes were different. Picloram reduced the ATP-dependent signal amplitude in proportion to its concentration. The amplitude was reduced by 50% with 20 μM picloram. In comparison, picloram concentrations as high as 50 μM did not reduce the signal amplitude from R protoplast suspensions by more than a few percent. Altering the ionic environment of the protoplasts indicated that calcium plays a role in the resistance mechanism. For example, pre-loading the protoplasts with calcium by using the calcium ionophore A23187 reduced inhibitory effects of picloram on S protoplasts. Conversely, the addition of verapamil, a calcium channel blocker, increased the inhibitory effects of picloram on R protoplasts. These results were confirmed using right angle scattering measurements to study the kinetics of volume changes of R and S protoplasts in the presence of the previously mentioned auxinic herbicides (Deshpande & Hall, 1996).

In this paper, we determine if previously reported effects of calcium and verapamil on protoplasts (Deshpande & Hall, 1995) could be reproduced using intact seedlings. We found that calcium ions ameliorated the inhibitory effects of auxinic herbicides on R and S seedlings. Conversely, verapamil increased the inhibitory effect of the herbicides on R biotype seedlings. Therefore, these results support our previous findings using protoplasts.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of R and S biotypes of *S. arvensis* were surface sterilized using 5% (v/v) sodium hypochlorite for 5 minutes and rinsed in distilled water. Twenty-five seeds were placed onto a filter paper disk placed in a petri dish containing 3 ml distilled water. Seeds were germinated in the dark at 25°C, 65% RH for one to two days. The imbibing solution containing herbicides or other chemicals was added to the petri plates immediately after germination of the seeds.

Chemicals and reagents

Dicamba (3,6-dichloro-2-methoxy-benzoic acid; 99.6% purity) was supplied by Sandoz Crop Protection. Mecoprop (2-(4-chloro-2-methylphenoxy) propanoate, 99% purity) was obtained from Riedel-de Haëu. Verapamil (HCl), A23187 (calcium ionophore), sodium hypochlorite, and calcium chloride were purchased from Sigma. Nanopure distilled water was used in all experiments.

Seedling root length determination data statistical analysis

Each set of experiments consisted of at least four replicates (petri dishes with 25 seeds) of each treatment with its corresponding control. The seedling root-length was measured 72 hours after treatment. The experiment was replicated and data was pooled prior to statistical analysis.

RESULTS AND DISCUSSION

The sensitivity of the S and R biotypes to the auxinic herbicides can be exemplified by their dose response patterns to dicamba (Figure 1). When the two biotypes were compared, there was at least an order of magnitude difference between the dicamba doses required to cause inhibition of growth. Regardless of the biotype, mecoprop was effective at lower concentrations than dicamba (data not shown). For example, seedling growth was inhibited by about 40% (S) and 20% (R) with 10^{-8} M mecoprop. The IC_{50} values calculated from the dose-response curves were 1.0 μM (S) and 50 μM (R) with dicamba, and, 0.1 μM (S) and 0.5 μM (R) with mecoprop.

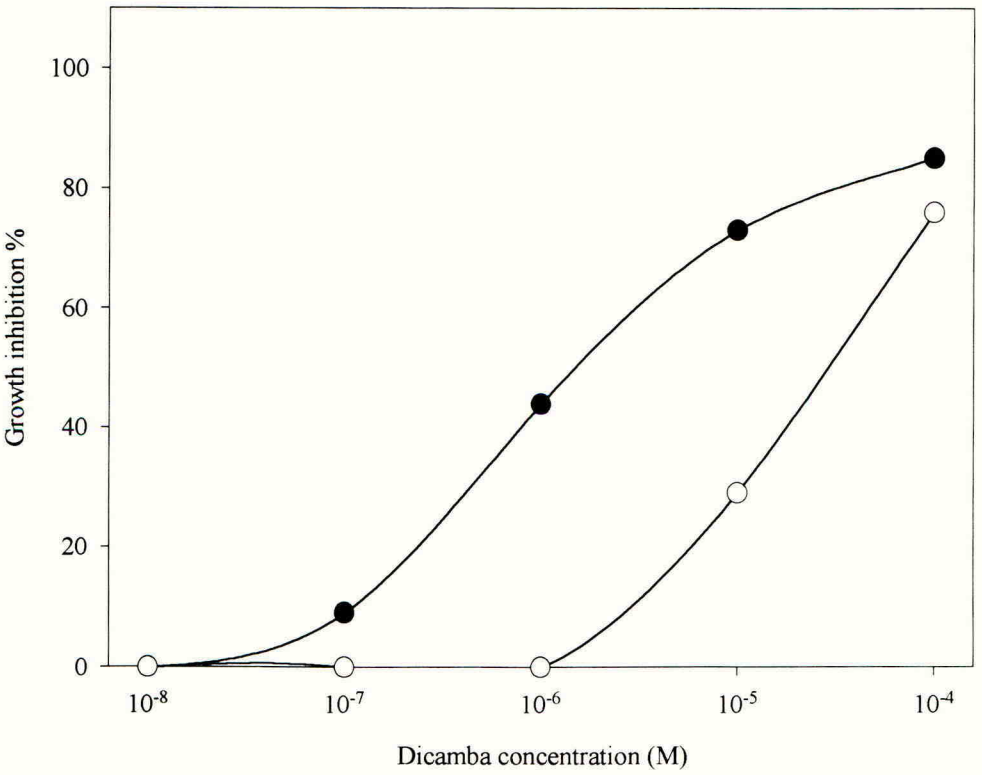

Figure 1. The dose response of S (●) and R (○) seedlings of *S. arvensis* treated with dicamba. Decreases in the root length of the seedlings treated with dicamba are expressed as a percentage of the untreated control. Each point represents the mean of 100 observations (4 petri plates (blocks) containing 25 seedlings (replicates)). In all cases, error bars are smaller than the symbols at each concentration of herbicide.

Based on our previous research, calcium loading using the ionophore A23187 reduced the inhibitory effects of picloram on S protoplasts. Conversely, we found that the addition of verapamil, a calcium channel blocker, increased the inhibitory effects of picloram on R protoplasts. Since the R seedlings were also found to be less sensitive to the auxinic herbicides, calcium/A23187 or verapamil was added to determine if the seedling response was similar to that observed with protoplasts.

In control experiments using R seedlings, verapamil was found to inhibit seedling growth at concentrations above 100 μM; therefore, the concentration used was 100 μM. Calcium concentrations as high as 1 mM did not affect the seedling growth. However, to limit the effect of the calcium salt on ionic strength of the solutions, a concentration of 50 μM was used. The calcium ionophore A23187 did not affect the growth of seedlings at the concentration (1 μM) required to load calcium.

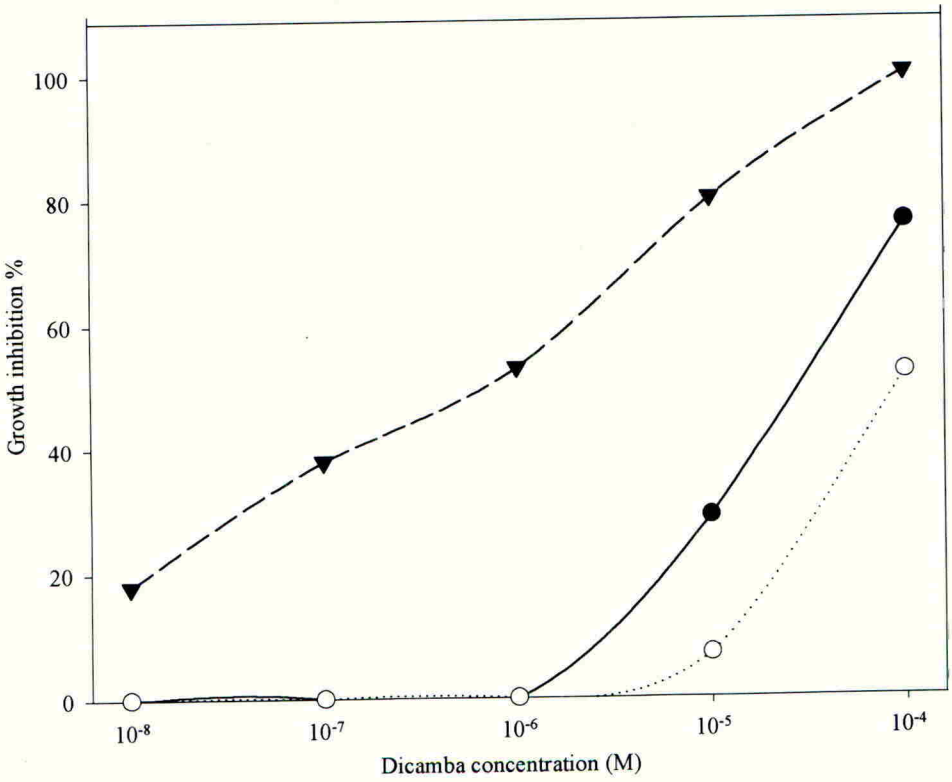

Figure 2. The effect of dicamba applied alone (●) and in the presence of 100 μM verapamil (▼), or calcium/A23187 (○; 50/1 μM, respectively) on the growth of R seedlings of *S. arvensis*. Decreases in the root length of the seedlings treated with dicamba are expressed as a percentage of the untreated control. Each data point represents the mean of 100 observations (4 petri plates (blocks) containing 25 seedlings (replicates)). In all cases, error bars are smaller than the symbols at each concentration of herbicide.

The effects of calcium/A23187 and verapamil on R seedling growth in the presence of dicamba are shown in Figure 2. In the presence of calcium/A23187 (Figure 2), the sensitivity of the R seedlings to dicamba was reduced when compared to the control (dicamba only). For example, inhibition of seedling growth by 10^{-5} M dicamba was 30 and 7%, respectively, in the absence and presence of calcium/A23187. In the presence of verapamil, sensitivity to dicamba was increased by several orders of magnitude (Figure 2). Furthermore, a dicamba concentration of 10^{-6} M, which has no effect on R biotype seedlings, inhibited growth by about 50% in the presence of verapamil.

The effects of calcium/A23187 and verapamil on R seedling growth in the presence of MCPP are shown in Figure 3. Mecoprop had a greater effect on seedling growth than dicamba (IC_{50} 0.1 and 0.5 μM, respectively). Furthermore, the addition of verapamil did not increase the

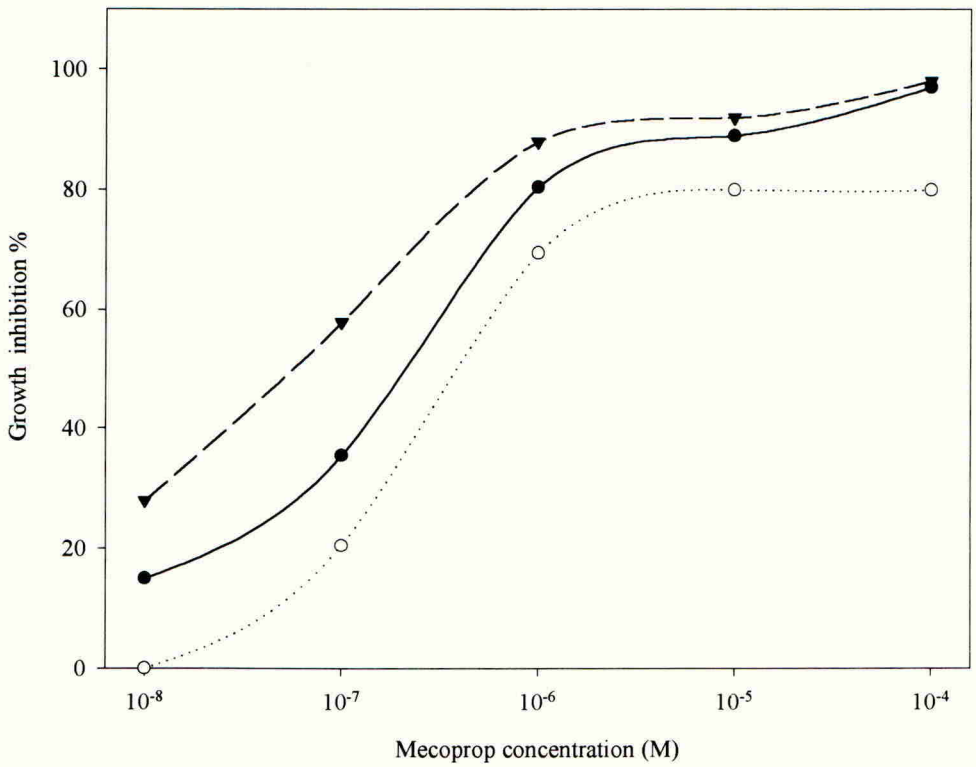

Figure 3. Effect of mecoprop applied alone (●) and in the presence of 100 μM verapamil (▼), or calcium/A23187 (○; 50/1 μM, respectively) on the growth of R seedlings of *S. arvensis*. Decreases in the root length of the seedling treated with mecoprop are expressed as a percentage of the untreated control. Each data point represents the mean of 100 observations (4 petri plates (blocks) containing 25 seedlings (replicates)). In all cases, error bars are smaller than the symbols at each concentration of herbicide.

efficacy nor did the addition of calcium/A23187 decrease the efficacy of mecoprop to the same extent as they did with dicamba. The addition of calcium eliminated the inhibition of growth caused by mecoprop at 10^{-8} M. However, at higher concentrations of mecoprop, calcium/A23187 did not significantly reduce inhibition of growth by the herbicide.

The auxinic herbicides have been used for over 50 years without clear knowledge of their mechanism(s) of action. Therefore, our goal continues to be the elucidation of the mechanism of resistance of the R biotype of *S. arvensis* to the auxinic herbicides in order to determine the mechanism(s) of action of these herbicides. The results of this study, using seedlings (whole plants), agree with those obtained using light scattering spectroscopy on protoplasts, and unequivocally link calcium to the resistance mechanism of the R biotype of *S. arvensis* to auxinic herbicides. Our next objective is to determine the differences in gene expression between the R and S biotypes before and after treatment with auxinic herbicides.

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CHEMICAL CATALYSIS OF THE ISOMERISATION OF A PEROXIDISING HERBICIDAL THIADIAZOLIDINE

I JABLONKAI
Central Research Institute for Chemistry, Hungarian Academy of Sciences, H-1525 Budapest, PO Box 17, Hungary

T KÖMIVES
Plant Protection Institute, Hungarian Academy of Sciences, H-1525 Budapest, PO Box 102, Hungary

P BÖGER
Department of Plant Physiology and Biochemistry, University of Konstanz, D-78434 Konstanz, Germany

Y SATO, K WAKABAYASHI
Department of Agricultural Chemistry, Faculty of Agriculture, Tamagawa University, Machida-shi, Tokyo 194, Japan

ABSTRACT

Chemical catalysis of the isomerisation of the herbicide BW 78 was studied in non-aqueous media. Various -OH, -NH, and -SH nucleophiles were found to be efficient catalysts in acetonitrile in the presence of tertiary nitrogen bases. Significant isomerisation was initiated by glutathione in an aqueous system as well, when the nucleophilic buffer Tris was used. Based on the effects of the chemical structures of the catalysts on the reaction rate a mechanism of the reaction is proposed.

INTRODUCTION

Some 5-arylimino-3,4-tetramethylene-1,3,4-thiadiazolidin-2-ones (thiadiazolidines) are peroxidising bleaching herbicides which interrupt chlorophyll biosynthesis, inhibit the activity of protoporphyrinogen oxidase (protox, EC 1.3.3.4) leading to the accumulation of protoporhyrin IX (Sato *et al.*, 1994). The bioconversion of these molecules into their isomeric 4-aryl-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thiones (triazolidines) yielded stronger inhibitors of protox. Short-term phytotoxicity of thiadiazolidines and triazolidines also differed markedly demonstrating that isomerisation resulted in bioactivation of thiazolidines. Further studies revealed that the isomerisation requires the catalytic action of an isoenzyme of glutathione *S*-transferases (GST, EC 2.5.1.18) in plants (Senoo *et al.*, 1996, Nicolaus *et al.*, 1996). In addition, it was found that isomerase activity of maize glutathione *S*-transferase isoform was dependent on reagents containing a thiol group such as dithiothreitol, thioglycolate or glutathione (GSH) (Iida *et al.*, 1995). These results suggested the participation of a thiol as a cofactor in the isomerisation. While a non-enzymatic interconversion of a related tetrahydroisophthalimide to a tetrahydrophthalimide in water has been observed (Sato *et al.*, 1997), interestingly, regardless of pH no structural changes of thiadiazolidines and triazolidines were found in buffer solution (Sato *et al.*, 1994). Also, non-enzymatic conversion was hardly detectable at basic pH (Nicolaus *et al.*, 1996).

The objective of our study was to characterise the nature of this enzymatic conversion by carrying out model reactions with BW 78 (5-[4-bromophenylimino]-3,4-tetramethylene-1,3,4-thiazolidin-2-one) in aprotic solvents in presence of nucleophiles and a low molecular weight catalyst.

MATERIALS AND METHODS

Chemicals

BW 78 and BW 85 (4-[4-bromophenyl]-1,2-tetramethylene-1,2,4-triazolidin -3-one-5-thione) were products of syntheses described previously (Sato *et al.*, 1994). All chemicals used were of the highest purity available from Sigma-Aldrich (Budapest, Hungary). Acetonitrile was HPLC grade from Carlo Erba (Rodano, Italy).

Isomerisation assays

For non-aqueous solvent, isomerisation reactions of BW 78 with various nucleophiles and bases were carried out in acetonitrile as solvent. In 2-ml vials mixtures of BW 78 (1 mg, 3 μmole), nucleophile (3 μmole), and base (3 μmole) were stirred in 400 μl of solvent at 25 ± 1 °C. In order to follow the kinetics of the reaction subsamples (5 μl) were taken at defined time periods and quantitatively analysed by thin-layer chromatography (TLC). When the effects of L-cysteine were investigated, 800 μl of solvents were used due to the low solubility of this nucleophile in acetonitrile.

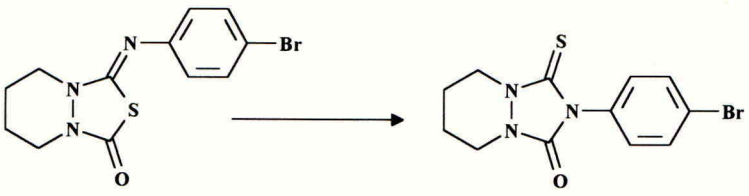
For aqueous medium, isomerisation of BW 78 was studied in potassium phosphate and Tris buffers using GSH as a nucleophile. BW 78 (1 mg, 3 μmole) was dissolved in 200 μl of ethanol and GSH (0.9 mg, 3 μmole) in 950 μl respective buffer was added. Mixtures were stirred at 25 ± 1 °C and 5 μl of subsamples were taken at regular intervals TLC analyses.

TLC analyses

Subsamples of isomerisation experiments taken from assay mixtures were analysed by TLC on silica gel plates (Kieselgel 60 F_{254}, 250 mm thickness, Merck, Darmstadt, Germany). Subsamples (5 ml) were applied to the plates by a Linomat III automatic sample applicator (Camag, Muttenz, Switzerland). Plates were developed in hexane-ethyl acetate (7:3, v/v) solvent. Respective R_f-values of standards were 0.43 for BW 78 and 0.12 for BW 85. After development, bands on plates were quantitatively evaluated by densitometry using a Shimadzu CS-920 high speed TLC scanner (Shimadzu Corp., Kyoto, Japan) in absorption mode at 254 nm.

