Session 3C

Biochemical Basis of Herbicide Action and Selectivity

Session
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BASIS OF SELECTIVITY OF THE HERBICIDE TRIFLUSULFURON METHYL IN SUGAR BEET

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ABSTRACT

Triflusulfuron methyl, formerly DPX-66037, is a new selective post-emergence sulfonylurea herbicide for the control of annual and perennial broad-leaf weeds and grasses in sugar beet (*Beta vulgaris*). This herbicide is unique in its safety to sugar beet, a crop that has often been used as an indicator of residual sulfonylurea in soils due to its high sensitivity to this class of herbicides. We investigated the mechanism of selectivity by comparing the response of sugar beet with that of sensitive and tolerant weeds. Studies of foliar uptake and *in vitro* inhibition of acetolactate synthase (ALS) across these species discount differential uptake or differential active site sensitivity as the basis for sugar beet tolerance to this herbicide. However, a good correlation between metabolism and plant tolerance does exist. Sugar beet metabolized triflusulfuron methyl very rapidly (half-life of < 1 h) while metabolism in *Chenopodium album* was intermediate (half-life of 7 h) and slow in sensitive weeds (half-lives of >35 h). The initial metabolism of triflusulfuron methyl in sugar beet involves glutathione conjugation. Although glutathione is known to have a role in the metabolism/detoxification of many xenobiotics in plants, this is the first known report in sugar beet. The 2,6-disubstituted sulfonamide appears essential for this metabolism, but surprisingly even minor changes on the triazine ring have a profound effect on sugar beet tolerance. Although the data strongly suggests enzymatic involvement, attempts to determine if glutathione-S-transferase was involved proved inconclusive.

INTRODUCTION

Triflusulfuron methyl (methyl 2-[[[[[4-(dimethylamino)-6-(2,2,2-trifluoroethoxy)-1,3,5-triazin-2-yl]amino]carbonyl]amino]sulfonyl]-3-methylbenzoate), formerly DPX-66037, (Fig. 1) is a sulfonylurea herbicide for weed control in sugar beet (*Beta vulgaris*). It is a highly active post-emergence herbicide that controls a diverse spectrum of broadleaf weeds and also has activity on a range of grasses (Peeples *et al.*, 1991) In addition to its low use rate of 10 to 20 g AI/ha, it is a short-residual herbicide, which breaks down rapidly in soil via chemical and, to a lesser degree, microbial mechanisms.

Like other sulfonylurea herbicides, the site of action of triflusulfuron methyl is acetolactate synthase (LaRossa & Schloss, 1984; Ray, 1984), an enzyme in branched-chain amino acid biosynthesis. This herbicide is unique in its safety on sugar beet, a crop that has often been used as an indicator of residual sulfonylurea in soils due to its high sensitivity to this class of herbicides. Because of the unique tolerance of sugar beet to triflusulfuron methyl, we have studied the mechanism of selectivity. In this paper we report the results of studies on plant and enzyme sensitivity, foliar uptake, and metabolism of ^{14}C-triflusulfuron methyl.

MATERIALS AND METHODS

Plant growth and treatment

The plants used and their stage of growth at the time of treatment were sugar beet, 3-4 leaf; *Brassica napus*, 4 leaf; *Veronica persica*, 4 leaf; *Matricaria inodora*, 6-8 leaf; and *Chenopodium album*, 5-6 leaf stage. Plant sensitivity to the herbicide was evaluated under greenhouse conditions. Visual herbicidal injury was evaluated approximately 16 days after treatment.

Herbicide uptake studies were initiated by foliar applications to each species, as a broadcast spray, of 8 ug ^{14}C[*triazine*]-triflusulfuron methyl in 100 uL of 0.1% citowet in water (specific activity of 146 KBq/mmole). This was equivalent to a rate of 10 g AI/ha. Plants were harvested 5 and 20 hr after treatment by excising the shoots at the soil level. The shoots were rinsed with acetone: water (1:1,V:V) and total radioactivity in the rinsate was determined by liquid scintillation counting . The washed shoots were dried and combusted to determine the quantity of radioactivity in the tissue.

Metabolism studies were carried out by placing shoot explants in quarter-strength Hoagland's nutrient solution containing 10 ppm of ^{14}C[*triazine*]-triflusulfuron methyl. Explants were allowed to take up the compound until uptake reached c. 1 ug/g fresh weight (1-2 h), so that the initial concentration of herbicide within each species was approximately equal. Next the explants were transferred to solutions containing only quarter-strength Hoaglands. Plants were then harvested at various times, extracted with 50 or 80% acetone, and analyzed using HPLC (H_2O:acetonitrile gradient, each containing 0.2% formic acid or 0.03% phosphoric acid and a DuPont RxC8 column) with a radioactive flow detector. Similar experiments were repeated with 14C[*ester carbonyl*]-triflusulfuron methyl in sugar beet only.

A major polar metabolite was isolated from sugar beet leaves and purified using HPLC. This metabolite was analyzed by fast atom bombardment (FAB) LC/MS.

Acetolactate synthase extraction and assay

Acetolactate synthase (ALS, EC 4.1.3.18) was extracted from seedlings of each species at the same stage of growth that the plants were used for the uptake and metabolism studies. The enzyme extraction and assay protocols were the same as those described by Ray, 1984.

RESULTS AND DISCUSSION

Selectivity studies

Sugar beet exhibits a high level of tolerance to triflusulfuron methyl as indicated by the high GR_{50} value when compared to the values of the four weed species (Table 1). To determine if ALS sensitivity had a role in this tolerance, the enzyme was extracted from the five species and tested *in vitro* for sensitivity to triflusulfuron methyl. Although there were some differences in I_{50} values, these differences were not correlated with plant sensitivities (Table 1).

TABLE 1. Triflusulfuron methyl uptake, metabolism and ALS I_{50} values for plants differing in sensitivity.

Plant species	Plant sensitivity GR_{50} (g/ha)[a]	Enzyme sensitivity I_{50} (nM)	% Foliar Uptake after 20h	Metabolic half-life (h)
Sugar beet	5917	33	6	<1
Chenopodium album	110	92	17	7
Matricaria inodora	17	37	8	80
Brassica napus	10	62	7	36
Veronica persica	25	26	15	61

[a] The rate of application required to inhibit plant growth 50% in the greenhouse. Values are the means of at least 4 different experiments.

Uptake of triflusulfuron methyl following foliar application was low (Table 1) but in a typical range for a sulfonylurea (11). Although there were differences in uptake among the five plant species, there was no apparent correlation between uptake (as a percentage of applied herbicide) and plant sensitivity.

Only the results from the metabolism study show a correlation (inverse correlation to metabolic half-life) with the whole plant response (GR_{50}). Sugar beet in the 3-4 leaf stage metabolized triflusulfuron methyl very rapidly, with a half-life of less than 1 h. The sensitive weeds, *B. napus*, *V. persica*, and *M. inodora* metabolized triflusulfuron methyl much more slowly, resulting in half-lives of greater than 35 h. *C. album* exhibited an intermediate half-life (7 h) consistent with its moderate tolerance to triflusulfuron methyl. These results indicate that tolerance in sugar beet is due to rapid metabolic inactivation of triflusulfuron methyl, which is the same mechanism for tolerance reported for other sulfonylurea herbicides (Beyer *et al.*, 1987; Brown *et al.*, 1990; Brown, 1990).

Sugar Beet Metabolism Studies
The rapid metabolism of triflusulfuron methyl by sugar beet cannot be explained by simple chemical hydrolysis. Triflusulfuron methyl has a half-life of 3.7 days in water at pH 5 and 25 $^{\circ}$C, and the half-life increases at higher pH values resulting in values of 32 and 36 days at pH 7 and 9, respectively (Peeples *et al.*, 1991). In addition, the rate of hydrolysis declines at lower temperatures with a Q_{10} of about 2 (data not shown). Thus the rapid metabolism by sugar beet is almost certainly enzyme catalyzed.

In sugar beet only one metabolite peak was observed with the ^{14}C-ester carbonyl-triflusulfuron methyl, but this peak actually represents both saccharin and its free acid, which could not be separated by our HPLC method. Metabolism of ^{14}C-triazine-triflusulfuron methyl in leaves yielded primarily the triazine amine and a more polar metabolite. This polar metabolite was isolated and purified by HPLC. LC/MS (FAB) of this metabolite yielded a strong parent ion of 571 and fragment ions at m/z 308, 264 and 238. The 571 parent ion is attributed to the glutathione conjugate, S-(N-triazinyl-carbamoyl)glutathione (STG), which has a MW of 570. This identification was confirmed by the synthesis of STG and comparison of the mass spectra and HPLC retention times with the polar metabolite. Although glutathione is known to have a role in the metabolism and detoxification of many xenobiotics in plants, this is the first known report for its involvement in sugar beet. Based on these results a proposed pathway for the initial metabolism of triflusulfuron methyl in sugar beet is shown in Figure 1.

Structure-activity studies

Structure-activity data supports the importance of the carboxylic ester at the 2-position and the methyl group at the 6-position of the phenyl ring (Table 2). The steric crowding caused by the methyl group in the 6-position may play a role in enhancing the conjugation reaction with glutathione. It appears, however, that the methyl group must have importance beyond size since replacement of a similar size group at the 6 position, e.g. Cl, does not result in sugar beet selectivity. Moreover, minor changes on the triazine ring (replacing trifluoromethyl with methyl and removing one of the methyl groups on the amine) can also abolish sugar beet selectivity. Furthermore, combining these minor changes on the triazine ring with removal of the 6-methyl group on the phenyl ring reverses selectivity, so rape is now tolerant and sugar beet is the sensitive species. The fact that these minor changes in structure can have such a dramatic effect on selectivity further supports the role for an enzyme in sugar beet metabolism of triflusulfuron methyl.

TABLE 2. Sugar beet tolerance to triflusulfuron methyl analogues

R1	R2	R3	R4	R5	Tolerance*
COOCH3	H	CH3	N(CH3)2	OCH2CF3	SB
COOC3H7	"	"	"	"	SB
COOCH3	CH3	H	"	"	None
"	H	"	"	"	"
"	"	Cl	"	"	"
"	"	CH3	NHCH3	"	Some SB
"	"	"	"	OCH2CH3	None
"	"	H	"	"	Rape

* Sugar beet (SB) vs. oilseed rape

The enzyme most likely to catalyze the formation of the glutathione conjugate STG from triflusulfuron methyl would be GST. Yet, attempts to use an inhibitor (tridiphane) to obtain further evidence for the involvement of GST proved inconclusive. Tridiphane had no effect on the rate of metabolism of triflusulfuron methyl in sugar beet leaves, but it is uncertain whether it reached the site of action. Alternatively, because GSTs exist in multiple forms with differing substrate specificities, it is possible that tridiphane does not inhibit the GST which metabolizes triflusulfuron methyl.
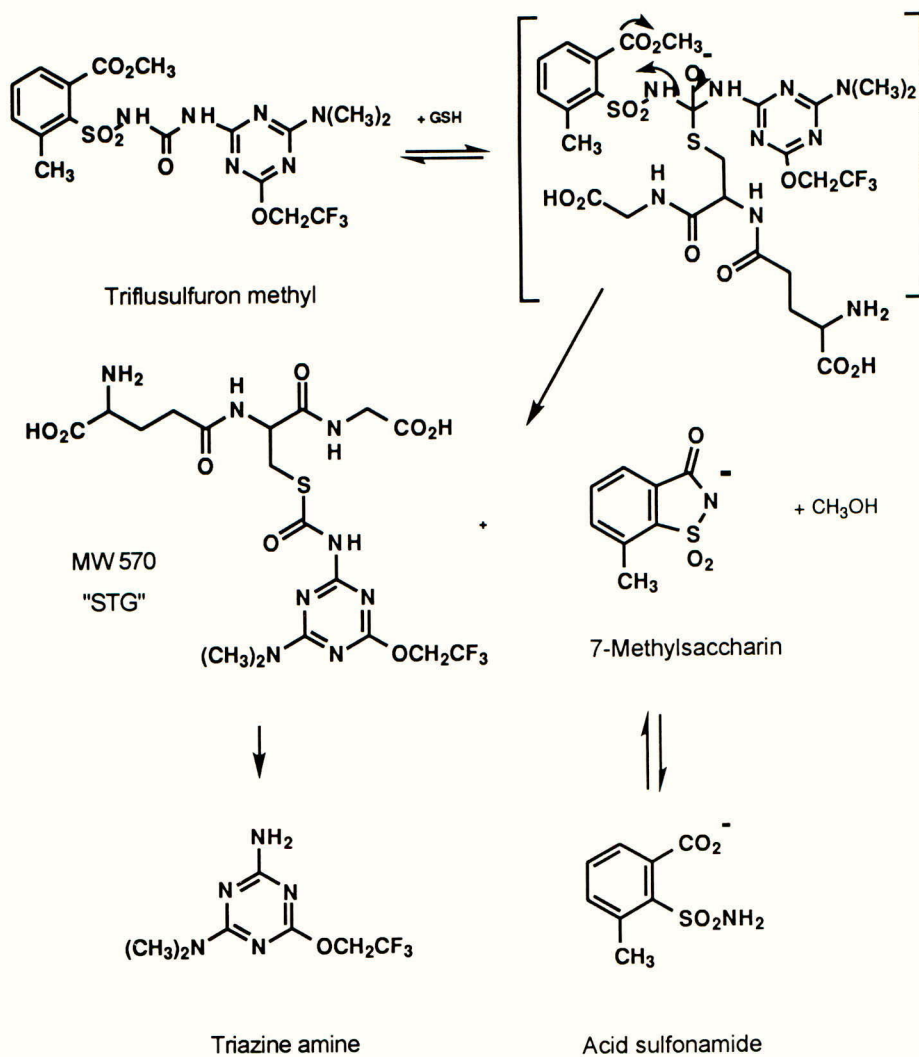
Figure 1. Summary of initial metabolism of Triflusulfuron methyl in sugar beet

In summary, sugar beet tolerance to triflusulfuron methyl is due to rapid metabolism of the herbicide, resulting in a half-life of about 1 h. This metabolism is initiated by nucleophilic attack by the glutathione sulfhydryl group on the urea carbonyl, producing the glutathione conjugate STG, 7-methylsaccharin and its free acid, which are all herbicidally inactive. These results support and extend previous studies, which have established that metabolic inactivation is the basis for selectivity of sulfonylurea herbicides on crop plants (Brown, 1990).

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THE BASIS OF THIOCARBAMATE ACTION ON SURFACE LIPID SYNTHESIS IN PLANTS

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ABSTRACT

The effects of the thiocarbamate herbicide, pebulate (S-propyl butylethylthiocarbamate) and its sulphoxide, were investigated on germinating peas *in vivo*. It was found that the sulphoxide caused a much greater inhibition of very long chain fatty acid (VLCFA) synthesis, than the parent molecule, suggesting that the sulphoxide was the active form of the herbicide. No inhibition was seen on *de novo* fatty acid synthesis. So that the inhibition of VLCFA synthesis could be studied in more detail, optimal conditions for *in vitro* microsomal assays were determined. Experiments using group selective reagents suggested that cysteine and arginine residues were important for the elongation reactions.

INTRODUCTION

Thiocarbamate herbicides have been reported to cause changes in the quality of plant surface lipid (i.e. wax, suberin, and cutin) (see Abulnaja *et al.*, 1992). These alterations result in an increased vulnerability to environmental factors and may ultimately cause the death of susceptible plants. A pre-emergent application of such compounds will allow for control of crop-associated weeds whilst safener (herbicide antidote) protected crops are not affected (Abulnaja & Harwood, 1991a). The thiocarbamate herbicide, pebulate (S - propyl butylethylthiocarbamate) and its sulphoxide were used in this project. It is believed that the latter is the active herbicidal form. This sulphoxide metabolite (like the thiocarbamates themselves) is thought to alter wax formation by having a selective effect upon the elongases involved in very long chain fatty acid (VLCFA) synthesis (i.e. fatty acids> C18) whilst having no effect on *de novo* synthesis of palmitate and stearate (Abulnaja & Harwood, 1991b). The elongases, responsible for the synthesis of VLCFAs, are present on both the endoplasmic reticulum and the Golgi apparatus. Evidence has shown that, in the synthesis of arachidic ($C_{20:0}$), behenic ($C_{22:0}$) and lignoceric ($C_{24:0}$) acids the elongase enzymes involved have different chain-length specificities (Harwood, 1991). In addition, data have shown that they may reside within differing subcellular compartments - stearoyl-CoA elongase has been shown to have greater activity within the endoplasmic reticulum, whilst the arachidoyl-CoA elongase appears to be more concentrated within the Golgi fraction (Cassagne *et al.*, 1987).

This paper describes the effect of pebulate and its sulphoxide derivative on fatty acid synthesis in germinating peas and barley leaves *in vivo*. It also details the assays we are using to investigate further the effects of these compounds on the individual elongases involved in VLCFA synthesis.

MATERIALS AND METHODS

In vivo experiments

Two systems, which have been previously shown to give good rates of fatty acid synthesis (including the rapid labelling of VLCFAs) were used - germinating peas and whole barley leaves (Harwood, 1991). [1-^{14}C]Acetate was used as a radiolabelled precursor and radiolabelled fatty acids were extracted using the method of Garbus *et al.* (1963).The thiocarbamate pebulate and its sulphoxide derivative were a kind gift from C.J. Spillner (ICI Americas). The herbicides were dissolved in ethanol (0.5%(w/v) final concentration). This ethanol concentration was shown to have no effect on either plant system with regard to fatty acid synthesis. Germinating peas were allowed to imbibe the herbicide and 1μCi [^{14}C]acetate (Amersham) for 24 h. Barley leaves were pre-incubated for 8 h with herbicide, followed by incubation in the presence of 2μCi [1-^{14}C]acetate for 15 h.

In vitro experiments

Microsomal preparations were made from 30 pea seeds (Harwood & Stumpf, 1972) which had been germinated in Vermiculite at 20°C for 24 h. Samples (3-4mg protein) were incubated for 3 h with arachidoyl - CoA (9μM) or stearoyl - CoA(9μM) and 0.05μCi of the radiolabelled precursor [2-^{14}C]malonyl - CoA (Amersham). Conditions for each assay were as follows: Arachidoyl-CoA assay - 5mM ATP, 0.5mM NADPH, 20mM potassium phosphate buffer, pH 6.6., Stearoyl-CoA assay - 3mM ATP, 0.2mM NADPH, 20mM potassium phosphate buffer, pH 7.0. Reactions were stopped by addition of 60% KOH followed by heating for 30 mins at 70°C (Bolton & Harwood, 1977). Fatty acids were extracted and analysed by radio-g.l.c. as detailed below.

Inhibitor studies

Microsomal preparations were incubated with cysteinyl residue modifiers: (Iodoacetic acid, N-ethylmaleimide, *p*-chloromercuribenzoic acid, and dithiothreitol) and phenylglyoxal (specific modifier of argine residues) at the concentrations shown in table 2. No pre-incubation was found to be necessary.

Fatty acid analysis

Fatty acids were transesterified and the resultant methyl esters were analysed by radio-g.l.c. in glass columns packed with SP-2100, using a temperature programme (210°C for first ten minutes, then 4°C/min up to 250°C) on a Pye Unicam G.C.D. chromatogram. I$_{50}$ values were estimated, assuming that separate elongases were involved, using SigmaPlot 4.0, from graphs of the labelling of each individual fatty acid versus concentration of herbicide. Fatty acid methyl esters were also analysed by reverse-phase thin layer chromatography. Silica gel G plates were silanised by exposing them to dimethyldichlorosilane for 16 hours. The plates were run in the solvent system acetonitrile:methanol:water (6:3:1, v:v:v) (Ord & Bamford, 1966). After a double development the bands were visualised, scraped and the radioactivity quantified by lipid scintillation counting.

RESULTS AND DICUSSION

The I$_{50}$ curves for the inhibition of both pebulate and its sulphoxide of very long chain fatty acid synthesis by germinating peas are shown in Figures 1, 2 and 3. Assuming that separate enzyme systems are present for the synthesis of each fatty acid, it can be surmised that elongation of behenic acid is as sensitive as elongation of arachidic acid. The inhibition of the elongation of stearic acid was also studied. However the I$_{50}$ value for this reaction was difficult to estimate for pebulate because of its low sensitivity. I$_{50}$ values for the action of pebulate on stearate, arachidate and behenate elongation were found to be $>10^{-3}$M, 3.9 x 10^{-6}M and 4.7 x 10^{-5}M, respectively. By contrast, I$_{50}$ values for pebulate sulphoxide on the same elongations were 8.7 x 10^{-6}M, 1.6 x 10^{-7}M and 1.1 x 10^{-7}M, respectively. Thus, the sulphoxide derivative itself is a more potent inhibitor of very long chain fatty acid synthesis, at comparable concentrations to that of its parent thiocarbamate. This is in agreement with the postulate that thiocarbamates are activated to their herbicidal sulphoxide derivatives (Abulnaja & Harwood, 1991b). Similar results were obtained with barley leaves (data not shown). In neither plant system did pebulate or its sulphoxide affect palmitate or stearate synthesis (by the *de novo* fatty acid synthase).

In order to study the effect of pebulate and its sulphoxide in more detail we examined the elongation of stearoyl-CoA and arachidoyl-CoA by microsomal fractions. First, optimal conditions were determined (Table 1). In both instances 9μM of the respective acyl-CoAs were required for optimal very long chain fatty acid synthesis to occur. It was found that larger concentrations of acyl-CoAs actually inhibited synthesis (data not shown). ACP was not necessary for elongation (which use acyl-CoA substrates) and its inclusion diverted

TABLE 1. Optimal assay conditions for very long fatty acid synthesis by microsomal fractions from pea seeds.

	Arachidoyl - CoA elongation				Stearoyl - CoA elongation			
pH	6.4	**6.6**	6.8	7.0	6.6	6.8	**7.0**	7.2
	943.2 ±164	**1148.2 ±133**	904.0 ±122	859.0 ±183	354.1 ±16	369.1 ±5	**490.1 ±74**	352 ±29
NADPH (mM)	0	0.25	**0.5**	0.75	0	0.1	**0.2**	0.3
	2676 ±460	3004 ±397	**3594 ±394**	3540 ±224	613.3 ±24	980.7 ±557	**1106.8 ±69**	985.5 ±139
NADH (mM)	**0**	0.25	0.5	0.75	**0**	0.1	0.2	0.5
	1736 ±80	1191 ±250	811 ±238	1290 ±1	**4672.9 ±104**	3526.7 ±153	3668.4 ±26	2992.7 ±453
ATP (mM)	0	2.5	**5**	7.5	0	2	**3**	4
	1758 ±387	1955 ±458	**2713 ±339**	955 ±93	1219.6 ±129	1265.1 ±180	**1741.0 ±293**	1220.7 ±86

Results (VLCFA d.p.m.) show means ±S.D.s for duplicate determinations

FIGURE 1. The effect of pebulate and its sulphoxide on total VLCFA synthesis in germinating pea seeds

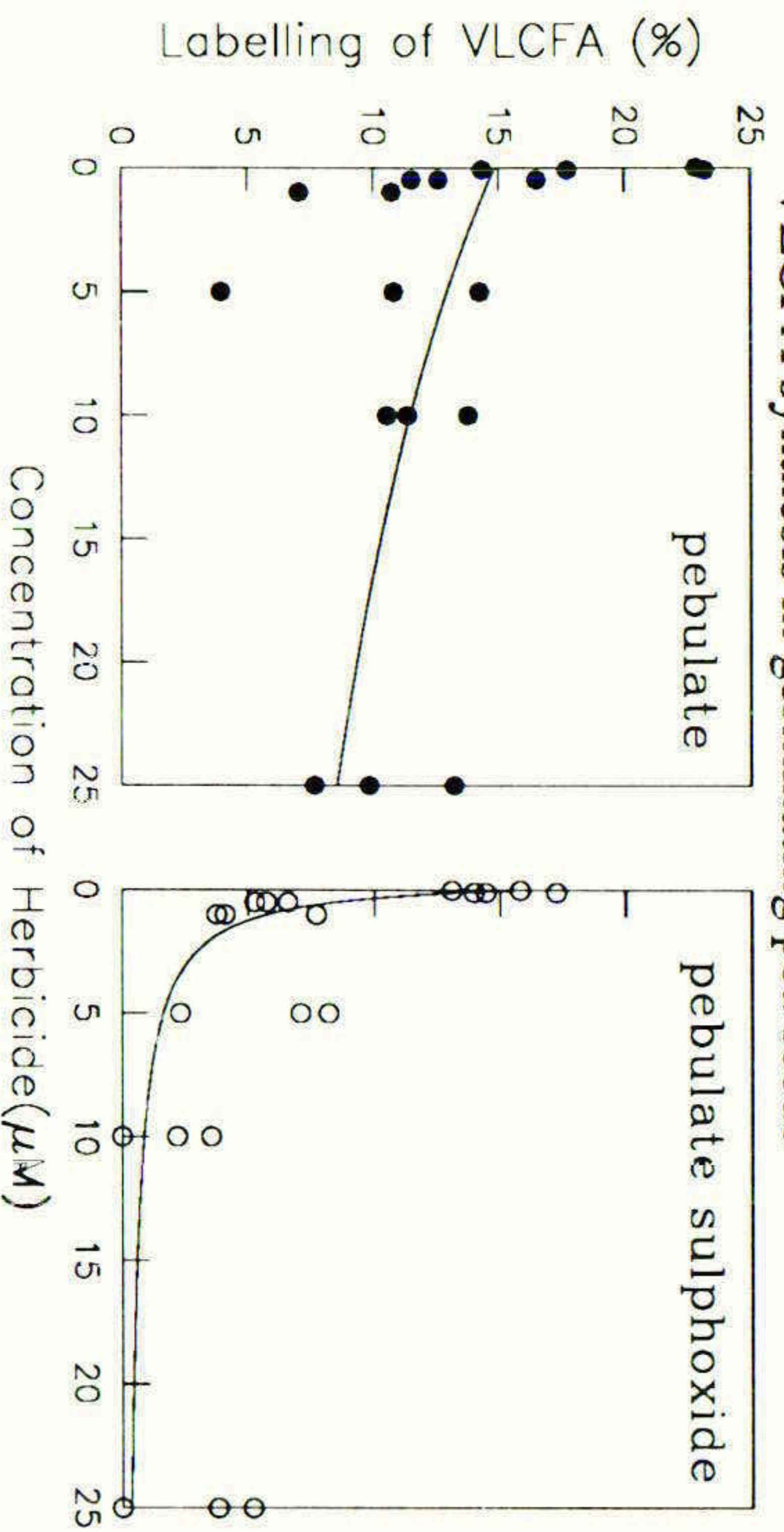Labelling of VLCFA (%)

Concentration of Herbicide(μM)

pebulate

pebulate sulphoxide

FIGURE 2. The effect of pebulate and its sulphoxide on the synthesis of arachidic acid in germinating pea seeds
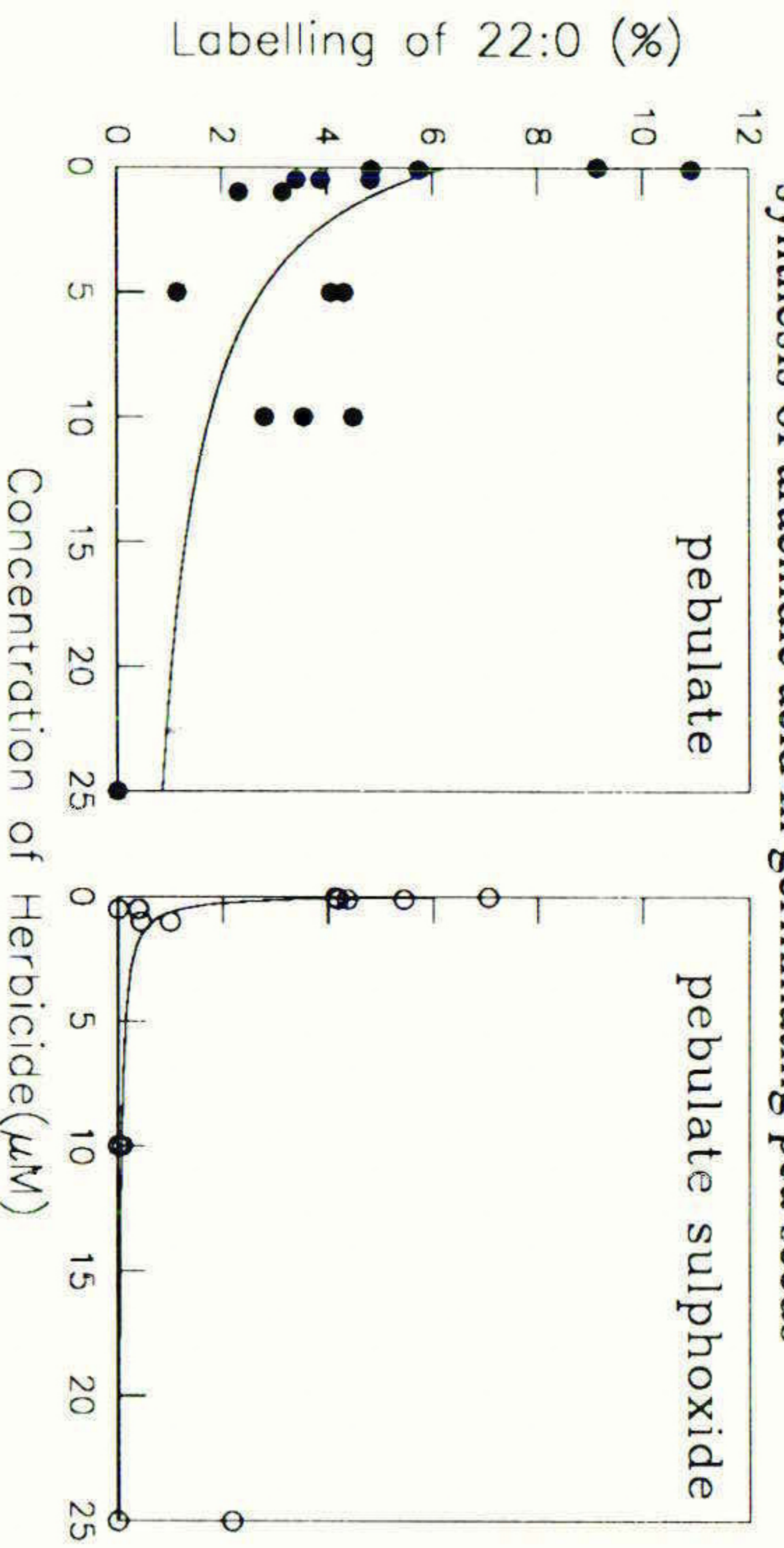
Labelling of 22:0 (%)

Concentration of Herbicide(μM)

pebulate

pebulate sulphoxide

FIGURE 3. The effect of pebulate and its sulphoxide on the synthesis of lignoceric acid in germinating pea seeds

Labelling of 24:0 (%)

Concentration of Herbicide(μM)

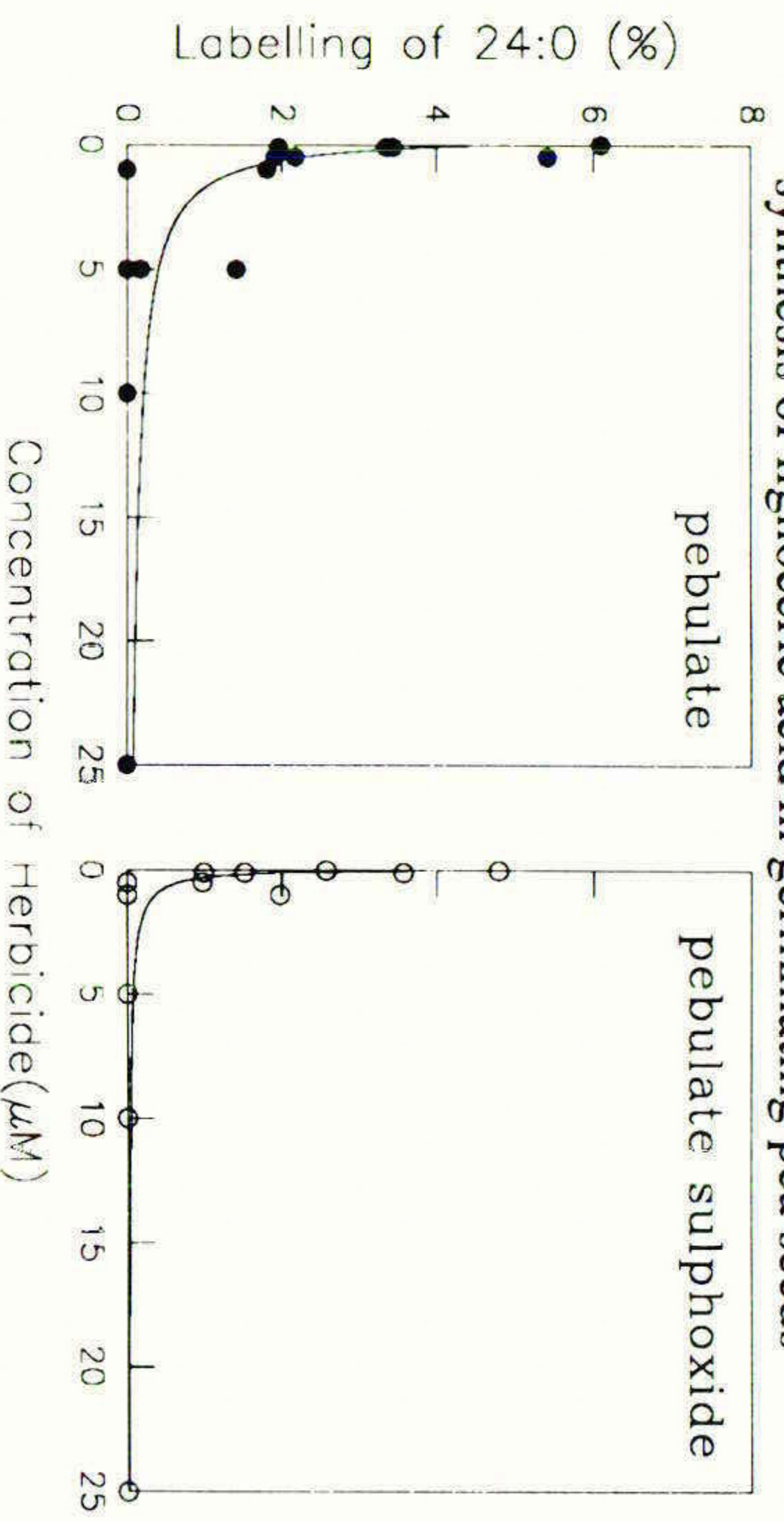pebulate

pebulate sulphoxide

Table 2. The effects of various inhibitors/group modifiers on total lipid counts
(d.p.m.) from microsomal preparations

Modifier	Assay	Inhibitor Concentration			
		0mM	1mM	2mM	10mM
phenylglyoxal	C_{18} elong.	792.5 ±291	588.9 ±137	856.1 ±140	4143.9 ±221
	C_{20} elong.	883.1 ±74	542.3 ±19	749.8 ±37	1773.1 ±974
p-CMB	C_{18} elong.	2309.6 ±469	225.7 ±25	192.9 ±115	29.0 ±5
	C_{20} elong	848.9 ±125	83.7 ±5	60.3 ±14	128.7 ±28
N-ethylmaleimide	C_{18} elong.	397.4 ±96.1	172.4 ±54.1	126.0 ±22.9	85.1 ±7.0
	C_{20} elong.	292.9 ±145.5	121.1 ±9.5	81.5 ±17.2	76.2 ±8.6
iodoacetic acid	C_{18} elong.	562.9 ±59.8	445.9 ±17.6	253.4 ±79.6	122.4 ±9.0
	C_{20} elong.	754.2 ±62.1	242.1 ±37.8	176.4 ±30.6	72.5 ±34.6
dithiothreitol	C_{18} elong.	2765.4 ±78	not known	not known	5279.7 ±1870
	C_{20} elong.	648.2 ±142	1155.6 ±311	999.9 ±456	648.9 ±48.4

Results show means ±S.D.s for duplicate determination
C_{18} elong. = stearate elongation; C_{20} elong. = Arachidate elongation

[14C]malonyl-CoA into *de novo* synthesis (data not shown). The elongases required NADPH but did not appear to utilise NADH. ATP was found to be stimulatory in both cases, probably because it allowed regeneration of acyl-CoA substrates. The slightly different optimal requirements for stearate compared to arachidate elongation confirmed the postulate that different chain length-specific elongases are present in pea seeds.

In order to characterise stearate and arachidate elongases further, we have used different group selective inhibitors (Table 2). The cysteinyl group inhibitors, N-ethylmaleimide, iodoacetic acid and p-chloromercuribenzoic acid, when added to either assay, caused a dramatic decrease in the incorporation of radiolabel within fatty acids. Analysis of radiolabelled fatty acids by radio-g.c. or reverse-phase thin layer chromatography, confirmed that the inhibition was on the respective elongation systems. The small amount of *de novo* fatty acid synthesis which was also present was less affected by the compounds. Dithiothreitol, which maintains cysteine residues in a reduced condition, had a protective effect on elongation.

The action of phenylglyoxal, an arginyl residue modifier, is also shown in Table 2. In both assays, overall fatty acid synthesis appeared to be increased at very high concentrations (10mM). However, when the radiolabelled fatty acids were analysed further it was found that phenylglyoxal caused a severe inhibition of very long chain fatty acid production in both assays. By contrast, incorporation of radioactivity from [^{14}C]malonyl-CoA into short chain fatty acids was increased.

Thus, the results of experiments using group-selective reagents show that stearate and arachidate elongases both contain functional cysteine and arginine residues. Further work is now underway to characterise the effects of pebulate and its sulphoxide on these two elongation systems.

ACKNOWLEDGMENTS

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BENZISOTHIAZOLE ARYL ETHERS - A NOVEL CLASS OF HERBICIDAL PROTOPORPHYRINOGEN OXIDASE INHIBITORS

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ABSTRACT

3-Alkyl and 3-alkoxy-5-aryloxybenzisothiazoles are a novel class of diaryl ether herbicides, which may be synthesised from 2-amino-5-methoxybenzoic acid. These compounds are inhibitors of maize etioplast protoporphyrinogen oxidase, with I50 values $\geq$ 18 nM, and are herbicidal against both broad leaf species and grasses post-emergence at an application rate of 16 g/ha. Both *in vivo* and *in vitro* activities depend on the nature of the substituents at positions 3 and 5 on the benzisothiazole ring.

INTRODUCTION

Diaryl ethers, eg acifluorfen-methyl (**1**) (Matringe *et al.*, 1989), are one of several classes of herbicides that act by inhibiting protoporphyrinogen oxidase, the last common enzyme in the biosynthetic pathway to haem and chlorophylls (Duke *et al.*, 1991; Scalla and Gauvrit, 1991; Nandihalli and Duke, 1993). Some of these herbicides have been shown to be competitive inhibitors with respect to the substrate of protoporphyrinogen oxidase from the maize etioplastic membrane, maize mitochondrial membrane and mouse liver mitochondrial membrane (Camadro *et al.*, 1991; Nandihalli *et al.*, 1992a; Nicolaus *et al.*, 1993). Inhibition of this enzyme in plants causes a build up of protoporphyrinogen, that diffuses away from the site of biosynthesis and undergoes chemical oxidation to form protoporphyrin IX, which in turn accumulates (Matringe *et al.*, 1990). The differential susceptibility of some plant species to acifluorfen (**2**) has been correlated with differences in protoporphyrin accumulation, which in several species reflects differences in the activity of the porphyrin biosynthetic pathway (Sherman *et al.*, 1991).

(1) R = CO_2 Me   (8) R = OEt
(2) R = CO_2 H   (9) R = CONHSO_2 Me
(3)
(4)
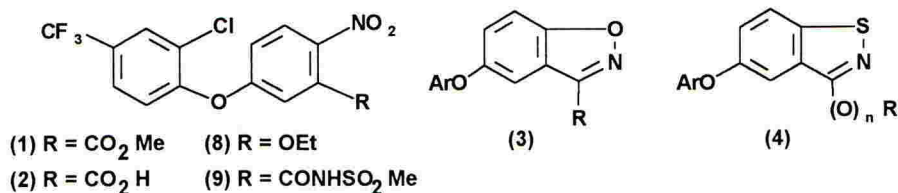
Protoporphyrin IX can act as a photosensitiser and promote the formation of singlet oxygen. The singlet oxygen generated by this process is the ultimate toxic species which can initiate the lipid peroxidation chain reaction. This leads to a loss of both cellular and

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organelle membrane integrity and eventually cell death (Nandihalli and Duke, 1993; Duke *et al.*, 1991; Halliwell, 1991; Kouji *et al.*, 1988; Haworth and Dan Hess, 1988).

Following the synthesis and evaluation of a series of benzisoxazole aryl ethers (**3**) as herbicides and protoporphyrinogen oxidase inhibitors (Chrystal *et al.*, 1991), a novel series benzisothiazole aryl ethers (**4**) was investigated.

SYNTHESIS

The 3-alkyl (**5**) and 3-alkoxybenzisothiazoles (**6**) were prepared from 2-amino-5-methoxybenzoic acid (**7**) by complimentary routes (Figure 1) (Chrystal *et al.*, 1993).

FIGURE 1: Synthetic routes to the 3-substituted-5-aryloxybenzisothiazoles
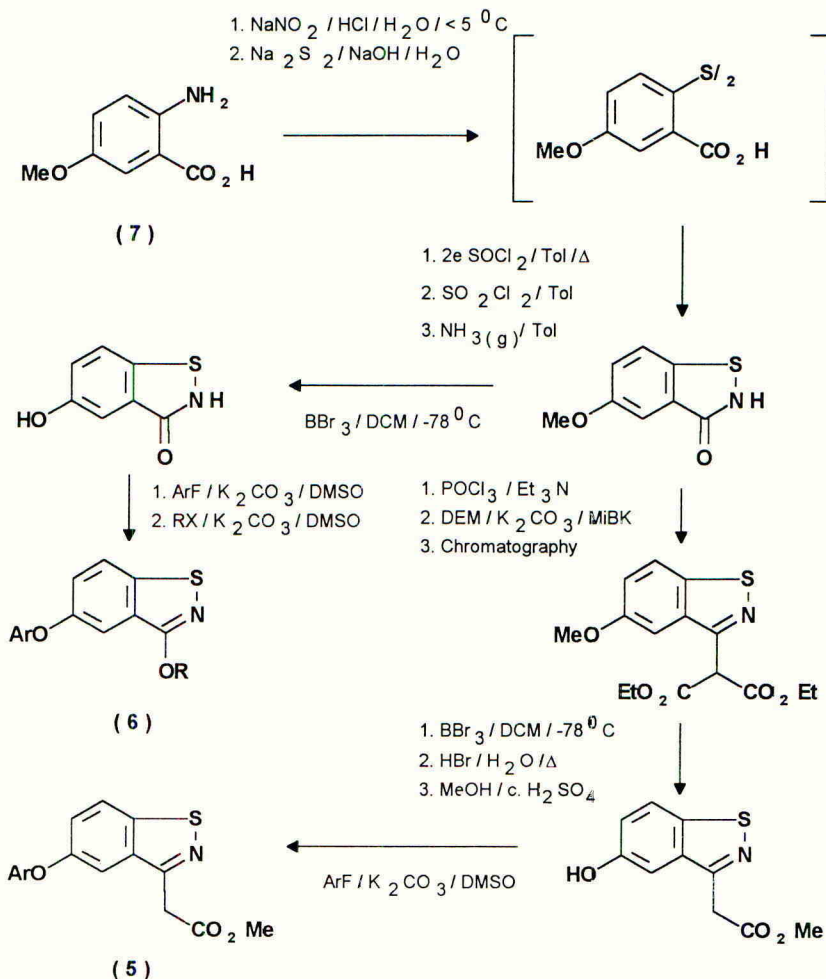
PROTOPORPHYRINOGEN OXIDASE INHIBITION STUDIES

The 3-substituted 5-aryloxybenzisothiazoles were tested as inhibitors of protoporphyrinogen oxidase using a maize etioplast preparation (Camadro *et al.*, 1989). Enzyme activity was assayed using δ-amino levulinic acid as described by Matringe *et al.* (1989). The percentage inhibition of the enzyme was measured at three concentrations of the test compound; these values were used to estimate a dose response curve and an I50 value for inhibition of protoporphyrinogen oxidase. The standards, acifluorfen (**2**), oxyfluorfen (**8**) and fomesafen (**9**) , had I50 values of 891, 14 and 29 nM respectively in this assay system.

The inhibitory activities of some benzisothiazoles are summarised in Table 1. Several analogues, (**10**), (**14**), (**15**), (**16**) and (**17**) possessed activity comparable to the more active standards and the corresponding benzisoxazole aryl ethers (**21**), (**22**), (**23**) and (**24**). The 3-alkylbenzisothiazoles were generally stronger inhibitors than the 3-alkoxybenzisothiazoles, except for compound (**10**) which showed similar activity to its 3-alkyl analogue (**14**). Replacement of the 3-methoxycarbonylmethoxy substituent (**18**) with methoxy (**19**) significantly decreased inhibitory activity. 3-Alkoxy substituted benzisothiazoles were more sensitive to the nature of the position 3 substituent. Larger substituents reduced activity.

Considering the 5-aryloxy substituent, the trisubstituted phenyl 5-(2-chloro-6-fluoro-4-trifluoromethylphenoxy)-benzisothiazoles, (**10**) and (**14**) are more active than the corresponding disubstituted phenyl 5-(2-chloro-4-trifluoromethylphenoxy)-benzisothiazoles, (**18**) and (**20**). Thus the inhibitory activity of the benzisothiazole aryl ethers depends on the nature of both the position 3 and 5 substituents. In the diphenyl ether variant of the diaryl ethers, substitution of the phenoxy group corresponding to the position 5 substituent effects both herbicidal activity and mode of action (Ohnishi *et al.*, 1993).
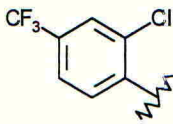
HERBICIDAL ACTIVITY

The post-emergence herbicidal activities of these benzisothiazoles were determined using standard glasshouse procedures against broad leaf species and grasses (Chrystal *et al.*, 1993). These are summarised in Table 1 as average damage assessed 13 days after treatment for broad leaf species and grasses post-emergence at a 16 g/ha application rate.

In general, activity against grasses was less than that against broad leaf species at the same application rate. The benzisoxazole analogues were usually more active than the corresponding benzisothiazoles. The corresponding 3-alkyl and 3-alkoxybenzisothiazoles exhibit similar herbicidal activities, except when the position 3 substituent is methoxy (**19**), then activity is reduced even at a higher application rate of 62.5 g/ha. A major modifier of herbicidal activity is the nature of the 5-aryloxy substituent. The 3-alkoxy substituted 5-(2-chloro-6-fluoro-4-trifluoromethylphenoxy)-benzisothiazole (**10**) is more active than the corresponding 5-(2-chloro-4-trifluoromethylphenoxy)-benzisothiazole (**18**). However, the corresponding 3-alkylbenzisothiazoles, (**14**) and (**20**), show similar herbicidal activity.

TABLE 1: I50 values for the inhibition of protoporphyrinogen oxidase and average post-emergence herbicidal activity, as percentage damage versus control 13 days after treatment at an application rate of either 16 g/ha, or [1] 62.5 g/ha.

Ar Group A   Ar Group B

Compound Number	X	Ar Group	R	I50 (nM)	Herbicidal Activity	
					Broad Leaf Species	Grasses
10	S	A	-OCH$_2$CO$_2$Me	18	85	44
11	S	A	-OCH$_2$CO$_2$Et	63	78	57
12	S	A	-OCHMeCO$_2$Me	85	79	30
13	S	A	-OCHMeCO$_2$Et	155	76	29
14	S	A	-CH$_2$CO$_2$Me	22	66	49
15	S	A	-CH$_2$CO$_2$Et	37	80	48
16	S	A	-CHMeCO$_2$Me	30	81	55
17	S	A	-CHMeCO$_2$Et	38	74	55
18	S	B	-OCH$_2$CO$_2$Me	174	49	24
19	S	B	-OMe	537	28[1]	12[1]
20	S	B	-CH$_2$CO$_2$Me	56	65	30
21	O	A	-CH$_2$CO$_2$Me	74	97	55
22	O	A	-CH$_2$CO$_2$H	20	75	31
23	O	A	-OCH$_2$CO$_2$Me	42	74	31
24	O	A	-CHMeCO$_2$Me	3	95	80

CONCLUSIONS

There is a general relationship between protoporphyrinogen oxidase inhibitory activity and herbicidal activity for the benzisothiazole aryl ether class of diaryl ether herbicides. However, there is sufficient variation to implicate other factors such as uptake, translocation and metabolism as influencing *in vivo* activity. Enzyme inhibitory activity depends on the nature of both the 3-alkyl/alkoxy substituent and the 5-aryloxy substituent of the benzisothiazoles. Herbicidal activity depends mainly on the nature of the 5-aryloxy substituent.

Recent analyses of the activities of diphenyl ether herbicides have found that variation in the electronic properties (partial charge, superdelocalisability of the lowest unoccupied molecular orbital, and dipole moment) and lipophilicity accounted for the variation in enzyme inhibiting activity, while the molecular bulk and overall electrostatic potentials correlated with the variation in herbicidal activity (Nandihalli, *et al.*, 1992a). Other studies have also found a correlation between lowest unoccupied molecular orbital properties (shape and energy level) for inhibitors of protoporphyrinogen oxidase from several different structural classes (Akagi and Sakashita, 1993). Further studies on phenopylate and its analogues found that protoporphyrinogen oxidase inhibition, protoporphyrin IX accumulation and herbicidal activity were all correlated with the van der Waals volume, electrophilic superdelocalisability and the energy of the lowest unoccupied molecular orbital (Nandihalli *et al.*, 1992b). The relationship between biological activities and molecular lipophilicity (Log P) was not linear. Similarly studies on the pyridone carboxanilide class of protoporphyrinogen oxidase inhibitors and diphenyl ethers found herbicidal activity was correlated with lipophilicity (Log P) and molecular steric and electrostatic properties (Osabe *et al.*, 1992). A difference in the property accounting for the variation in enzyme inhibitory activity (substituent lipophilicity) and the variation in herbicidal activity (substituent steric dimensions) has also been noted for the cyclic imide class of protoporphyrinogen oxidase inhibitors (Nicolaus *et al.*, 1993). Thus the observation that the enzyme inhibitor activities and the herbicidal activities of the benzisothiazole aryl ethers do not correlate perfectly is consistent with the results of other workers, ie the optimisation of the two activities can depend on different molecular properties and that the relationship between these biological activities and molecular properties is often complex.

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ENHANCEMENT OF AC 263222 METABOLISM BY THE HERBICIDE SAFENER NAPHTHALIC ANHYDRIDE

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ABSTRACT

In glasshouse experiments, naphthalic anhydride (NA) was found to protect maize cv. Monarque, from injury by the imidazolinone herbicide AC 263222. Uptake, distribution and metabolic studies, where [^{14}C]-AC 263222 was applied through the roots of hydroponically-grown maize, have shown that safening is associated with retention of the radiolabel in the roots and accelerated degradation of the herbicide to the hydroxylated metabolite. The lower mobility of this compound accounts for its accumulation in the roots while its hydroxylated nature suggests that NA enhances the level of a cytochrome P_{450} or other oxygenase system. In agreement with this theory, the cytochrome P_{450} inhibitor, aminobenzotriazole, was found to synergize AC 263222 activity by inhibiting its hydroxylation. Furthermore, NA significantly promoted total cytochrome P_{450} content of microsomes extracted from etiolated maize seedlings. Attempts to demonstrate microsomal metabolism of AC 263222 are also described.