RESULTS AND DISCUSSION

Non-enzymatic conversion of the thiadiazolidine herbicide BW 78 to BW 85 according to Scheme 1 was studied in aprotic solvent with various -SH, -OH, and -NH nucleophiles listed in Table 1. The use of aprotic solvent instead of water was required to mimic the environment of the hydrophobic pocket of GST. Also, insolubility of GSH in acetonitrile demanded the use of soluble nucleophiles. In preliminary experiments we found that both alkyl- and aryl-thiols alone were unable to trigger



BW 78 **BW 85**

Scheme 1. Isomerisation of BW 78 to BW 85

detectable isomerisation: neither butane-1-thiol or thiophenol were able to induce conversion of BW 78 up to 2 days (data not shown). In order to increase the nucleophilicity of both thiols we added 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) as a powerful base to the assay mixture thus facilitating

Table 1. Influence of nucleophiles and bases on isomerisation of BW 78 to BW 85

Cofactor	Time required for 50% isomerisation (h)	pK_a in water
Nucleophile[a]		
Butane-1-thiol	0.06	10.65
Thiophenol	0.25 (31.30[b])	6.50
2-Mercapto-ethanol	0.07	9.44
Cysteine	5.60	8.53
GSH	not studied[c]	8.66
n-Butanol	38.60	16.50
Phenol	21.60	9.39
Pyrrolidine	0.07[d]	11.27
Imidazole	12.30	6.95
Indole	46.50	-3.63
Base[e]		
DBU	46.90	not available
DMAP	44.70[f]	9.70
2,6-Lutidine	no isomerisation	6.15

[a] Isomerisation of BW 78 with nucleophiles in the presence of DBU
[b] Isomerisation rate of BW 78 with thiophenol in the presence of DMAP
[c] Not studied because of the insufficient solubility of GSH in acetonitrile
[d] Isomerisation rate of BW 78 with pyrrolidine alone
[e] Isomerisation of BW 78 only with respective base
[f] Time required for 10% isomerisation

the formation of the stronger nucleophile anionic form. In the presence of this "non-nucleophilic base" (Barton *et al.*, 1972) rapid isomerisation of thiadiazolidine occurred with both thiols as indicated by the time required for 50% isomerisation (Table 1, Figure 1). DBU also catalysed the formation of BW 85 with other thiols such as 2-mercaptoethanol and cysteine. In addition, -OH and -NH nucleophiles were able to catalyse the transformation of BW 78 (Table 1). Using -OH compounds lower isomerisation rates were detected as compared with thiols, as indicated by higher half life values (Table 1). In the amine nucleophile series we used pyrrolidine, imidazole and indole which are backbones of histidine, proline and tryptophan amino acids, important building blocks of several GST enzymes (Jepson *et al.*, 1994). These amines showed a wide range of reactivity in the conversion of BW 78. Pyrrolidine was able to rapidly isomerise BW 78 even without the assistance of DBU. In presence of DBU, imidazole exhibited much slower reactivity as compared to pyrrolidine alone and indole was extremely sluggish in the isomerisation reaction.

These results indicate the relative importance of basicity and nucleophilicity of cofactors involved in the isomerisation reaction. Among thiols, it seems that increasing basicity (higher pK_a) enhances the isomerisation rate (Table 1). Similar conclusions cannot be drawn regarding role of isomerisation reactivity of thiols and alcohols because both butanol and phenol are more basic than their thiol analogs (Figure 1). In turn, thiols are much stronger nucleophiles (Edwards & Pearson, 1962). The amines studied have -NH groups with remarkable differences in their basicity. In this series increasing basicity enhances the reaction rate. In case of the very weak base indole about 2 days were necessary for 50% conversion of BW 78 even in the presence of DBU. In control experiments DBU also

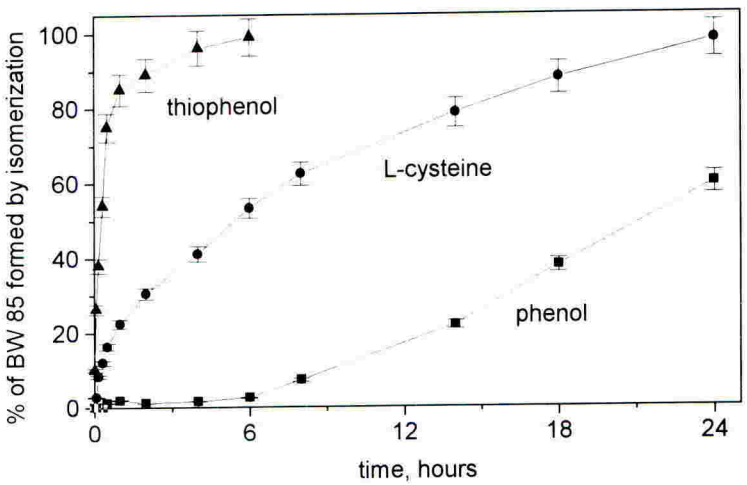


Figure 1. Time course of the conversion of BW 78 to BW 85 catalysed by mixtures of nucleophiles and DBU

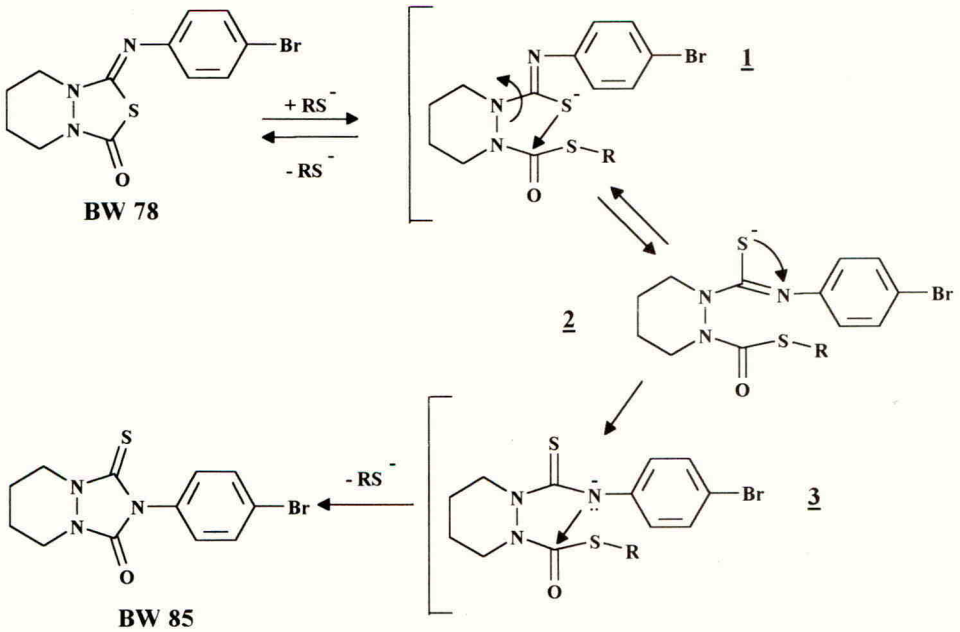
showed some ability to isomerise BW 78 (Table 1): during a 5-day exposure to DBU complete isomerisation was detected. Since the time required for 50% isomerisation of BW 78 was the same with or without DBU in case of indole we can assume that the isomerisation can exclusively be

attributed to the action of DBU. Studies with structurally different tertiary bases revealed that 4-dimethylaminopyridine (DMAP) which is also a strong base was barely able to catalyse the isomerisation without using any other nucleophile. When applied with thiophenol a much slower reaction took place compared with the DBU-catalysed isomerisation. However, no rearrangement occurred with 2,6-lutidine either alone or in mixtures with thiophenol or butane-1-thiol.

Table 2. Isomerisation of BW 78 with GSH in phosphate and Tris buffers

	% of BW 85 formed			
	K-phosphate buffer		Tris buffer	
Time (h)	pH 7.2	pH 7.8	pH 7.2	pH 9.0
1	0	0	0	0
17	0	traces	6.9	12.9

Since the above-mentioned results suggested that the basicity and the nucleophilicity of cofactors are of prime importance in the isomerisation we attempted to initiate the reaction in an aqueous solution using a buffer other than the non-nucleophilic phosphate. Tris (tris[hydroxymethyl]aminomethane) has a free primary amino group which can promote isomerisation by nucleophilic catalysis. Indeed, in Tris buffer GSH could give rise to slow isomerisation even in the absence of GST enzyme (Table 2). The reactions were more rapid at the higher pH value (Table 2).



Scheme 2. Possible mechanism of the isomerisation of BW 78 to BW 85

In summary, these findings suggest that the GST-catalysed rearrangement of thiazolidine herbicides may involve a nucleophilic attack by the anionic form of the thiol at the carbonyl group followed by a ring opening and formation of intermediate **1** (Scheme 2). Rotation around the N-C bond in **1** presumably gives intermediate **2** from which **3** is formed by tautomerisation. Finally, the highly nucleophilic negatively charged nitrogen atom of **3** attacks the thiolester part of the molecule yielding the triazolidin-one-thione end product.

The steps of this rearrangement mechanism are likely to be very rapid because no intermediate can be detected during the whole isomerisation process. The role of the specific GST-II isoform in the isomerisation (Nicolaus *et al.*, 1996) is probably the promotion of formation of intermediate **2**.

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A ROLE FOR GLUTATHIONE-S-TRANSFERASE IN HERBICIDE RESISTANCE IN BLACK-GRASS (*ALOPECURUS MYOSUROIDES*)

J P H READE, M R HULL, A H COBB
Department of Life Sciences, The Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, UK

ABSTRACT

In the UK, biotypes of black-grass (*Alopecurus myosuroides*) showing resistance to both chlorotoluron and aryloxyphenoxypropionate graminicides are being increasingly observed. However, the molecular bases of this resistance remain to be established. Whilst enhanced metabolism via cytochrome P_{450} has been implicated in the chlorotoluron resistant biotype Peldon, we have also observed elevated glutathione-S-transferase (GST) activity in these plants. The GST activity measured in resistant plants was approximately twice that of susceptible plants. Treatment with field-rate chlorotoluron did not induce further GST activity in either biotype.

Fourteen biotypes showing a variety of responses to fenoxaprop-ethyl (FE) and clodinafop-propargyl (CP) have also been studied with respect to *in vitro* GST activities. Five biotypes have been identified which show elevated GST activities and resistance to FE. A further four biotypes have been identified which have low GST activities and are susceptible to both herbicides. However, resistance in some biotypes could not be correlated to elevated rates of GST. We postulate that high GST activity may play a role in herbicide resistance in *A.myosuroides*. The enzyme has been subsequently purified and *in vitro* kinetic studies have been carried out in order to further study the role of GST in resistance in this important grass weed.

INTRODUCTION

Black-grass (*Alopecurus myosuroides*) is a major weed in cereal crops in the UK. Since the identification of resistance in this species to the herbicide chlorotoluron (CTU) in the early 1980s (Moss & Cussans, 1985) many cases of resistance and cross resistance have been identified (Clarke *et al.*, 1994). Enhanced metabolism has been implicated in this resistance, with much emphasis being placed on elevated levels or activities of cytochrome P_{450} monooxygenases (Hyde *et al.*, 1996). However, some evidence suggests that elevated activity of the detoxification enzyme glutathione-S-transferase (GST) may also play an important role in both weed resistance (Sharples *et al.*, 1997) and crop tolerance (Dixon *et al.*, 1997) to herbicides. GST activity towards the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) measured in the CTU-resistant *A.myosuroides* biotype Peldon was approximately double that of susceptible plants,

with a comparative ED_{50} of 42 on a fresh weight basis, using greenhouse grown plants (Sharples *et al.*, 1997).

GSTs are a group of enzymes responsible for the conjugation of the tripeptide glutathione to a variety of electrophilic substrates and their distribution and functions in plants have recently been reviewed by Marrs (1996). In plants they are generally found as hetero- or homodimers with subunit molecular weights of between 23 and 29 kDa. In *A.myosuroides* the spectrum of GSTs present is yet to be characterised. In this study, the resistance profiles for a range of *A.myosuroides* biotypes with respect to fenoxaprop-ethyl (FE) and clodinafop-propargyl (CP) sensitivity, and *in vitro* GST activity, were determined. GST was purified from a susceptible biotype and initial kinetic studies of this enzyme are reported.

MATERIALS AND METHODS

Plant material

Seed stocks of *A.myosuroides* biotypes were obtained from Herbiseed Ltd., Berkshire, UK (susceptible), Peldon, Essex, UK (resistant) and Novartis Crop Protection UK (of varying resistance and susceptibility to CP and FE). Seeds were sown in J Arthur Bowers multipurpose peat-based compost, watered from above and grown under glasshouse conditions (20°C day, 15°C night ± 5°C, 14h photoperiod with a photosynthetic flux density always above 120µmol m^{-1} sec^{-1}). Plants were harvested at the 2-3 leaf stage for all subsequent analyses.

Characterisation of resistance

Plants were sprayed with herbicides according to Sharples *et al.* (1997) and Mills and Ryan (1995) and fresh weights recorded.

Glutathione-S-transferase (GST) assay

Typically, 0.5g of leaf tissue was ground to powder in liquid N_2, thawed in 100mM potassium phosphate buffer pH 7.0 containing 5mM diethylenepentaacetic acid (DTPA), 10mM sodium ascorbate and 4% (w/v) polyvinylpolypyrrolidone, and homogenised on ice. The extract was centrifuged and the supernatant desalted on a Sephadex G-25 PD-10 column (Pharmacia Biotech Ltd., Hertfordshire, UK). The GST activity of a desalted leaf extract was assayed using the substrate 1-chloro-2,4-dinitrobenzene (CDNB) in 100mM phosphate buffer, pH. 6.5 containing 0.2mM DTPA, 1mM glutathione (GSH), 1mM CDNB and 50µl of the crude enzyme extract. For kinetic studies, each substrate concentration was varied whilst the other was held constant, and 100µl of purified GST was used in place of the cell free extract. In both cases the final assay volume was 1ml. The increase in absorption at 340nm was measured spectrophotometrically at 20°C.

GST purification from the biotype Herbiseed

The protocol for GST purification involved ammonium sulphate precipitation followed by ion exchange chromatography (Macro-prep high Q, Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts., UK). Further purification utilised a gel filtration step (Sephadex G-75) and affinity chromatography (Glutathione agarose, Sigma-Aldrich Co. Ltd., Poole, Dorset, UK). After each chromatography step fractions were assayed using CDNB and those with GST activity were pooled prior to the next step. Purity was assessed using SDS-PAGE. Further details will be provided elsewhere (Reade & Cobb, submitted for publication).

RESULTS

Fenoxaprop-ethyl (FE)

Of a total of 14 biotypes studied five biotypes have been identified which showed high GST activity and resistance to FE, numbered no.4,no.60,no.89,no.200 and Peldon in Figure 1. A further 4 biotypes with lower GST activity that are susceptible to FE have also been identified, namely no.83,no.120,no.234 and Herbiseed (Figure 1). Biotype no.1712 also showed a lower GST activity but did show some resistance to FE treatment.

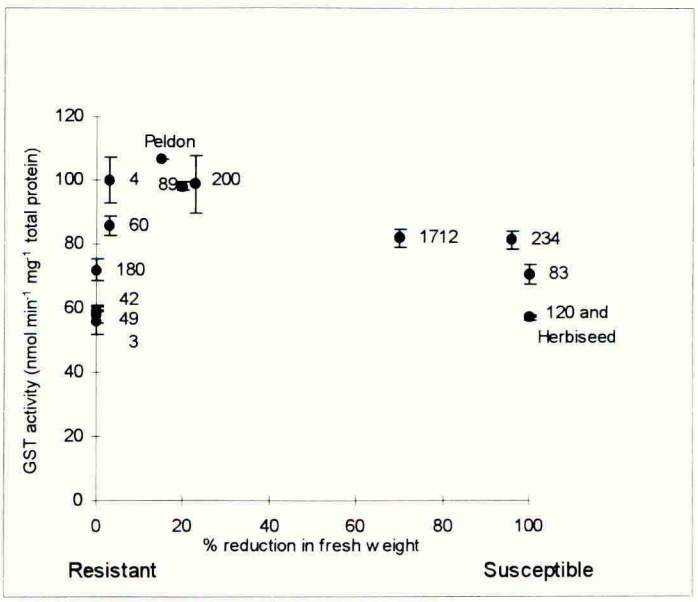

Figure 1: A plot of GST activity against plant fresh weight following application of fenoxaprop-ethyl (Cheetah Super, 27.5g a.i. ha^{-1}) (each number represents a separate biotype). Results are from a single experiment, but are typical of at least 3 observations.

Clodinafop-propargyl (CP)

Of a total of 14 biotypes studied, four biotypes have been identified which showed resistance to CP, numbered no.3,no.42,no.49 and no.180 in Figure 2. These did not show raised GST activities. Biotypes susceptible to CP showed a wide range of GST activities, both relatively high (no.4,no.60,no.89,no.200 and Peldon) and low (no.83,no.120,no.234 and Herbiseed) (Figure 2). Biotype no.1712, which demonstrated some resistance to CP treatment, also showed relatively low GST activity.

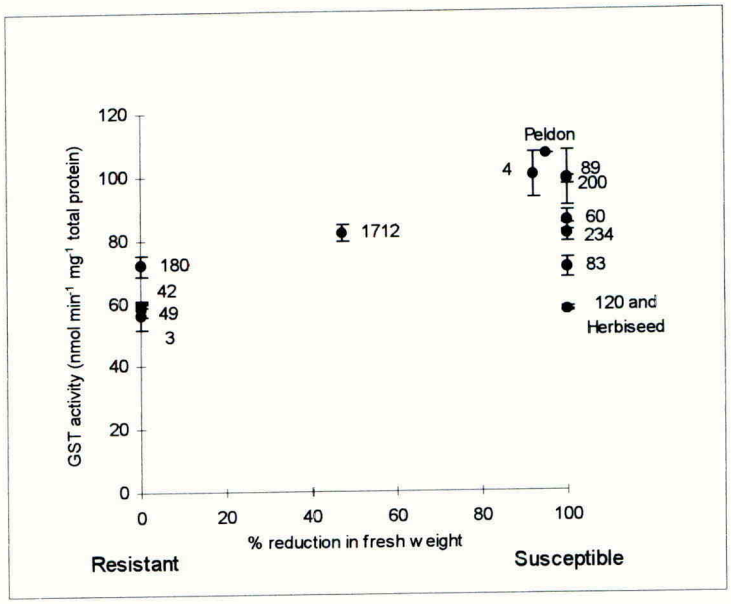

Figure 2: A plot of GST activity against plant fresh weight following application of clodinafop-propargyl (Topik, 30g a.i. ha^{-1})(each number represents a separate biotype). Results are from a single experiment, but are typical of at least 3 observations.

Protein purification and kinetic data

GST was purified as described and kinetic studies with respect to both substrates were carried out and values presented in Table 1.

Table 1: K_m and V_{max} values for GST purified from *A.myosuroides* (Herbiseed).