INTRODUCTION

The herbicide safener, naphthalic anhydride (NA), enhances the tolerance of maize (*Zea mays*) to several chloroacetanilide, thiocarbamate, sulphonylurea and imidazolinone herbicides. As the selectivity of these herbicides is largely determined by differential metabolism, it is postulated that NA may accelerate their degradation to less active or immobile metabolites. This would involve NA in the induction of the appropriate metabolic enzymes, such as the cytochrome P_{450} mono-oxygenases believed to be responsible for some reactions of Phase I herbicide metabolism. Several authors have presented data consistent with this mode of action, including Sweetser (1985) and Shaner (1991) who demonstrated NA-induction of chlorsulfuron and imazethapyr hydroxylation, respectively. Furthermore, Frear *et al*. (1991), Mougin *et al*. (1991) and McFadden *et al*. (1990) have shown that pre-treatment with NA can promote hydroxylation of chlorsulfuron, chlorotoluron and bentazon, respectively, by the microsomal fraction with which cytochrome P_{450} is associated. However, investigations beyond this point are restricted by a lack of direct evidence implicating cytochrome P_{450}, or indeed other oxygenases, in the detoxification of many of the herbicides in question. Consequently, the precise mode of action of NA still remains unclear.

Here, the effects of NA on the activity and metabolism of the imidazolinone herbicide, AC 263222, in maize are demonstrated while interactions with the cytochrome P_{450} inhibitor, 1-aminobenzotriazole (ABT) will be used as indirect evidence for the involvement of cytochrome P_{450} in the metabolism of this herbicide. Furthermore, attempts to associate herbicide metabolism with the maize microsomal fraction will be discussed.

METHODS

Effects of NA on AC 263222 activity

Seeds of the maize cultivar Monarque were dressed with 0, 0.25, 0.5 or 1.0% w/w NA, and sown at a depth of 3cm in a sandy loam soil amended with 30% grit. After 2 days, the pots were sprayed with AC 263222 (396g a.i. l^{-1} EC) at 6 doses and returned to glasshouse conditions, i.e. 26°C with watering by capillary action, until harvest 14 days after treatment (DAT). At this time assessments were made of 3rd leaf length, fresh and dry shoot weights.

Effect of NA and ABT on AC 263222 metabolism

NA-treated and untreated seed was sown on damp filter paper and germinated in a growth cabinet maintained at 26°C and 90% r.h. in the dark. After 2 days, seedlings were suspended in 5ml half-strength Hewitt's nutrient solution with 0.1% acetone containing 0.4-0.5μCi [^{14}C]-AC 263222 (6.1 μCi μmol^{-1}) with or without 40μM ABT. Seedlings were maintained for 24h with a 14h photoperiod before being transferred to polystyrene seed trays floating on 500ml nutrient with continued ABT treatment as required. Plants were harvested 3, 4 or 7 days after sowing, weighed and cut into 0.5cm lengths before freezing in liquid nitrogen.

Tissue samples were extracted in a volume of acetone - methanol - water (1:1:1,v:v:v) corresponding to 10 times tissue weight, using an Ultra-Turrax homogenizer for 3 x 1 min intervals. The homogenate was then centrifuged, the resulting supernatant decanted and the pellet resuspended. Following a second extraction, the remaining pellet was combusted using a Harvey Biological Oxidizer OX500, for determination of unextractable radiolabel. Supernatants were combined and filtered through Whatman No 1 paper before being reduced to dryness by rotary evaporation. Residues were resuspended in 200μl methanol, 100μl of which was dispensed into OptiPhase HiSafe 3 scintillant for liquid scintillation counting. The remaining extract was spotted onto TLC plates (Merck, Kieselgel 60 F_{254}) to be developed in propan-1-ol - dichloromethane - formic acid (4:5:1,v:v:v). AC 263222 and its metabolites were located using an Isomess radio-TLC scanner and the corresponding areas of silica gel scraped into vials and eluted with 300μl methanol in preparation for liquid scintillation counting. Data presented is based on 4 replicates of 2 plants for root extractions and 2 replicates of 4 plants for shoot extractions.

Microsome extraction from etiolated seedlings

NA-treated and untreated seed was sown in vermiculite, watered and germinated under conditions described for hydroponic experiments. After 3 days in the dark, roots and shoots were harvested and homogenized by Ultra-Turrax for 3 min with a volume of extraction buffer equivalent to 2 times tissue weight. The buffer consisted of 0.1M sodium phosphate, pH 7.4, containing 1mM EDTA, 2% soluble polyvinylpyrrolidone and 12mM mercaptoethanol. The resulting pulp was filtered through 2 layers of muslin before centrifuging at 6,000 g for 30 min followed by centrifugation of the supernatant at 100,000 g for 1h. The resulting microsomal pellet was then resuspended in 1-2ml 0.1M sodium phosphate buffer, pH 7.4, containing 30% glycerol, and stored at -80°C. Microsomal protein contents were estimated using the Pierce BCA (bicinchoninic acid) assay system with bovine serum albumin as standard.

Microsomal enzyme assays

Microsomal cytochrome b_5 and P_{450} contents were determined using the methods of Omura and Sato (1964). Microsomes were diluted to a protein concentration of 1-2 mg ml^{-1} with 0.1M sodium phosphate buffer, pH 7.4, and divided between sample and reference cuvettes. A base-line spectrum was then generated between 400 and 600nm and the sample cuvette subsequently reduced for 1 min with sodium dithionite before production of a reduced difference spectrum. This was used to calculate cytochrome b_5 content assuming an extinction coefficient of 185 mM^{-1}cm^{-1} for $A_{409-424}$. The reference was then also reduced and a new base-line produced before the sample was bubbled with CO for 45 sec. A final scan produced the CO reduced difference spectrum from which cytochrome

P_{450} content was calculated using an extinction coefficient of 91 mM^{-1}cm^{-1} for $A_{450-490}$.

Cytochrome c reductase and cinnamic acid-4-hydroxylase activities were assayed according to the methods of Strobel and Dignam (1978) and Reichhart et al. (1980), respectively. Possible in vitro metabolism of AC 263222 was assayed in a volume of 200μl 0.1M sodium phosphate buffer, pH 7.4, containing 0.5-1.0 mg microsomal protein, 10mM NADPH and 0.02μCi [^{14}C]-AC 263222 (6.1 μCi μmol^{-1}). After incubation in a shaking water bath at 26°C for 20 min, the reaction was terminated by the addition of 100μl acetone. Samples were then centrifuged to remove precipitated protein before 150μl supernatant was subject to TLC as described previously. In all experiments, control samples were assayed in which either NADPH and/or microsomes were omitted. Data presented is based on the mean of at least 6 replicates, each obtained using microsomes extracted on separate occasions.

RESULTS AND DISCUSSION

Despite 20% inhibition of shoot growth, seed treatment with NA significantly reduced the phytotoxicity of AC 263222 to maize in glasshouse experiments. For example, the GR$_{50}$ (as estimated by fitting parallel dose response curves with a non-zero asymptote) was increased significantly from 108 g ha^{-1} in unsafened plants to >300 g ha^{-1} in safened maize (Figure 1). Similar safening responses were obtained in hydroponically-grown maize exposed to AC 263222 for 24h when 2 days old. In this case, 0.5% NA increased the GR$_{50}$ by more than 10-fold.

FIGURE 1 : Effect of NA on maize tolerance to AC 263222 injury
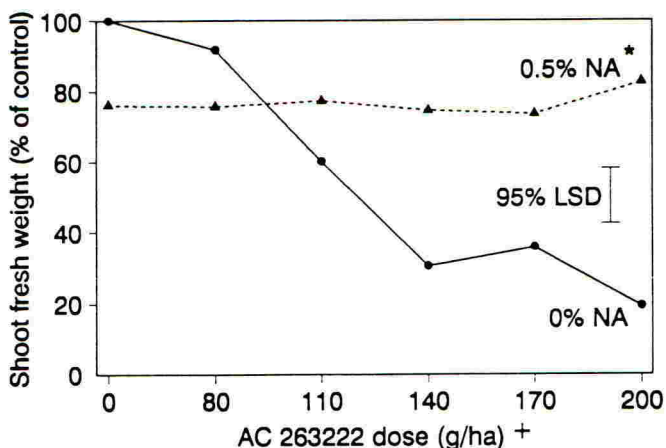

* NA applied as a seed-dressing
\+ AC 263222 applied as a pre-emergence spray 2 days after sowing

Seed-dressing with 0.5% NA did not alter uptake of [^{14}C]-AC 263222, expressed in terms of dpm per unit root weight, or levels of unextractable radiolabel. However, it did change the distribution of the radiolabel within the seedlings such that a greater proportion was retained in the root system (Figure 2). For example, although treated and untreated seedlings contained similar amounts of radiolabel in their roots at 3 days, by day 7 only 9% had been translocated to the shoots of safened plants while 30% had been translocated in unsafened plants.

FIGURE 2 : Effect of NA on the distribution of radiolabelled AC 263222
between maize seedling roots and shoots
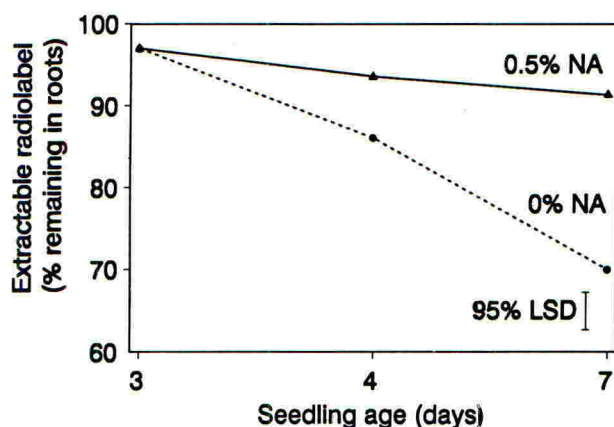
Metabolic studies indicated that maize seedlings metabolized AC 263222 to a single metabolite which was identified by co-chromatography as the pyridinyl ring methyl-hydroxylated product (Figure 3). Furthermore, the rate of hydroxylation was approximately doubled by seed treatment with 0.5% NA (Figure 4). For example, at 4 days >90% of AC 263222 had been hydroxylated by roots of safened seedlings while only 48% had been converted by roots of untreated plants. A similar difference was observed in the composition of ^{14}C present in shoot tissue. Experiments conducted with isolated maize shoots produced identical metabolic profiles thus suggesting that hydroxylated AC 263222 appearing in the shoot was produced *in situ* rather than solely by translocation from the roots. Indeed data regarding the mobility of the imidazolinones (Little and Shaner, 1991) indicates that the increased hydrophilicity of AC 263222 following hydroxylation would reduce mobility of the metabolite relative to the parent molecule. This suggests that increased retention of radiolabel in the root system of NA-treated plants is a result of accelerated hydroxylation. Similar observations have been made by Barrett (1989) and Shaner (1991) investigating the effect of NA on the distribution and metabolism of imazethapyr.

FIGURE 3 : Metabolism of AC 263222 in maize

AC 263222   hydroxylated AC 263222
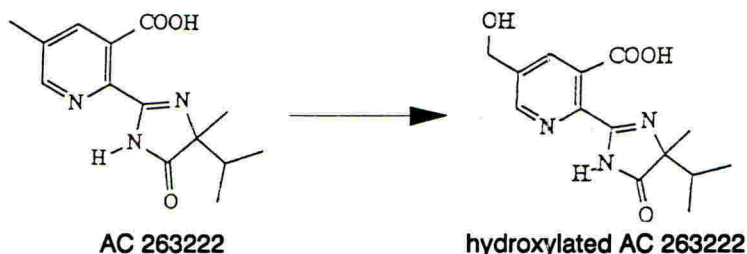
In contrast to the effect of NA, ABT was found to synergize AC 263222 activity in hydroponically-grown maize and reduce the rate of hydroxylation. For example, while 44% of the parent molecule had been hydroxylated in untreated roots, only 24% was metabolised in those receiving $40\mu M$ ABT treatment. The status of ABT as a known cytochrome P_{450} inhibitor, suggests that hydroxylation of AC 263222 may occur *via* a P_{450} mono-oxygenase. In light of this observation, the effects of NA on various P_{450} activities are illustrated in Figure 5.

FIGURE 4 : Effect of NA on the metabolism of radiolabelled AC 263222 in maize seedlings
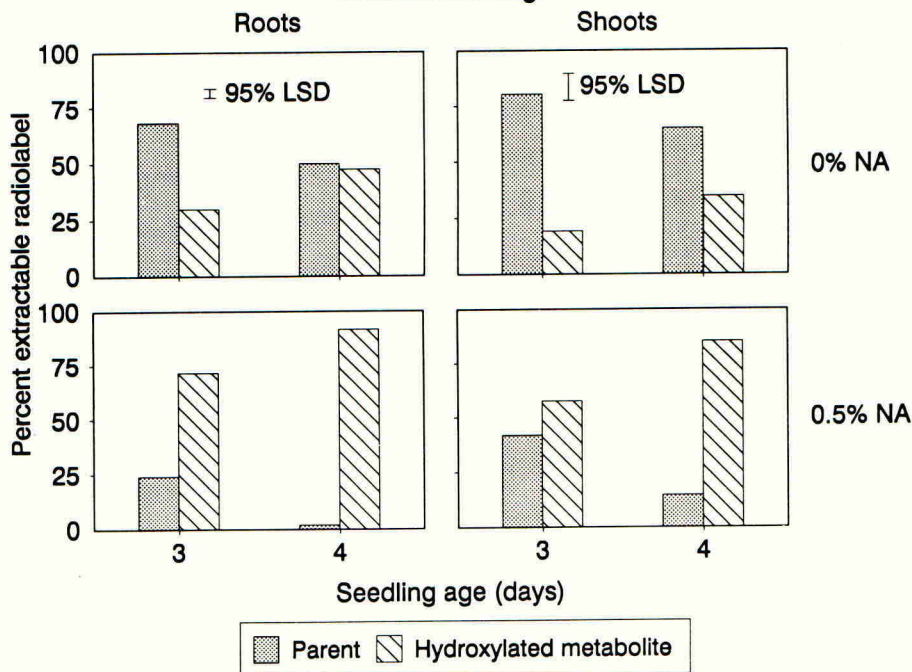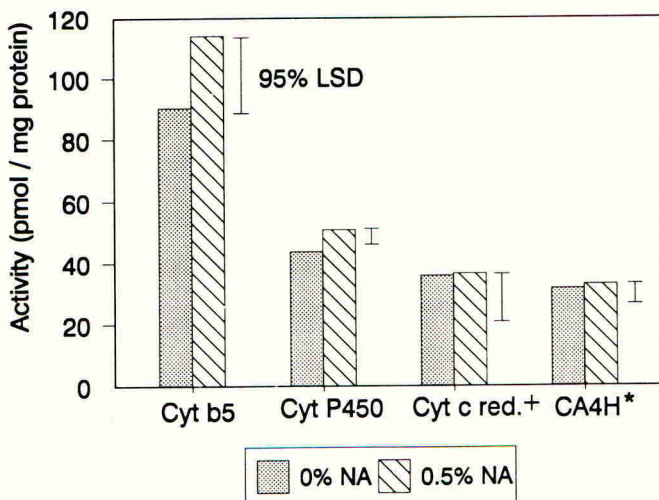

FIGURE 5 : Cytochrome P450 activities of microsomes prepared from etiolated maize shoots

+ NADPH cytochrome c (P450) reductase (nmol/min/mg protein)
* cinnamic acid hydroxylase (pmol/min/mg protein)

As components of the microsomal electron transport reactions of cytochrome P_{450}, increased NADPH cytochrome c (P_{450}) reductase activity and cytochrome b_5 content could contribute to a rise in the metabolic capacity of the P_{450} system. However, 0.5% NA did not significantly alter these factors in microsomes extracted from etiolated maize seedlings exhibiting similar metabolic capacity and safening response as chlorophyllous seedlings. Similarly, NA had no effect on the activity of the P_{450} enzyme cinnamate-4-hydroxylase (CA4H). In contrast, total cytochrome P_{450} content was significantly increased from 42.7 to 51.0 pmoles mg protein^{-1} by 0.5% NA. Although small, this difference may reflect an increase in the specific isozyme responsible for AC 263222 metabolism at the expense of others. Consequently, attempts were made to demonstrate microsomal herbicide metabolism. To date, these have been unsuccessful, although attempts are continuing by manipulation of microsome extraction procedures and assay conditions.

CONCLUSIONS

NA protection of maize from AC 263222 injury has been linked to accelerated hydroxylation of this herbicide *in vivo*. Indirect evidence, namely inhibition of hydroxylation by ABT and the ability of NA to increase total cytochrome P_{450} content, suggests that metabolism occurs *via* a cytochrome P_{450} system. However, doubt over possible secondary effects of ABT combined with failure to demonstrate microsomal metabolism, suggests that the involvement of other oxygenase systems cannot be eliminated.

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THE BASIS OF RESISTANCE DISPLAYED TO FLUAZIFOP-BUTYL BY BIOTYPES OF
ELEUSINE INDICA

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ABSTRACT

A biotype of *Eleusine indica* from Malaysia, which had reportedly survived
field treatment with the aryloxyphenoxypropionate (AOPP) graminicide
fluazifop-butyl, was found to show up to 200-fold resistance to the herbicide
under glasshouse conditions compared with reference plants from a susceptible
population. In addition, the biotype showed resistance to other members of the
AOPP and cyclohexanedione (CHD) classes of herbicides. The resistance was
not found to be related to differences in metabolic detoxification or
translocation of the herbicide at the whole plant level. *In vitro* assays, which
employed leaf-discs to measure inhibition of fatty acid biosynthesis by
fluazifop acid, showed that the resistant biotype was less sensitive at this level.

INTRODUCTION

Populations of *Eleusine indica*, a C4 annual grass weed, which showed evidence of
resistance to fluazifop-butyl, were reported in Malaysian vegetable gardens in 1989. Fluazifop-
P-butyl is a member of the aryloxyphenoxypropionate (AOPP) class of herbicides and acts
through inhibition of acetyl CoA-carboxylase which catalyses the first committed step in fatty
acid biosynthesis (Secor & Cseke, 1988). All of the areas where failure of control was noted
were thought to have undergone treatment with fluazifop-butyl at recommended rates
approximately 2-3 times per year for the preceding 4-5 years. Seeds from putative resistant
plants were taken from one of the affected sites in 1989.

The resistance was confirmed in the field by the treatment of plants grown from this
seed with fluazifop-butyl (0.25 - 16 kg ha^{-1} AI). The surviving plants set seed (R-type) and
were comparable with local reference susceptible plants (S-type) for their growth
characteristics (Marshall et al., 1993) and the ba sis of resistance (present study).

MATERIALS AND METHODS

Multi-graminicide screen and dose rate responses of *E. indica* biotypes to fluazifop-P-butyl

E. indica plants were raised from seed which was surface-sown into seed trays
containing a fine textured, peat-based compost (Fisons F2). The trays were placed in
controlled environment growth-rooms at 22°C (+/- 3°C) with a 16h photoperiod provided by
fluorescent tubes (photosynthetic photon flux density = 350-450 mmoles m^{-2}s^{-1}). After
approximately 21 days, the plants were transplanted into 10 cm pots (five plants per pot) and

maintained in the environment rooms for a further week (growth stage 2-3 leaf) before being sprayed using a Mardrive (Marine Engineering, Stockport) bench-mounted track-sprayer. Application was made using an 80° flat fan nozzle (TeeJet) and an application rate of 185 l ha^{-1} in a single pass of the head.

Plants were treated with a range of commercially formulated graminicides from the AOPP and CHD classes, all at 50 g ha^{-1} AI. The herbicides used were: fluazifop-P-butyl; diclofop-methyl; fenoxaprop-ethyl; sethoxydim; tralkoxydim and clethodim. Agral 90 (0.1% v/v) was added to the spray solution as wetter. Each pot was regarded as a single replicate with four replicate pots per biotype per herbicide. The plants were harvested 21 days after treatment and dry weight yields assessed.

A dose rate response experiment in which plants were treated with the range 0, 50, 100, 250, 500, 1000 and 2000 g ha^{-1} AI of fluazifop-P-butyl was also performed. In all cases Agral 90 (0.1%) was added as wetter. Again, five plants were grown per pot with each pot regarded as a single replicate. There were four replicate pots per biotype per dose. The plants were harvested 21 days after treatment and dry weight yields measured. The number of dead plants in the sample was also noted.

Translocation studies

Translocation experiments were carried out using [14C]-*phenoxy*-labelled herbicide. Plants of *E. indica* were raised as above and treated at the 3-leaf (fully expanded) growth stage. Immediately prior to treatment with radiolabel, all plants were sprayed at 400 l ha^{-1} using a rate of 100 g ha^{-1} AI fluazifop-P-butyl. The leaves that were intended for treatment with [14C]-herbicide were covered with slips of parafilm during this pre-spray procedure. Both the standard and [14C]-herbicide were applied to the plant surface in 10% blank, commercial, emulsifiable concentrate formulation, without additional wetter. [14C] *phenoxy*-labelled herbicide (sp. act. 2.16 GBq mmol^{-1}) was applied to the youngest fully expanded leaf using a Micro-lab-P pipette, as 5 x 0.2 ml drops (0.8 kBq per plant). The leaf blade was held horizontally throughout the application of the [14C]-herbicide and until the surface of the leaf appeared dry. Harvest was carried out at 1,3 or 7 days after treatment. At each date, 6 replicate treated plants of each biotype were selected at random from the stock of treated plants in the growth cabinet. The plants were divided into treated and non-treated portions and these were placed in glass vials and oven-dried at 40°C for 48 h prior to oxidation (Packard oxidiser: model B306). The amount of activity present in each portion was then assessed by liquid scintillation counting.

Analysis of metabolites from apical meristems

Plants were raised as above for use in apical meristem extractions. The two youngest fully expanded leaves were treated with [14C]-pyridyl-labelled herbicide (sp. act. 2.15 GBq mmol^{-1}) Each plant received 1.6 kBq. Twenty-four replicate plants of each biotype were treated for each experiment. The plants were maintained in a growth room under fluorescent tube lighting and a temperature of 25°C+/-1°C.

At 1 DAT, the apical meristems were dissected out of the plants and macerated with acetonitrile which was decanted from the debris and collected. The debris was rinsed with further fresh solvent. The solvent fractions were pooled and dried under nitrogen and analysed by tlc under the solvent system: diethylether:hexane:acetic acid (60:40:2 v v:v). Non-radiolabelled standards of fluazifop-butyl and fluazifop acid were co-chromatographed for purposes of metabolite identification. The chromatographic analyses were carried out at Zeneca

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Agrochemicals, Jealott's Hill, Berks.

[^{14}C]-Acetate incorporation in leaf discs

The method used was adapted from Bjelk & Monaco (1992). Leaf-discs were taken from the middle of the youngest fully expanded leaf of *E. indica* plants which had been raised under the conditions described above. Ten leaf discs (each *c*. 19.6 mm^2 surface area) were taken from each plant and placed in a test-tube containing 1.85ml of 25 mM tricine-KOH buffer, pH 7.9, containing 1.0 mM MgCl$_2$, 1.0 mM CaCl$_2$ and 10 mM NaHCO$_3$. The tubes were degassed under vacuum for 30 min before 100ml of herbicide or blank buffer was added to the tubes. The herbicide used was technical grade fluazifop acid which was applied to the assay tubes in a buffer (9% acetone; 25 mM tricine and 10 mM MgCl$_2$, pH 7.9). This delivered a final acetone concentration of 0.45% in both treatment and control tubes; the latter received buffer without herbicide. Each treatment consisted of five replicate tubes, each of which contained ten leaf-discs taken from a single plant. The tubes were placed under light (approx. 21.7 klx) at 28°C for 30 min. The acetate incorporation reaction was initiated by the addition of 9 nmol [^{14}C]-acetate (2.08 GBq mmol^{-1}) per tube to give final concentration of 4.6 mM (c.18.7 kBq per tube). Thereafter the tubes were placed under the light for a further 2 h. After the 2 h incubation period the bathing solution was removed and the leaf-discs were extracted using chloroform : methanol (1:1, v:v). The activity present in the organosoluble fraction was assessed by liquid scintillation counting.

RESULTS

Multi-graminicide screen and dose rate responses of *E. indica* biotypes to fluazifop-P-butyl

All the herbicides used in the screen reduced dry weights of the treated plants of both biotypes, but significant ($P = 0.05$) reductions in the biomass yields were attained by all compounds only in the case of the S-type; reductions in the biomass yields of the R-type were not significant at this level for fluazifop, diclofop or fenoxaprop. These results are shown in Figure 1.

The effects of the application rates 0-2000 g ha^{-1} of fluazifop-P-butyl on dry weight yields of R and S-types are shown in Figure 2. The dose that yielded 50% growth reduction for the S-type *E. indica* with fluazifop-butyl over 21 days was below 50 g ha^{-1} whilst that from the R-type was approximately 750 g ha^{-1}. The 50 and 100 g ha^{-1} rates of fluazifop-P-butyl caused 100% mortality in the S-type population after three weeks whilst 2 kg ha^{-1} caused only 75% mortality in the R-type in the same period. From similar experiments conducted at lower application rates (data not shown), it was found that 10 g ha^{-1} AI of fluazifop-P-butyl caused 100% mortality of the S-type at 21 DAT.

Metabolism studies

The tlc and analysis performed on the apical extracts of [^{14}C]-herbicide-treated plants identified only fluazifop-acid, the phytotoxic, de-esterification product of fluazifop-butyl at 1 DAT. The extracts from both biotypes showed traces of unidentified polar products. There was no apparent difference in the content of the extracts of the two biotypes.