Substrate	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1} total protein)
GSH	1.07	5.27
CDNB	1.56	6.99

DISCUSSION

Fourteen biotypes of *A.myosuroides* studied showed a range of GST activities between 57nmol min^{-1} mg^{-1} total protein (Herbiseed) and 107nmol min^{-1} mg^{-1} total protein (Peldon). *In vitro* GST activity appeared to correlate with resistance to FE in some biotypes , and no biotype with high GST activity has been identified which is susceptible to FE (Figure 1). Since GST has been implicated in the metabolic detoxification of FE in cereal crops (Tal *et al*, 1993), it is not too surprising that elevated activity of this key enzyme can be correlated to resistance in a grass weed. However, there was no cross resistance in these biotypes to CP. In other biotypes resistance to FE was not accompanied with high GST activities, and these biotypes were resistant to CP (Figure 2). Thus high GST activities may play a role in the resistance mechanism towards FE in some *A.myosuroides* biotypes. However, this does not appear to be the only resistance mechanism present in this weed. A separate mechanism appears to confer resistance to both FE and CP. Whether this is due to raised activities of other enzymes involved in herbicide metabolism (e.g. cytochromes P_{450}) or a target site resistance has yet to be demonstrated.

Purification of GSTs from the Herbiseed biotype revealed one protein of molecular weight 28.5 kDa on SDS PAGE (Reade & Cobb, submitted for publication). Initial kinetic data are presented in Table 1. The K_m value for GSH compares favourably with that obtained in crude extracts from this biotype (Sharples *et al.*, 1997) and is similar to theoretical estimates of intracellular GSH concentrations (Hausladen & Alscher, 1993).The higher K_m value for CDNB probably reflects the fact that this is a model substrate. The K_m values for herbicide substrates remain to be determined.
The molecular weight obtained for GST from *A.myosuroides* compares well with molecular weights for other monocot weed and cereal GSTs (Marrs, 1996). In cereal and weed crops so far studied GSTs are reported to be homo- or hetero- dimers (Marrs, 1996). The native configuration of the GST in blackgrass remains to be established.
It hence appears that GSTs may play an important role in the mechanism of resistance to FE in *A.myosuroides*. It is hoped that further *in vitro* study of GST purified from *A.myosuroides* will provide a greater understanding of its role in resistance to FE, and its possible role in resistance to other herbicides.

CONCLUSION

GST activity can be correlated with resistance to FE in five of the biotypes of *A.myosuroides* studied. This resistance does not impart additional tolerance to CP. Biotypes resistant to both herbicides studied did not show raised GST activities and it therefore appears that this cross resistance is independent of a role for GST. Initial studies of GST purified from this weed suggest the methodology provides an excellent opportunity to study the factors affecting GST activity *in vitro*.

ACKNOWLEDGEMENTS

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CHARACTERISTICS OF ACETYL-CoA CARBOXYLASES FROM GRAMINICIDE-TOLERANT GRASSES

L J PRICE, J L HARWOOD
School of Molecular and Medical Biosciences, University of Wales Cardiff, PO Box 911, Cardiff CF1 3US, UK

D J COLE
Rhône-Poulenc Agriculture Ltd., Ongar, Essex CM5 OHW, UK

S R MOSS
IACR-Rothamsted, Harpenden, Herts. AL5 2JQ, UK

ABSTRACT

Graminicides of the aryloxyphenoxypropionate or cyclohexanedione classes act on acetyl-CoA carboxylase (ACCase). Resistance is a problem either in inherently insensitive weed species or when acquired following herbicide use in the field. In an effort to understand the molecular mechanisms responsible for herbicide-insensitivity, we have studied acetyl-CoA carboxylases from maize (sensitive), *Festuca rubra* (inherently-insensitive) and resistant or sensitive populations of *Alopecurus myosuroides*. These studies have included acetyl-CoA carboxylases isolated from both leaves and cell cultures. Isozymes have been separated from a tolerant cell culture of *A. myosuroides*, which differ in their pH optima, and sensitivity to quizalofop. The major and minor isozymes are 1,300-fold and 300-fold less sensitive to quizalofop, respectively, than the major ACCase isozyme isolated from maize leaves. Kinetic characteristics of the various purified ACCase isozymes have been evaluated and are reported here. Our results show clearly that resistance in the Notts A1 population of *A. myosuroides* to quizalofop and sethoxydim is due to an altered form of ACCase which is much less sensitive to inhibition. The data also show the utility of using tissue cultures where leaf material is limited.

INTRODUCTION

Graminicides of the aryloxyphenoxypropionate (AOPP), and cyclohexanedione (CHD) classes are used for the post-emergence control of grasses in broad-leaved crops. The site of action of these herbicides is acetyl-CoA carboxylase (ACCase), and selectivity in broad leaved crops is normally high, being attributable to altered target sites in susceptible and tolerant plant species. However, diclofop, fenoxaprop and clodinafop are all members of the AOPP family which are used for weed control in cereal crops due to the ability of the crops to detoxify the compounds.While most annual and perennial grasses are susceptible to compounds from both chemical classes, some grass species are known to be inherently resistant to some, or all graminicides. Among these are several of the species of the genus *Festuca* which are used as turf or forage and *Poa annua*, a problem weed in turf grasses.

As well as those grasses that show inherent resistance to the graminicides, a number of species are now giving rise to populations which have acquired the ability to tolerate these compounds at normal application rates. Resistance to diclofop, a member of the AOPP class of graminicides, was first reported in 1982, just five years after its introduction in 1977 (LeBaron, 1991). The wide and persistent use of these graminicides since then has led to the appearance of resistant populations of several grass species including *Lolium multiflorum* (Betts *et al.*, 1992), *Setaria viridis* (Marles *et al.*, 1993), and *Alopecurus myosuroides* (Hall *et al.*, 1993). While resistance to many herbicides can be attributed to decreased uptake or translocation, or increased metabolism, there have been reported cases of graminicide resistance shown to be at the level of acetyl-CoA carboxylase (Parker *et al.*, 1990; Tardiff *et al.*, 1993). One population of *A.* myosuroides, Lincs. E1, in addition to non-target site cross-resistance conferred by enhanced herbicide metabolism of diclofop-methyl and fenoxaprop-ethyl, included individuals (approximately 15% of the population) with resistant ACCase (Hall *et* al., 1993). In most cases of target site resistance, tolerance is thought to be conferred by a single point nuclear mutation encoding a herbicide-insensitive ACCase.

Our studies have focused on the Notts A1 biotype of *A. myosuroides* which was discovered in Nottinghamshire, England in 1992 as showing resistance to the AOPP herbicides, following its first exposure to this class of herbicide in 1989. In previous studies, this Notts A1 population showed very high resistance to five ACCase inhibiting herbicides in glasshouse dose response assays (Moss & Clarke, 1995). The resistance indices (the ratios of ED_{50} values relative to a susceptible standard) were: sethoxydim >400; tralkoxydim >14; fluazifop 37; fenoxaprop 191; diclofop 13. We present here, evidence that resistance in this biotype is due to an altered acetyl-CoA carboxylase enzyme.

MATERIALS AND METHODS

Plant material and growth conditions

Seed of resistant (Notts A1) *A. myosuroides* were collected from a field in Nottinghamshire UK where weed control had been poor, and susceptible (Rothamsted) *A. myosuroides* seeds were collected from a field at IACR-Rothamsted, UK. *F. rubra* seeds were purchased from Herbiseed Ltd., UK, and maize (cv. Celebration) seeds purchased from Nickerson Seeds Ltd., UK. Seeds were grown in a soil-less compost, under a covering of *c.*1cm. Plants were then grown in a growth cabinet (Birchover Instruments Ltd) at 20°C with a 14 hour light period.

The maize suspension culture used was of Black Mexican Sweetcorn. Callus cultures of *F. rubra* and *A. myosuroides* were initiated from sterilised seeds on Murashige & Skoog basal salts media (Sigma, UK), containing 2mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 20g/l sucrose. Suspension cultures were derived by placing in a similar liquid medium and were then maintained in 250ml media in 500ml flasks, rotating at 200 rev/min on an orbital shaker at 27°C under fluorescent lighting. Cells were sub-cultured into fresh media every 7 days.

Hydroponic studies

Seeds of *A. myosuroides* were germinated in vermiculite at 20°C. When the seedlings were 4-5cm long, they were carefully removed and transferred to glass vials with the roots suspended in 20ml of inorganic salts nutrient solution (Hewitt, 1966) containing the appropriate concentrations of sethoxydim or quizalofop in 1% ethanol. The vials were then covered in foil. Seedling height and herbicide injury were recorded 7 DAT.

^{14}C-Acetate incorporation studies

Leaf blades from maize seedlings of uniform height, (*c.*3cm) were cut underwater and placed in glass vials containing herbicide solutions in 1% ethanol (v/v), a concentration found not to be inhibitory to lipid synthesis. Triplicate samples were pre-incubated in the herbicide solution, overnight (15h) at room temperature, with a constant draught to aid transpiration. After this period, the seedlings were transferred to clean tubes containing 37kBq (5µl) [1-^{14}C]acetate in 0.25ml of the appropriate herbicide solution, and incubated for a further 4h with supplementary illumination. A two-phase lipid extraction (Garbus *et al.*, 1963), was performed following denaturation of the endogenous lipases by heating in isopropanol. The method was modified for the finer leaves of *A. myosuroides*, by taking 0.1g of young leaf tissue (7 days following emergence) and floating on 8ml of herbicide solution in 1% ethanol in a petri dish, which was shaken for 4h. 128kBq (20µl) [1-^{14}C]acetate was then added to the herbicide solution, and shaking was continued for a further 4h, under lighting. Suspensions of grass cell cultures (25ml in 50ml flasks) were sub-cultured into fresh medium using a 20% inoculum. Three days after sub-culture, herbicide was added in 1% ethanol. After 24 hours, 128kBq [1-^{14}C]-acetate was added, followed by a 4h incubation under lighting. The cells were filtered under vacuum before lipid extraction.

Lipid analysis

Lipid classes were separated for analysis on TLC silica gel 60 plates (Merck, Germany), resolved in either petroleum ether (60-80°C b.p. fraction)/diethyl ether/acetic acid (80:20:2) or chloroform/methanol/glacial acetic acid/water (170:30:20:7). Lipids were revealed by spraying with a 0.02% solution of 8-anilino, 4-naphthosulphonic acid. Radiolabelled bands were observed either by autoradiography in a spark chamber (Birchover Instruments, UK) or by imaging using a Fujix Bas1000 phosphoimager (Fuji) with "Tina" software from Raytek Scientific.

Acetyl-CoA carboxylase assay

Acetyl-CoA carboxylase activity was measured using the method of Burton (1991), by measuring the rate of incorporation of [^{14}C]-bicarbonate, into acid-stable material, at 30°C, over 20 minutes. Samples were evaporated to dryness on a heating block, before adding 50µl distilled H_2O, and Opti-Fluor scintillant (Canberra Packard) and counting for 1min in a Beckman 1209 Rackbeta liquid scintillation counter. Quench correction was by the external standard channels ratio method.

Purification of acetyl-CoA carboxylase

ACCases were partially purified in a three-step procedure, employing ammonium sulphate fractionation, gel filtration chromatography, and Q-Sepharose anion-exchange chromatography., achieving up to 100-fold purification of the enzyme. Leaves were used at the one-leaf stage (7 days following emergence), and cells were used 7 days after sub-culturing. The purification method was comparable to that outlined in Herbert *et al.* (1996).

RESULTS AND DISCUSSION

Hydroponic growth of the *A. myosuroides* seedlings in the presence of the two herbicides showed the resistant Notts A1 population to be more than 200-fold less susceptible to quizalofop, and more than 100-fold less susceptible to sethoxydim (not shown). Similarly, at concentrations of quizalofop and sethoxydim that inhibited acetate incorporation into total lipids in the susceptible population by 70% and 60% respectively, we found just 30% and 0% inhibition of acetate incorporation, in the tolerant population. Enhanced detoxification or metabolism of the herbicides has been ruled out as the main basis of resistance to the graminicides in the Notts A1 population, as tolerance to both quizalofop and sethoxydim was also observed in partially purified extracts of ACCase from leaf tissue (Table 1).

Table 1. Inhibition of the incorporation of radioactivity from [^{14}C]bicarbonate into malonyl-CoA by partially purified acetyl-CoA carboxylases from leaf tissues.

	Sethoxydim I_{50} (μM)	Quizalofop I_{50}(μM)
Maize ACCase1	n.m.	0.03
Maize ACCase2	n.m.	60
A.myosuroides (Rothamsted	4	0.1
A.myosuroides (Notts A1)	>100	28
F.rubra	n.m.	40
P.annua	n.m.	10

Abreviation: n.m.,not measured

Evaluating the use of cell cultures as a model for plant systems

Due to the fineness of the *A. myosuroides* leaf blades and the limited supply of the Notts A1 population of seeds, the extent of studies performed on the leaf tissue, or leaf extracts, was very limited. This led us to initiate callus from the resistant seed line. The rapidly dividing cells provided a good source of ACCase, with the enzyme from cultures possessing similar properties to that from leaves.

In vivo studies with hydroponic systems, and on whole cells, showed that graminicide resistance observed in the whole plant is mirrored in the cell cultures. Also, purified

ACCase from leaves and cell cultures showed similar susceptibilities to graminicides with an I_{50} value of 26μM for quizalofop in the Notts A1 leaf ACCase and 28μM in the cell ACCase. Both quizalofop and sethoxydim affected the uptake of [^{14}C]acetate in both the leaf and cell culture systems with similar effects on the lipid classes and constituent fatty acids, as observed using thin layer chromatography and gas chromatography. A blanket reduction in incorporation into all acyl lipid classes was observed in both cases, with an increase in the incorporation of acetate into palmitate compared to the 18C fatty acids with both herbicides, due to a reduction in the available malonyl-CoA.

Initial attempts at purification of ACCase from tissue culture focused on maize cells, due to our previous experience with maize leaf tissue. The maize cell extract behaved exactly as the leaf tissue extract (Herbert *et al.*, 1996), when subjected to gel filtration chromatography, affinity chromatography and anion exchange chromatography. The two maize ACCase isoforms were separated on an anion exchange column, and were found to be present in the same proportions as in maize leaf tissue, with the minor, extraplastidic isoform representing *c.* 20% of the total activity (see Herbert *et al.*, 1996). Inhibition of the two isoforms from cell cultures with quizalofop was also similar to that in the leaf isoforms, with the major isoform being *c.* 2000-fold less sensitive to quizalofop than the minor. Two isoforms have subsequently been isolated from the Notts A1 cell line, which differ in their pH optima and sensitivity to quizalofop.

Km values have been obtained for each of the substrates of the ACCase reaction in each of the partially purified enzyme extracts. These values vary between the species, demonstrating the diversity of the ACCase enzymes (Table 2).

Table 2. Apparent Km values for the substrates of the ACCase reaction in various purified grass ACCases

Substrate	*F.rubra*	Notts A1 (major)	Notts A1 (minor)	*Maize ACCase1	*Maize ACCase2
Acetyl-CoA (μM)	0.20	0.34	0.10	0.12	0.11
ATP (μM)	0.25	0.42	0.49	0.08	0.08
HCO_3^- (μM)	0.12	1.36	1.13	1.09	0.39

These apparent Km values were obtained by fitting data to an Eadie-Hofstee plot (V vs V/S).
*These values were obtained from Herbert *et al*, 1995, and refer to ACCase obtained from leaf tissue.

The results reported here confirm the resistance of the *A. myosuroides* Notts A1 population to the graminicides as being attributable to an insensitive form of acetyl-CoA carboxylase. The use of cell cultures as a potential source of ACCase has also been evaluated, and promises to be a useful tool for studying the mechanisms of graminicide resistance, given the depleted state of the Notts A1 seed supply.

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PURIFICATION AND CHARACTERISATION OF GLUTATHIONE TRANSFERASE ENZYMES FROM SOYBEAN SEEDLINGS

M SKIPSEY, C J ANDREWS, R EDWARDS
Department of Biological Sciences, University of Durham, South Road, Durham, DH1 3LE, UK

J K TOWNSON, I JEPSON
Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, RG42 6ET, UK

ABSTRACT

Soybean (*Glycine max*) is known to contain multiple GSTs but their role in herbicide detoxification and endogenous metabolism has not been well defined. In this study we have purified several GST isoenzymes from 5-day-old soybean seedlings, and determined their activity towards chemically diverse xenobiotic substrates including the soybean selective herbicides; metolachlor and chlorimuron-ethyl. GST isoenzymes were purified by a combination of hydrophobic interaction chromatography, affinity chromatography using *S*-hexyl-glutathione, and anion exchange chromatography. Using the different herbicide substrates it was possible to resolve the GST activities into at least three isoenzymes composed of polypeptides with molecular masses in the range 25kDa to 29kDa.

INTRODUCTION

Glutathione transferases (GSTs, EC 2.5.1.18) catalyse the detoxification of a diverse range of electrophilic, and often hydrophobic compounds by conjugation with the tripeptide glutathione (GSH, gamma-glutamyl-L-cysteinyl-L-glycine). These enzymes are found in a wide variety of organisms. As is the case in animals, plants contain multiple GST isoenzymes, composed of two subunits, each encoded by a distinct gene (Marrs 1996).

A number of herbicides used to control weeds selectively in soybean are rapidly detoxified by conjugation with homoglutathione (hGSH, gamma-glutamyl-L-cysteinyl-ß-alanine), for example, chloroacetanilide herbicides (Breaux 1986), acifluorfen (Frear *et al.*, 1983), chlorimuron-ethyl (Brown and Neighbors 1987) and fomesafen (Evans *et al.*, 1987). In soybean, hGSH rather than GSH is the predominant thiol and as such is used for conjugation. Our recent studies have suggested that the conjugation of these herbicides is catalysed by GSTs (Andrews *et al.*, In press) and soybean has recently been shown to contain multiple GSTs (Flury *et al.*, 1996) but their role in herbicide detoxification and endogenous metabolism has not been defined.

Previous studies have isolated a soybean GST with activity toward 1-chloro-2,4-dinitrobenzene (CDNB) and metolachlor (Flury *et al.*, 1995). A cDNA clone which we isolated using a probe based on the amino acid sequence of this purified protein has proven to show activities toward CDNB, metolachlor, fluorodifen, fomesafen and acifluorfen when expressed as a recombinant protein (Andrews *et al.*, 1997). A distinct stress-inducible

soybean GST has also been cloned (Ulmasov *et al.*, 1995; Czarnecka *et al.*, 1988) and we have demonstrated that the recombinant enzyme is active as a homodimer towards diverse xenobiotics (Skipsey *et al.*, 1997). However, analysis of recombinant GSTs cannot define their relative importance in the detoxification of herbicides in whole plants. To address this question a defined fraction of the GSTs from soybean seedlings have been purified and the complexity of GST subunits and their relative concentrations determined.

MATERIALS AND METHODS

Plant material

Seeds of soybean (cv. D297) were supplied by Zeneca Agrochemicals. For the determination of enzyme activities, seeds were imbibed in tap water for 3 hours, sown in vermiculite, and grown in a controlled environment chamber at 25°C with a 16h photoperiod and a light intensity of 140 µmols/m/s. Plants were watered daily. At 5 days after sowing the plants were uprooted, washed free of vermiculite with tap water, blotted dry, weighed and frozen in liquid nitrogen.

Purification of active GSTs

Frozen plant tissue was ground to a fine powder with a mortar and pestle using liquid nitrogen. The powder was then thawed in 3ml/g 0.1M Tris-HCl pH 7.5 containing 2mM EDTA, 1mM dithiothreitol and 150g/kg polyvinylpolypyrrolidone. The slurry was further homogenised with the aid of acid-washed sand, prior to being filtered through a double thickness of muslin. The homogenate was centrifuged (17,000*g*, 20 min, 4°C) and the supernatant decanted and mixed with an equal volume of hexane to remove the lipid portion of the extract. The aqueous phase was then repartitioned with hexane prior to adjusting to 80% saturation with respect to ammonium sulphate. The protein pellet was then collected by centrifugation as above, dissolved in buffer A (10mM potassium phosphate buffer pH 7.4 containing 1mM dithiothreitol), and dialysed overnight against the same buffer containing 500mM ammonium sulphate and 200mM potassium chloride. The extract was loaded onto a phenyl Sepharose CL-4B column and the column 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, to elute the more polar GSTs. The bound hydrophobic proteins were recovered from the column by washing with 50% ethylene glycol and 50% buffer A. The ethylene glycol was removed from this fraction by loading onto a Q Sepharose column. After washing with buffer B (20mM Tris-HCl pH 7.8 containing 1mM dithiothreitol) the protein was eluted with buffer B + 0.5M NaCl. The polar protein fraction was loaded onto a *S*-hexyl-glutathione affinity column. Following loading, the column was first washed with buffer A until no more protein was eluted, and then with buffer A + 200mM potassium chloride. The affinity bound protein was eluted by the final wash of buffer A + 200mM potassium chloride + 5mM *S*-hexyl-glutathione.

Analysis of affinity bound proteins

Following overnight dialysis against buffer B, the affinity bound protein was analysed by anion exchange chromatography. The protein was loaded onto a Q Sepharose column in

buffer B and eluted with a 50ml linear gradient of increasing NaCl concentration up to 0.5M in buffer B. Each of the resulting fractions (1ml) were assayed for GST activities. Those fractions showing activities of interest were analysed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Denatured protein extracts were loaded onto a 0.8mm-thick mini-gel composed of 17.5% acrylamide and 0.1% N'N'-*bis*-methylene-acrylamide. Following electrophoresis at 200V the gel was stained with B/T-blu stain as described by the manufacturer. Fractions were also analysed by HPLC using a VYDAC C18 column equilibrated at 0.5ml/min with 10% acetonitrile in 0.1% trifluoroacetic acid (TFA). A sample was injected onto the column and the following linear gradient used to separate peptides: 10% to 80% acetonitrile over 45 min followed by 100% acetonitrile for 5 min to clean the column. The UV absorbance (at 280nm) of the column eluant was monitored.

GST assays

During purification procedures GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) was determined using a spectrophotometric assay (Habig *et al.*, 1974).

For GST assays with the herbicides, incubations consisted of 10µl of 10mM herbicide dissolved in either acetone (chlorimuron-ethyl) or methanol (metolachlor), 20µl of 100mM GSH or hGSH and 25-125µl of enzyme extract, made up to a final volume of 200µl of the relevant buffer. With chlorimuron-ethyl as substrate the buffer used was 0.4M Tris-HCl pH 9.5 and the thiol was hGSH, and for metolachlor 0.1M potassium phosphate pH 6.8 was used with GSH. In all cases the assays were initiated with the addition of the thiol, and the samples incubated at 37°C for 1 hour. The reaction was terminated by the addition of 10µl of concentrated HCl, and stored at -20°C until required. The precipitated proteins were removed by centrifugation (12,000*g*, 5 min) prior to analysis of 50µl of the supernatant by HPLC (Hatton *et al.*, 1996).

RESULTS AND DISCUSSION

Purification of soybean GSTs

In initial studies investigating the complexity of GST isoenzymes in soybean, crude protein extracts were prepared from whole 5-day-old soybean seedlings. Processing of the crude extract proved difficult due to the presence of large amounts of lipids, which had to be removed by solvent extraction with hexane. The defatted extract was then applied to a phenyl Sepharose column in the presence of salt. All of the GST activity toward CDNB was retained on the column suggesting that soybean GSTs are relatively hydrophobic proteins. The majority of the GST activity (75%) was then recovered by removing the salt from the eluting buffer. A further 25% of the activity was recovered after washing the column with ethylene glycol. The partitioning of the GST activity on the phenyl Sepharose column demonstrates that soybean contains GST isoenzymes which differ in their hydrophobicities. Interestingly, when extracts from soybean cell cultures were applied to a similar column, the majority of the GST activity (87%) was recovered in the ethylene glycol wash rather than in the low salt wash (unpublished data). In the case of the extract from soybean seedling, the major fraction of the activity eluting from the phenyl Sepharose column in the low salt fraction was applied

to an affinity column of *S*-hexyl-glutathione. Using the affinity column, again the GST activity could be partitioned into multiple fractions. The majority of the activity toward CDNB (60%) was unretained on the column. Similarly, the unretained fraction did not bind to a fresh affinity column of *S*-hexyl-glutathione confirming that the GSTs in this fraction had a poor affinity for this ligand. Of the remaining 40% of the GST activity retained on the first affinity column, one half was weakly-bound, being eluted in the presence of salt, while the other half was tightly bound, being recovered using *S*-hexyl-glutathione in the eluting buffer. SDS-PAGE analysis showed that the unretained and weakly-bound GST fractions contained multiple polypeptides and their purification was not attempted. However, when analysed by SDS-PAGE the affinity-bound GSTs were found to be enriched with polypeptides of molecular masses in the range 25kDa to 29kDa (data not shown). Resolution of the polypeptides present in this fraction by reversed-phase HPLC revealed a number of UV absorbing peaks, consistent with the presence of multiple GST subunits (Figure 1).

When analysed on Q-Sepharose, the absorbance of the eluate at 280nm suggested the presence of two UV absorbing peaks, the larger first peak encompassed fractions 18 to 27 and the second relatively smaller peak contained fractions 29 to 36 (data not shown). The individual fractions were then assayed for GST activity toward CDNB and the herbicide substrates. All of the GST activities eluted between fractions 17 and 36 and this portion of the eluant was analysed in detail (figure 2). Three peaks of GST activity could be determined. Peak 1, which coincided with a peak of UV absorbance showed high activity toward CDNB and some activity toward metolachlor, while Peak 2 was associated with further conjugating activity toward metolachlor. The late eluting peak 3 contained measurable activity toward chlorimuron-ethyl only.

Multiplicity of GST isoenzymes in soybean seedlings

Our results demonstrate that GST activities in soybean seedlings can be fractionated on the base of their hydrophobicities, affinities for *S*-hexyl-glutathione and isoelectric points. In this initial report we have concentrated on the more polar GSTs which bind to *S*-hexyl-glutathione. This fraction was composed of some seven polypeptides and contained at least three distinct peaks of activity. Significantly, these peaks differed in their relative activities toward CDNB and the herbicides metolachlor and chlorimuron-ethyl. This result suggests that different GST isoenzymes are responsible for detoxifying the selective herbicides and that the respective isoenzymes are specific for different classes of herbicide substrate. We have previously demonstrated that crude extracts from soybean plants show GST activity toward metolachlor (Andrews *et al.*, In press). However no GST activity toward chlorimuron-ethyl could be detected. The observation that a peak of GST activity toward chlorimuron-ethyl could be determined in the purified enzyme fraction confirms that soybean contains GSTs active in detoxifying this sulphonylurea, supporting our previous suggestion that soybean requires such GSTs to detoxify this herbicide (Andrews *et al.*, In press)

Having characterised a relatively minor fraction of the GSTs present in 5-day-old soybean plants we will now study the remaining GSTs and determine their importance in herbicide metabolism.

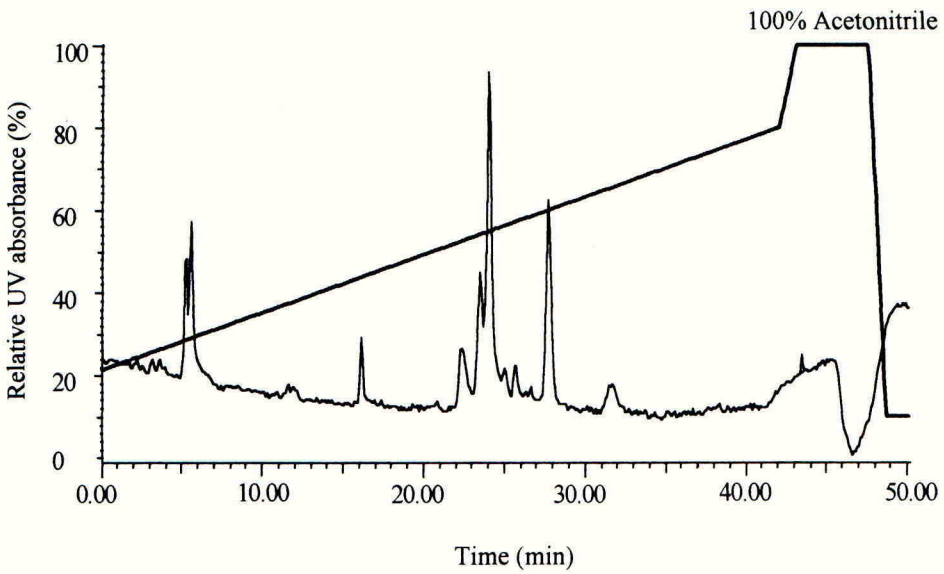

Figure 1. Reversed phase HPLC analysis of the affinity-bound GSTs used for anion exchange. The y-axis represents an arbitrary scale of UV absorbance and the x-axis represents time of the run in minutes.

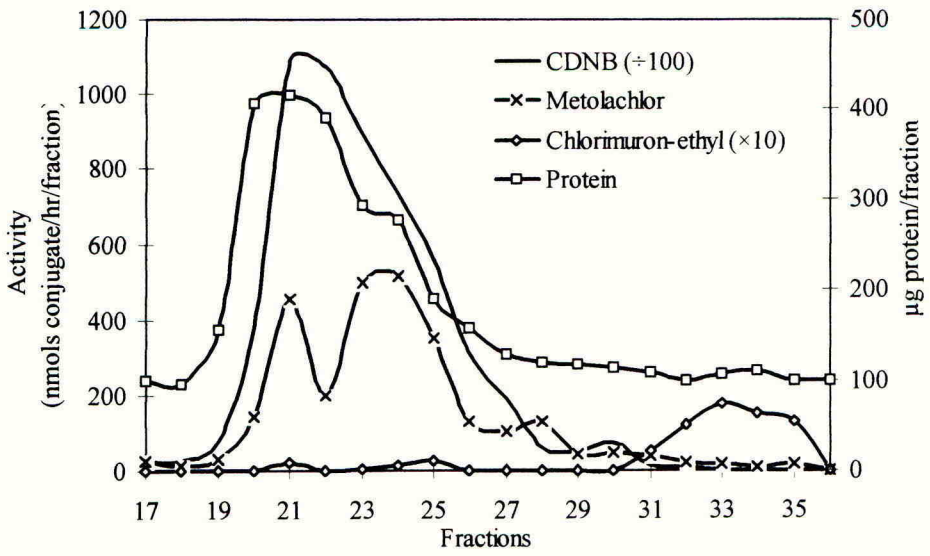

Figure 2. Protein content and GST activities towards a range of substrates in different fractions eluted from a Q-Sepharose column with a NaCl gradient. Conversion factors used to plot the enzyme activities to the same scale are given in parentheses.

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PHYTOTOXICITY OF AUSTRALIFUNGIN AND FUMONISINS TO WEEDS

H K ABBAS
Southern Weed Science Laboratory, USDA-ARS, Stoneville, Mississippi 38776, USA

W T SHIER
College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455, USA

ABSTRACT

Fumonisin B_1 (FB_1) and AAL-toxin are phytotoxic to susceptible plants by inhibition of the enzyme ceramide synthase. Australifungin is a structurally unrelated fungal metabolite that also inhibits ceramide synthase. FB_1 and AAL-toxin at 1 μM caused cellular leakage and chlorophyll loss from duckweed (*Lemna pausicostata*) plants, and from leaf discs of jimsonweed (*Datura stramonium*) and black nightshade (*Solanum nigrum*), beginning at 24 hr. Australifungin caused phytotoxicity at higher concentrations including cellular leakage and chlorophyll loss from *L. pausicostata* at 5 and 10 μM, and from *D. stramonium* and *S. nigrum* at 10 and 20 μM. An analog, australifunginol, caused no phytotoxicity at any concentration.

INTRODUCTION

Fumonisin B_1 (FB_1) and AAL-toxin are structurally related toxins produced by fungi, mainly *Fusarium* spp. and *Alternaria alternata*, respectively (Abbas *et al.*, 1997b). FB_1 was originally described as a mammalian toxin, causing mycotoxicoses such as equine leukoencephalomalacia and pulmonary edema in swine. AAL-toxin was first described as a host-specific phytotoxin to *asc/asc* tomatoes. Because of structural similarities, FB_1 and AAL-toxins were tested for their phytotoxicity to various plants and found to be herbicidal to a variety of plant species (Abbas & Boyette, 1992; Abbas *et al.*, 1995).

Further studies have shown that both toxins act by disruption of sphingolipid synthesis through inhibition of the enzyme ceramide synthase (Abbas *et al.*, 1994). Inhibition of ceramide synthase by FB_1 and AAL-toxin is believed to be related to the striking structural analogy they bear with sphinganine and sphingosine, which are both substrates for ceramide synthase (Riley *et al.*, 1996). The known mammalian toxicity of FB_1 makes it unsuitable as a herbicide but there has been interest in developing other compounds that retain this unique site of action, but are not toxic or are minimally toxic to humans and animals.

Australifungin, produced by *Sporomiella australis,* also acts by inhibition of ceramide synthase. It is, however, structurally unrelated to FB_1 and AAL-toxin. It was therefore decided to compare the responses of susceptible plants, including *L. pausicostata*, *D. stramonium* and *S. nigrum* to ceramide synthase inhibitors that are sphingosine analogues with those which are not.

MATERIALS

Fumonisins fall into 4 series, FA, FB, FC, and the 3-hydroxypyridine series (FP). The B series, of which FB_1 is most abundant in nature, include FB_1, FB_2, FB_3 and FB_4 (Gelderblom *et al.*, 1988). The C-series (Braham & Plattner, 1993; Seo *et al.*, 1996) have one less carbon in the backbone than the B series. Recently, the pyridine group, FP_{1-4}, has been discovered (Musser *et al.*, 1996). These contain a 3-hydroxypyridinium functional group where the FB and FC series have an amine group and FA has an acetylated amine (Figure 1). We have also tested the hydrolysis products, HFB_1 and HFB_2 (Figure 1). AAL-toxin exists in five forms, T_A, T_B, T_C, T_D and T_E, each with two isomers (Abbas *et al.*, 1997a). T_C has not been available. Australifungin and australifunginol, a related compound isolated from the same fungus, were also tested (Figure 1).

BIOASSAY SYSTEMS

A number of bioassays are useful for determining phytotoxicity of the fumonisins to plants and mammalian tissue. *L. pausicostata* is a small aquatic plant which shows chlorosis and necrosis when exposed to fumonisins and their analogues. This assay lends itself to measurement of increases in conductivity of the bathing media and determination of chlorophyll loss, as well as quantitation of sphingoid bases. Leaf discs of susceptible plants allow similar measurements. Intact plants are used to demonstrate the effects of the toxins on the entire organism. This assay allows measurement of mobility and application of the toxin in *in vivo* situations.

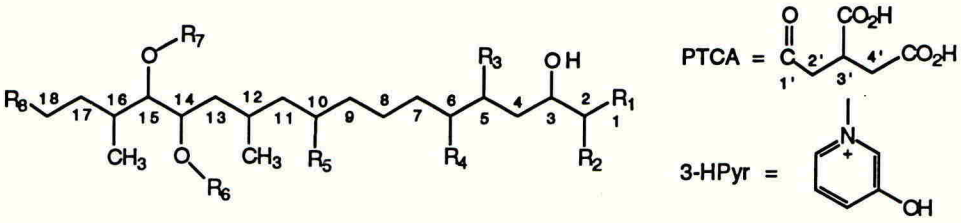
Mammalian toxicity can be estimated by screening in mammalian tissue cell cultures. We have used H4TG rat hepatoma and MDCK dog kidney cells which are susceptible to the fumonisins and NIH3T3 mouse fibroblasts which are naturally resistant to these compounds.

PHYTOTOXICITY AND CYTOTOXICITY

The fumonisins are toxic to a number of broadleaf weeds, including *D. stramonium*, *S. nigrum*, etc, (Abbas & Boyette, 1992; Abbas *et al.*, 1995). FB_1 is active on plant tissue by contact, but it is also xylem mobile (Abbas *et al.*, 1997b). The B series of fumonisins are the most toxic in a *L. pausicostata* bioassay. FA_1 and FA_2 were not found to be phytotoxic in any bioassay. The C series, although present in small amounts, are as active as the B series. FP_1 has slight activity at high concentrations (Abbas *et al.*, unpublished data). AAL-toxins Type A and B are the most active compounds in the *L. pausicostata* bioassay. The two isomers are equivalent. T_D and T_E caused phytotoxicity at high concentrations (Abbas, *et al.*, 1997c).

Australifungin is 8 to 10 times less active than AAL-toxin. It also causes somewhat different symptoms in the *L. pausicostata* bioassay, causing clumping of the plants and photobleaching of the central leaflet only. Australifunginol has no detectable activity (Abbas *et al.*, 1997c). In *L. pausicostata* assays, HFB_1 at 1 μM caused cellular toxicity measured by electrolyte leakage and chlorophyll content loss, with HFB_2 being somewhat less toxic. Both derivatives caused less cellular toxicity than FB_1 and AAL-toxin at 1 μM (Tanaka *et al.*, 1993).

In *S. nigrum* and *D. stramonium* leaf disc assays, FB_1 and AAL-toxin at 1 μM caused measurable cellular toxicity determined by electrolyte leakage and decrease in chlorophyll content at 48 hrs after the initial treatment. Cellular toxicity increased with increasing concentration of toxins to



Toxin	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
FA_1	CH_3	$NHCOCH_3$	OH	H	OH	PTCA	PTCA	C_2H_5
FA_2	CH_3	$NHCOCH_3$	OH	H	H	PTCA	PTCA	C_2H_5
FB_1	CH_3	NH_2	OH	H	OH	PTCA	PTCA	C_2H_5
FB_2	CH_3	NH_2	OH	H	H	PTCA	PTCA	C_2H_5
FB_3	CH_3	NH_2	H	H	OH	PTCA	PTCA	C_2H_5
FB_4	CH_3	NH_2	H	H	H	PTCA	PTCA	C_2H_5
FC_1	H	NH_2	OH	H	OH	PTCA	PTCA	C_2H_5
FC_2	H	NH_2	OH	H	H	PTCA	PTCA	C_2H_5
FC_3	H	NH_2	H	H	OH	PTCA	PTCA	C_2H_5
FC_4	H	NH_2	H	H	H	PTCA	PTCA	C_2H_5
FP_1	CH_3	3-HPyr	OH	H	OH	PTCA	PTCA	C_2H_5
FP_2	CH_3	3-HPyr	OH	H	H	PTCA	PTCA	C_2H_5
FP_3	CH_3	3-HPyr	H	H	OH	PTCA	PTCA	C_2H_5
HFB_1	CH_3	NH_2	OH	H	OH	H	H	C_2H_5
HFB_2	CH_3	NH_2	OH	H	H	H	H	C_2H_5
AAL-T_{A1}	H	NH_2	OH	OH	H	PTCA	H	H
AAL-T_{A2}	H	NH_2	OH	OH	H	H	PTCA	H
AAL-T_{B1}	H	NH_2	OH	H	H	PTCA	H	H
AAL-T_{B2}	H	NH_2	OH	H	H	H	PTCA	H
AAL-T_{C1}	H	NH_2	H	H	H	PTCA	H	H
AAL-T_{C2}	H	NH_2	H	H	H	H	PTCA	H
AAL-T_{D1}	H	$NHCOCH_3$	OH	H	H	PTCA	H	H
AAL-T_{D2}	H	$NHCOCH_3$	OH	H	H	H	PTCA	H
AAL-T_{E1}	H	$NHCOCH_3$	H	H	H	PTCA	H	H
AAL-T_{E2}	H	$NHCOCH_3$	H	H	H	H	PTCA	H

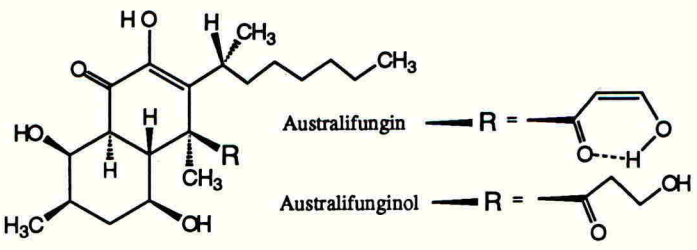


Figure 1. Structures of fumonisins, AAL-toxins, australifungin and australifunginol.