Translocation studies

Plants treated on a single leaf with [^{14}C]-herbicide were harvested, sectioned and

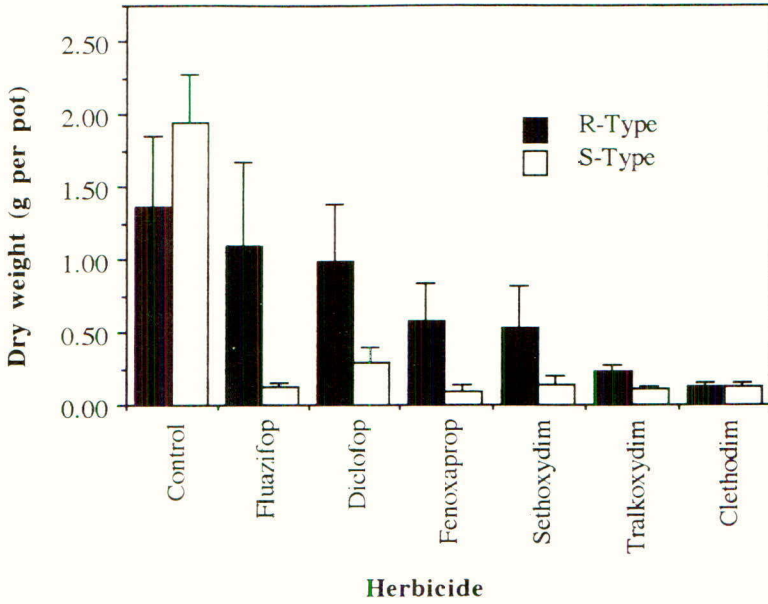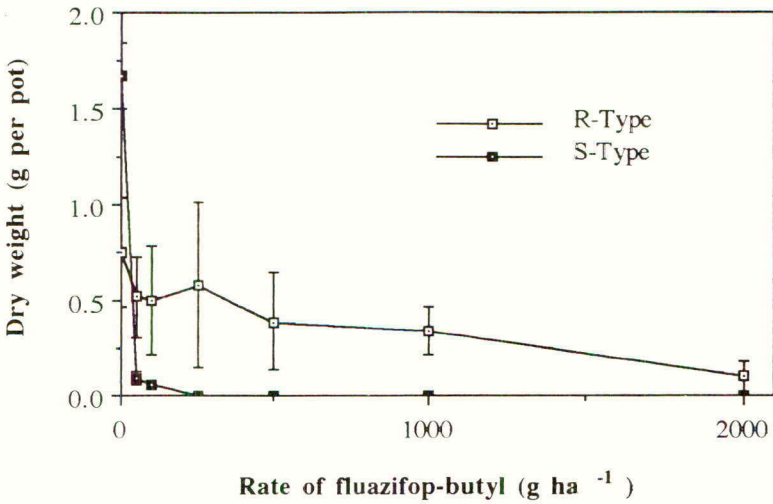**FIGURE 1.** Dry weight yield of *E. indica* (21 DAT) treated with a range of graminicides (50 g ha $^{-1}$)

FIGURE 2. Dose rate response of *E. indica* (21 DAT) treated with a range of rates of fluazifop-butyl (0-2000 g ha $^{-1}$)

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combusted. The percentage of the recovered activity which was translocated by each biotype is shown in Table 1. From the results of these experiments, it was apparent that, in both biotypes, the majority of the applied herbicide (>80%) remained on or in the treated leaf. The proportion of the herbicide delivered to the meristematic area appeared to be similar in each of the two biotypes ($P = 0.05$). When tillers were present, the amount of herbicide to reach them was similar in each biotype.

TABLE 1. Percentage of recovered radio-activity from regions of [^{14}C]-fluazifop-butyl treated-*E. indica* outwith the treated leaf.

| DAT	R-Type	S-Type
1	2.73 [1.76]	2.70 [2.02]
3	4.72 [2.64]	2.76 [1.30]
7	7.71 [5.52]	4.15 [2.64]

[ ] = standard error

[^{14}C]-Acetate incorporation

The results of the *in vitro* leaf disc dose response are shown in Figure 3. The concentration of fluazifop acid which caused 50% inhibition of incorporation of [^{14}C]-acetate into the organic fraction (I50) after two h was approximately 2.5 mM for the S-type leaf-discs. The I50 of the R-type was calculated from this graph by extrapolation of the linear regression line and this indicated an I50 of c. 24 mM fluazifop acid, ie. a ten-fold decrease in sensitivity.

DISCUSSION

Since it is known that the two graminicide classes to which resistance has been confirmed are structurally and chemically dissimilar but share the same target enzyme, the involvement of a specific mechanism involving the target site of action in the resistance does appear to be a possibility. The presence of a lower level of resistance to members of the CHD class in addition to that towards the AOPPs may be due to the presence of an ACCase which was selected on the basis of its resistance toward an AOPP (fluazifop-butyl) and not on its ability to evade inhibition by CHDs. The results that have been presented with regard to fatty acid biosynthesis by leaf discs treated *in vitro* with fluazifop, indicate that the site of action (ie. ACCase) may be involved in the resistance mechanism of R-type plants. The R-type leaf-discs showed a reduction of sensitivity of c. 10-fold compared to that of the susceptible type in the absence of differences in translocation or metabolism. However, the leaf disc system did not remove considerations of possible subcellular localisation or of possible involvement of membrane effects.

FIGURE 3. Incorporation of [^{14}C]-acetate by leaf-discs from R & S types of *E. indica* treated *in vitro* with fluazifop acid.
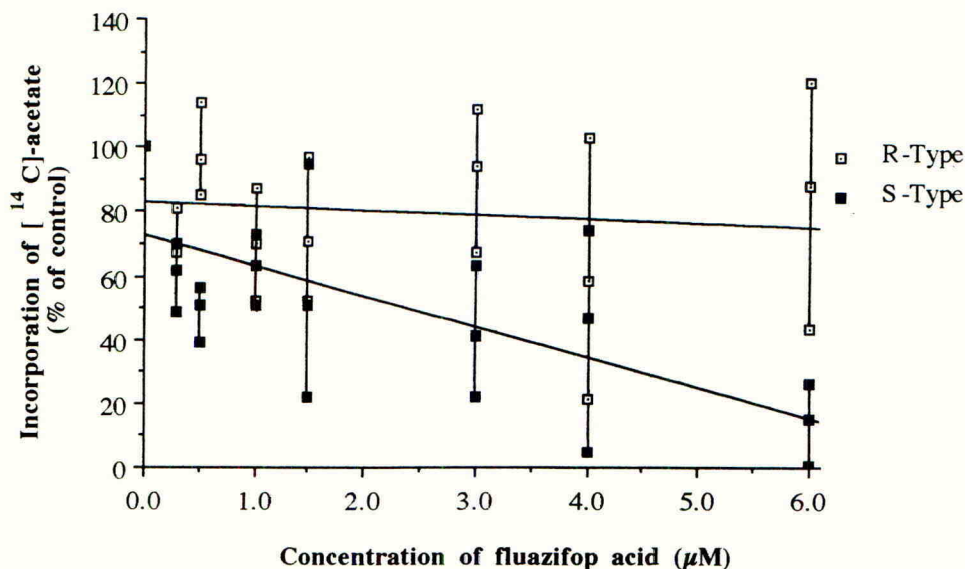
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INVESTIGATION OF THE SAFENING OF EPTC ON SEVERAL GRASSY CROPS AND WEEDS BY
VARIOUS SAFENERS

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ABSTRACT

Tolerance to EPTC and the safening effects of MG-191, dichlormid,
AD-67, BAS-145138 and flurazole on seven grassy crops and six
weeds were determined in sand culture under growth-chamber
conditions. Practically all species examined could be safened to
some extent at EPTC concentrations which caused c. 50% inhibition
of shoot growth (GR_{50}). As sensitive and moderately sensitive
species, oat (GR_{50} c. 0.005 mM EPTC) and barley (GR_{50} c. 0.02 mM
EPTC) were chosen to measure glutathione (GSH) levels and
glutathione-S-transferase (GST) activity (determined with
chlorodinitrobenzene and [^{14}C]EPTC sulfoxide as substrates). Data
were compared to those of maize (GR_{50} c. 0.1 mM EPTC). Safener
treatment significantly raised both GSH levels and GST(CDNB)
activities depending on the structure of the safener. No direct
correlation was found, however, between sensitivity to EPTC and
GSH level or GST activity of the three species either with or
without safener treatment. GST([^{14}C]EPTC sulfoxide) activity was
markedly increased by safener treatment in maize but not in barley
and oat.

INTRODUCTION

Herbicide safeners (antidotes or protectants) are compounds that
eliminate the injury to crops by herbicides without reducing the herbicidal
potential. Commercialized safeners are members of diverse chemical groups
and are especially effective in protecting large seeded grassy crops.

A number of safeners selectively protect maize against EPTC injury at
herbicide rates required for effective weed control. At these rates other
monocots are heavily damaged or completely killed.

The question, however, whether plant species more sensitive to EPTC
than maize can be protected to some extent by the same safeners at
decreased herbicide doses has not been investigated in detail. Therefore we
have carried out studies to investigate whether EPTC-sensitive crops can be
safened against EPTC at lower herbicide dose rates. We report some
biochemical information on these interactions and compare them with those
found in maize.

MG-191 is a highly effective EPTC safener to maize. Like other
safeners its protecting activity may be attributed, at least partly, to its
ability to elevate the glutathione (GSH) level and glutathione-S-
transferase (GST) activity in maize (Dutka & Kőmíves, 1987). Since GSH
levels and GST activities may vary in different plant families and species,
it is envisaged that different chemical structures may exhibit a high
degree of specificity for different plant species (Ebert & Gerber, 1989).
The use of MG-191 in our present work was of interest because its chemical
structure differs fundamentally from the other safeners studied.

MATERIALS AND METHODS

Chemicals

EPTC (b.p. 112°C/2.1 kPa) was purified from commercial samples. MG-191 (2-dichloromethyl-2-methyl-1,3-dioxolane), dichlormid (R-25788) and carbonyl-labeled [^{14}C]EPTC sulfoxide (sp. act. 37 MBq/mmol) were prepared in this laboratory by literature methods. AD-67 (N-dichloroacetyl-1-oxa-4-azaspiro-4,5-decane) was provided by Nitrokémia Chemical Works (Füzfőgyártelep, Hungary). Flurazole was provided by the North-Hungarian Chemical Works (Sajóbábony, Hungary) and BAS-145138 (1-dichloroacetyl-hexahidro-3,3,8a-trimethylpyrrolo-(1,2a)pyrimidin-6(2H)-one) was provided by BASF AG (Ludwigshafen, Germany). All active ingredients used in the experiments were technical grade (purity > 95%). GSH, CDNB (1-chloro-2,4-dinitrobenzene), DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)), PVPP (polyvinylpolypyrrolidone) and Coomassie Blue G-250 were commercially purchased.

Glasshouse experiments

Seeds of seven grassy crops [maize (Zea mays L. cv. Pannonia SC), oat (Avena sativa L. cv. G-3)), barley (Hordeum vulgare L. cv. GK-33), grain sorghum (Sorghum vulgare L. cv. GK Alföld), wheat (Triticum aestivum L. cv. Jubilejnaja 50), proso millet (Panicum miliaceum L. cv. Fertődi 2), ryegrass (Lolium perenne L. cv. Georgikon) and six weeds [Bromus secalinus L., Setaria glauca (L.) P.B., Avena fatua L., Sorghum halapense (L.) Pers., Echinochloa crus-galli (L.) P.B., Apera spica-venti (L.) P.B.] were planted in plastic boxes (12x17 cm, 8 cm deep) in foundry sand (1750 g/box) well mixed with 250 cm^3 aqueous solutions of chemicals. The conditions in the growth room were: 60-70% relative humidity, 16 h light period, light intensity of 10 klux, and temperatures of 23°C and 18°C during the light and dark periods, respectively. The plants were watered every two days with half strength Hoagland solution. Shoot heights of the plants were measured two weeks after planting in order to compare the effect of the different treatments on plant growth. All experiments were repeated twice in a completely randomised design with three replicates per treatment.

Non-protein thiol (NP-SH) content

Non-protein thiol content of leaves and roots of 4-day-old etiolated seedlings was measured spectrophotometrically (412 nm), using DTNB reagent at pH=7.0-7.1 [Ekler & Stephenson, 1989]. Since in grass species the predominant NP-SH is thought to be glutathione (GSH), we have referred to this component as GSH in the remainder of this report.

Glutathione S-transferase (GST) activity

GST(CDNB) activity in leaves and roots of 4-day-old etiolated seedlings was determined spectrophotometrically with CDNB as the substrate (Mozer et al.,1983). GST([^{14}C]EPTC sulfoxide) activity from roots of 4-day-old seedlings was quantified by liquid scintillation spectrometry with [^{14}C]EPTC sulfoxide (0.12 mM) as the substrate (Lay & Casida, 1976). The protein content of the plant material was measured spectrophotometrically (Bradford, 1976) at 595 nm with Coomassie G 250, using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Safener effectiveness

The selected species showed different EPTC sensitivities. The EPTC concentration causing 50% shoot height reduction (GR_{50}), was calculated for each species on the basis of dose-response curves.

These critical herbicide dose rates for the most sensitive plants (oat, ryegrass, A. spica-venti, B. secalinus, S. glauca, A. fatua,) were c. 0.005 mM, for the moderately sensitive plants (proso millet, sorghum, wheat, E. crus-galli, S. halapense) c. 0.01 mM and for barley 0.02 mM. For maize, the most EPTC resistant, GR_{50} was 0.1 mM.

In preliminary studies a 0.03 mM concentration of safeners gave the optimum protection effects. The extent of safening activity of the safeners studied against EPTC is given in TABLE 1.

TABLE 1. Effect of safeners against EPTC damage.

Species	Safening rate * Safener (0.03 mM)				
	MG-191	Dichlormid	AD-67	BAS-145138	Flurazole
Maize	++++	++++	++++	+++	+++
Sorghum	++	++	+	+	++
Wheat	+++	++	++	++	+
Oat	+++	++	++		+
Barley	+++	+	+++	+	
Millet	++	++	++		
Ryegrass	+	+	+		
B. secalinus	++	+	+	++	++
A. spica-venti	+	+			+
S. glauca	+	+	+		++
E. crus-galli	+	+	+	+	+
A. fatua	+	+	+		+
S. halapense	+	+	+		+

* Safening rating scale: ++++ = complete safening effect, equal to untreated control; +++ = pronounced safening effect, up to 75% reduction in phytotoxicity; ++ = moderate safening effect, up to 50% reduction in phytotoxicity; + = slight safening effect, up to 25% reduction in phytotoxicity.

The data show that all species examined could be safened to some extent. These results indicate that the safening effects of the compounds studied are not necessarily confined to maize, when lower herbicide dose

rates are used. Complete safening of EPTC was observed only for maize. However, some safeners enabled moderate or pronounced safening on grassy crops which are moderately sensitive to EPTC. MG-191 was particularly effective in safening EPTC on oat which is very sensitive to EPTC. Lesser safening was seen on ryegrass and the weed species which are very sensitive to EPTC. Although in some cases no safening was seen, a slight safening effect often occured and occasionally moderate safening was seen. These results confirm earlier findings that plants more sensitive to EPTC than maize can be safened to a certain extent (Hatzios, 1991).

Glutathione (GSH) content and GST activity

Differential metabolic detoxification in tolerant and susceptible plant species is the basis of selectivity of a number of herbicides. The importance of GSH conjugation as the key metabolic pathway of carbamothioates has long been recognized. Safeners enhance the GSH conjugation of sulfoxidized carbamothioates by elevating the levels of GSH and by inducing the activity of GST enzymes.

To investigate the biochemical basis of the safener effect we chose oat and barley as examples of species sensitive and moderately sensitive to EPTC. Their GSH levels (TABLE 2.) and GST activities (TABLES 3. and 4.) were determined, and the data were compared to those of maize.

Each safener was found to increase GSH levels significantly in all three species (TABLE 2.).

TABLE 2. Effect of EPTC and safener treatments on glutathione content of maize, barley and oat seedlings.

Safener	Glutathione (% of untreated control*)					
	Maize		Barley		Oat	
(0.03 mM)	EPTC		EPTC		EPTC	
	0	0.01 mM	0	0.02 mM	0	0.005 mM
No safener	100 a**	106 ab	100 a	194 bc	100 a	129 ab
MG-191	119 ab	130 b	168 b	278 d	133 ab	158 b
Dichlormid	153 bc	176 c	175 b	322 e	136 ab	182 bc
AD-67	122 ab	137 ab	221 c	330 e	120 ab	199 c
BAS-145138	156 bc	163 bc	147 ab	265 d	111 ab	146 b
Flurazole	122 ab	137 b	134 ab	258 cd	149 b	196 c

* Control values (nmol/g fresh wt) for GSH were: maize: 488±38, barley: 103±9, oat: 228±15.
** Means in case of one species followed by the same letter are not significantly different at 5% level according to the LSD.

Safener plus EPTC treatments caused higher GSH levels than safeners alone in barley and in oat but not in maize where the normal GSH content is the highest. However, the GSH levels of plants, either untreated or treated can not be correlated directly with their EPTC tolerance (TABLE 1.). Oat as

the most sensitive plant contained much more GSH than the less susceptible barley. Even though safener treatment resulted in greatest increase in the GSH levels in barley, the most elevated GSH level was much higher in oat (453 nmol/g fresh wt) than in barley (339 nmol/g fresh wt). Thus, the enhanced GSH content is not correlated with decreasing EPTC sensitivity.

Safeners also markedly increased GST with respect to CDNB activities (TABLE 3). This is a broadly specific GST substrate used to estimate total GST content and does not discriminate between different isoforms of the enzyme.

TABLE 3. Effect of EPTC and safener treatments on glutathione-$\underline{S}$-transferase activity with respect to CDNB.

Safener	Glutathione-$\underline{S}$-transferase (% of untreated control*)					
	Maize		Barley		Oat	
(0.03 mM)	EPTC		EPTC		EPTC	
	0	0.1 mM	0	0.02 mM	0	0.005 mM
No safener	100 a**	116 ab	100 a	145 b	100 a	114 ab
MG-191	168 bc	135 b	160 bc	222 d	153 bc	180 c
Dichlormid	257 e	203 cd	172 c	234 d	110 ab	134 ab
AD-67	174 c	180 c	158 bc	167 bc	133 b	220 cd
BAS-145138	168 bc	220 d	98 a	138 ab	130 ab	234 d
Flurazole	116 ab	175 bc	170 c	220 d	226 d	268 d

* Control values (nmol/mg protein·min) for GST(CDNB) were: maize: 305±46, barley: 374±49, oat: 125±24.
** Means in case of one species followed by the same letter are not significantly different at 5% level according to the LSD.

Safener plus EPTC combinations also proved to be more effective enzyme activators in barley and oat than in maize. The higher enzyme activity both in untreated and treated barley correlates with its lower sensitivity to EPTC compared to oat.

GST activity when [^{14}C]EPTC sulfoxide is specifically utilized as a substrate is also increased by safener treatments in maize (TABLE 4.) and to a greater extent than GST(CDNB) activity (TABLE 3.). Safener plus EPTC combinations are, however, similar or less effective enzyme inducers than is the case for GST(CDNB). Similar enzyme changes in barley and oat were not observed.

From these data it may be concluded that a range of herbicide safeners can promote the content of GSH and GST in oat and barley as well as in maize. There is, however, no clear correlation between safener effect-iveness and either GSH content or GST activity. However, the fact that safeners elevated the GSH levels and GST(CDNB) activities in barley and oat, as well as in maize, suggests that similar safening mechanism can occur in these plants. Nevertheless, barley and oat can be safened less effectively than maize probably because their GST([^{14}C]EPTC sulfoxide) which conjugates EPTC sulfoxide to GSH was not induced by the same safeners.

TABLE 4. Effect of EPTC and safener treatments on glutathione-s-transferase activity with respect to [^{14}C]EPTC sulfoxide in maize.

			Glutathione-s-transferase (% of untreated control*) Safeners (0.3 mM)				
	EPTC						
(mM)	0	MG-191	Dichlormid	AD-67	BAS-145138	Flurazole
0	100 a**	249 b	449 d	360 c	305 bc	260 b
0.1	125 a	220 b	303 bc	293 bc	234 b	229 b

* Control value for GST([^{14}C]EPTC sulfoxide) was 14.8 ± 2.9 nmol/mg protein·hr.
** Means followed by the same letter are not significantly different at 5% level according to the LSD.

Safeners which have shown to exhibit identical or nearly identical protecting effects on maize caused different increases in GSH levels and GST activities indicating that some biomechanism other than GST conjugation may also participate in the protection process.

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PENETRATION, TRANSLOCATION AND METABOLIZATION OF DICLOFOP-METHYL IN CHLOROTOLURON-RESISTANT AND -SUSCEPTIBLE BIOTYPES OF *Alopecurus myosuroides.*

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ABSTRACT

A chlorotoluron-resistant *Alopecurus myosuroides* biotype found in winter wheat fields in Northeastern Spain showed a higher level of tolerance to the aryloxyphenoxypropionate herbicide diclofop-methyl than the susceptible wild biotype, as indicated by the survival rate at 1 Kg ha^{-1} dose of diclofop-methyl (90% for the chlorotoluron-resistant biotype and 35% for the wild susceptible biotype). The resistant biotype was also more tolerant to other aryloxyphenoxypropionates tested, namely, fluazifop-p-butyl, haloxyfop-methyl.

Both chlorotoluron-resistant and -susceptible biotypes showed similar retention ability of diclofop-methyl (524.2 and 497.5 μg g^{-1} dry weight, when treated with a 6.6 mg l^{-1} solution of diclofop-methyl). The uptake kinetics for diclofop-methyl were similar for both susceptible and resistant blackgrass biotypes, and the extent of penetrated herbicide was maximal at 12-24 h after herbicide application (65-75% when treated with a 6.6 mg l^{-1} solution of diclofop-methyl). Addition of a terpenic alcohol in the herbicide formulated solution slightly increased the penetration rate and the amount of diclofop absorbed, and this effect was more acute when a parafinic oil was used as adjuvant. The herbicide was distributed homogeneously throughout all the treated leaves in chlorotoluron-resistant and susceptible plants. The following metabolites of diclofop-methyl have been found in both chlorotoluron R and S plants: diclofop, polar diclofop conjugates and a third compound whose chemical structure is unknown. Differences between the biotypes relate to the rate of conversion of diclofop acid to phenolic or ester conjugates. The amounts of these compounds were 75.82% (R-biotype) and 39.38% (S-biotype) 72 h after the herbicide application.

INTRODUCTION

Alopecurus myosuroides Huds. populations which escape to the action of the photosynthetic-inhibiting herbicide chlorotoluron have been found in winter wheat fields in Northeastern Spain. The resistant character of a biotype to chlorotoluron has been assessed and characterized (De Prado *et al.*, 1991a, b; Jorrín *et al.*, 1992; Menéndez *et al.*, 1993a, b). For this biotype an ED_{60} value of 2.43 Kg a.i. ha^{-1} has been found. Chlorophyll fluorescence and Hill reaction analysis support the view that the mechanism of resistance of this biotype to chlorotoluron is due to degradation/detoxification processes (De Prado *et al.*, 1991a, b; Menéndez *et al.*, 1993a). Metabolic studies with the resistant biotype have revealed a higher metabolization capacity of chlorotoluron than the susceptible wild biotype (Jorrín *et al.*, 1992; Menéndez *et al.*, 1993b). The aim of the work des cribed here relates to the effects of other herbicides, especially the aryloxyphenoxypropionate diclofop-methyl, on the Spanish chlorotoluron-resistant *Alopecurus myosuroides* biotype, in order to establish alternative

herbicides which control this weed-resistant biotype and to detect and study cross-resistance phenomenons.

Cross-resistance is the phenomenon whereby, following exposure to a herbicide, a weed population evolves resistance to herbicides from chemical classes to which it has never been exposed (Holt *et al.*, 1993). Examples of cross-resistance have been described for an ample number of weed biotypes and families of herbicides (Holt *et al.*, 1993).

Diclofop-methyl is an aryloxyphenoxypropionate graminicide which is used as a postemergent herbicide to remove grass weeds. This herbicide, among other secondary effects, inhibits the enzyme Acetyl coenzyme A carboxylase (ACCase) (Rendina *et al.*, 1988). A modified target site and enhanced herbicide metabolism are, among other proposal mechanisms, the most important factors proposed to explain the tolerant or resistant character of several crops and weeds to diclofop-methyl (Gronwald *et al.*, 1992; Holtum *et al.*, 1991). Hydrolysis of diclofop-methyl to diclofop acid, aryl hydroxylation and conjugation to sugar phenolic or sugar esters derivatives are the main steps proposed for diclofop-methyl detoxification (Shimabukuro *et al.*, 1979)(Figure 1).
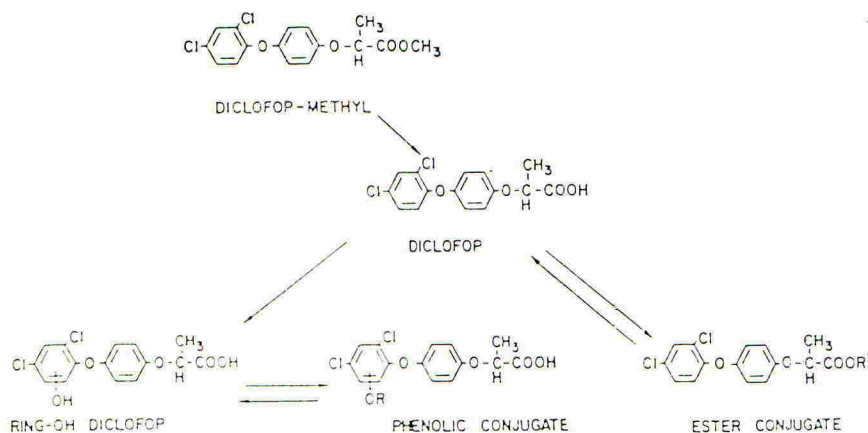

FIGURE 1. Proposed metabolic pathway for diclofop-methyl (taken from Shimabukuro *et al.*, 1979).

MATERIALS AND METHODS

Chemicals

The following herbicides were used: diclofop-methyl('Illoxan' 360 g AI l^{-1} EC); fluazifop-p-butyl ('Fusilade' 125 g AI l^{-1} EC); haloxyfop-methyl ('Gallant' 125 g AI l^{-1} EC); clotinafop-propargil ('Topik' 240 g AI l^{-1} EC) and propaquizafop ('Agil' 100 g AI l^{-1} EC). ^{14}C-labelled diclofop-methyl (specific activity 2.58 mCi mmol^{-1}) and their non-radioactive metabolites were provided by Dr. H. Köcher (Hoechst AG, Germany). A terpenic alcohol ('Heliosol' 665 g AI l^{-1} EC) and a parafinic oil (Schering 684 g l^{-1}) were used as adjuvants for penetration studies. Fluorescein (disodium salt) was obtained from Merck. All the other reagents utilized were of analytical grade.

Plant material

Seeds of the chlorotoluron-resistant biotype of *Alopecurus myosuroides* were collected from winter wheat fields in Northeastern Spain (Menéndez *et al.*, 1993a). Seeds of the wild, susceptible, biotype were taken from neighbouring marginal areas that had never received herbicide treatment. Seeds were germinated in Petri dishes (containing filter paper moistened with a 20 mM KNO_3 solution) under continuous illumination (350 μmol m^{-2} s^{-1}) at 25°C and 80% relative humidity, for 5 days. Germinated seeds were planted in fertilized peat-sandy loam mixture (1:2, by weight) and grown in a growth chamber (16 h photoperiod, 350 μmol m^{-2} s^{-1}, 25/18°C day/night temperature and 80% relative humidity). Plants were watered when required.

Growth assays

Chlorotoluron-resistant and -susceptible seeds were germinated and planted as described above in 40x55-cm, 8-cm deep trays (20 seeds per tray). At the two-leaf stage, plants were sprayed with formulated herbicides using a laboratory track sprayer delivering 350 l ha^{-1} at 200 kPa. The herbicides and doses used were: diclofop-methyl, 1.0 kg ha^{-1}; fluazifop-p-butyl, 100 g ha^{-1}; haloxyfop-methyl, 50 g ha^{-1}; clotinafop-propargil, 80 g ha^{-1}; propaquizafop, 10 g ha^{-1}. Treatments were repeated three times and for each treatment the number of surviving plants after two weeks was recorded and expressed as a percentage of the controls (plants grown in the absence of herbicide). The viability percentage of control plants was 100% of the planted seedlings.

Retention studies

Chorotoluron-resistant and -susceptible seedlings of *A. myosuroides* were planted in 6 cm deep plastic pots, 6 cm in diameter, (5 plants per pot) containing the above soil mixture. At the 3.5-4 leaf stage, plants were sprayed with a 6.6 g l^{-1} solution of diclofop-methyl (equivalent to 1 Kg ha^{-1}) containing 0.01% (m/V) fluorescein disodium salt as dye indicator, using a pot sprayer that delivered 150 l ha^{-1} at 300 kPa. Five minutes after herbicide application, the foliar portion of the plants was removed and placed in a test tube (in batches of 15 plants) with 50 ml of 5 mM NaOH, and shaken for 30 seconds. According to Richardson (1984), the amount of herbicide retained was determined fluorimetrically by measuring the amount of fluorescein (λ excitation 490 nm and λ emission 510 nm) in the washing solution , using a Perkin-Elmer MPF-43A Fluorescence Spectrophotometer. Fluorescence values were transformed into herbicide concentration by using a standard curve of fluorescence intensity vs μg of diclofop-methyl in a herbicide-dye mixture prepared in 5 mM NaOH. After washing the plants, foliage was dried at 80°C for 48 h and dry-weight was determined. The amount of diclofop-methyl retained was expressed as μg g^{-1} dry weight. This experiment was repeated three times, with fifteen plants each time.

Penetration and translocation studies

Germinated seeds of the chlorotoluron-R and -S biotypes of *Alopecurus myosuroides* were planted in 6-cm deep plastic pots of 6 cm diameter (5 plants per pot) containing the above soil mixture and placed in a growth chamber under the conditions described for the growth assays. Plants in the 3.5-4 leaf stage were used. [^{14}C] Diclofop-methyl was diluted with the commercial herbicide to final specific radioactivity of 37.87 Bq μg^{-1} (absorption studies) and 378.7 Bq μg^{-1} (translocation studies); the final diclofop concentration was in both cases 6.6 g l^{-1} (equivalent to an agronomical dosis of 1.0 kg ha^{-1} in a carrier volume of 150 l ha^{-1}). The herbicide was applied, using a Hamilton microapplicator, to the adaxial surface of the third leaf in four 0.5 μl droplets containing a total of 500 Bq (absorption) and 5000 Bq (translocation). Penetration studies were performed as described by Gauvrit and Dufour (1990), with several modifications. Diclofop-methyl was applied with the terpenic alcohol 'Heliosol' (0.4% V/V) or a parafinic oil (6.5% V/V). Plants were harvested 0, 3, 6, 12 and 24 h after treatment and separated into treated leaves and the rest of the shoot. Roots were discarded because herbicide translocation has not been detected from leaves to roots, something previously described by Brezeann *et al.* (1976). Treated leaves were washed first with acetone and then with chloroform in order to eliminate the herbicide remaining on the leaf surface (acetone wash) and cuticle (chloroform wash). Washes of five plants were pooled and analyzed. Radioactivity in the acetone and chloroform

washes was quantified by scintillation counting using a Beckman LS 6000TA scintillation counter. Plant tissue was dried at 80°C for 48 h and combusted in a Model 307 Packard Tri-Carb sample oxidizer. The CO_2 was trapped in 3 ml of CO_2 absorber (Carbosorb E, Packard Instruments Co.) and mixed with 7 ml of scintillation fluid (Permafluor E⁺). Radioactivity was determined by liquid scintillation counting. This experiment was repeated three times, with five plants each time.

Absorption data are presented as percentages of recovered radioactivity, according to the following formula:

$$\% \text{ absorption} = \frac{\text{dpm combusted tissue}}{\text{dpm acetone and chloroform washes} + \text{dpm combusted tissue}} \times 100$$

In translocation studies, plants were removed from pots four days after treatment. Roots were carefully washed, whole plants pressed against X-ray films, stored for three weeks at -10°C and then developed.

Metabolization studies

Germinated seeds of the R and S biotypes of *Alopecurus myosuroides* were grown as in absorption/translocation studies. The metabolism of ^{14}C-diclofop-methyl in leaf tissues was studied in the two-leaf stage for plants of both biotypes. ^{14}C-diclofop-methyl was diluted as in absorption/translocation studies in order to obtain a herbicide solution of 2.75 g l^{-1} diclofop-methyl and 62.5 Bq μg^{-1} specific activity. 1720 Bq were applied in 20 0.5-μl droplets on the adaxial surface of the second leaf with a microapplicator. Treated leaves were washed with acetone in order to eliminate the ^{14}C-diclofop-methyl remaining on the leaf surface, and the radioactivity was measured by liquid scintillation counting. Shoots and roots were then separated and shoots were frozen with liquid nitrogen and then stored at -70 °C for subsequent analyses. Roots were dried at 80 °C for 48 h and combusted in a biological oxidizer and the ^{14}CO$_2$ formed was quantified by liquid scintillation counting. Frozen leaf tissue from each plant was pulverized in liquid nitrogen using a mortar and pestle. The powder was extracted with 4 ml of 80% methanol and the homogenate centrifugated at 12000 g during 20 min. Pellet was reextracted until no more radioactivity was detected (usually three times). Pellet was dried at 80 °C during 48 h and combusted and the radioactivity measured as previously described. Supernatants were pooled and evaporated at 40°C under nitrogen steam (2 p.s.i.). The residue was redissolved in 1 ml 80% methanol and diclofop-methyl and metabolites separated and analyzed by tlc on 250 μm silica gel plates developed in toluene:ethanol:acetic acid (150:7:7). The radioactive zones were detected with a radiochromatogram scanner and their chemical nature identified by comparing their Rf values with those of standards. Radioactive zones were scrapped off and the metabolites were extracted with 80% methanol and quantified by liquid scintillation counting. Data are expressed as percentage of the total extracted radioactivity. This experiment was repeated five times (one plant each time).

RESULTS

Bioassays

Diclofop-methyl (DFM) at 1 Kg ha^{-1} was very effective in controlling the chlorotoluron-susceptible wild biotype of *Alopecurus myosuroides*; thus, at this dose only 35% of the treated plants survived after two weeks' treatment (Table 1). In contrast, the chlorotoluron-resistant biotype was more tolerant of this herbicide, with a 90% of survival rate at the same diclofop-methyl dose (Table 1).

The chlorotoluron-resistant biotype was more tolerant than its susceptible counterpart to some aryloxyphenoxipropionates, i.e. fluazifop-p-butyl (FPB) and haloxyfop-methyl (HFM), but not to others, i.e. clotinafop-propargil (CFP) and propaquizafop (PF; Table 1). According to the percentage of surviving plants and the herbicide doses utilized, the following order of efficacy can be established: PF > CFP > HFM > FPB > DFM.

TABLE 1. Effect of diclofop-methyl (DFM), fluazifop-p-butyl (FPB), haloxyfop-methyl (HFM), clotinafop-propargil (CFP) and propaquizafop (PF) on chlorotoluron-resistant and -susceptible biotypes of *A. myosuroides*. Values are expressed as percentage of surviving plants of the planted seedlings at the herbicide doses indicated ± SE. Survival in the control assay was 100%. Doses are expressed in g ha^{-1}. The experiment was repeated three times.

Herbicide	Assayed Doses	Field doses	% Surviving plants	
			Resistant	Susceptible
DFM	1000	900-1450	90 ± 10	35 ± 15
FPB	100	156-250	85 ± 5	40 ± 10
HFM	50	105-157	85 ± 10	30 ± 15
CFP	80	70	30 ± 5	20 ± 5
PF	10	70	30 ± 10	25 ± 5

Retention of diclofop-methyl

A. myosuroides biotypes resistant and susceptible to chlorotoluron did not show appreciable differences in the diclofop-methyl retention ability. When plants of both biotypes were sprayed with a 6.6 mg l^{-1} chlorotoluron solution, the retention values found were 524.2 ± 76.29 and 497.5 ± 71.47 μg g^{-1} dry weight for, respectively, the chlorotoluron-R and -S biotypes.

Penetration and translocation of diclofop-methyl

The uptake kinetics for ^{14}C-diclofop-methyl dissolved in a commercial formulation were similar for both chlorotoluron-susceptible and -resistant blackgrass biotypes (Figure 2A). The extent of herbicide penetration was maximal 12-24 h after herbicide application with 50% uptake having occurred by about 4 h and maximal penetration of 70.7% (resistant biotype) and 72.8% (susceptible biotype) after 24 hours. Addition of a terpenic alcohol in the herbicide formulated solution slightly increased the penetration rate and the amount of diclofop absorbed after 24 h treatment (85.4% and 88.9% for, respectively, the R and S biotypes; Figure 2B). This effect was also observedd with the parafinic oil formulation (90.0% and 88.0% for the resistant and susceptible biotypes; Figure 2)
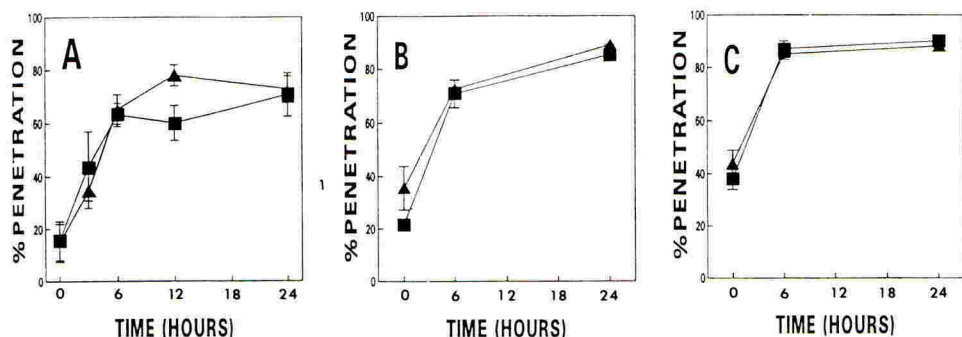

FIGURE 2. Penetration of diclofop-methyl in chlorotoluron-resistant (■) and -susceptible (▲) biotypes of *A. myosuroides* 0, 6, 12 and 24 h after treatment. Plants were treated with a commercial herbicide preparation containing ^{14}C-diclofop-methyl. (A) Herbicide alone; (B) Terpenic alcohol is included in the formulated herbicide; (C) Parafinic oil is included in the formulated herbicide. Values are expressed as percentage of the recovered radioactivity and are means of 3 trials ± SE.

Autoradiographies of R and S plants treated with ^{14}C-diclofop-methyl revealed a homogeneous distribution of the herbicide along all the treated leaves with no appreciable differences between both biotypes (data not shown).

Metabolization of diclofop-methyl

Besides diclofop-methyl (Rf = 0.63), the following metabolites of this herbicide have been found in plants of the chlorotoluron-resistant and -susceptible biotypes of *A. myosuroides*: diclofop (Rf = 0.42), polar diclofop-conjugates (Rf = 0.00), whose nature is being studied in our laboratory, and another unknown compound (Rf = 0.09) (Figure 3).
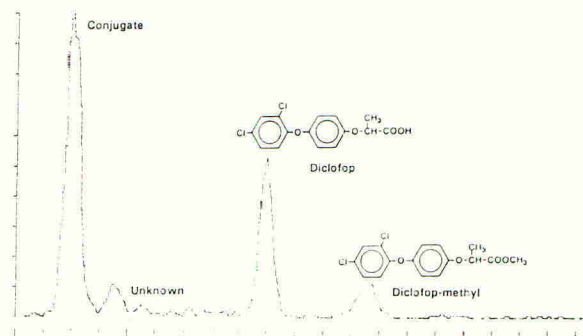

FIGURE 3. Thin layer chromatography of diclofop-methyl and metabolites. The sample corresponds to chlorotoluron-resistant plants after 48 h treatment.
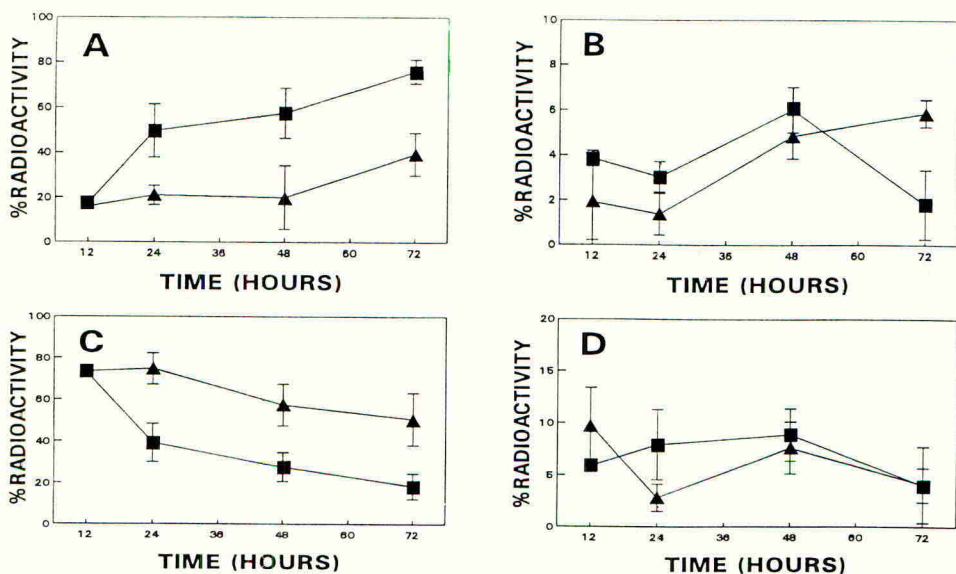

FIGURE 4. Metabolism of diclofop-methyl in chlorotoluron-resistant (■) and -susceptible (▲) biotypes of *A. myosuroides* 12, 24, 48 and 72 h after treatment. Plants were treated with a commercial herbicide preparation containing ^{14}C-diclofop-methyl. (A) Polar conjugate; (B) Unknown; (C) Diclofop-acid; (D) Diclofop-methyl. Values are expressed as percentage of the recovered radioactivity and are means of five trials ± SE.

Most of the radioactivity extracted corresponded to diclofop and diclofop-conjugates (almost 90% of the recovered radioactivity at the different incubation times), with the amount of diclofop-methyl and the unknown compound lower than 10% of the recovered radioactivity (Figure 4). The amount of diclofop was maximal after 12 h treatment (close to 75%), indicating a very rapid hydrolysis of diclofop-methyl to diclofop acid, and decreased at longer times up to 72 h (50.5% and 18.5% of the extracted radioactivity for, respectively the chlorotoluron-S and -R biotypes). The amount of diclofop-conjugates increased in both biotypes from 18%, after 12 h treatment, to 75.8% (R biotype) and 39.5% (S biotype), after 72 h treatment (Figure 4). According to our data, differences between both biotypes can be established in terms of rate of conversion of diclofop to phenolic conjugates or ester conjugates, with the amount of these non-toxic derivatives of diclofop significatively higher in the chlorotoluron-resistant than in the chlorotoluron-susceptible biotype.

DISCUSSION

A chlorotoluron-resistant biotype found in winter wheat fields in Lerida (Spain) (De Prado *et al.*,1991a) was more tolerant to the aryloxyphenoxypropionate herbicide diclofop-methyl than the wild chlorotoluron susceptible biotype as revealed by survival percentage in presence of 1 kg ha^{-1} diclofop-methyl. This property which is also extended to some of the aryloxyphenoxypropionates assayed, namely fluazifop-p-butyl, haloxyfop-methyl, is under investigation in our laboratory and is a typical example of cross-resistance, a phenomenon also described for a number of weeds and family of herbicides (Holt *et al.*, 1993). The physiological and biochemical bases of such cross-resistance have partially been described earlier in results. Differences have not been found in the extent of diclofop-methyl retention (524.2 ± 76.29 and 497.5 ± 71.47 µg g^{-1} dry weight for, respectively, the chlorotoluron-R and -S biotypes penetration) and translocation for all the treated leaves. Differences have been detected in the rate of diclofop-methyl metabolization, especially in the conversion of diclofop acid to the phenolic or ester conjugates. Other proposed mechanisms to explain the resistant character to diclofop-methyl in crops and weeds, such as insensitivity of the acetyl-CoA carboxilase enzyme and membrane depolarization-repolarization processes, need to be investigated in our *A. myosuroides system*. However, differences of tolerance to diclofop-methyl between the Spanish chlorotoluron-R and -S biotypes can be explained, at least in part, because of the higher metabolization ability of diclofop-methyl found in the chlorotoluron-resistant plants. It is important to emphasize that the resistant character to chlorotoluron of the Spanish biotype is also due to an enhanced metabolization of this herbicide (*De Prado et al.*, 1991b; Menéndez *et al.*, 1993b) and that cytochrome P450 monooxygenases are implicated in both chlorotoluron and diclofop-methyl metabolization. Because of this, cross-resistance to diclofop-methyl in the chlorotoluron-resistant biotype is not susprising.

ACKNOWLEDGEMENTS

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BEHAVIOUR OF METRIBUZIN IN MAIZE PLANTS: BIOAVAILABILITY FACTORS GOVERNING TOLERANCE

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ABSTRACT

Metribuzin-5-^{14}C was applied to the roots of one week old maize plants for 7 h. After this pulse the plants were transferred to water and the uptake and distribution of the radiolabel among roots and leaves was determinated for up to 162 h (7 days). Metribuzin was metabolized in maize plants by conjugation. The metabolism rate after 1-3 days was only 40-50 % in the roots and 20-40 % in the leaves (total soluble metabolites = 100 %). Further metribuzin metabolism occurred only very slowly. This metribuzin content did not inhibit photosynthesis, as was shown by parallel fluorescence measurements. The fraction of this metabolically inactive metribuzin was presumably contained in or bound to an "inactive cell compartment", possibly the cell walls, from where it can only very slowly be liberated. The experiments suggest that the level of tolerance in corn for metribuzin rests in part on detoxification by conjugation, but additionally on deposition of the unchanged herbicide molecule in an inert cell compartment.

INTRODUCTION

Metribuzin, a photosystem II inhibitor herbicide, is well known for its tolerance in soybean. Metribuzin tolerance in soybean is mediated by metabolic conjugation and by deamination (Fedtke, 1991). Metribuzin tolerance in some wheat cultivars appears to be caused exclusively by rapid conjugation (Fedtke & Schmidt, 1988). The present work has been done to investigate the mechanism of tolerance for metribuzin in maize plants. Weed control in maize with metribuzin is a recent and valuable application for this herbicide (Cobia et al., 1992; Sorensen, 1993).

MATERIALS AND METHODS

Maize plants cv. "Mutin" were grown for one week in vermiculite. The roots of whole plants were then immersed in 3 ml distilled water containing 37 kBq (1 MBq/mmol, concentration 12.5 μM) 5-^{14}C-metribuzin for 7 h. After this pulse the roots were washed once with water, transferred to water, and returned to the growth cabinet (day/night, 12/12 h, 24/18°C, 60/90 % r.h., 30,000 lx).

Aerial plant parts and roots were extracted separately with methanol. The extracts were centrifuged, and the supernatants were dried under nitrogen at 50°C. The samples were then redissolved in 50 % ethanol

and chromatographed in chloroform and dioxan (9+1) on silica gel thin layer plates. The procedure has already been described (Fedtke, 1991).

Fluorescence was measured after 1 h dark adaption with a "Plant Productivity Fluorimeter" (SF-10, Brancker, Ottawa) attached to a data interface (PCI-6380, Datalog) and a computer (SX-64, Commodore). Ten parallel measurements were done for each plant. The variable fluorescence was defined as $F_{var} = (F_{max}-F_i)/F_{max}$, with F_{max} for the maximum fluorescence within the 5 s illumunation time and F_i for the inflection point.

Each incubation was carried out in parallel with 4 plants each. The data are therefore averages with standard deviations of four individual measurements, except for the fluorescence data where each point is the average of 40 individual measurements.

RESULTS AND DISCUSSION

Two similar experiments differing only in their duration were done in sequence. The results are reported in Table 1 and Figs. 1 and 2 and in Table 2 and Figs. 3 and 4, respectively, for the two experiments. Metribuzin uptake by the plants, as calculated from the difference in the

TABLE 1. Uptake of ^{14}C-metribuzin into maize plants during a 7 h pulse, and extractable radioactivity in roots and leaves after additional 0-96 h in water. The averages and standard deviations are given for four parallel incubations (plants) each (Experiment 1).

| Time in water following herbicide pulse (h)	Extracted tissue	^{14}C-Content		Metribuzin parent compound (ng per plant)
		Uptake (10^3 dpm)	Extracts (10^3 dpm)	
0	Roots	1122 ± 384	267 ± 93	972 ± 292
	Leaves	-	203 ± 39	833 ± 195
18	Roots	917 ± 66	51 ± 33	123 ± 78
	Leaves	-	272 ± 66	700 ± 97
42	Roots	1034 ± 124	54 ± 14	105 ± 32
	Leaves	-	362 ± 60	701 ± 299
66	Roots	958 ± 147	49 ± 11	86 ± 18
	Leaves	-	298 ± 52	697 ± 248
96	Roots	903 ± 133	47 ± 16	71 ± 15
	Leaves	-	266 ± 57	441 ± 64
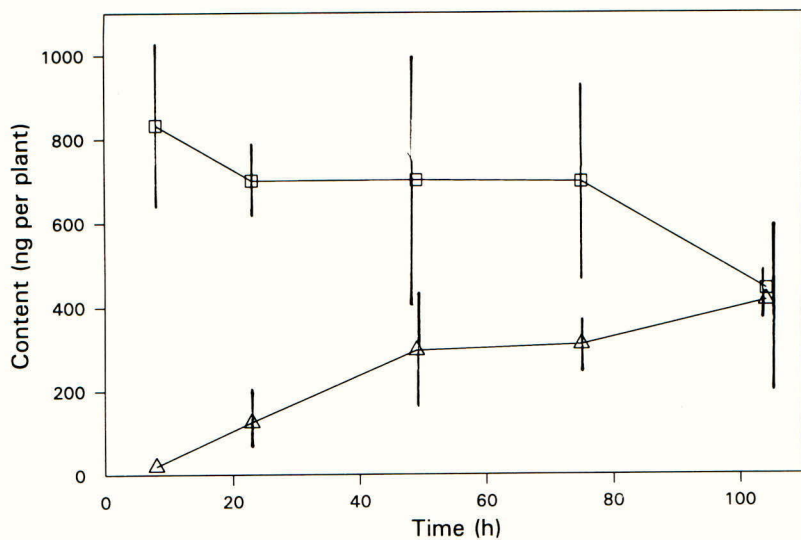
FIGURE 1. Amounts of metribuzin (□—□) and conjugates (△—△) (given as ng metribuzin equivalents) in maize leaves after a 7 h pulse of ^{14}C-metribuzin followed by 0 - 96 h in water (Experiment 1).
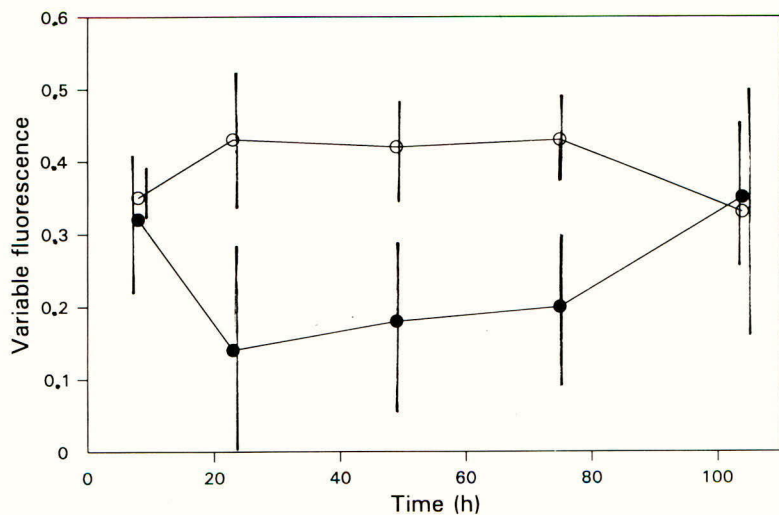

FIGURE 2. Variable fluorescence in leaves of control (o—o) and metribuzin treated (●—●) maize plants (Experiment 1).

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TABLE 2. Uptake of ^{14}C-metribuzin into maize plants during a 7 h pulse, and extractable radioactivity in roots and leaves after additional 0-162 h in water. The averages and standard deviations are given for four parallel incubations (plants) each (Experiment 2).