10 μM and more during the time course of the experiment. Australifungin at 1 μM caused no cellular toxicity on *S. nigrum* and *D. stramonium* leaf discs, but caused measurable cellular toxicity at 10 and 20 μM. However, the cellular toxicity caused by australifungin was less by 3 to 6 fold than FB_1 and AAL-toxin in *S. nigrum*, and by 20 to 30 fold in *D. stramonium* (Abbas *et al.*, unpublished data).

Fumonisin structure-activity relationships for mammalian cytotoxicity are similar to those for phytotoxicity (Abbas *et al.*, 1997a), consistent with fumonisins acting by inhibiting ceramide synthase in both systems. The structural requirements for activity are a free amino group, a long alkyl chain and probably a hydroxyl group on the carbon adjacent to the one bearing the amino group. Not required is a methyl group on the carbon bearing the amino group, or any of the substuents on the long alkyl chain beyond carbon 4, including the propanetricarboxylic acid side chains which constitute about half the molecular weight of the toxin. Notably higher phytotoxicity than mammalian cytotoxicity (a requirement for an effective bioherbicide) was observed only in side chain methyl ester derivatives, which could be due to the presence of higher levels of non-specific carboxylesterase activity in plant tissues. For all mammalian cell lines that were examined, australifungin was cytotoxic at concentrations similar to those observed with FB_1 in sensitive cell lines. Analog australifunginol was cytotoxic at about two-fold lower concentrations.

REVERSIBILITY

Ceramide synthase inhibitors were tested for reversibility of toxicity with *L. pausicostata* (Figure 2). In a preliminary experiment australifungin at 5 μM showed toxic effects after 72 hr treatment, but when the toxin-containing medium was removed and replaced with toxin-free medium, tissues began to recover within 7 days of initial treatment. At 14 days plants initially treated with australifungin at 1 or 5 μM contained significantly more chlorophyll than controls ($P < 0.05$, Student's unpaired t-test). No recovery was noted in plants initially treated for 72 hrs with 10 μM

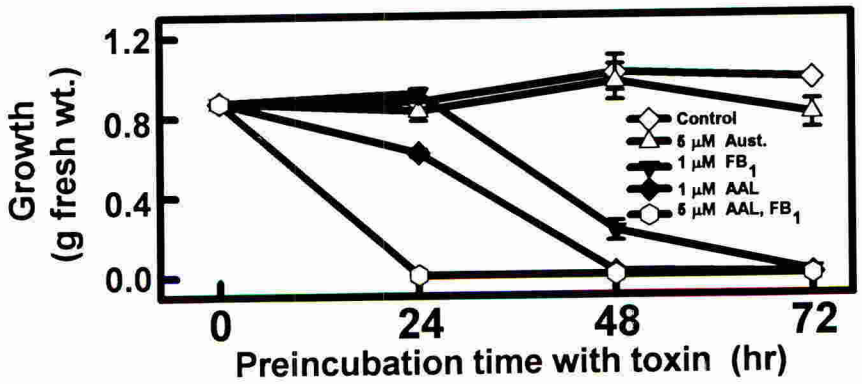


Figure 2. Time course of reversibility of the toxic effects of various toxins in *L. pausicostata* pretreated for 0 to 72 hr with australifungin (5 μM), FB_1 (1 and 5 μM) or AAL-toxin (1 and 5 μM), followed by wash-out and culturing for 14 days in toxin-free medium. Recovery is shown as growth determined by the gain in fresh weight. Values are the means of three replicates ± standard deviation.

australifungin or with 1μM or greater FB_1 or AAL-toxin. In a second reversibility experiment, tissues pre-treated 24, 48, or 72 hr with 5 μM australifungin initially exhibited toxic effects (photobleaching, increased conductivity in the media), but began to recover within 7 days after removal from the toxin-containing medium. Tissues were not affected by exposure to FB_1 or AAL-toxin at 1 μM for 24 hr, but after the same treatment for 48 hr they showed limited recovery within 14 days (Figure 2). No recovery was seen in tissues treated with FB_1 or AAL-toxin at 1 or 5 μM for 72 hr (Figure 2) (Abbas *et al.*, 1997b; 1997c).

MECHANISM OF ACTION

Fumonisins act on the enzyme sphinganine (sphingosine) N-acyltransferase (ceramide synthase). The inhibition of this enzyme causes a rise in the levels of sphinganine and sphingosine, the precursors to the acylation step, and a decrease in the formation of more complex sphingolipids in cells. Riley *et al.* (1996) demonstrated that the changes in sphingoid bases occurred before, or at the same time, as ultrastructural lesions. The same group noted a dose-response relationship between the amount of FB_1 in the diet and the degree of elevation of free sphingoid bases. The elevation of free sphinganine can be measured at low concentrations of FB_1 (5 ppm) in pigs, at which time no other biochemical or histological lesions are seen in lung or liver. Therefore, the elevation of free sphinganine is a useful screening test for fumonisin exposure.

Being aware of the mechanism of action of FB_1 in animals, we undertook parallel studies in plants to determine if the same mechanism is involved in the phytotoxicity. Significant increases of free phytospingosine and sphinganine can be detected as early as 2 hr after exposure to AAL-toxin or FB_1. In *L. pausicostata*, tomato leaf discs, and tobacco callus cell cultures, marked increases in phytosphingosine and sphinganine were measured after exposure to 1 μM FB_1 or AAL-toxin for 24 hr. The rise in sphingoid bases precedes the indicators of cell membrane destruction, namely the slower rise in increased conductivity and chlorophyll loss. Thus, it seems likely that the phytotoxicity of the fumonisins and AAL-toxin is also a result of the inhibition of ceramide synthase (Abbas *et al.*, 1994).

FB_1, australifungin, and AAL-toxin at 1, 5, and 1 μM, respectively, caused increases in free phytosphingosine (Pso) and free sphinganine (Sa), while the controls and australifunginol at 50 μM did not change the level of free sphingoid bases. All three toxins caused increases in Pso greater than Sa. The level of both sphingoid bases rose within 12 hr of initial treatment and increased with time, reaching their peak at 24 hr for Sa and 48 hr for Pso, and dropping at 72 hr for all toxins. The increases in Pso and Sa in *L. pausicostata* treated with FB_1 or AAL-toxin at 1 μM were higher than when treated with australifungin at 1 μM by 5 to 8 fold. Australifungin caused an increase in the level of free sphingoid bases as early as 12 hr and an increase in electrolyte leakage at 24 hr, as in the case of FB_1 or AAL-toxin (Abbas *et al.*, 1997c).

CONCLUSION

The phytotoxicity of ceramide synthase inhibitors, including the fumonisins, AAL-toxins and australifungin has been extensively studied. The B and C series fumonisins and AAL-toxins T_A and T_B are markedly toxic to a number of plant species. As has been demonstrated earlier in animals, these toxins act by inhibiting ceramide synthase. Australifungin, although structurally

unrelated, also inhibits this enzyme. The phytotoxicity of australifungin has been shown to be reversible at early stages, while the other toxins cause irreversible effects. FB_1, as a representative of this class of toxins, is primarily a contact phytotoxin but it is xylem mobile.

It is hoped that by further study of fumonisins, AAL-toxins and their analogues that it will be possible to exploit inhibition of ceramide synthase as a molecular site of action for a commercially viable herbicide. However, obstacles remain with the most important being significant mammalian toxicity.

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GLUTATHIONE AND CYSTEINE CONJUGATES INHIBIT GLUTATHIONE *S*-TRANSFERASE ENZYMES MEDIATING GSH CONJUGATION OF THE HERBICIDE ACETOCHLOR

I JABLONKAI, A HULESCH, I C BARTA
Central Research Institute for Chemistry, Hungarian Academy of Sciences, H-1525 Budapest, PO Box 17, Hungary

ABSTRACT

Glutathione (GSH) and cysteine conjugates of acetochlor, deethoxymethyl-acetochlor and a chloro-acetylated indoline were found to be inhibitors of glutathione *S*-transferase enzymes of maize, *Avena fatua*, and *Echinochloa crus-galli* catalyzing the GSH conjugation of acetochlor. The cysteine conjugates of these molecules were less effective inhibitors compared to their GSH analogs. The GSH conjugate of acetochlor appeared to be competitive with the substrate GSH, while the GSH conjugates of the other two chloroacetamides were non-competitive with respect to glutathione.

INTRODUCTION

Glutathione (GSH) conjugation has been established as the major detoxication reaction in the metabolism of chloroacetanilide herbicides in higher plants (Cole, 1994). Conjugation of these electrophilic molecules with cysteine can also contribute to their detoxication in certain plant species (Jablonkai & Dutka, 1985). However, much less is known about the intracellular toxicity and the inhibitory effects of GSH and cysteine conjugates on glutathione *S*-transferase enzymes (GSTs) mediating the conjugation of GSH and electrophilic substrates. *S*-(2-chloro-4-nitrophenyl) glutathione was found to be a potent inhibitor of rat liver GST A with a K_i of 5 μM (Pabst *et al.*, 1974). The structurally similar *S*-(2,4-dinitrophenyl)glutathione which is the product of conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH inhibited crude maize GSTs by 50% at 67 μM (Jablonkai & Dutka, 1996). The GSH conjugates of tridiphane (2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl)oxirane) and its sulfoxide were very effective inhibitors of GST enzymes from maize and *Setaria faberii* which catalyzed the conjugation of CDNB and GSH (Lamoureux & Russness, 1986).

The objective of this study was to evaluate the inhibitory action of GSH and cysteine conjugates found during the plant metabolism of the herbicide acetochlor (2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(ethoxymethyl)acetamide) in the conjugation reaction of acetochlor and GSH catalyzed by GST activities from maize, *Avena fatua*, and *Echinochloa crus-galli*.

MATERIALS AND METHODS

Chemicals

GSH and cysteine conjugates of acetochlor (AC), deethoxymethyl-acetochlor (DEMA, 2-chloro-*N*-(2-ethyl-6-methylphenyl)acetamide), and 1-(chloroacetyl)-7-methyl-2,3-dihydro-1*H*-indole) (CAIN)

were synthesized and purified as described previously (Jablonkai & Dutka, 1992). [Carbonyl-^{14}C]acetochlor (sp. act. 37 MBq/mmol) was prepared as reported earlier (Jablonkai & Dutka, 1985). Reduced GSH and all other chemicals were from Sigma-Aldrich (Budapest, Hungary).

Plant materials

Seeds of maize, *A. fatua*, and *E. crus-galli* were germinated on water-saturated filter papers in Petri dishes at 27 °C in a dark incubator. Seeds of 4-day-old maize and 6-day-old weed seedlings were removed and the whole plants were used for isolation of the enzyme.

GST isolation and assay

Etiolated seedlings (20 g) were homogenized with a mortar and pestle after freezing in liquid nitrogen. Then 40 ml of cold 0.1 M potassium phosphate buffer (pH 7.2) containing 5% (w/v) polyvinylpolypyrrolidone and 1 mM sodium metabisulfite was added to the mortar and the slurry was briefly ground again. The suspension was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA, USA) and centrifuged for 20 min at 10,000*g* at 4 °C. The supernatants were used as crude extracts for the GST assay.

GST activities using [^{14}C]acetochlor as a substrate were radioassayed in 5 ml glass vials. The reaction mixture contained 0.375 ml of 0.1 M phosphate buffer (pH 7.2), 0.125 ml of crude GST extract (about 0.2 mg protein content) preincubated for 15 min at 25 °C, 50 µM [^{14}C]acetochlor, and 0.125, 0.25, 0.5, and 1 mM concentrations of the respective conjugate dissolved in the buffer pH 7.2. The conjugation reaction was initiated by the addition of various amount of GSH dissolved in deaerated buffer to obtain 0.5, 1, 3, and 5 mM final GSH concentrations. Assay mixtures in vials were incubated at 25 °C. After 1 h, the non-conjugated herbicide substrate was removed from the aqueous assay mixture by extracting the mixtures twice with 1.5 ml of ethyl acetate. The radioactivity remaining in the aqueous phase was determined by liquid scintillation counting.

The enzymatic rate of the conjugation was calculated from data obtained with enzyme extract by subtracting the non-enzymatic rate determined by the conjugation between GSH and the herbicidal substrate in the absence of GST.

RESULTS AND DISCUSSION

In vitro catalytic activities of GSTs from etiolated seedlings were measured at various concentrations of GSH substrate and of GSH- and cysteine conjugates with acetochlor at 50 µM. GSH and cysteine conjugates of acetochlor, deethoxymethyl-acetochlor and the chloroacetylated-indoline were found to be inhibitors of maize and weed GSTs (Figure 1). Conjugates of deethoxymethyl-acetochlor were the most powerful inhibitor of maize GST: 67% inhibition was detected at 0.5 mM of its GSH conjugate at 0.5 mM GSH (Figure 1a). The other two conjugates were also inhibitory. At higher GSH concentrations the inhibitory effects were less (data not shown). Enzyme inhibitory activity of GSH conjugates were dependent on weed species and exhibited more or less the same range of activity in the conjugation reaction regardless of plant species. GSTs of *A. fatua* were more sensitive to the GSH conjugate of the indoline derivative, while the acetochlor-GSH exhibited the least inhibitory effect. In studies with GSTs from *E. crus-galli*, acetochlor and deethoxymethyl-acetochlor GSH conjugates showed comparable activity while the indoline GSH derivative was only a very

weak inhibitor. These results indicate that the inhibitory action of GSH conjugates does not seem to be a major selectivity factor because similar levels of inhibition were obtained for the maize and weeds. In contrast, several GSH conjugates were reported to be four times more effective as inhibitors of a GST from *S. faberii* than from a GST from maize in CDNB/GSH conjugation (Lamoureux & Rusness, 1986). Due to the lower activity of weed GSTs and the lower levels of inhibition Figure 1b displays the inhibitory activity of cysteine conjugates at 1 mM of GSH at which more reliable data were obtained. In general, cysteine conjugates were less effective inhibitors of GSTs compared with the GSH conjugates. The cysteine conjugate of acetochlor and the chloroacetylated indoline had only marginal inhibitory effects on maize GST. No difference in

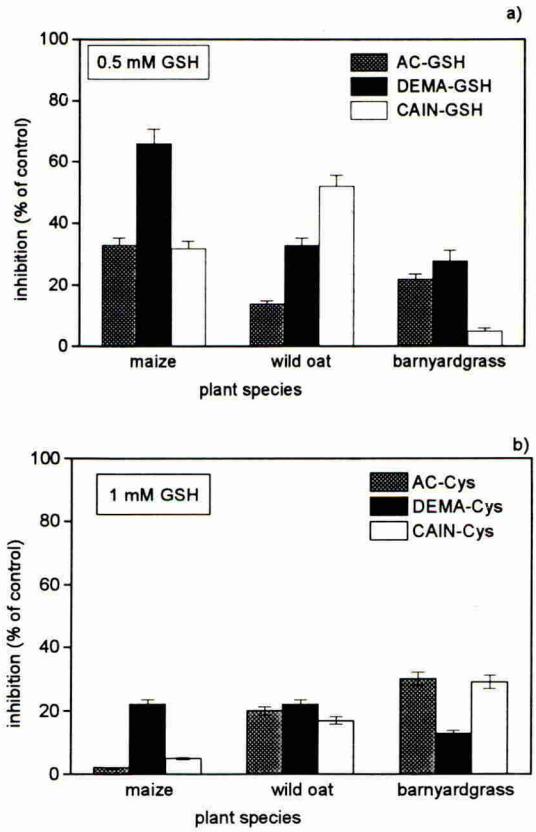

Figure 1. Inhibitory activity of 0.5 mM GSH (a) and cysteine (b) conjugates to GSTs from various plant species

activity of cysteine conjugates was detected for GSTs from *A. fatua*. The GSTs of *E. crus-galli* were least inhibited by the deethoxymethyl-acetochlor cysteine conjugate.