Time in water following herbicide pulse (h)	Extracted tissue	^{14}C-Content		Metribuzin parent compound (ng per plant)
		Uptake (10^3 dpm)	Extracts (10^3 dpm)	
0	Roots	880 ± 198	201 ± 19	687 ± 75
	Leaves	-	46 ± 20	147 ± 68
18	Roots	594 ± 176	23 ± 4	58 ± 17
	Leaves	-	60 ± 11	182 ± 20
66	Roots	638 ± 88	24 ± 2	36 ± 4
	Leaves	-	64 ± 6	164 ± 29
90	Roots	462 ± 154	19 ± 9	29 ± 14
	Leaves	-	79 ± 10	178 ± 41
114	Roots	660 ± 44	23 ± 5	34 ± 8
	Leaves	-	60 ± 33	167 ± 57
162	Roots	506 ± 264	34 ± 9	29 ± 26
	Leaves	-	81 ± 1	136 ± 52

treatment solution before and after the pulse, was 41-51 % of available herbicide in the first (Table 1) and 21-40 % in the second (Table 2) experiment. Total extractable radioactivity found in leaves ("Extracts" in Tables 1 and 2) increased slightly during the 18 h immediately following the pulse but did not increase further after longer incubation times. The extractable radioactivity in roots decreased markedly during the first 18 h after the pulse and remained at this low level thereafter. Extractable metribuzin in leaves ("Metribuzin" in Tables 1 and 2, after chromatographic separation of the extracts) was already present after the 7 h pulse and stayed at a constant level for most of the experiment time. The "Metribuzin" disappearing from the roots after the 7 h pulse did not appear in the leaf, nor was the equivalent radioactivity found in the leaf "Extracts". Therefore it may be strongly bound to the root matrix. Metabolism seems improbable because of the steady metribuzin level during the remaining duration of the experiment.

Metribuzin was very slowly metabolized into conjugates in leaves (Figs. 1 and 3) and roots (not shown). No other metribuzin metabolites were found. The extractable metribuzin observed immediately after the pulse did not cause inhibition of photosynthesis, as measured by the variable fluorescence signal (Figs. 2 and 4, 7 h). This suggests that at
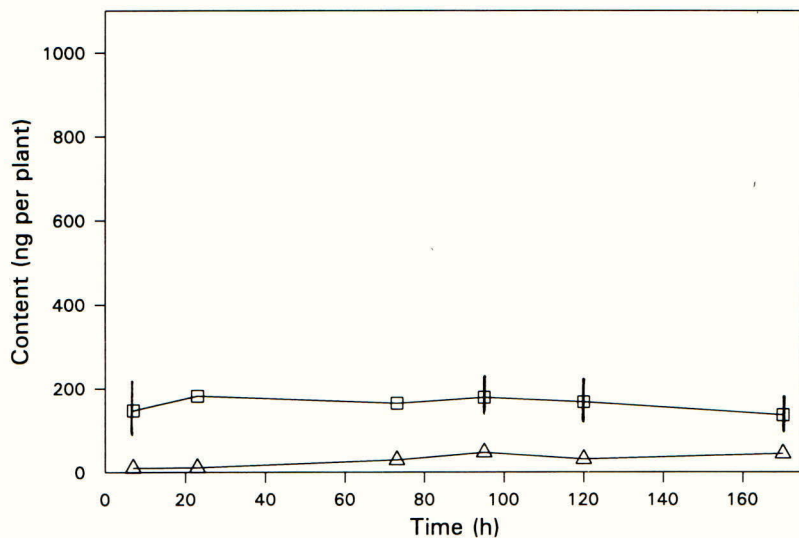
FIGURE 3. Amounts of metribuzin (□–□) and conjugates (△–△) (given as ng metribuzin equivalents) in maize leaves after a 7 h pulse of ^{14}C-metribuzin followed by 0 - 162 h in water (Experiment 2).
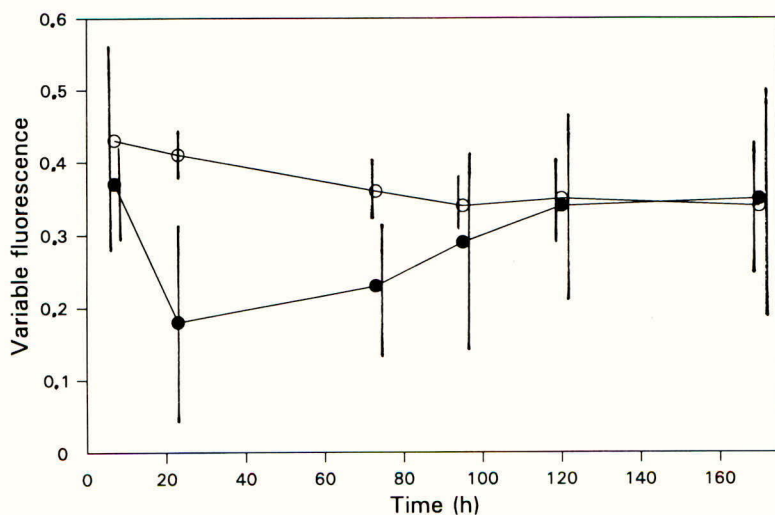

FIGURE 4. Variable fluorescence in leaves of control (O–O) and metribuzin treated (●–●) maize plants (Experiment 2).

this stage, metribuzin was still largely confined to the xylem tissue. Partial photosynthesis inhibition was, however, apparent after 18-66 h in water in both experiments. After about 100 h total incubation time no further photosynthesis inhibition was observed, whereas the extractable metribuzin content in the leaves was still considerable after these times (Tables 1 and 2, Figs. 1 and 3). Photosynthetic electron transport in isolated chloroplasts is inhibited ca. 30 % by 0.1 μM metribuzin, and ca. 90 % by 1 μM. Since the aerial parts of one plant weighed about 1 g, even the lower concentrations of extractable metribuzin (164 ng/g fresh weight) would be expected to be inhibitory in vivo, provided that the herbicide was evenly distributed and freely available in the metabolic pool. The recovery of photosynthetic electron transport from the original inhibition means that metribuzin was not available in the chloroplasts after about 100 h. However, since metribuzin could still be extracted from the leaves at concentrations sufficient for photosynthesis inhibition (214 ng in the 0.8 ml aqueous fraction of 1 g fresh weight would give 1.25 μM), it is concluded that the herbicide molecule is firmly bound to the plant cell matrix, possibly the cell wall. In conclusion, it is suggested that maize tolerance to metribuzin is caused in part by conjugation and in part by binding to a metabolically inert cell compartment.

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COMPARATIVE EFFECTS OF DIFFERENT GRASS-SPECIFIC HERBICIDES ON
FATTY ACID SYNTHESIS IN RESISTANT WEED SPECIES

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ABSTRACT

The cyclohexanediones and aryloxyphenoxypropionates are two classes of post-
emergence herbicide which are selective for grass species (the *Poaceae*). They act
at the level of acetyl-CoA carboxylase and thus inhibit fatty acid (and membrane)
synthesis. We have studied the action of different herbicides from these two classes
on fatty acid synthesis in sensitive crops and in grass weed species reported to be
resistant. Our results show clearly that the mechanism of resistance in the weeds
studied is due to differences in the protein structure of acetyl-CoA carboxylase.

INTRODUCTION

Cyclohexanedione and aryloxyphenoxypropionate herbicides are used mainly post-
emergence to control annual and perennial grass weeds in broadleaved crops (Worthing,
1991). Selectivity for grasses (the *Poaceae*) is high; other monocots., as well as broadleaved
plants, are little affected. Grass crops such as maize (*Zea mays.*, L.) are generally
susceptible, as are most annual and perennial grass weeds. *Poa annua* (annual meadowgrass
(U.K.) or annual bluegrass (U.S.A.) and *Festuca rubra* (red fescue) are two grass species
which have been reported to be resistant or partially resistant to compounds from both of the
above herbicide classes in experiments performed *in vivo* and in *in vitro* (Stoltenberg *et al.*,
1989; Lichtenthaler *et al.*, 1992). *P. annua* is able to germinate throughout the year and is a
problem weed in horticultural situations and in turf grass. It is very common in the U.K. and
temperate regions of Europe (Roberts, 1982).

Acetyl-CoA carboxylase (see Stumpf, 1987) is the primary target site for these
herbicides, as demonstrated using [14C]-labelled precursors of fatty acid biosynthesis
(Harwood, 1991). Indeed, it is at the level of acetyl-CoA carboxylase that grass selectivity of
cyclohexanediones and aryloxyphenoxypropionates exists in most cases (Walker *et al.*,
1989), since uptake, translocation and metabolism of these compounds does not generally
differ between susceptible and resistant species (Owen, 1991). Where graminaceous crops
are resistant to such herbicides this is because the crop can rapidly metabolize the herbicide.

We used *P. annua* and *F. rubra* as two examples of resistant *Poaceae* and maize as our
susceptible species. The aryloxyphenoxypropionate herbicides used were quizalofop ((RS)-

2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionic acid) and fluazifop ((RS)-2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propionic acid) and the cyclohexanedione was sethoxydim ((±)-(E2)-2-(1-ethoxyiminobutyl)-5-[2-(ethylthio)propyl]-3-hydroxycyclohex-2-enone). Susceptibility was evaluated at the whole tissue (leaf blade), isolated chloroplast and acetyl-CoA carboxylase enzyme levels.

MATERIALS AND METHODS

Plant material and growth conditions

Maize (*Zea mays* L., cv. Champ) seeds were purchased from Nutting Seeds Ltd., U.K. *P. annua* and *F. rubra* seeds were purchased from Herbiseed Ltd., U.K. Seeds were soaked overnight in tap water to aid germination and then sown in soil-less compost (Fisons Levington multi-purpose) with a light covering of soil. Plants were grown at 20°C with 650 $\mu E \cdot s^{-1} \cdot m^{-2}$ illumination and a 12h-day/12h-night cycle. Tap water was used for watering, when required. For experiments on leaf blades and for acetyl-CoA carboxylase assays, all plant species were used between 4-and 6-days after germination. Chloroplasts were isolated from 8-day old maize and *P. annua* plants.

Leaf blade experiments

Seedling leaf blades, selected for uniformity of height (*c.*3.5cm) and weight (*c.*0.08g), were cut close to soil level and placed in glass tubes containing solutions of herbicide in 1% ethanol (v/v) (1% ethanol was used for controls and had been found previously not to affect lipid synthesis). Triplicate samples were taken for each treatment. The leaf blades were pre-incubated overnight (15h) at room temperature in the presence of herbicide, with a constant draught (*c.*5m/s) to aid transpiration and, hence, uptake. After 15h, the leaves were transferred to clean tubes containing 1µCi (5µl) [1-^{14}C]acetate in 0.25ml of the appropriate solution of herbicide (1% ethanol for controls) and incubated for 4h under the above conditions, but with supplementary illumination (800 $\mu E \cdot s^{-1} \cdot m^{-2}$) provided by warm-white lights. Incubation was terminated by a two-phase lipid extraction system (Garbus *et al.*, 1963) on leaf material. The lower organic phase was washed 3 times with synthetic upper phase and aliquots taken for radioactivity counting to measure the incorporation of radiolabel into total plant lipids.

Chloroplast incubations

Chloroplasts were isolated from maize and *P. annua* leaves by the method of Mills and Joy, (1980). A single layer of Percoll (Pharmacia) was used, 40% for maize and 22% for *P. annua*. Intactness was *c.*70% for maize (yield *c.*2%) and 40-50% for *P. annua*. Incubation conditions were as described previously (Walker *et al.*, 1988). Chloroplasts were pre-incubated for 3min in the presence of herbicide before incubations were started by the addition of 1µCi (5µl) [1-^{14}C]acetate. Incubations were terminated by a two-phase lipid extraction system (Garbus *et al.*, 1963) and incorporation of radiolabel into total chloroplast lipids determined. Labelling was linear for up to 20min (maize) and 10min (*P. annua*). All

incubations were terminated at 10min. Maximal incorporation of radiolabel into lipid was 3.6% for maize and 1% for *P. annua* chloroplasts. Duplicate samples were used.

Acetyl-CoA carboxylase assays

Acetyl-CoA carboxylase was assayed directly in desalted (PD-10 column, Pharmacia) leaf homogenates using the [^{14}C]bicarbonate-fixation assay (Burton *et al.*, 1991). Background activity (radiolabel incorporated in the absence of acetyl-CoA substrate) was subtracted from each duplicate treatment. Desalting reduced background activity from *c.*50% (*P. annua*) to *c.*8% of control activity. All radioactivity incorporated was incorporated into malonyl-CoA, as determined by HPLC.

Radioactivity counting

All samples were evaporated to dryness under N_2 before adding Opti-Fluor scintillant (Canberra Packard) and counting for 4min in a Beckman 1209 Rackbeta liquid scintillation counter (Wallac Oy, Turku, Finland) with automatic quench correction by the external channels ratio method.

RESULTS AND DISCUSSION

When seedling leaf blades were incubated in the presence of [^{14}C]acetate, all herbicides inhibited incorporation of radiolabel into lipids in the sensitive species, maize (Table 1). This inhibition was not caused by a reduction in uptake of [^{14}C]acetate into leaves. Leaves from *P. annua* proved more resistant and leaves from *F. rubra* highly resistant to the herbicides. Increasing herbicide concentrations did not increase the levels of inhibition beyond those shown below. In the case of maize it did not matter whether total or acyl lipids were counted for radioactivity, the inhibitions shown below were the maximal attainable under the experimental conditions used.

TABLE 1.   Inhibition of the incorporation of radioactivity from [^{14}C]acetate into the lipids of leaf blades.

lipid labelling (% of control)

	Maize	*P. annua*	*F. rubra*
Fluazifop 5µM	62*	82*	105
Quizalofop 0.05µM	37*	67*	89
Sethoxydim 5µM	52*	75*	103

An asterisk (*) indicates that the result was significant by Student's *t* test (P<0.05)

In order to check for the effects of herbicides at the subcellular site of fatty acid synthesis, chloroplasts were isolated. For inhibitor studies it is customary to pre-incubate the preparation with the reagent before carrying out an assay of activity. Because of the labile nature of chloroplasts, we were concerned that long pre-incubation times might result in low percentage intactness and, hence, poor lipid synthesis rates. However, it was found that inhibition was virtually instantaneous (Table 2). Accordingly, only a short (3min) pre-incubation was used for our experiments (see MATERIALS AND METHODS).

TABLE 2.   Effect of pre-incubation time with herbicide on inhibition of lipid labelling from [^{14}C]acetate in maize chloroplasts.

Figures are % of control incorporation of radiolabel

Pre-incubation time	0min	1min	3min	5min
Fluazifop 1µM	14.6	17.2	--	15.1
Quizalofop 0.05µM	--	9.7	9.1	7.6

When isolated chloroplasts were tested for the effects of herbicides it was found that total lipid labelling from [^{14}C]acetate in maize chloroplasts was very sensitive to both fluazifop and quizalofop. *P. annua* chloroplasts were 10-20 times less sensitive to each of these compounds (Table 3).

TABLE 3.   Inhibition of the incorporation of radioactivity from [^{14}C]acetate into the lipids of isolated chloroplasts.

Estimated I_{50} value (µM) for lipid labelling

	Maize	*P. annua*
Fluazifop	0.3	20
Quizalofop	0.003	0.070

The target site of the grass herbicides is acetyl-CoA carboxylase. Before testing the enzyme activity directly and because inhibition shows mixed kinetics against acetyl-CoA substrate (see Harwood, 1991), we checked how long a pre-incubation with herbicide was

necessary. There was no further increase in inhibition in the period 1 to 5min (Table 4) and the latter time was adopted for inhibitor experiments.

TABLE 4.   Effect of pre-incubation time with quizalofop on inhibition of maize acetyl-CoA carboxylase activity.

% of control activity

(Pre-incubation time)	(1min)	(5min)
Quizalofop 0.025µM	52.6	54.5

When acetyl-CoA carboxylase was assayed directly (Table 5) the highly insensitive nature of the enzyme from *F. rubra* to all the herbicides tested was clearly seen. From the I_{50} values, acetyl-CoA carboxylase from *P. annua* appeared to be less sensitive to fluazifop than to quizalofop. However, some degree of insensitivity to quizalofop was shown at higher herbicide concentrations. For example, at 0.25µM quizalofop maize acetyl-CoA carboxylase activity was inhibited to *c*.18% of control, whereas the *P. annua* enzyme was *c*.40% active at this inhibitor concentration.

TABLE 5.   Inhibition of acetyl-CoA carboxylase from different grass species

[^{14}C]bicarbonate-fixation (Estimated I_{50} value in µM)

	Maize	*P. annua*	*F. rubra*
Fluazifop	2	20	>50
Quizalofop	0.020	0.040	15
Sethoxydim	5	20	>1000

The results reported here confirm the resistance of *P. annua* and *F. rubra* to the aryloxyphenoxypropionate and cyclohexanedione herbicides. The resistance appears to be at the level of the target enzyme, acetyl-CoA carboxylase, which presumably has a different protein structure to the enzyme from susceptible species. Acetyl-CoA carboxylase from *F. rubra* appeared to be especially insensitive and, of the aryloxyphenoxypropionates tested, quizalofop appeared more potent than fluazifop.

ACKNOWLEDGEMENTS

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1,8-NAPHTHYRIDIN-4-ONES: NOVEL INHIBITORS OF PHOTOSYSTEM II
DISPLAYING SECONDARY BINDING CHARACTERISTICS

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ABSTRACT

A series of 1,8-naphthyridin-4-ones has been investigated as herbicides. In an
in vitro isolated chloroplast assay these compounds inhibit photosystem II,
weakly at first, but then show slow tighter binding kinetics. We have called
this time-dependent phenomenon *secondary binding*, and have devised a
convenient parameter (an I_{SB} value) for quantifying it. Correlations between
this parameter and whole plant activity for a series of compounds are
discussed, and reasons for some discrepancies are suggested.

INTRODUCTION

The 1,8-naphthyridin-4-one (1) was identified as a herbicide lead from a random
screening programme. This compound controlled a number of broad-leaved and grass weeds
post-emergence, though at relatively high rates (>1000g/ha), with some evidence of soyabean
tolerance. The symptoms caused by (1) are similar to those displayed by inhibitors of
photosystem II, ie photodestruction of pre-existing pigments (Ridley, 1982), and develop over
a period of 1-2 weeks. This compound was subsequently shown to inhibit photosystem II in
an *in vitro* assay, though it displayed some unusual slow binding kinetics. In this paper we
describe work carried out to explore this lead area, particularly with respect to structure-
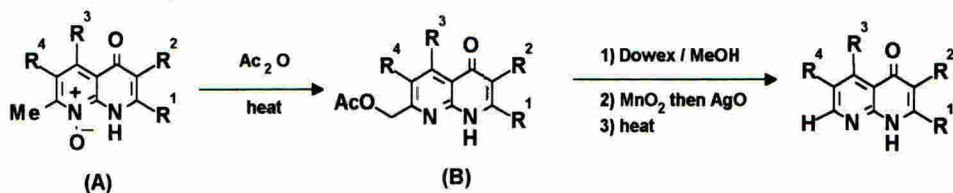activity studies *in vitro* and *in vivo*.

(1)
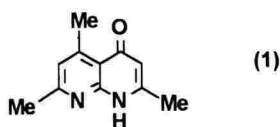
PREPARATION OF 1,8-NAPHTHYRIDIN-4-ONES

The 1,8-naphthyridin-4-one (hereafter referred to as naphthyridinone) ring system was
constructed by the following general two step procedure (Hermecz *et al.*, 1977):
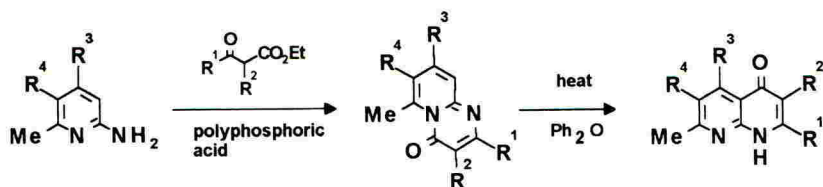
Compounds (1)-(9), (12), (13) and (16) were prepared by this method.

A limitation of this synthesis is that it cannot be used to prepare 7-unsubstituted naphthyridinones, since if the starting 2-aminopyridine does not bear a 6-substituent, the second (rearrangement) step does not proceed. We have developed a general route to these compounds which involved the selective functionalisation of the 7-methyl substituent of a readily available 7-methyl naphthyridinone. Thus, treatment of a N-oxide (A) with acetic anhydride afforded the 7-acetoxymethyl naphthyridinone (B) in good yield. Hydrolysis of this followed by oxidation to the corresponding 7-carboxylic acid and thermal decarboxylation afforded the required 7-unsubstituted derivatives:

$$\text{(A)} \xrightarrow[\text{heat}]{Ac_2O} \text{(B)} \xrightarrow[\substack{\text{1) Dowex / MeOH} \\ \text{2) MnO}_2 \text{ then AgO} \\ \text{3) heat}}]{}$$

(A)   **(B)**

Compound (18) was prepared by this method.

Alternatively, 7-amino napththyridinones were prepared from the 7-carboxylic acid by Curtius type chemistry (diphenyl phosphoryl azide); these were readily mono- and di-alkylated using standard techniques. Compounds (19)-(21) were prepared in this manner.

Naphthyridinones lacking a 3-substituent could be cleanly brominated (bromine/acetic acid) or chlorinated (sulphuryl chloride/acetic acid) to give the corresponding 3-bromo- and 3-chloro-compounds in high yields. In cases where the 3-position was already substituted, bromination occurred predominantly at the 6-position. Compounds (10), (11), (14), (15) and (17) below were prepared by such halogenation reactions.

PHOTOSYSTEM II ELECTRON TRANSPORT INHIBITION

Methods

Chloroplasts were isolated from pea and spinach leaves as described by Walker (1983). Photosystem II activity was measured in 2ml resuspension medium (Ridley, 1983) with a Hansatech O_2 electrode DW-2 at 20°C using saturating red light; the chloroplasts contained 20μg of chlorophyll. Potassium ferricyanide was the electron acceptor, and electron transport was uncoupled with 4mM ammonium chloride.

A convenient parameter has been devised for quantifying time-dependent inhibition or *secondary binding* (an I_{SB} value) in structure-activity comparisons that is simple to determine, although it is not a true kinetic constant. Photosystem II (O_2 evolution) activity versus time was monitored over a period of 8 to 10 min in the presence of 7 or 8 different concentrations of inhibitor from 10mM stock solutions in dimethyl sulfoxide (range depending on potency). As a measure of activity, the time taken to achieve 50% inhibition (tI_{50}) was determined for each dose of compound using a curvefit analysis programme. These activity values were replotted on a Dixon plot ($1/tI_{50}$ versus compound concentration) and gave a straight line. The intersection on the negative X-axis provided a measure of *secondary binding* (I_{SB} value). High inhibition concentrations tended to vary from a straight line relationship and were not used in I_{SB} determinations.

Results

When tested on isolated chloroplasts from peas or spinach in an uncoupled assay, compound (1) was found to give an initial rate I_{50} of 1.3μM. By comparison with other photosystem II inhibitor herbicides measured under the same conditions, this was weak (c/f I_{50} for diuron at 38nM [Ridley, 1983]) and, by itself, was not likely to be the primary cause of herbicidal action.

Oxygen evolution kinetics for photosystem II in the presence of compound (1) not only showed an inhibition of the initial rate, but also an additional time dependent inhibition indicative of a slow binding interaction; this we have called *secondary binding*. Such characteristics were not found with diuron or atrazine. We believe this *secondary binding* inhibition of photosystem II provides the essential contribution to the herbicidal activity of compound (1).

TABLE 1. Effect of substitution in the pyridone ring on PSII inhibition characteristics of 1,8-naphthyridin-4-ones
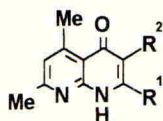
Compound	R^1	R^2	Initial Rate Inhibition I_{50} (nM)	Secondary Binding Inhibition I_{SB} (nM)
(1)	Me	H	1300	273
(2)	Et	H	700	∞
(3)	^{i}Pr	H	2100	∞
(4)	Me	Me	275	20
(5)	Me	Et	140	28
(6)	Me	^{n}Pr	360	21
(7)	Me	^{i}Pr	750	44
(8)	Me	^{i}Bu	750	194
(9)	Me	^{i}Pen	3200	532
(10)	Me	Cl	170	18
(11)	Me	Br	105	6
(12)	-(CH$_2$)$_3$-		600	34
(13)	-(CH$_2$)$_4$-		180	13

Table 1, entries (1)-(3), show that the size of the substituent R^1 is critical; when changed from methyl (1) to either ethyl (2) or isopropyl (3), the time-dependant *in vitro* activity is lost completely. A substituent at R^2 appears to be essential for good activity [entries (4)-(11)], and this can be varied whilst still retaining high intrinsic *secondary binding* activity, though larger alkyl groups do tend to reduce activity. Compounds in which R^1 and R^2 are joined to form a ring also retain good *secondary binding* activity [entries (12)-(13)].

TABLE 2. Effect of substitiution in the pyridine ring on PSII inhibition of 1,8-naphthyridin-4-ones

Compound	R^2	R^3	R^4	R^5	Initial Rate Inhibition I_{50} (nM)	Secondary Binding Inhibition I_{SB} (nM)
(14)	Me	Me	Br	Me	40	19
(4)	Me	Me	H	Me	275	20
(15)	Et	Me	Br	Me	30	20
(5)	Et	Me	H	Me	140	28
(16)	Et	H	H	Me	2800	3223
(17)	Et	H	Br	Me	80	6
(18)	Et	Me	H	H	nd	nd
(19)	Et	Me	H	NH_2	2700	765
(20)	Et	Me	H	NHEt	1120	119
(21)	Et	Me	H	NEt_2	540	99

nd - not determined

Table 2 shows that the effects of substitution in the pyridine ring are more complex, and are to some degree interdependent. The introduction of a bromo substituent at R^4 appears consistently to give rise to high *secondary binding* activity, but also gives a high initial rate inhibition [entries (14), (15) and (17)]. The presence of a methyl substituent at R^3 seems to be required for *secondary binding* [c/f (5) with (16)], except when a bromo substituent is present at R^4 [c/f (15) with (17)]. Introduction of an amino group at R^5 significantly reduces activity [entry (19)]; N-alkylation results in a modest recovery of activity [entries (20) and (21)]. Compound (18) in which R^5 is hydrogen was not tested, but was much less active than its methyl counterpart (5) in whole plant tests.

Investigation of time-dependent inhibition

Bromacil, which bears some structural similarity to the naphthyridinones, also shows the characteristics of *secondary binding*, giving an I_{SB} value of 16nM, and it has been previously reported to give a slow inhibition binding (Ducruet *et al.*, 1990). Diuron shows no *secondary binding*. When diuron is mixed in a low dose (50nM) with compound (5) the slow binding by (5) is totally lost, indicating that *secondary binding* depends on the initial or primary binding, and is not a separate process. Investigations are continuing.

WHOLE PLANT ACTIVITY

All of the compounds were tested on our standard primary glasshouse screens for herbicidal activity. The compounds are generally more active post-emergence, are active

against a range of broadleaved weeds and some grasses, and some show a degree of selectivity towards soyabean. In order to correlate the *in vitro* and *in vivo* activities, post-em LD_{90} values (ie the rate of compound required to achieve 90% control) were estimated for each of a series of broadleaved weeds (*Abutilon theophrasti, Chenopodium album, Xanthium strumarium, Ipomoea hederacea*), and then averaged to a single LD_{90} value which was used as a relative potency figure (rate in g/ha) for herbicidal activity. Representative values for a subset of compounds (1)-(21) are given in Table 3, together with *secondary binding* figures.

TABLE 3. *In vivo* and *in vitro* activities of representative naphthyridinone herbicides

Compound	Average LD_{90}* (g/ha)	Secondary Binding Inhibition I_{SB} (nM)
(1)	2120	273
(2)	>>4000	∞
(4)	560	20
(5)	170	28
(6)	180	21
(8)	410	194
(9)	2050	532
(11)	>3000	6
(14)	>>1000	19[#]
(15)	>1000	20[#]
(16)	>>1000	3223
(17)	inactive	6[#]
(18)	820	-
(21)	>1000	99

(*) Average of rate of compound required for 90% control of each of *Abutilon theophrasti, Chenopodium album, Xanthium strumarium* and *Ipomoea hederacea*

(#) These compounds also show potent initial binding

A comparison of the weed LD_{90} values in Table 3 with the *in vitro* data reveals some interesting trends. For compounds in which the pyridone substituents are varied, (2) shows little whole plant activity, in keeping with the *secondary binding* data. For the series (4)-(9) in which R^2 is alkyl, *in vivo* and *in vitro* activities correlate well, with the exception of (4) which is somewhat less active than anticipated from the *secondary binding* data. However, the bromo derivative (11) is significantly less active than the *in vitro* data would suggest; this is also true for the chloro analogue (10).

For the series of compounds in which the pyridine ring substituents are varied, results for compounds (16) and (21) corellate reasonably well with the *secondary binding* data. However, the poor whole plant activity displayed by the series of analogues (14), (15) and (17), all of which are substituted with bromine at R^6, is surprising given their potent *in vitro* activities.

Though a reasonable correlation between *in vitro* and *in vivo* activity is observed for some members of this series of naphthyridinones, there are also a number of discrepancies, primarily with the halogenated derivatives, which are all poorly active on whole plants whereas they are amongst the most potent compounds *in vitro*. Possible reasons for these discrepancies include the effects of physical properties on uptake and/or movement within the plant, and metabolism of the herbicide. Such factors may also contribute to the low *in vivo* activity of compound (4) relative to other close analogues with comparable *in vitro* activities.

Uptake and metabolism studies

A comparison of the physical properties of compound (17) [logP 3.0, m.p. >300°C, water solubility 2.5ppm] with those of compound (5) [logP 2.6, m.p. 235°C, water solubility 129ppm) suggested that poor foliar uptake and/or movement within the plant could well be factors limiting the whole plant activity of (17). Good evidence for this was obtained from a hydroponic study, in which both compounds showed similar levels of activity (Baylis, unpublished). It is likely that such factors also limit the *in vivo* activity of other halogenated derivatives which have physical properties similar to those of compound (17).

A series of naphthyridinones was tested for oxidative metabolism in a rat liver microsomal assay (Foxon, unpublished). All of the compounds were readily oxidised. These studies clearly demonstrated that a potential for oxidative metabolism of naphthyridinone herbicides does exist, though discrepancies between *in vivo* and *in vitro* data (or crop selectivity) did not correlate with the relative rates of oxidation. .

CONCLUSION

The naphthyridinones show variable initial rate inhibition of photosystem II; when weak, this is unlikely to be the sole cause of herbicidal activity. However, a time dependent inhibition has been revealed which indicates an additional slow binding interaction (*secondary binding*). We believe that this contributes to the herbicidal activity in cases where initial binding is weak. A reasonable correlation is seen between *in vitro* and *in vivo* data, and discrepancies may be explained qualitatively in terms of poor uptake/movement and/or oxidative metabolism.

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MECHANISMS OF SELECTIVITY OF AC 322,140 IN PADDY RICE, WHEAT AND BARLEY

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ABSTRACT

AC 322,140 is a herbicide which is selective in rice, wheat, and barley and is an in vitro inhibitor (1 nM) of acetohydroxyacid synthase. Selectivity in paddy rice is a function of rapid metabolic detoxification of the herbicide by rice shoots, concentration of herbicide in the upper soil layer of the rice paddy, and the placement of rice seedlings during transplanting. AC 322,140 binds moderately tightly to paddy soils, with K_{OC} values of about 2200. After 3 days of leaching, 85 % of the herbicide applied to the soil remains in the upper 3 cm. Radiolabel applied in the flood water also partitions into the upper soil layer within 3 days of application. Target weeds absorb more herbicide on the basis of plant weight than does rice and are unable to redistribute it as well. Selectivity in wheat and barley following a foliar application is due to a slow rate of absorption and to rapid metabolism by the shoots, while Galium aparine rapidly absorbs the herbicide and can not metabolize it.

INTRODUCTION

AC 322,140 (1-{[2-(cyclopropylcarbonyl)phenyl]sulfamoyl}-3-(4,6-dimethoxypyrimidin-2-yl)-urea) is a new, sulfamoylurea herbicide with selectivity in small grains (Condon et al., 1993; Murai et al., 1993; Quakenbush et al., 1993). In Japanese paddy rice it is particularly active on annual and perennial sedges and on broadleaf weeds. The herbicide can be applied at rates of 45 to 60 g/ha within 0 to15 days after the rice is transplanted. In barley and wheat AC 322,140 controls important broadleaf weeds, including Galium aparine. Herbicide is applied in barley and in wheat at rates of 25 to 35 g/ha, either preemergence or post emergence. The mechanism of action of this herbicide is to interfere with the synthesis of branched-chain amino acids by inhibiting the enzyme acetohydroxyacid synthase (AHAS or ALS) with an in vitro I_{50} value of about 1 nM. The mode of action is the subject of this report.

AC 322,140 is selective on rice through a variety of factors related to the translocation of herbicide in rice versus the target weeds, metabolism of herbicide in rice shoots, placement of rice seedlings during transplanting, and soil binding properties which retain the herbicide in the upper soil layer of the paddy. AC 322,140 is selective postemergence on wheat and barley because wheat and barley absorb a relatively small amount of herbicide and are able to rapidly metabolize it.

RICE SELECTIVITY

Uptake, translocation and metabolism of ^{14}C-AC 322,140 were compared for rice vs. Echinochloa crus-galli. Seedlings at the early 3-leaf stage were transferred from a synthetic potting mix (Metro 350) to glass vials for hydroponic labelling in one-half strength Hoagland's mineral solution. AC 322,140 [2-^{14}C-pyrimidine, 20 μCi/mg, 7 μM] was added to the vials for up to 24 hrs, after which the solution was replaced daily with mineral solution alone. Seedlings of E. crus-galli absorbed and retained somewhat more herbicide than did rice (Figure 1). Of the radiolabel absorbed, a somewhat greater portion was translocated to the shoots of E. crus-galli than to the shoots of rice (Figure 2). Rice shoots rapidly metabolized the herbicide (Table 1), but the 24 hr half-life of AC 322,140 in rice roots would suggest that roots might be injured if in continuous contact with the herbicide. Shoots of E. crus-galli also metabolized the herbicide, although the rate was slower than that of rice. This may explain why E. crus-galli is only moderately susceptible to AC 322,140.
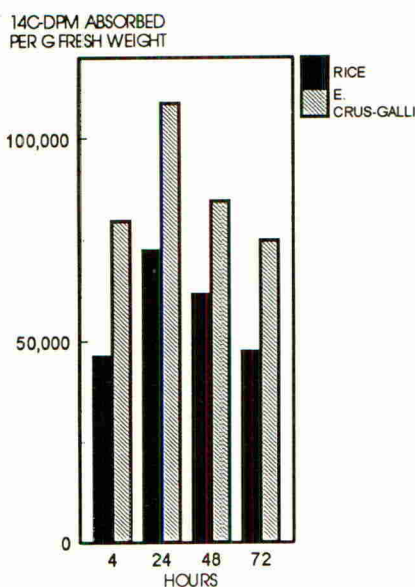
14C-DPM ABSORBED PER G FRESH WEIGHT

RICE
E. CRUS-GALLI

100,000

50,000

0

4   24   48   72
HOURS

14C-TRANSLOCATED TO SHOOTS (% OF ABSORBED)

RICE
E. CRUS-GALLI

40

30

20

10

0

4   24   48   72
HOURS

FIGURE 1. Absorption by rice and *E. crus-galli* of ^{14}C-AC 322,140 from hydroponic solutions. Radiolabel was provided for a maximum of 24 hrs, then was withdrawn.
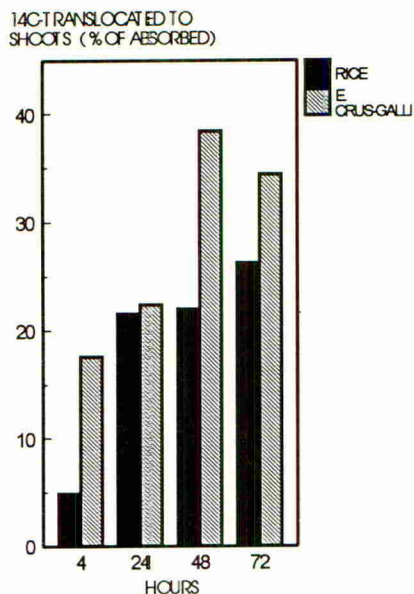
FIGURE 2. Translocation of root-applied ^{14}C-AC 322,140 to shoots of rice and *E. crus-galli*.

TABLE 1. Metabolic half-lives of ^{14}C-AC 322,140 in seedlings of rice and *E. crus-galli*. Herbicide was added to hydroponic solutions for a pulse of 24 hrs, followed by withdrawal of herbicide.

	Half-life (hours)	
	Root	Shoot
Rice	24	1
E. crus-galli	24	4

Given that rice roots are slow to detoxify the herbicide, how does rice escape exposure to AC 322,140? In transplanted rice, most herbicides are applied to the water of the paddy (the "flood") and thus the herbicide partitions into many compartments, including the flood water, the soil, and the water in the submerged soil; and the roots, shoots, seeds and tubers as they contact the zones of herbicide concentration in the paddy system. This has led us to investigate herbicide distribution in all of these compartments.

We determined the distribution of herbicide in relation to the water and soil in the paddy system, and then we determined the distribution of radiolabel in the plants in the system. A batch slurry method was used to determine Freundlich adsorption isotherms (K_f) based on the equilibrium between AC 322,140 in solution (μg/litre) and adsorbed to the soil (μg/kg) at increasing concentrations of herbicide. K_f values were determined as the intercepts of the curves at 1 μg/litre, and K_{oc} values were determined by dividing the K_f value by the organic carbon content of the soils. Included were three Japanese paddy soils: an alluvial sandy loam (2.8 %

organic matter), a deluvial loam (3.0 % OM), and a volcanic loam (10.4 % OM). In comparison to bensulfuron methyl, AC 322,140 bound consistently more strongly to the paddy soils (Figure 3).

These K_{oc} values also suggest that downward flow of water in the paddy would not leach AC 322,140 out of the uppermost soil layers. Soil columns (10 x 10 x 8 or 15 cm deep) were treated with ^{14}C-AC 322,140 applied to the top of the soil prior to establishing a head of water (analogous to the flood) on top of the soil. The soil columns were allowed to leach at the rate of 3 cm per day for 2 days, then were drained to the water holding capacity of the soil, sectioned into 1 cm slices, and combusted to release the ^{14}C as $^{14}CO_2$. As predicted by the adsorption isotherms, AC 322,140 was found to concentrate in the upper soil layer (Figure 4). The total radiolabel collected in the leachate contained less than 1 % of the applied ^{14}C. All three soils produced nearly identical results.

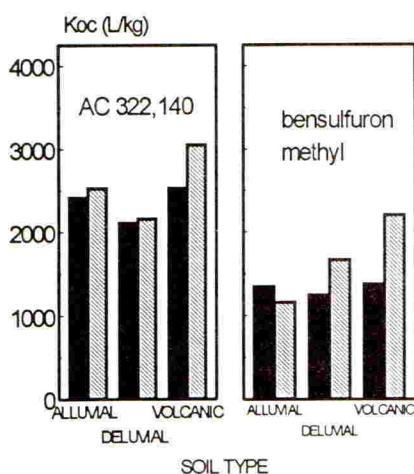FIGURE 3. Calculated K_{oc} values based on experimental K_f determinations for ^{14}C-AC 322,140 and ^{14}C-bensulfuron methyl on three paddy soils. Solid bars represent 1 day of adsorption, hatched bars, 6 days of adsorption.
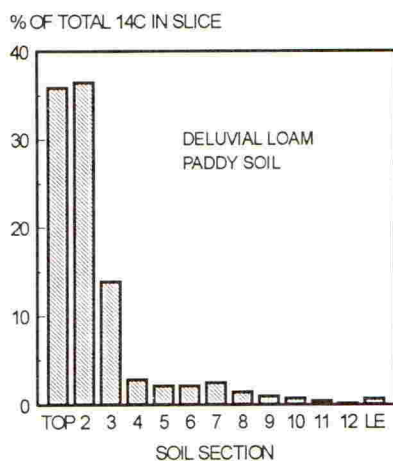
FIGURE 4. Distribution of ^{14}C-AC 322,140 following leaching of a column containing a deluvial loam paddy soil. Sections are 1 cm slices of the soil column. LE represents the total radioactivity in the leachate.

Does herbicide applied in the flood water remain in the flood? Water-saturated soil columns were prepared containing rice transplants and germinated propagules of three species of paddy weeds. A 2 cm flood was established on top, to which was added an aqueous solution of ^{14}C-AC 322,140. Columns were allowed to leach at the rate of 2 cm per day for three days, after which the flood was removed with a pipette, then the plants were removed with a gentle upward pull. The columns were drained, then the soil was sectioned into an upper layer (3 cm) and a lower layer (5 cm). All of the fractions, flood, plants, and upper and lower soil layers, were assessed for distribution of ^{14}C-radioactivity. During the three days between application of herbicide to the flood and the time when the flood was removed, 90 % of the AC 322,140 had become bound to the soil. At least two-thirds of this ^{14}C was in the upper 3 cm of the soil profile (Figure 5). Although some radiolabel was recovered in the lower soil layer, much of this was an artifact of disturbance caused by uprooting the seedlings. Relatively little of the original ^{14}C-AC 322,140 remained in the flood water.
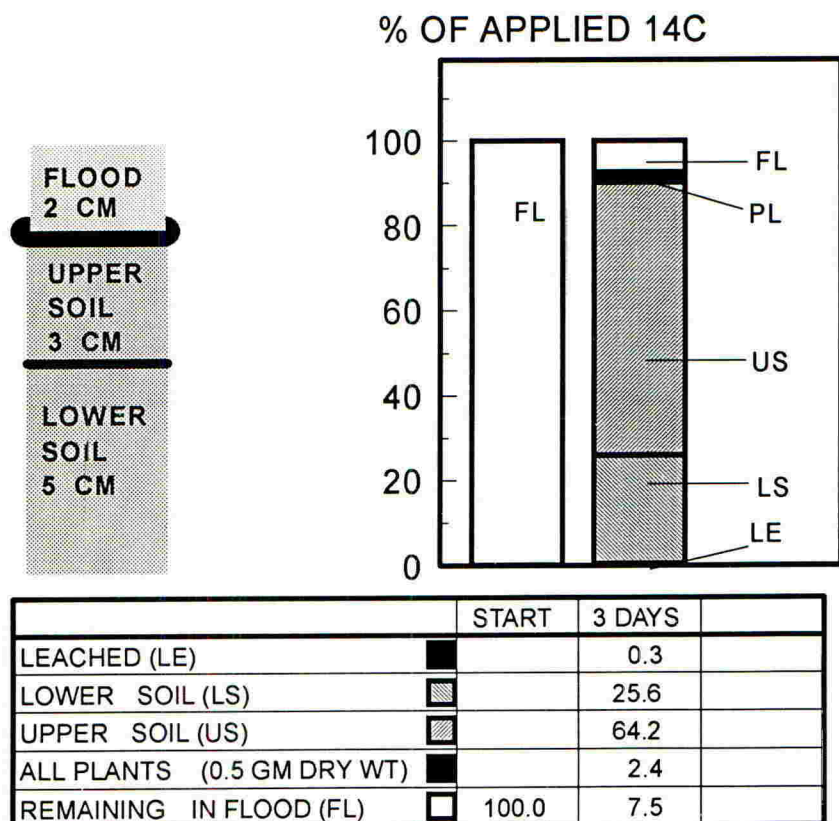
% OF APPLIED 14C

		START	3 DAYS	
LEACHED (LE)	■		0.3	
LOWER SOIL (LS)	▧		25.6	
UPPER SOIL (US)	▨		64.2	
ALL PLANTS (0.5 GM DRY WT)	■		2.4	
REMAINING IN FLOOD (FL)	□	100.0	7.5	

FIGURE 5. Redistribution of ^{14}C-AC 322,140 applied in the flood water to other plants and soil following 3 days of incubation. Soil columns were planted with seedlings of rice, *S. pygmaea*, *C. serotinus*, and *E. crus-galli*. Radioactivity in the lower soil was partially an artifact of uprooting the plants prior to harvest.

How does herbicide distribute in the paddy plants? The soil columns were planted with *Sagittaria pygmaea*, *Cyperus serotinus*, and *E. crus-galli* by pregerminating the tubers, tubers, and seed, then placing them at a depth of 1 cm in the soil. Rice at the 3-leaf stage was transplanted into the columns, and all four species were allowed to grow on for 4 days in the absence of a flood layer. By this time, the rice was 30 cm tall (3.3-leaves), *S. pygmaea* shoots were 1 cm tall, *C. serotinus* shoots were 4 to 10 cm tall, and *E. crus-galli* was 4 cm tall (1.8-leaves). The flood was established, and after 1 day ^{14}C-AC 322,140 was applied in the flood water. It was necessary that the weeds be allowed to grow to these sizes so that absorption and translocation could be quantified, and in the short course of this study there were no lethal effects of AC 322,140. At the end of the 3 day leaching period, the plants were uprooted, their roots were rinsed with water, and the plants were subsequently sectioned according to their exposure to the herbicide. Included groupings were: leaf tissue above the flood and not in direct contact with any herbicide; the lower part of the leafy shoots, primarily the bottom of the leaf sheaths, which is where the flood contacts the shoot; the upper 3 cm of soil (known via Figures 4 and 5 to develop the greatest concentration of herbicide); and the lower soil zone. All plant tissue was dried, weighed, and combusted to determine the total radiolabel per plant and per plant segment.

On the basis of total plant dry weight, rice absorbed the least amount of herbicide per gram of tissue (Figure 6). Similar results were obtained from soil columns of two different soils including a model soil (Sassafras sandy loam) obtained from our fields at Princeton, NJ. When plants were examined for distribution of herbicide in the plant in relation to the distribution of the herbicide in the paddy system (Figure 7), it was clear that rice was the only species to translocate a significant amount of its radiolabel outside (white bars) of the major zones of herbicide concentration. The result was that rice not only absorbed a lower concentration of radiolabel on a whole plant basis, it diluted the herbicide further by translocating it throughout the plant. Most of the radiolabel in rice transplants was translocated to the leaf tissue above the flood. There AC 322,140 could be rapidly metabolized (see Table 1) and its phytotoxicity diminished. In addition it is noted that the outermost leaf sheath of rice, that of the first leaf, was so tightly clasping around the younger leaves that none of the flood water was in direct contact with any of the younger leaf tissue.
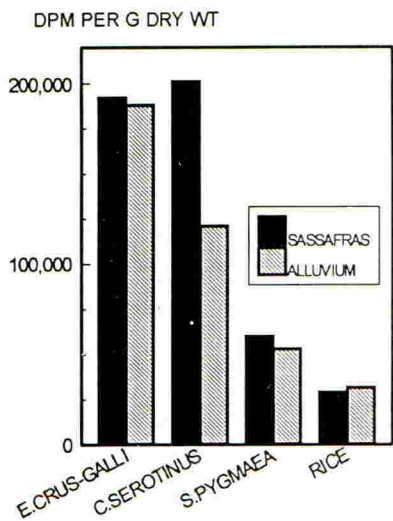
FIGURE 6. Absorption of ^{14}C-AC 322,140 by rice as compared to three paddy weeds. Values are normalized to plant dry weight. Data are for two soil types.
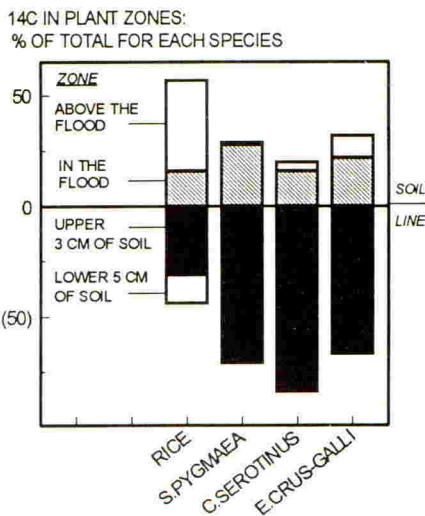
FIGURE 7. Distribution of radiolabel from ^{14}C-AC 322,140 into various plant zones. Seedlings were grown in soil columns that simulated paddies, and the indicated zones correspond to areas of concentration of herbicide. Values add to 100 % for each species.

The selective activity of AC 322,140 in paddy rice is explained as follows. AC 322,140 applied in the flood becomes rapidly bound to the paddy soil, moving only into the upper soil layer. It is from this upper soil layer that weed seeds and tubers emerge, and these are therefore in direct contact with the greatest concentration of herbicide as they germinate. Since the herbicide is applied preemergence or early postemergence to the weeds, weed roots and shoots emerge in a zone of high herbicide concentration, and all tissues are exposed to the herbicide. Rice on the other hand is transplanted into the paddy when the rice seedlings are relatively large. From the beginning, a portion of the rice root is always below the herbicide concentration zone, and most of the leaf mass is above. This placement of the rice transplants, with considerable tissue outside of the zone of herbicide concentration, ensures that rice absorbs the least herbicide on a weight basis. Much of the radiolabel is translocated to the leaves, where the half-life of the herbicide is very short. Root tissues developing in the lower soil, where herbicide levels are low (see Figures 4 and 5), can support plant growth. E. crus-galli escapes injury when roots and shoots grow beyond the zone in which the herbicide is concentrated.

SELECTIVITY IN BARLEY AND WHEAT

Post emergence applications of AC 322,140 to barley and wheat involve uptake of herbicide that is almost exclusively by the foliage. This is a direct result of the high soil K_{OC} values and thus low soil leaching potentials which restricts the herbicide to the upper soil zone. Established plants or seedlings would have most of their root mass below that zone and therefore be protected from herbicide reaching the soil. For a Sassafras sandy loam soil with a soil pH of 6.6 and an organic matter content of 1.2 % we calculated a K_{OC} of 5500.

Foliar absorption was compared for barley, wheat, and *G. aparine*, a troublesome weed during harvest. Herbicide was applied as 10 µl droplets to the first leaf of barley and wheat when the third leaf was just visible. *G. aparine* was treated on two leaflets of the first whorl, at the time the second whorl was first visible and the first axillary buds were released. Of the ^{14}C-AC 322,140 applied , less than 4 % was taken up by barley or wheat, while *G. aparine* absorbed over 40 % of the applied within 48 hrs (Figure 8 A). These results were obtained using Tween 20 as surfactant. *G. aparine* also translocated much of the herbicide outside of the site of application of the radiolabel (Figure 8 B), while barley and wheat did not (Figure 8 C, D). Nearly all of the translocated ^{14}C in *G. aparine* remained in the shoot, where it was widely distributed away from the treatment zone (results from autoradiography, not shown).
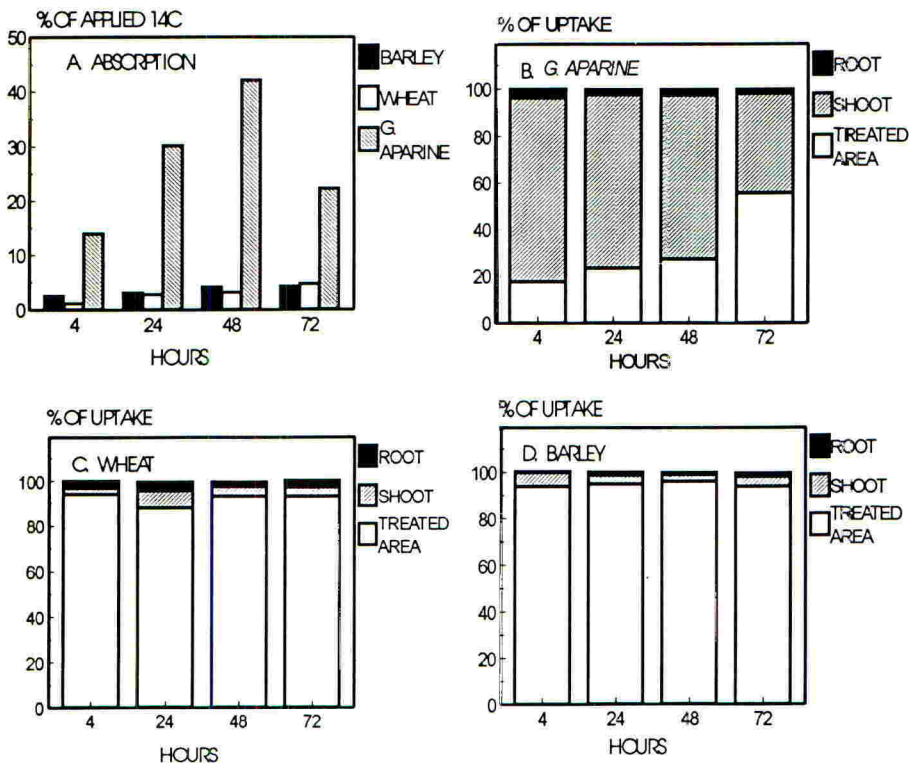

FIGURE 8. Absorption and translocation of foliarly applied ^{14}C-AC 322,140 in barley, wheat, and *G.aparine*.