Since increasing the concentration of GSH in the assays could reverse the observed inhibitory effects we attempted to study the kinetics of inhibitory action of GSH conjugates at fixed concentration of acetochlor substrate and variable concentrations of the substrate GSH and the conjugates. Graphical representations of kinetic data according to the equations of Lineweaver-Burk plotting reciprocal

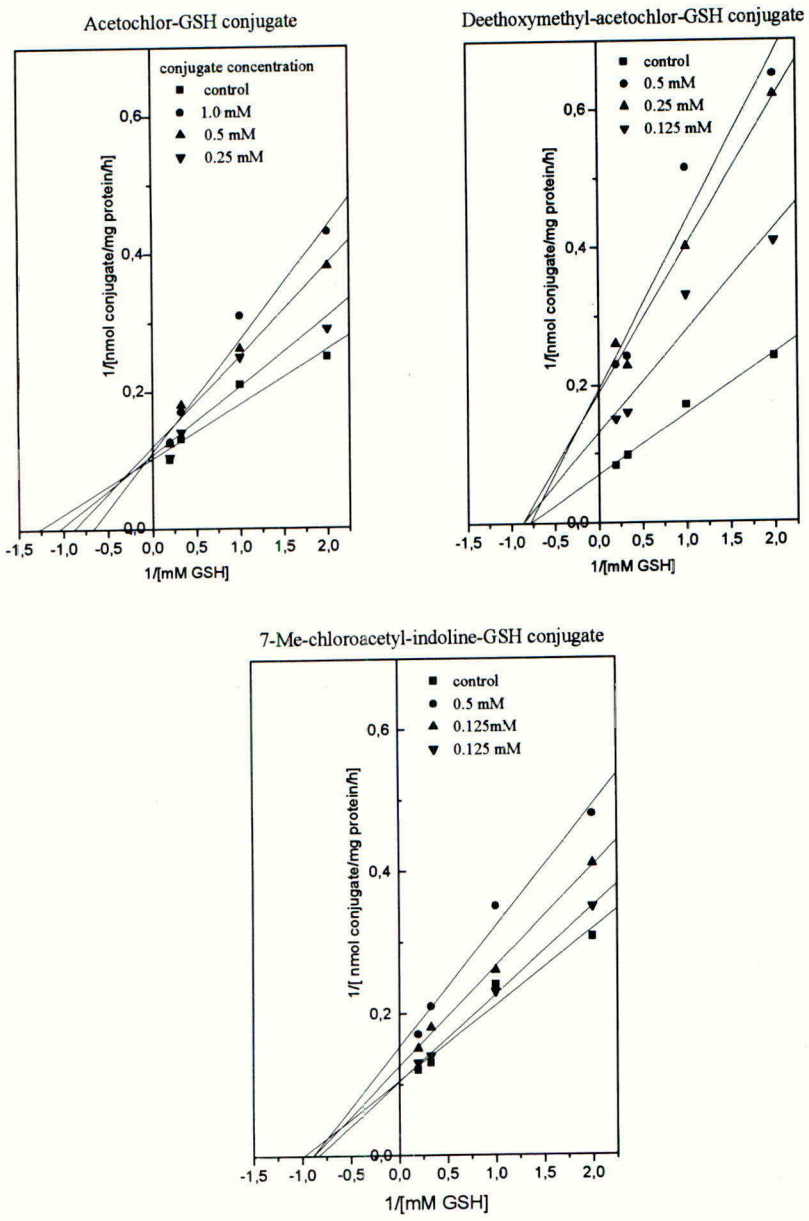

Figure 2. Lineweaver-Burk plots of GSH conjugate inhibition of acetochlor conjugation with glutathione as function of GSH concentration . Linear regression analyses of plots showed correlation of grater than 0.98 in all cases

velocities versus reciprocal GSH concentrations for maize GSTs are shown on Figure 2. The glutathione conjugate of acetochlor appears to be competitive with GSH. Non-competitive inhibition by both the deethoxymethyl-acetochlor and the chloroacetyl-indoline GSH conjugate can be concluded from the evaluation of these plots. Based on the kinetic data for both weed species (data not shown) the type of inhibition by the acetochlor GSH conjugate was also competitive. From the analyses of data sets obtained for deethoxymethyl-acetochlor and chloroacetyl-indoline GSH conjugate with weed GSTs we can assume non-competitive inhibition for both molecules with respect to glutathione. The apparent K_m and K_i values calculated from the kinetic data are shown in Table 1. The estimated K_i values confirm that the deethoxymethyl-acetochlor glutathione conjugate (DEMA-GSH) is about three times more effective inhibitor of maize GST than acetochlor glutathione (AC-GSH) conjugate. Also, the GSH conjugate of chloroacetyl-indoline (CAIN-GSH) is approximately 2-3 times stronger an inhibitor of GST from *A. fatua* as compared to the effectiveness of the other two conjugates.

Table 1. Kinetic analysis of inhibition of glutathione conjugation of acetochlor by glutathione conjugates

	Maize		*A. fatua*		*E. crus-galli*	
Inhibitor	K_m (mM)	K_i (mM)	K_m (mM)	K_i (mM)	K_m (mM)	K_i (mM)
AC-GSH competitive	1.3±0.3	0.8±0.3	3.0±0.9	1.7±0.6	2.0±0.6	2.3±0.7
DEMA-GSH non-competitive	1.3±0.3	0.3±0.03	2.3±0.3	1.2±0.2	2.0±0.5	1.3±0.5
CAIN-GSH non-competitive	1.2±0.1	0.9±0.1	3.6±1.5	0.5±0.1	3.8±1.2	3.2±1.2

Nevertheless, no explanation for the differential inhibitory activity of these conjugates is offered. Also, *S*-(propachlor)glutathione was 2.5 times more effective inhibitor of GSH conjugation of CDNB catalyzed by maize GSTs as compared to that of *S*-(atrazine)glutathione (Lamoureux & Rusness, 1986). However, we should note that several simplifications were made when calculating the inhibitory mechanism because this enzyme displays a complex kinetic mechanism. For interpreting our data we used a kinetic model of the one-substrate reaction but in fact the GSH conjugation is a two-substrate reaction producing two products as well. The kinetic mechanism proposed for GSH conjugation mediated by GST A of rat liver is a biphasic model with different K_m values depending on GSH concentrations (Pabst *et. al.*, 1974). At high levels of GSH, approximately 0.15 to 5 mM, the kinetic analysis indicated an ordered sequential pathway in which GSH is the first substrate to react with the enzyme. Under low GSH conditions (less than 0.1 mM) the pattern suggested is a ping-pong mechanism in which the electrophilic substrate is the first to bind to the enzyme. However, this biphasic reaction has not been observed for other isozymes of GST (Jakoby, 1978). The product *S*-(2-chloro-4-nitrophenyl)glutathione was competive with GSH and non-competitive with 1,2-dichloro-4-nitrobenzene. Also, *S*-(tridiphane)glutathione was competitive with GSH and non-competitive with CDNB (Lamoureux & Rusness, 1985).

The physiological significance of the product inhibition is not fully understood. Glutathione conjugates formed either enzymatically or non-enzymatically in the cytosol are transported to the vacuoles by an ATP-dependent pump (Martinoia *et al.*, 1993) where a specific carboxypeptidase can cleave the glycine moiety (Wolf *et al.*, 1996). These processes are limiting factors in their accumulation and therefore can decrease their inhibitory action. In turn, high rates of the non-enzymatic conjugations of some commercial chloroacetanilides (Jablonkai & Hatzios, 1993) could continously increase the amounts of GSH conjugates leading to the inhibition of the enzyme.

In summary, our studies demonstrate that glutathione and cysteine conjugates of chloroacetamides can cause product inhibition of the detoxifying plant GST isoenzymes *in vitro*. For further characterization of the inhibition mechanism of plant GSTs by GSH conjugates and to clarify the biological significance of the observed inhibitory effects additional studies need to be conducted with highly purified isozymes of the enzyme, since GSTs in plants occur in multiple forms which may be differently regulated (Cole, 1994).

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IS DICLOFOP RESISTANCE IN *ALOPECURUS MYOSUROIDES* ASSOCIATED WITH AN ALTERED EFFECT ON THE PLASMA MEMBRANE ELECTROGENIC POTENTIAL?

R A DE PRADO
Departamento Quimica Agricola-Edafologia E.T.S.I.A.M., Universidad de Cordoba, Apdo. 3048, Cordoba, Spain

R H SHIMABUKURO
United States Department of Agriculture, Agricultural Research Service, Biosciences Research Laboratory, Fargo, North Dakota 58105, USA

ABSTRACT

Diclofop-methyl (DM) caused irreversible damage to apical meristems in a susceptible (S) biotype of *A. myosuroides* within 22-24 hours after treatment (HAT) when sprayed at 10 mM. The apical meristems of a resistant (R) biotype were unaffected. DM induced ethylene evolution (224% of control) within 22-24 HAT in the (S) biotype of *A. myosuroides* at 10 mM, but little ethylene induction occurred in the R biotype. The cell membrane potentials (E_m) of both R and S biotypes were unaffected by 10 μM diclofop (acid). The E_m of both R and S was depolarized at 25 μM diclofop (acid). However, repolarization of E_m differed significantly between R and S biotypes upon the removal of 25 μM diclofop with repolarization occurring in the R biotype but not the S biotype. The correlation between the rapid injury to the apical meristem, ethylene induction and response of E_m to diclofop was consistent with the resistance or susceptibility of *A. myosuroides* biotypes. The three factors appear to be correlated with oxidative stress known to cause rapid lethality in plant senescence involving signal perception, membrane disassembly and ethylene evolution due to free radical formation.

INTRODUCTION

Alopecurus myosuroides is a cross-pollinating weed present in Europe and is found in wheat and legume crops. Several graminicides such as chlorotoluron and diclofop-methyl (DM) have been successfully used to control this weed. Differences in physiological and biochemical effects between diclofop-methyl and diclofop (acid) may be due to the dynamics of absorption, metabolism and interactions with different target sites and between *in vitro* and *in vivo* experiments (Devine, 1977, Shimabukuro & Hoffer, 1996, Shimabukuro *et al.*, 1997). In Spain, the use of DM alone or in combination with phenylurea herbicides for control of grass weeds in wheat has resulted in the appearance of several DM-resistant populations of *Alopecurus myosuroides* (Menendez & De Prado, 1996). A correlation appears to exist between the depolarization of the membrane potential (E_m) by diclofop and an apparent oxidative stress mechanism, resulting in the collateral induction of ethylene as an indicator of free radical formation (Paliyath & Droillard, 1992, Shimabukuro & Hoffer, 1996, Shimabukuro *et al.*, 1997). However, the signal transduction pathway from perception of

diclofop at the membrane to the formation of reactive oxygen species is unknown. Therefore, the objective of this research was to determine if whole plant resistance to DM in *A.myosuroides* biotypes is correlated with their sensitivity to membrane depolarization and induction of ethylene formation. A positive correlation between these responses would indicate that membrane depolarization may be the signal that induces oxidative stress (Paliyath & Droillard, 1992, Thompson *et al.*, 1991) as a major mechanism of lethality of DM and other aryloxyphenoxypropionic acid herbicides (Bana *et al.*,1993, Shimabukuro & Hoffer, 1996, 1997).

MATERIALS AND METHODS

Plant material

Seeds of slender foxtail (*Alopecurus myosuroides*) were collected from sites where chlorotoluron had failed to control it under normal practices (R biotype). Seeds were collected during 1990 from winter wheat fields continously treated with chlorotoluron for the last 5 yr (two application per year, one preemergence and one postemergence). Seeds of control plants of slender foxtail (S biotype) were taken from neighbouring marginal areas that had never been treated with herbicides. Both biotypes were located near Lerida (Northeastern Spain)(Menendez *et al.*, 1994). Seeds of R and S biotypes of *A. myosuroides* were germinated as previously described (Shimabukuro & Hoffer, 1992). The seeds were exposed to 3 h of red light per 24-h period to inhibit elongation of mesocotyls. The coleoptiles of germinated seedlings were used to determine effects of DM on E_m.

Seeds of *A. myosuroides* were planted in individual 5-cm pots with Sungro Sunshine Mix #1. Seedlings were grown in the glasshouse in Fargo, ND supplemented with sodium and mercury lamps for a 16-h photoperiod. Seedlings reached the 2.5 to 3-leaf stage in approximately 15 to 20 days. These seedlings were used to determine the effects of DM on the induction of ethylene formation and the antagonism or reversal of DM phytotoxicity by 2,4-D.

Membrane potential

Depolarization of E_m was determined with the microelectrode technique as previously described (Wright & Shimabukuro, 1987). The epidermis of coleoptiles from seedlings of *A. myosuroides* germinated as described was removed before incubation in Higinbotham's 1X solution (pH 5.7) for at least 30 min. A parenchyma cell was impaled with a microelectrode for measuring E_m changes. Three to four measurements were made on separate coleoptiles for each biotype and representative results on E_m changes are presented in Figure 2. Treatment solutions of diclofop were prepared in 1X solution with a final acetone concentration of 1% (v/v).

Ethylene induction by diclofop-methyl

Seedlings of *A. myosuroides* (R and S biotypes) in at the 2- to 3-leaf stage were sprayed using an atomizer to cover the foliage with a fine uniform spray of DM solution (10 mM) in water. Twenty two to 24 hours after treatment (HAT), five or more seedlings per pot were excised

at the soil level to give approximately 350 to 400 mg shoot fresh weight. The shoots were placed in the body of a 10-mL syringe with 1.0 mL distilled water and sealed as described (Shimabukuro & Hoffer, 1996). The syringes were placed in a dark incubator at 27° C for 4 h. One mL of the headspace gas following the 4-h incubation period was analyzed for ethylene by gas chromatography as described (Shimabukuro & Hoffer, 1996). The evolved gas from treated plants was confirmed previously as ethylene. The evolved ethylene was expressed as nL/g fresh weight (f.w.)/h. There were three to five replicates per treatment and the experiments were conducted twice.

Replicates of whole plants grown as described above for ethylene assay were sprayed similarly with DM and a combination of DM plus 2,4-D. Sprayed whole plants were observed for DM damage and its antagonism by 2,4-D and photographed between 7-10 days after treatment (DAT) (Figure 1).

Herbicide damage to meristematic tissues was determined by observing apical regrowth (new growth) from seedling whose shoots were excised 22-24 HAT for ethylene assay. About 0.5 cm of the stem above the root-shoot transition zone had remained after excision. The regrowth responses were observed and photographed 10 days after shoot excision. These observations confirmed the resistance and susceptibility of the different biotypes and the extent of irreversible herbicide damage to meristematic tissues within 22-24 HAT.

RESULTS AND DISCUSSION

Effects of diclofop-methyl and 2,4-D on whole plants

Differential responses between the R and S biotypes of *A. myosuroides* were observed when treated with 5 and 10 mM (photographs not shown). Regrowth of new apical shoots from the base of excised shoots occurred very rapidly. Significant regrowth diffferences between the S and R biotypes weren evident within 24 h following shoot excision from whole plants sprayed with 10 mM DM and excised 22 HAT. The regrowth response of meristem tissue to DM treatment correlated with whole plant sensitivity. The herbicide action of DM on the S biotype was antagonized by a simultaneous application of DM (5mM) and 2,4-D (4mM) (Figure 1) as demonstrated also for *Lolium rigidum* (Shimabukuro & Hoffer, 1992). The antagonism by 2,4-D was effective on whole plants as well as apical meristem tissues.

Depolarization of membrane potential by diclofop

Both R and S biotypes of *A. myosuroides* were unaffected by 10 μM diclofop but depolarization of E_m occurred at 25 and 50 μM diclofop (Figure 2). However, partial recovery (repolarization) of E_m at 25 μM occurred in the R but not the S biotype upon removal of diclofop. No repolarization of E_m occurred in both biotypes at 50 μM diclofop. These results agree with those reported for R and S biotypes of *Lolium rigidum* (Hausler *et al.*, 1991, Shimabukuro & Hoffer, 1992) and *Avena fatua* (Devine *et al.*, 1993).

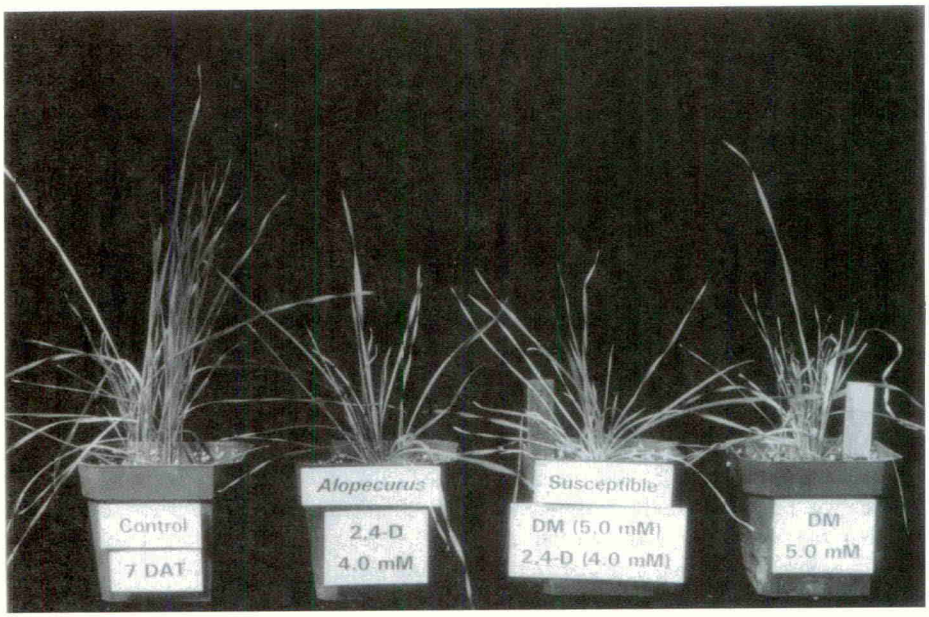

Figure 1. Response of *A.myosuroides* to diclofop-methyl, 2,4-D, and mixture of both compounds 7 DAT .

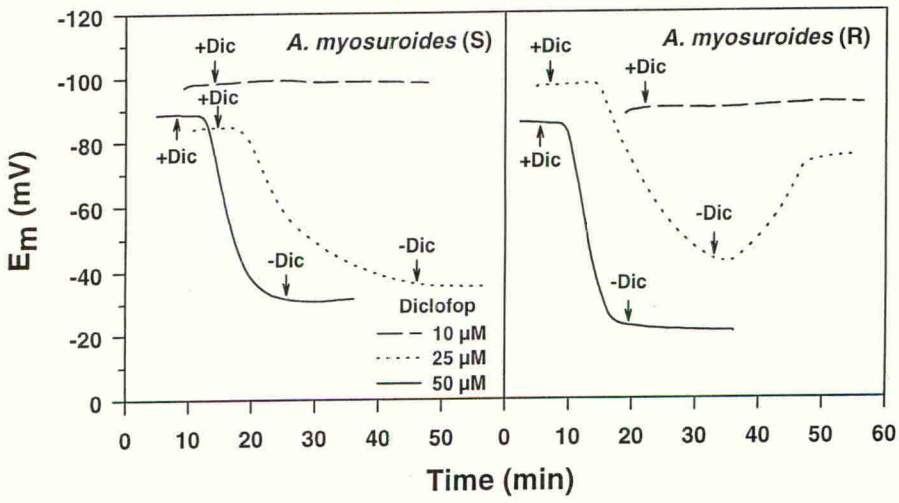

Figure 2. Response of DM-susceptible (S) and -resistant (R) *A. myosuroides* coleoptile parenchyma cells to diclofop. A single parenchyma cell was impaled with a microelectrode and changes in E_m were monitored continously as described in Materials and Methods. The arrows indicate addition (+Dic) and removal (-Dic) of diclofop.

Ethylene induction by diclofop-methyl

DM had a significant effect on ethylene induction in the S biotype (Figure 3). Ethylene evolution in the S biotype increased by 148% (5 mM) and 224 % (10 mM) of control within 22-24 HAT. DM caused little or no increase in ethylene evolution in the R biotype over the same period. The concentrations of DM used for determining ethylene induction were the same as those used to observe whole plant and apical meristem injury. The rapid temporal induction of ethylene by DM in *A. myosuroides* within 22-24 h correlated with its temporal damage to meristematic tissues.

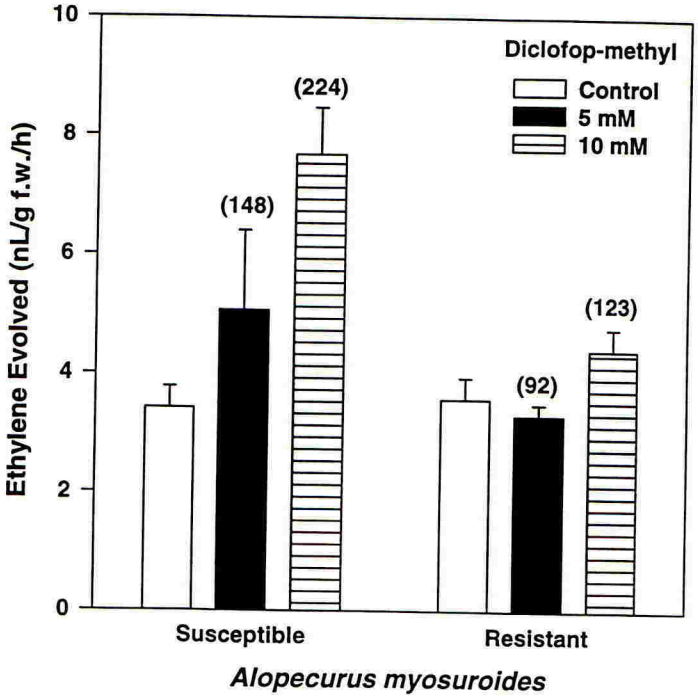

Figure 3. Evolution of ethylene induced by diclofop-methyl in *A. myosuroides*. Seedlings of *A. myosuroides* biotypes were treated with 5 and 10 mM diclofop-methyl as described in Materials and Methods. Seedlings were allowed to stand for 24 h before excision and analysis for ethylene. The rate of ethylene evolved is the average of six replicates ± S.E. with percent of control values in parenthesis.

SUMMARY

The correlation between the rapid effects of DM on plasma membrane, whole plants and meristematic tissues, and ethylene induction in biotypes of *A. myosuroides* appears to be similar to results on other susceptible species. These rapid effects and the associated mechanisms reversing DM phytotoxicity by 2,4-D, lipoxygenase inhibitors and several free radical scavengers, including α-tocopherol whose primary function is to prevent peroxidation of free polyunsaturated fatty acids, support the rapid and damaging action of the oxidative

stress mechanism (Thompson *et al.*, 1991, Paliyath & Droillard, 1992, Bana *et al.*,1993, Shimabukuro & Hoffer, 1996, Shimabukuro *et al.*, 1997).

ACKNOWLEDGEMENTS

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PURIFICATION OF GLUTATHIONE TRANSFERASES INVOLVED IN HERBICIDE METABOLISM FROM WHEAT

I CUMMINS, R EDWARDS
Dept. of Biological Sciences, University of Durham, DH1 3LE, UK

D J COLE
Rhône-Poulenc Agriculture Ltd., Fyfield Rd., Ongar, Essex, CM5 0HW, UK

ABSTRACT

Glutathione transferases (GSTs), involved in herbicide detoxification, have been purified from untreated wheat shoots and wheat shoots treated with the herbicide safener fenchlorazole-ethyl using a two-step procedure of hydrophobic-interaction and *S*-hexyl-glutathione affinity chromatography. GSTs partitioned during hydrophobic-interaction chromatography into two pools of activity. *S*-hexyl-glutathione affinity chromatography further resolved GSTs into immunologically distinct classes as determined using antibodies raised against purified wheat and maize GSTs. The immunologically distinct GST types showed differing overall detoxifying activities toward herbicides.

INTRODUCTION

Glutathione transferases (GSTs) have been intensively studied in maize, due to their importance in the detoxification of several herbicides including *s*-triazines and chloroacetanilides (Marrs 1996). Relatively little is known about the GST complement of wheat, although herbicides used as selective graminicides in this crop, such as fenoxaprop-ethyl and dimethenamid, together with chloroacetanilide compounds, have been shown to undergo glutathione conjugation (Tal *et al.*, 1993; Riechers *et al.*, 1996). Fenoxaprop-ethyl and dimethenamid are formulated with herbicide safeners to maximise selectivity in wheat and we have demonstrated that GST activities toward fenoxaprop-ethyl, the diphenyl-ether herbicide, fluorodifen and metolachlor, are increased in wheat by the application of the safener fenchlorazole-ethyl (Edwards and Cole, 1996). We have also purified the major GSTs from wheat shoots, which bind strongly to *S*-hexyl-glutathione and are enhanced by safener treatment (Cummins *et al.*, submitted for publication).

In the course of recent studies we have determined that maize GSTs can be resolved into distinct classes on the basis of their binding to *S*-hexyl-glutathione affinity columns (Dixon *et al.*, In press). The majority of GST activity in maize does not bind to *S*-hexyl-glutathione, but is retained by Orange-A agarose. All of the maize GSTs retained on Orange-A agarose contain the 29 kDa subunit (Dixon *et al.*, 1997). The minor fraction of GSTs which bind to *S*-hexyl-glutathione agarose contain a subunit termed *Zm*GST V, which is immunologically distinct from *Zm*GST I and has a coding sequence which is similar to the type III auxin-induced GSTs (Dixon *et al.*, In press). We were therefore interested to determine whether or not wheat contains such discreet classes of GST

isoenzymes and now report on the purification of the major GSTs involved in herbicide detoxification in this crop.

MATERIALS AND METHODS

Plant material

Wheat seed cv. Hunter was imbibed in a solution of the safener, fenchlorazole-ethyl (26.5 μM fenchlorazole-ethyl, diluted from a stock solution of 26.5 mM in acetone) for 3 h with constant agitation. Control plants were treated in a solution of 0.1% acetone (v/v). Plants were grown in the light for 7 d at 25°C under a 16 hr photoperiod at a light intensity of 510 μmol m^{-2} s^{-1}. Plants were watered with 0.1% (v/v) acetone (control plants) or with a 13.25 μM solution of fenchlorazole-ethyl (safener - treated).

Enzyme purification

Purification steps were carried out between 0°C to 4°C, using a Gradi-Frac low-pressure chromatography system (Pharmacia). Frozen tissue was homogenised in a mortar and pestle under liquid nitrogen and then extracted in 3 v/w 0.1 M Tris-HCl, pH 7.5, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT) and 5% w/v polyvinylpolypyrrolidone. The homogenate was centrifuged (17,000 ***g***, 20 min) and the protein in the supernatant was precipitated by the addition of solid $(NH_4)_2SO_4$ to 80% saturation. Precipitated proteins were resuspended in buffer A (20 mM potassium phosphate, pH 7.2, 1 mM DTT) and were applied to a column of phenyl-Sepharose CL-4B pre-equilibrated with buffer A containing 0.2 M KCl and 1 M $(NH_4)_2SO_4$. Bound proteins were eluted with a linearly-decreasing concentration of salt starting with 100% buffer A containing 0.2 M KCl and 1 M $(NH_4)_2SO_4$ and finishing with 100% buffer A. The column was then washed with buffer A to recover the major proportion of the GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) and this was termed the polar GST fraction. The remaining GST activity was then eluted from the column with the addition of 50% (v/v) ethylene glycol. Ethylene glycol was removed from the hydrophobic pool by anion-exchange chromatography as described (Dixon *et al.*, In press) and after dialysis against buffer B (10 mM potassium phosphate, pH 7.2, 1 mM DTT), polar and hydrophobic GST fractions were applied separately to an *S*-hexyl-glutathione affinity column (Dixon *et al.*, In press). The weakly-bound GST activity was then removed by washing the column with buffer B containing 0.2 M KCl and the tightly-bound GSTs were recovered with buffer B containing 0.2 M KCl and 5 mM *S*-hexyl-glutathione. All purified fractions were then dialysed against buffer B prior to further analysis.

Enzyme assays

Proteins from the weakly-bound and tightly-bound GST pools eluted from the affinity column were then assayed for GST activity toward CDNB spectrophotometrically. GST activity toward the herbicide substrates fenoxaprop-ethyl, fluorodifen and metolachlor was determined by quantification of the herbicide-GSH conjugates by reversed-phase HPLC as previously described (Edwards and Cole, 1996).

SDS-PAGE and Western blotting

The protein content of fractions was determined using a dye-binding reagent (Bio-Rad) with gamma-globulin as the standard. Polypeptides were separated on 15% polyacrylamide gels and were electroblotted onto nitrocellulose membranes as described (Dixon *et al.*, In press). Blots were probed with polyclonal antisera raised in rabbits against the combined *Zm*GST I and II subunits (Dixon *et al*, In press) and against the major wheat GST, *Ta*GST I, purified from the GST fraction bound to *S*-hexyl-glutathione (Cummins *et al.*, submitted for publication). Polypeptides on the Western blots were visualised using a secondary antibody coupled to alkaline phosphatase (Dixon *et al.*, In press).

RESULTS AND DISCUSSION

Immunoreactivity of polypeptides in crude extracts from untreated and fenchlorazole-ethyl-treated wheat shoots

Identical amounts of protein from untreated and safener-treated 7 day old wheat shoots were analysed by Western blotting of polypeptides resolved by SDS-PAGE. The anti-maize GST serum recognised three polypeptides of Mr 24 kDa, 27 kDa and 28 kDa. These polypeptides were determined in untreated shoots but were enhanced by treatment with fenchlorazole-ethyl. In contrast, the anti-wheat GST serum only recognised a distinct 25 kDa subunit, which was strongly expressed in the untreated shoots and was also enhanced by safener application (Table 1).

Table 1. Enhancement of GST subunits by fenchlorazole-ethyl

Mr of polypeptide	Maize GST Antibody		Wheat GST Antibody	
kDa	Control	Safener	Control	Safener
28	++	+++	-	-
27	+	++	-	-
25	-	-	+++	+++++
24	+++	++++	-	-

- = not detected, + = just visible, ++ = clearly visible, +++ = strong reaction, ++++/+++++ = dominant band.

The results from these studies with crude extracts suggested that wheat contained multiple GST polypeptides, three of which were immunologically related to maize GSTs. Using these antisera, the purification of the different immunoreactive polypeptides was monitored.

Purification of wheat polypeptides recognised by antisera raised to maize GSTs and a wheat GST

The total protein extract from wheat shoots treated with fenchlorazole-ethyl was first applied to a phenyl-Sepharose column as reported previously (Cummins *et al.*, submitted for publication). Wheat GSTs were partitioned into a polar class (65% recovered activity toward CDNB), which eluted in low salt and a hydrophobic class (35% recovered activity toward CDNB) requiring 50% ethylene glycol for recovery. The polar and hydrophobic GSTs were then separately applied to an *S*-hexyl-glutathione agarose affinity column. The weakly-bound GSTs were eluted with KCl and the tightly-bound GSTs with *S*-hexyl-glutathione. The distribution of the immunoreactive polypeptides is shown in Table 2. Polypeptides recognised by the maize GST antiserum bound to the *S*-hexyl-glutathione affinity matrix with lower affinity than those recognised by the wheat *Ta*GST I antiserum, since they eluted from the matrix in 0.2 M KCl, prior to application of *S*-hexyl-glutathione. The 25 kDa GST recognised by the anti-wheat GST serum was mostly associated with the tightly-bound GST fraction from the affinity column. The two types of GSTs also differed in their relative hydrophobicities, with the 'maize-type' GSTs enriched in the hydrophobic fraction and the *Ta*GST I being more dominant in the polar GST fraction.

Table 2. Separation of GST subunits by hydrophobic interaction and affinity chromatography, identified by Western blotting

	Polar GSTs		Hydrophobic GSTs	
	Weakly-bound	Tightly-bound	Weakly-bound	Tightly-bound
		Anti-wheat GST serum		
kDa				
25	+	++++	+	++
		Anti-maize GST serum		
kDa				
28	-	-	+++	-
27	+	-	++	-
24	++	-	++++	-

The distribution of GST activities toward herbicides which partitioned between the weakly-bound and affinity-bound GST fractions is summarised in Table 3. Activities were determined for the herbicides fenoxaprop-ethyl, fluorodifen and metolachlor.

Table 3. Percentage distribution of polar and hydrophobic GST activities toward herbicides in the weakly-bound and tightly-bound fractions from *S*-hexyl-glutathione affinity columns

	% Recovered GST activity toward herbicides		
	Fenoxaprop-ethyl	Fluorodifen	Metolachlor
Polar GSTs	(35)	(61)	(32)
Weakly-bound	15	16	11
Strongly-bound	20	45	21
Hydrophobic GSTs	(65)	(39)	(68)
Weakly-bound	40	13	42
Strongly-bound	25	26	26
Total recovered activity	100	100	100

Several conclusions could be drawn by correlating the distribution of the immunologically-distinct types of wheat GSTs with the enzyme activities toward herbicides. The 25 kDa *Ta*GST I - containing fractions, notably the tightly-bound GSTs from the affinity column, were associated with the majority of the activity toward fluorodifen. In contrast, the weakly-bound GSTs in the hydrophobic fraction which are enriched with the 'maize-type' GSTs, contained the largest proportion of recovered activity toward fenoxaprop-ethyl and metolachlor. Although the presence of additional unrecognised GSTs in these fractions remains a possibility, our results suggest that wheat contains immunologically distinct classes of GSTs which differ in their relative activities toward the herbicides fenoxaprop-ethyl, fluorodifen and metolachlor.

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THE EFFECT OF GRAMINICIDES ON PLANT PLASMA MEMBRANE H^+-ATPase ACTIVITY *IN VITRO*

M R HULL, A H COBB
Department of Life Sciences, The Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, UK.

ABSTRACT

In this study we have screened the H^+-ATPase activity of purified plasma membrane vesicles from the dicotyledon sugar beet *(Beta vulgaris* cv Celt) and the monocotyledon black-grass (*Alopecurus myosuroides*) against different graminicide classes. The thiocarbamates, diallate and triallate had the greatest effect on H^+-ATPase activity with a 70-80% inhibition at 100 μM in both species. A third thiocarbamate EPTC, did not inhibit activity. Aryloxyphenoxypropionates such as diclofop-methyl inhibited H^+-ATPase activity by approximately 40-50% in both species. Cyclohexanediones and chloroacetamides generally had no effect. Results are discussed in relation to proposed modes of action of graminicides.

INTRODUCTION

The plasma membrane (PM) H^+-ATPase acts as a primary transporter by pumping protons out of the cell, thereby creating pH and electrical potential differences across the membrane that play a key role in the regulation of multiple physiological functions, solute transport and auxin-induced growth. The study of herbicide effects on the PM is limited and as yet none have been shown to have a direct inhibition of PM function as their primary mechanism of action. However, there are various secondary effects including changes in permeability, enzyme activity, transport inhibition and the alteration of the hormonal and environmental regulation of membrane function that often occur in parallel to the primary mode of action (Balke, 1985).

A functional PM can regulate herbicide uptake and subsequent accumulation at the target site to cause phytotoxicity. Sethoxydim uptake in wheat (*Triticum aestivum*) was reduced by vanadate, an inhibitor of PM H^+-ATPase, but stimulated by fusicoccin, a known activator, suggesting that uptake was dependent on both ATP production and an operative H^+-ATPase (Couderchet & Retzlaff, 1991). The PM also plays a role in the interaction between different herbicides (Barnwell & Cobb, 1994). The efficacy of the aryloxyphenoxypropionate (AOPP) diclofop-methyl for example, is reduced on mixing with an auxin-type herbicide such as 2,4-D. Several theories regarding this antagonism have been proposed and some evidence points to an interaction at the membrane level. The AOPPs may act as anti-auxins such that auxin-induced growth is antagonised and active membrane transport mediated through PM H^+-ATPase ceases (Barnwell & Cobb,

1994). Other cell membranes may determine the intracellular fate of a herbicide. A tonoplast-bound, glutathione *S*-conjugate dependent ATPase has been demonstrated that is involved in the transport of conjugated secondary products and herbicides into the vacuole during the third phase of herbicide detoxification (Martinoia *et al.*, 1993).

Clearly the PM is an important cellular site that can affect herbicide uptake, mode of action and antagonism between different herbicides. The objective of this study was to screen the effect of different graminicide classes on PM H^+-ATPase activity extracted from green leaves of the monocotyledon, *A. myosuroides* and the dicotyledon, sugar beet (*Beta vulgaris* cv Celt).

MATERIALS AND METHODS

Plant material

Sugar beet, cv. Celt (British Sugar PLC, Norfolk, UK) and *A. myosuroides* (Herbiseed Ltd., Berkshire, UK) plants were raised in glasshouse conditions (20-40°C day, 9-15°C night) at 60-70% relative humidity as described (Hull *et al.*, 1995). The first true pair of sugar beet leaves and *A. myosuroides* plants (1-2 leaf stage, lamina and sheath) were harvested 15 d after sowing, frozen in liquid nitrogen and stored at -70°C until required.

Plasma membrane purification

Microsomal membranes were prepared from leaves of sugar beet and *A. myosuroides* (Hull *et al.*, 1995). Aqueous two phase partitioning was carried out according to Widdell *et al.*, (1982). The procedure was carried out for a total of 3 (sugar beet) and 4 partitions (*A. myosuroides*). Once purified, PM vesicles were frozen in liquid nitrogen and stored at -70°C until needed.

Enzyme and protein assays

H^+-ATPase activity was measured as the rate of liberation of orthophosphate (Pi) after 1 h incubation at 37°C (Ohnishi *et al.*, 1975). H^+-ATPase activity was measured in the presence of Mg^{2+} and K^+ having subtracted the activity in the absence of these cations. 5 µg membrane protein was included in each assay. Protein was determined according to Bradford (1976) using bovine serum albumin (Sigma Fraction V) as standard.

Herbicides

Herbicides represented four graminicide classes: AOPPs, cyclohexanediones, chloroacetamides and thiocarbamates. All herbicides (>95% purity; Greyhound/Chemservice, Birkenhead, UK) were initially dissolved in acetone and diluted such that the final concentration of acetone was 0.5% (v/v) and which did not affect H^+-ATPase activity. Herbicides were added 30 min prior to starting the assay with 2 mol m^{-3} ATP.

Statistical analysis

Data was analysed using the students t-test to test the null hypothesis of zero mean difference at the 95% confidence level. Dose response curves were computed using the mathematics function in Slidewrite (Advanced Graphics Software Inc., Carlsbad, USA).

RESULTS AND DISCUSSION

Herbicides could be ranked according to inhibition of H^+-ATPase activity from isolated PM vesicles in both sugar beet and *A. myosuroides*: thiocarbamate > AOPP > cyclohexanedione > chloroacetamide (Table 1).

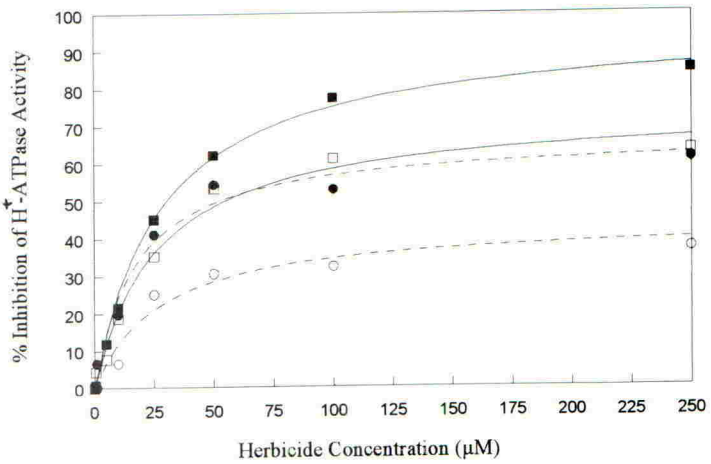
Table 1. The effect of different graminicides on PM H^+-ATPase activity (μmol mg^{-1} protein h^{-1}) in sugar beet and *A. myosuroides*. The percentage change in control, untreated activity after incubation with 100 μM herbicide for 30 min is shown. The effect of sodium vanadate, added to a final concentration of 200 μM, is also indicated. Data were calculated from means of 3 enzyme assays each conducted on 3 separate PM fractions. Figures in parentheses indicate statistical significance, not significant (NS).

Herbicide	Graminicide Class	% Change in H^+-ATPase Activity after 30 Min Incubation with 100 μM Herbicide			
		Sugar beet		*A. myosuroides*	
Control (μmol mg^{-1} protein h^{-1}) ± SE		39.05 ± 1.15		40.55 ± 2.01	
Sodium vanadate		-67		-68	
Triallate	Thiocarbamate	-80	(0.001)	-63	(0.001)
Diallate	,,	-70	(0.001)	-49	(0.001)
EPTC	,,	-3	(NS)	-3	(NS)
Diclofop-methyl	AOPP	-54	(0.001)	-37	(0.001)
Diclofop-acid	,,	-19	(0.01)	-8	(NS)
Haloxyfop-methyl	,,	-46	(0.001)	-35	(0.001)
Fenoxaprop-ethyl	,,	-22	(0.001)	-12	(0.001)
Quizalofop-ethyl	,,	-21	(0.001)	-25	(0.001)
Cycloxydim	Cyclohexanedione	-31	(0.001)	-4	(NS)
Sethoxydim	,,	-1	(NS)	-7	(NS)
Alachlor	Chloroacetamide	-9	(NS)	-5	(NS)

Two thiocarbamates, triallate and diallate inhibited H^+-ATPase activity in a concentration dependent manner, up to 80% at 100 μM although no inhibition was observed with EPTC (Table 1, Figure 1). The concentration of triallate required to inhibit H^+-ATPase activity by 50% was 15 and 17 μM in sugar beet and *A. myosuroides* respectively suggesting that activity was

highly sensitive to inhibition by triallate in both species (Table 2). Maximum inhibition was observed at 50-100 μM with no further inhibition at 250 μM (Figure 1). Consequently, there

Figure 1. The inhibition of PM H^+-ATPase activity by triallate (■, □) and diclofop-methyl (●, O) extracted from sugar beet (closed symbols) and *A. myosuroides* (open symbols). Data were calculated from means of 3 enzyme assays each conducted on 3 separate PM fractions from both species.



was a proportion of H^+-ATPase activity that was not inhibited by herbicide treatment. However, since H^+-ATPase activity attributable to the PM is defined as that proportion sensitive to vanadate (Hall & Williams, 1991), which in this study accounted for 70% of the H^+-ATPase activity measured, it is proposed that inhibition of PM H^+-ATPase activity by triallate and diallate was virtually complete at 100 μM. The primary mode of action of the thiocarbamates has not been identified although inhibition of very long chain fatty acids leading to a change in the quantity and quality of leaf surface lipids is thought to leave plants vulnerable to environmental stress (Wilkinson, 1988). However, evidence is largely indirect and it is unlikely that impaired surface lipid synthesis is phytotoxic in itself. An effect at the PM, particularly a rapid inhibition of H^+-ATPase activity, may play an important role in the herbicidal activity of these herbicides. We speculate that if H^+-ATPase activity is inhibited then the the export of epicuticular wax intermediates from the epidermal cells to the cuticle may be impaired. It is unclear why EPTC did not inhibit H^+-ATPase activity, but the sulphoxide metabolite of EPTC is thought to be more toxic than the parent herbicide (Barrett & Harwood, 1993). The chloroacetamide alachlor is similar to the thiocarbamates in type of application, efficacy and selectivity, although the primary mode of action is uncertain. Alachlor did not inhibit H^+-ATPase activity in either species suggesting that unlike thiocarbamate action, chloroacetamide treatment does not involve a response at the PM.

Table 2. Herbicide concentration (μM) required to inhibit PM H^+-ATPase activity by 50%. Data was calculated as means ± SE from dose reponses curves similar to those shown in Figure 1. ND not determined.

Herbicide	Herbicide Concentration (μM) Required to Inhibit H^+-ATPase Activity by 50%	
	Sugar Beet	*A. myosuroides*
Triallate	14.6 ± 2.98	16.6 ± 2.17
Diallate	48.0 ± 2.93	70.6 ± 4.30
Diclofop-methyl	17.0 ± 2.00	30.0 ± 3.00
Haloxyfop-methyl	11.8 ± 3.00	24.7 ± 2.60
Diclofop-acid	124.7 ± 1.24	ND

The AOPPs also inhibited H^+-ATPase activity in both species, and the methyl-esters were more effective compared to the ethyl-esters or the free acid (Table 1). There was a concentration dependent inhibition by diclofop-methyl with maximum inhibition observed at 100 μM (Figure 1). The concentrations required to inhibit H^+-ATPase activity by 50% were low indicating a high sensitivity to AOPP treatment (Table 2). Although inhibition was not to the same degree as the thiocarbamates, inhibition by the AOPPs accounted for a large proportion of H^+-ATPase activity attributable to the PM. The mechanism of action of diclofop-methyl and other AOPPs is unclear due to the absence of definitive evidence implicating a specific mechanism. With few exceptions, the *in vitro* inhibition of acetyl-CoA carboxylase correlates with whole plant responses to AOPPs (Powles & Preston, 1995). However, it is not certain whether a limitation of the supply of long chain fatty acids alone in intact plants is the cause of whole plant responses and the rapid lethality observed in susceptible species treated with diclofop-methyl. According to Shimabukuro & Hoffer (1996), diclofop-methyl action is due to induced senescence that may be initiated by an effect on the PM. A partial inhibition of H^+-ATPase activity and subsequent collapse of the transmembrane proton gradient have been implicated as a possible mechanism for the induction of senescence (Shimabukuro & Hoffer, 1996). These observations may shed further light on the selective action of graminicides by implying differences in H^+-ATPase sensitivity to herbicides. In the field, cyclohexanediones cause similar symptoms to AOPPs suggesting that they share a common mode of action. However, although cycloxydim inhibited H^+-ATPase activity in sugar beet, it had no effect in *A. myosuroides*. Similarly, sethoxydim had no effect in either species.

Clearly there are differences in response of the PM H^+-ATPase to *in vitro* treatment with several classes of graminicides. A high degree of inhibition by the thiocarbamates, triallate and diallate suggests that the mode of action of these herbicides may involve an effect at the PM. Similarly, an effect at the PM may be important in the mode of action of the AOPPs, but is less likely with both the cyclohexanediones and chloroacetamides.

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CHANGES IN GLUTATHIONE TRANSFERASE ACTIVITIES IN SOYBEAN IN RESPONSE TO TREATMENT WITH HERBICIDES AND SAFENERS

C ANDREWS, M SKIPSEY, R EDWARDS
Department of Biological Sciences, University of Durham, Durham, DH1 3LE, UK

G HALL, J TOWNSON, I JEPSON
Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, RG42 6ET, UK

ABSTRACT

Herbicide safeners play an important role in preventing herbicide damage in cereals such as maize and wheat by enhancing the levels of enzymes involved in herbicide detoxification, such as glutathione transferases. It was therefore of interest to determine whether or not safeners can exert a similar effect in important dicotyledonous species such as soybean (*Glycine max*).

Three-week-old soybean plants were sprayed with formulated herbicide safeners used in cereals and the diphenylether herbicides, fomesafen and oxyfluorfen, at a range of concentrations. GST activities toward 1-chloro-2,4-dinitrobenzene (CDNB) and fomesafen were then determined in crude leaf extracts. Both oxyfluorfen and fomesafen enhanced GST activity toward CDNB, with visible herbicide injury occurring at the rates used. The herbicide safeners dichlormid, naphthalic anhydride and BAS 145-138 gave a more modest enhancement of GST activity toward CDNB, but this was not associated with phytotoxicity. In contrast to the CDNB-conjugating activity, the GST activity toward fomesafen was unresponsive to all treatments.

INTRODUCTION

Herbicide safeners continue to play an important role in cereal crop protection, being used in herbicide formulations to prevent damage in crops such as maize and wheat (Hatzios, 1991) by certain selective herbicides. One way in which these compounds have been shown to exert their effect is by increasing the level of herbicide-metabolising enzymes, including glutathione transferases (GSTs), in the crop plant (Hatzios, 1991). There is also evidence that GSTs may be induced in cereals following herbicide treatment at sub-toxic application levels (Mauch & Dudler, 1993). In this study we have examined the effect of a number of safeners and herbicides on GST activities in soybean. Little is known regarding the efficacy of safeners in dicotyledonous species, and it was therefore of interest to establish whether safeners which are active in monocotyledonous species were able to exert similar, enhancing effects on GSTs in the agronomically important crop, soybean.

Three safeners were selected for the trial. Dichlormid, BAS 145-138 and naphthalic anhydride (Figure 1) can all be used in maize to safen against a wide variety of herbicides (Hatzios, 1991). Both BAS 145-138 and naphthalic anhydride safen against acifluorfen and

chlorimuron-ethyl damage in maize (Boger & Miller, 1994, Lamoureux & Rusness, 1991). Both of these herbicides are also used selectively in soybean. Dichlormid has been shown to safen against damage caused by the chloroacetanilide herbicides, such as metolachlor (Fuerst & Lamoureux, 1992). The effect of herbicide application on soybean GST activities was determined using the diphenylether herbicides fomesafen and oxyfluorfen. Fomesafen is known to be detoxified in soybean by conjugation with homoglutathione, the major available free thiol in soybean (Evans *et al*, 1987), while oxyfluorfen is non-selective and not detoxified by the action of glutathione transferases.
Changes in soybean GST activities toward the model GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) and the herbicides fomesafen, metolachlor and chlorimuron were assessed.

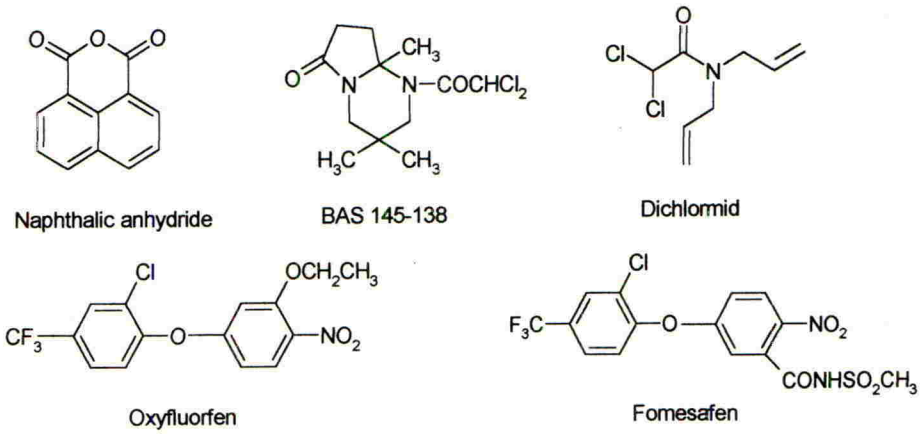

Figure 1. Structures of agrochemicals used in the trial.

MATERIALS AND METHODS

Spray trial

Soybean plants (cv D297) were germinated and grown in John Innes No. 3 Potting Compost in a glasshouse until they reached the three tri-foliar leaf stage (*c.* three weeks). The plants were then sprayed using a track sprayer fitted with a 8002E T-jet nozzle delivering an equivalent of 200 l ha^{-1}. Both herbicides and safeners were formulated with a non-ionic surfactant and organic solvent. Oxyfluorfen was applied at rates of 2500, 312 and 39g ai ha^{-1}. The other compounds were applied at 4000, 1000 and 125 g ai ha^{-1}. However, at the higher application rates, several of the formulated herbicides became non-homogeneous and the results from these experiments have not been included. Controls were treated with formulation only, water or received no treatment.
Following application, the plants were returned to the glasshouse for 48hr, after which the foliar tissue was harvested, frozen in liquid nitrogen and stored at -80°C.

Protein extraction

The plants were ground to a fine powder under liquid nitrogen using a mortar and pestle and suspended in three volumes (w/v) of ice cold 0.1mM Tris-HCl pH 7.4, containing 2mM EDTA, 1mM DTT and 5%(w/v) PVPP. The homogenisation was continued using sand as an abrasive. Extracts were adjusted to 80% saturation with respect to ammonium sulphate to precipitate the protein, centrifuged (17,000g, 20min) and the pellets stored at -20°C. Prior to use, proteins were desalted by dialysis against 20mM Tris-HCl pH 7.4, 1mM DTT and protein content adjusted to approximately 10mg ml^{-1}. Protein assays were performed using the Bio-Rad dye binding reagent according to the manufacturer's guidelines.

GST activity assays

GST activities toward CDNB were determined by monitoring the increase in absorbance at 340nm. 0.1M potassium phosphate buffer pH 6.8 (0.9ml) was incubated in a 1ml cuvette with 30mM CDNB (33µl) prepared in 80:20 MeOH:H_2O at 30°C in a water bath. After equilibrating for five minutes, 100mM glutathione (33µl) was added to the cuvette, followed by protein extract (33µl). The change in absorbance at 340nm was observed over 30s, with the specific activity expressed in nkatals mg^{-1} protein after correction for the non-enzymatic rate. Each extract was assayed in duplicate.
HPLC based assays were designed to monitor and quantify GST activity toward fomesafen. 10mM fomesafen (10µl) was added to an 1.5ml eppendorf tube containing 0.4M Tris-HCl pH 9.5 (50µl). Enzyme extract (120µl) was added and the reaction started by the addition of 100mM homoglutathione (20µl), prepared as described previously (Skipsey *et al.*, 1997). After incubation for 60 min at 37°C, the reaction was stopped with 10µl of 6M HCl. The sample was freeze/thawed and centrifuged at 10,000g for two minutes. A sample of the supernatant (50µl) was injected onto a Fisons Spherisorb ODS1 HPLC column (250mm x 4.6mm). The column was equilibrated with 5% acetonitrile : 95% water containing 1% phosphoric acid at a flow rate of 0.8ml min^{-1} prior to use. The column was eluted using the gradient shown in Figure 2. Products eluted from the column were detected by their absorbance at 280nm. Authenticity of the fomesafen homoglutathione conjugate was confirmed by synthesis and fast atom bombardment mass spectrometry in comparison with published data (Andrews *et al.*, In press).

RESULTS & DISCUSSION

GST activities toward CDNB and the herbicide fomesafen (Figure 2) could be readily determined in crude extracts from three-week-old soybean plants. Unfortunately, in the older plants used in this study, the HPLC-based assay was not suitable for assaying other herbicides such as metolachlor and chlorimuron-ethyl due to the presence of UV-absorbing natural products which co-chromatographed with the herbicide conjugate.

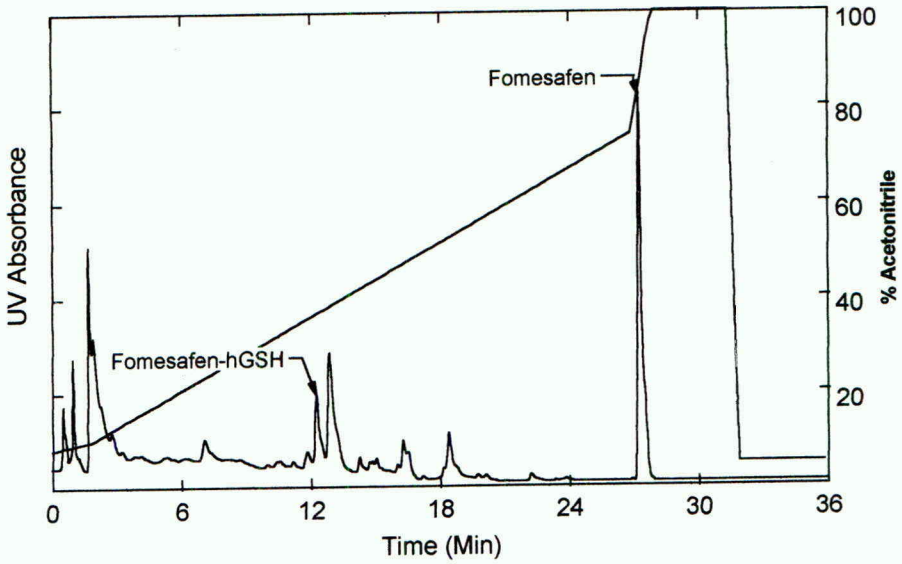

Figure 2. HPLC traces showing fomesafen and the homoglutathione conjugate of fomesafen.

Table 1. GST activities in soybean following application of test compounds at various rates. Results are ± SD.

Treatment	Rate g ai ha^{-1}	Phytotoxicity	CDNB nkat mg^{-1}	Fomesafen nmol hr^{-1} mg^{-1}
Oxyfluorfen	2500	+++	1.82 ± 0.33	4.60 ± 0.17
	312	+++	1.66 ± 0.19	
	39	+++	1.56 ± 0.13	
Fomesafen	1000	++	1.80 ± 0.07	3.58 ± 0.19
	125	+	1.24 ± 0.05	
Dichlormid	4000	0	1.37 ± 0.15	3.98 ± 0.21
	1000	0	0.99 ± 0.09	
	125	0	0.71 ± 0.08	
Naphthalic-	1000	0	1.25 ± 0.15	4.65 ± 0.33
Anhydride	125	0	0.89 ±0.03	
BAS 145-138	125	0	1.01 ± 0.08	5.58 ± 0.18
Formulation only		0	0.82 ± 0.08	3.72 ± 0.15
Water		0	0.78 ± 0.02	
No treatment		0	0.74 ± 0.11	4.34 ± 0.18

GST activity toward CDNB was enhanced in the foliage of soybean plants treated with the diphenylether herbicides, oxyfluorfen and fomesafen, at all application rates (Table 1). With oxyfluorfen, visible herbicide injury occurred at all rates. With fomesafen, severe phytotoxicity was only observed at the highest application rate, though lesser symptoms were observed at the lower rate. Oxyfluorfen has also been shown to increase GST activities in soybean cell cultures and it has been suggested that this enhancement is part of the antioxidant protective system of plants during the formation of reactive oxygen species (Knorzer *et al.*, 1996). Our results with soybean plants are consistent with this proposal. When applied to the foliage, use of all the herbicide safeners resulted in some enhancement of GST activity toward CDNB in the soybean leaves. BAS 145-138 gave a modest increase in GST activity at the lowest rate applied, but could not be effectively applied at higher concentrations due to its limited solubility. Dichlormid and naphthalic anhydride increased GST activity in a dose-dependent manner, though they were both less effective than fomesafen applied at similar rates. However, unlike the case with the diphenylether herbicides the herbicide safeners enhanced GST activities without any visible signs of phytotoxicity.

For each herbicide, or herbicide-safener, leaf extracts from the treatment shown to give optimal enhancement of GST activity toward CDNB, were also assayed for GST activity toward fomesafen. Treatment with BAS 145-138 gave a minor increase in GST activity toward the herbicide, but all the other treatments were ineffective (Table 1). The differential enhancement by the herbicides and herbicide-safeners of GST activities toward CDNB and fomesafen suggests that these two compounds are primarily detoxified by distinct GST isoenzymes.

GST enhancement in soybean

Our results suggest that the induction of GST activities by foliar-application of herbicide safeners is not unique to cereals, but can also be determined in dicotyledonous plants such as soybean. The safening response seen in soybean is less pronounced than for that determined in maize. However, many of the safeners used in this study are most commonly used as seed treatments and it will be of interest to determine whether or not these compounds induce GSTs more effectively in soybean when applied to the seed. A greater enhancement of GST activity was determined with the diphenylether herbicides, which is probably due to the oxidative stress placed on the plant as a function of the mode of action of these herbicides. It will now be of interest to determine whether diphenylether herbicides and safeners enhance GST activities in soybean using similar, or distinct, signalling mechanisms.

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