TABLE 2. Metabolic half-lives of translocated ^{14}C-AC 322,140 in seedlings of barley, wheat, and *G. aparine*. Herbicide was applied to the foliage as small droplets and plants were dissected after times up to 72 hrs. Treated areas were removed before analysis.

	Half-life (hours)	
	Root	Shoot
Barley	n.d.	4
Wheat	24	4
G. aparine	> 72	> 72

The redistribution of ^{14}C throughout the shoot of *G. aparine* did not promote its detoxification (as had occurred for rice and *E. crus-galli*) because *G. aparine* was unable to metabolize the herbicide in either the shoot or the root (Table 2). On the other hand, both barley and wheat shoots were able to metabolize ^{14}C-AC 322,140. In addition, phytotoxicity to *G. aparine* was strongly influenced by the choice of surfactant. A crop oil substantially improved the foliar absorption of AC 322,140 by *G. aparine* (Figure 9).
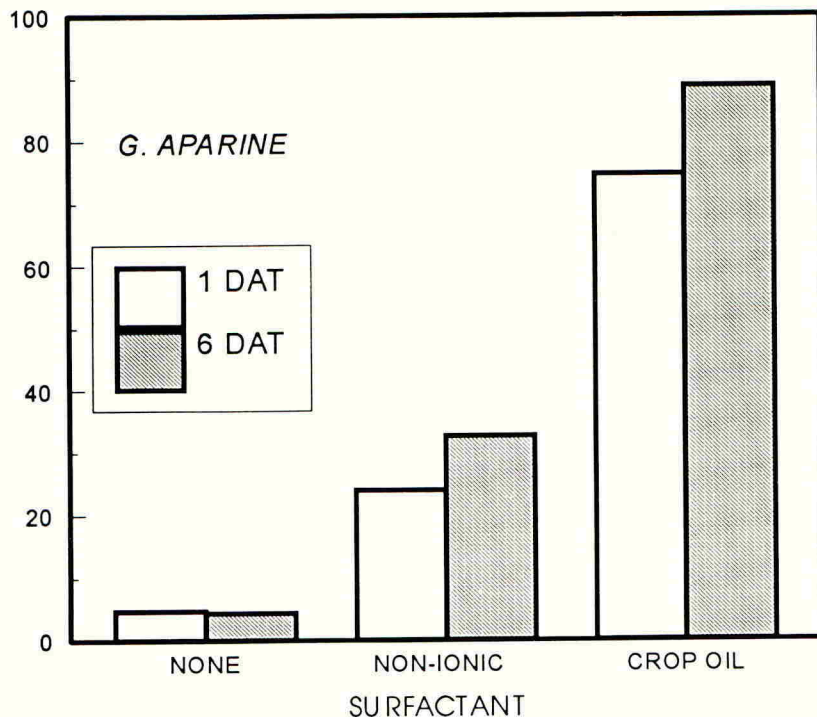
14C ABSORBED, % OF APPLIED

FIGURE 9. Effects of surfactant on absorption of ^{14}C-AC 322,140 by foliage of *G. aparine*. The non-ionic surfactant was 0.25 % X-77® (Valent Corp., Walnut Creek CA), and the crop oil was 1 % Scoil® (contains a methylated seed oil; Agsco Inc., Grand Forks ND). DAT is days after treatment.

The selectivity of AC 322,140 applied post emergence on barley and wheat is explained by the low rate of foliar uptake of the herbicide by barley or wheat, coupled to the rapid metabolism of foliar-applied herbicide by shoots of these species. Foliage of *G. aparine*, on the other hand, absorbs much more herbicide than foliage of either of these species. While *G. aparine* is able to translocate AC 322,140 throughout the shoot, its leaves are not able to metabolize the herbicide, leading to a herbicidal effect.

A combination of factors therefore contribute to the selectivity of AC 322,140 on paddy rice, on wheat and on barley. Contributing factors for herbicide safety are herbicide and crop placement, metabolic inactivation in the shoot, and the ability to translocate herbicide to sites of potential metabolic inactivation.

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Session 4A

New and Alternative Approaches to Weed Control: Risks and Benefits

Chairman   Dr E J EVANS

Session
Organiser   Dr T G A CLEMENCE

Papers   4A-1 to 4A-3

RISKS ASSOCIATED WITH MOLECULAR APPROACHES TO WEED
CONTROL

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ABSTRACT

Molecular approaches to weed control will involve the production of
genetically modified predators that are more effective for agronomic use,
the construction of crop plants that are tolerant to herbicides, and other
modifications to plants to make them immune to parasites or otherwise
interfere with the growth of weeds. The two main obstacles to
commercialisation are the speed with which useful genetically modified
plants might be produced and whether or not they will be considered safe
for the environment and for human use. This paper discusses these issues
briefly.

INTRODUCTION

Our ability to modify organisms genetically by introducing genes, or modifying
existing genes, has opened up a number of exciting opportunities for controlling
weeds in crops. Biological control, using microorganisms which cause disease, now
looks more attractive than it has in the past because it should be possible to modify
the microbes concerned to overcome limitations which reduce their effectiveness. It
is possible to make crops tolerant to particular herbicides which are not available for
them at present. It is also quite likely that in the near future plants will be modified
so that they inhibit the growth of weeds through the production of exudates, or for
parasitic weeds, such as *Striga*, by interfering with the ability of the parasite to infect
the crop.

While every effort should be made to encourage the development of plants which
facilitate the control of weeds, it is necessary to consider whether the introduction of
such genetically modified plants will give rise to environmental or health problems.
The intention of this article is to explore some of the concerns, taking into
consideration the emotional aspects of the development of this technology which may
in the end decide whether ideas are converted into agronomic practice.

BIOLOGICAL CONTROL

There is no doubt that the public perception of the crop protection industry is influenced strongly by pressure groups which have been very effective in propagating an image that protection is largely chemical, hazardous, and driven by industry to use larger and larger amounts of chemicals. I am of the opinion that the public have very little idea of the harm that weeds can cause and lament the loss of poppies and other colourful weeds in cereal crops. And, as a result of these feelings, the use of chemicals to control weeds is a cause of particular concern. The concept of utilising "natural" means of weed control is attractive to the public to obviate perceived problems with chemicals, and to academics because the science associated with biological control is so interesting.

Considerable work has been done to identify groups of insects, bacteria, fungi and viruses that can kill weedy species and a number of preparations are used routinely, albeit infrequently in relation to the total effort put into weed control. While it is simple to demonstrate that a predator is able to harm a weed, it is much more difficult to produce a commercial product because this requires an ability to produce large amounts of the predator cheaply, formulate it so that it will survive during marketing and release, and finally to have a product which will infect weeds in a crop sufficiently efficiently to control them. Most predators fail in one or more attributes, which is where molecular genetics offers opportunities. If a limitation can be defined biochemically there should be an opportunity to modify the existing metabolic pathway or to introduce a gene, or genes, from another organism to rectify the problem. A classic example of this approach has been the manipulation of baculoviruses to improve their ability to kill target insects by introducing genes for toxins into the viral genome to reduce the time it takes to kill the insect.

I must admit to being an enthusiast for the approach of using molecular techniques to improve biological methods for improving the growth of crops. However, it is necessary for enthusiasm to be tempered with a sense of reality. By definition predators harm the organisms they attack, which is an advantage when the host is a pest but a major problem when the host is not. Judicious selection of predators and careful testing on a range of potential hosts has been successful in preventing harm to non-target organisms with the biocontrol organisms presently in use, although there is always the chance that an introduction will cause unexpected damage. A problem for regulatory bodies, and for those involved in constructing genetically-modified organisms, is that the recombinant organism might differ from the parent in unexpected ways, and thus there is a debate about the extent to which they should be tested, or whether they need to be subject to scrutiny. Because we know so little biochemically about what causes an organism to be a pest it is very difficult to predict whether a particular genetic modification will have a significant effect on the phenotype of the host, particularly in relation to whether it will become a problem. In most cases to date when organisms have been genetically modified

there has been a long history of safe use of the parent strain, which provides confidence that the modified variant will be unlikely to cause problems. This is particularly true when the organism in question has been subjected to extensive programmes of mutagenesis and crossing. It is important, therefore, to recognise that organisms which do not have a history of safe use will be very difficult to assess for safety, whether they be genetically modified or not.

HERBICIDE TOLERANT PLANTS

There have been many applications to release herbicide tolerant plants which makes it appear from the outside that the large multinational agrochemical companies are concentrating on this aspect of plant gene manipulation. While it is reasonable easy to understand from a commercial viewpoint why such plants would be attractive, from a public relations point of view the concentration on this type of development has been very harmful because it appears to be directed towards the expansion of the use of chemicals in agriculture. Perhaps one of the commonest arguments heard against plant molecular genetics research is that it will lead to an increased dependence of farmers on seed suppliers and chemical manufacturers, because there will in future only be certain herbicide tolerant varieties available from a limited range of supplies linked to agrochemical companies. The objectors are also very concerned that herbicide tolerance will spread from the crops to other species which will then cause problems.

It is interesting to explore the questions raised about herbicide tolerance because they seem be epitomise the problems inherent in the development of a new technology. Industry is enthusiastic, the consumer is uncertain about potential benefits, pressure groups arise to articulate fears, and governments respond by developing regulations to control risks. Unfortunately for the development of new technologies within Europe there is a strong body of opinion which is antipathetic to industry and developments which might make industry more profitable. This has led to calls for a "Fourth Hurdle" to be introduced in regulatory processes to decide whether society "needs" a new technology or not. In the absence of such a hurdle at present it is inevitable that a technology which involves the interference with the genetic makeup of organisms should be criticised on the basis that the technology is hazardous and the risks are poorly understood. Furthermore, herbicide tolerance has provided an excellent opportunity for criticism of the technology on the basis that there is no simply-defined public benefit.

What is required to ease the position with respect to the development of herbicide tolerant plants is to find a way of informing the public about the need for herbicides, what selective herbicides do, and why it is in their interest that such plants be developed. There is little chance that the regulatory process will help to overcome public resistance to the development of such plants because the environmental issues

are not significant and there is every chance that herbicide resistant plants will be approved for commercial use this year. It is important that the existing European regulations for the release of genetically modified organisms are not used to ban the use of herbicide tolerant plants because there is public anxiety about their use. The regulations need to be used to ensure the safety of the public and the environment and should not be manipulated to include public acceptance. The latter task is the responsibility of governments using other forms of assessment of risk/benefit analysis.

PARASITE RESISTANCE

The importance of parasitic weeds, such as *Striga* and *Orabanche*, is often overlooked in the UK, but these plants can cause serious losses in some countries. The need for specific recognition between the parasite and host, and the limited range of alternate hosts, make these pests ideal targets for gene manipulation. What is needed is to modify host plants such that they are no longer recognised, and/or modify non-hosts so that they stimulate seed germination without providing the host on which the seedling is dependent. Whether or not such plants will become available soon is unknown. Should they be encouraged if they have the potential to eradicate *Striga* and *Orabanche*? Does it matter biologically if pest species are lost? Might the spread of resistance, or the virtual loss of these species, lead to loss of natural controls of weedy species? These and other related questions need to be addressed, hopefully by people with open minds and a good understanding of the biology of the systems concerned.

DISCUSSION

This brief overview of molecular approaches to weed control has, I hope, indicated some of the areas in which we might expect to see advances in our ability to control weeds in the near future. There is little doubt that developments in molecular biology will provide the methodologies to enable the construction of the genetically modified organisms that have been discussed. However, there are two major areas which will affect the rate at which such organisms will be introduced to trade. The first, and perhaps the most important, is the rate at which an understanding of the biochemistry and biology of weed/plant interactions develops. Resources for research of this nature are limiting, and it is most unlikely that the basic knowledge required to identify and manipulate the genes needed for weed control will keep pace with molecular genetics technology and scientists' aspirations for targets for gene manipulation.

The second major problem, public perception, has been discussed in this review. It would be foolish to make dogmatic predictions about the future impact of public opinion because humans have such an amazing capacity for self delusion. In looking at the whole area of the use of genetic engineering at the present time it appears that the development of vaccines, pharmaceuticals and products such as enzymes has broad public approval. Indeed there is very active pressure to develop things such as vaccines for AIDS as quickly as possible, often with demands that safety regulations be relaxed to speed their introduction. For foods the messages are more confused. Certain activists are very strongly against the use of the technology, but in general as long as the safety of such products has been determined by appropriate regulatory bodies it looks as though there will be general acceptance of new products. However, it is quite possible that manipulations which are done to facilitate the use of chemicals, such as herbicide resistance, may not be accepted so easily. A major problem from the point of view of public perception is that there are no genetically modified foods available at present to enable shoppers to see whether promises of improved quality are meaningful. To make matters worse, most of the first round of such foods are likely to be from herbicide resistant plants which have no obvious public benefits.

Another important concern for developed countries is the debate about the "Fourth Hurdle", and whether we should block the development of products on the basis that they are not needed, even though they are safe. Regrettably this is what is happening with bovine somatatropin (BST), which is not approved for use in the USA or Europe because of political concerns about the viability of small dairy farmers. We must hope that when politicians on both sides of the Atlantic wake up to the realisation of what they have done, BST will be seen to be the product which exemplifies the stupidity of political interference in the evolution of agricultural practice and human endeavour. However, experience with the Common Agricultural Policy suggests that political expediency far too often takes the place of common sense.

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USE OF BIOLUMINESCENCE MARKED BACTERIA TO ASSESS RISKS ASSOCIATED WITH USE OF GENETICALLY MODIFIED BIOCONTROL AGENTS

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ABSTRACT

To assess risks associated with use of exotic and genetically modified micro-organisms, as either herbicides or other biocontrol agents, release experiments must be carried out under contained conditions and using reliable cell marker systems.

This paper describes the use of bioluminescence-marked bacterial inocula to address important aspects of risk assessment, namely dispersal and persistence of biocontrol agents.

Dispersal of biocontrol agents through soils was found to be affected by cell type, antecedent matric potential, the water regime subsequent to inoculation, presence of plant roots and earthworms and soil structural properties which mediate preferential flow. The greater dispersal of spores as opposed to vegetative cells of marked bacilli suggests that, as with chemical herbicides, mobility and persistence are linked and must be considered together in risk assessment.

The potential of *lux*-based detection for investigation of aspects of risk assessment of use of biocontrol agents other than persistence and dispersal are discussed in summary.

INTRODUCTION

The potential use of microorganisms as agents of biological control of agricultural pests has been increasingly identified in recent years. With the development of microbial biocontrol systems, the potential to refine and enhance many of these systems through genetic modification has become apparent (Jones & Kerr, 1989; Ryder & Jones, 1991).

The commercial use of genetically modified biocontrol agents, although not common practice, has been carried out for more than a decade. For example, *Agrobacterium* strain K84 has been used to control crown gall disease on stone fruits, roses and other plants (Kerr, 1980) through production of the antibiotic agrocin 84, encoded on a plasmid, pAg K84.

The development of microbial herbicides, genetically modified or otherwise, although generally more recent than for other forms of microbial pest control, is an area of rapidly growing interest (TeBeest, 1991). The

technology generally involves utilising fungi capable of selectively
killing weed species. Two such mycoherbicides are currently registered by
the EPA for use in the United States. One, with the trade name DeVine,
consists of *Phytophthora palmivora* and was registered in 1981 for control
of strangler vine (milkweed) in citrus groves. It is applied either as a
soil additive or an aerial spray (TeBreest & Templeton, 1985). A second
product, originally registered in 1982, is a formulation of *Colletotrichum
gloeosporioides* for aerial spray control of northern joint vetch in rice
and soybeans (TeBreest & Templeton, 1985). Many other mycoherbicide
products are currently being developed, including *Alternaria cassiae* for
control of sicklepod and coffee senna in soybeans and peanuts (Klassen,
1987; Marking, 1988; Mycogen, 1990; Rich, 1988), *Fusarium lateritium* for
control of velvetleaf in soybeans (Klassen, 1987), and *Sclerotina
sclerotium* for control of spotted knapweed and the Canadian thistle
(Chemical Week, 1986). Bacterioherbicides are also being developed,
although to a lesser degree, and include strains of *Xanthomonas* (a genus of
the *Pseudomonoadaceae*) for control of annual blue grass in managed turf
(Mycogen, 1990).

Microbial herbicides may well be applied in conjunction with chemical
herbicides to produce a more potent and broad spectrum herbicide than is
possible from the two separate approaches (Allen, 1989).

Despite the currently high level of interest in microbial biocontrol
agents, genetically modified and non-modified, there are relatively few
reports with respect to the risk and safety considerations associated with
this technology. Recent contributions to providing information for risk
assessment on biocontrol include that by de Jong *et al.*, (1990), and
methods for estimating the risk associated with development of
bioherbicides have been considered by Weidermann (1991).

The safety issues associated with the use of genetically modified and
non-modified biocontrol agents include general considerations such as
survival, persistence and dispersal as well as gene flow and transfer.
More specific, potential considerations include possible trait effects
(e.g. antibiotic resistance, regulatory gene effects etc.), target effects
(e.g. establishment of competitive but poorly performing strains, decreased
growth of crop plants and other undesirable agronomic effects) and non-
target effects (effects on indigenous microbial populations, growth effects
on non-target plants, pathogenicity to animals and humans, effects on
nutrient cycling). A further safety consideration relates to inoculum
purity/contamination.

This paper will focus on general risk and safety considerations of
survival, persistence and dispersal of potential biocontrol microorganisms
by referring to release experiments involving plant-soil microcosms
inoculated with model, genetically-marked (bioluminescent) bacteria, and
maintained under controlled environmental conditions with biological
containment.

MATERIALS & METHODS

Preparation of microbial inocula

Biocontrol inocula were prepared by growing *lux*-modified *Pseudomonas fluorescens* in L-broth containing kanamycin and rifampicin (20 and 10 μg ml^{-1}, respectively) and *lux Bacillus subtilis* in LB broth containing chloramphenicol (10 μg ml^{-1}) and glucose (1 mg ml^{-1}). The cells were centrifuged (1400 g, 30 min) and washed twice in an equal volume of sterile Ringer's solution (1/4 strength) (Oxoid). After centrifugation, the bacterial pellet was resuspended in an equal volume of Ringer's solution and a dilution series prepared in Ringer's solution for determination of viable cell concentration by plating on to LB agar containing kanamycin and rifampicin, and incubating at 25°C for 48 h.

Microcosm characterisation of dispersal of model biocontrol agents

The contained microcosms consisted of intact soil cores in PVC pipes (50 cm long, internal diameter 15 cm) placed under a light bank and supported at the base with nylon (500 μm mesh) and fitted to a funnel for leachate collection. The soils used were a freely draining loamy sand (Craibstone), a sandy loam (Insch) and a clay loam (Cruden Bay). The properties of the soils are shown in Table 1.

Table 1. Soil characteristics

	Craibstone	Insch	Cruden Bay
Series	Countesswells	Insch	Tipperty
Texture	Loamy sand	Sandy loam	Clay loam
Drainage	Freely draining	Freely draining	Impeded
pH ($CaCl_2$)	5.4	6.2	4.7
CEC (cmol kg^{-1})	7.40	9.31	6.08
Base saturation (%)	88.43	92.34	78.27
Organic matter (%)	4.25	3.75	7.38

Soils were sampled from the surface layer (0 - 15 cm depth). CEC - Cation exchange capacity.

Sterile artificial rain was delivered to each core at a rate of 63 mm h^{-1} via a multichannel peristaltic pump under a light bank. The drops were nebulised with compressed air supplied through separate lines to facilitate even application of rain across the surface of the cores. Each rainfall event consisted of a 150 min shower, equivalent to 8.9 mm of rain. Throughout the duration of the experiment, a repeating rainfall regime was used, with a rainfall event applied every third day, equivalent to an annual rate of 1084 mm. This total rainfall and frequency are representative of lowland parts of the U.K.

The cores were equilibrated with respect to water content prior to inoculation, so that differences in water status between the soils were not responsible for variations in leaching and differences could be correctly

attributed to the treatments applied. Equilibration was achieved by
applying an 8.9 mm rainfall event each day until each core produced a
constant volume of effluent. This was followed by 2 weeks during which an
8.9 mm rainfall event was applied every third day. This process brought
all of the cores to approximately field capacity prior to inoculation,
meaning that the water content and matric potential of the soils could be
determined from their respective moisture release characteristics. The
cores were inoculated by applying 6.25 ml of the final bacterial suspension
across the surface of each core in 10 μl fractions using a micropipette.
This was carried out 2 days after the preceding rainfall event with the
first post-inoculation event approximately 18 h later.

Rainfall events (8.9 mm) were applied to the cores every third day,
with the first event applied the day after inoculation. Following each
rainfall event, the cores were allowed to drain for 3 h, which was
sufficient for almost complete drainage of the freely draining Insch and
Craibstone soils, and most of the drainage from the more poorly draining
Cruden Bay soil.

The concentrations of *lux*-modified cells in the leachate were
determined by dilution plating on to LB agar (with 1 g l^{-1} glucose)
supplemented with 20 mg ml^{-1} kanamycin monosulphate and 10 μg ml^{-1}
rifampicin for *P. fluorescens*, and with 10 μg ml^{-1} chloramphenicol for
B. subtilis. Spores were enumerated independently, using a pasteurisation
step (90°C for 20 min). The total volume of leachate produced by each core
following each rainfall event was also measured so that the total number of
cells leached could be calculated. This procedure was repeated throughout
the 2 month duration of the experiment (21 rainfall events).

Use of a charge-coupled device to confirm counts of *lux*-modified inocula

To confirm the presence of luminescent, viable colonies on agar
plates, a nitrogen cooled, slow-scan charge coupled device (CCD; 385 x 578
pixels, each 22 μm square) camera was used (Model 1 Nitrogen-Cooled Camera,
Wright Instruments Ltd., Enfield, UK) with associated computer imaging
system (Dell system 310). The CCD enabled rapid detection of luminescent
colonies against a background of antibiotic resistant indigenous bacteria
on agar plates. For bright field visualization, exposure time was 0.01 s.
Dark field exposure time was 2 min, after application of 2 ml (1:100
dilution) dodecanal to the lid of the Petri dish.

RESULTS & DISCUSSION

The pattern of leaching of the *lux*-marked model biocontrol agent
P. fluorescens through the Cruden Bay clay loam contrasted markedly with
that through the lighter textured soils (Craibstone and Insch) (Fig. 1).
Immediate and prolonged breakthrough occurred for Cruden Bay, whereas a
pulse-type breakthrough after 4 - 5 rain events occurred for Craibstone and
Insch soils.

The rainfall regime prior to inoculation equilibrated the cores at
field capacity. Consequently, the surfaces of the 35-cm cores drained to a
matric potential of approximately -3.4 kPa. This means that the model
biocontrol agents were introduced into large, water-conducting pores

(approx 90 μm neck diameter), since $d = \dfrac{0.3}{\psi_m}$ (where ψ_m = matric potential in kPa, d = neck diameter of largest water-filled pore in mm).

The pulse-type breakthrough observed through the Insch and Craibstone soils can then be explained as the progressive displacement of the inoculum through the conducting fraction of water-filled pores in these soils. This type of preferential flow is well documented for structured soils (Bevan and Germann 1982; Jardine et al., 1990). From moisture-release characteristics, the total volumes of pores between 10 and 100 μm in the cores were estimated at approximately 535 and 545 cm^3 for the Craibstone and Insch soils, respectively. These are the pores that are small enough to be water-filled, yet large enough to be conducting, and since the pulse of breakthrough for both soils occurred with between 450 and 750 cm^3 of applied rain, the breakthrough was consistent with piston-like displacement through this pore size class.

The numbers of colony-forming units of the model biocontrol agents present in the leachates were very low compared to those inoculated on to the cores, indicating that the majority of cells were not leached through the soils. This may be due to a number of factors, including adsorption (Marshall 1971), sieving (Griffin and Quail 1968; Gannon et al., 1991), predation (Danso et al., 1975; Casida 1982) and non-culturability (Grimes and Colwell 1986). Additionally, cells that have dispersed into the immobile fraction of the soil solution can only escape during the short drainage intervals when the conducting channels are water-filled. The lack of detectable breakthrough of surviving cells after the initial pulse is thus explicable in terms of the rapid drainage through these soils, which would provide minimal opportunity for cells adsorbed or entrapped in small pores to disperse into the conducting pore-size fraction.

The very early breakthrough of cells through the Cruden Bay soil (Fig. 1) may have been related to the small volume of pores between 10 and 100 μm in the soil. The calculated volume of 245 cm^3 per core was lower than for Insch or Craibstone, but did not wholly account for the observed breakthrough before 150 cm^3 of rain had been applied. However, this pore-size class may have been over-estimated using moisture release, which is less accurate for such clay soils, which shrink and swell (Haines 1923). The initial breakthrough may also have been partly due to by-passing flow through macropores (> 100 μm), since the smaller pore classes in this soil may not have been sufficiently interconnected to transport the rain at the observed rate. The macropores would not be water-filled before each rainfall event and so displacement of soil solution would not accompany movement through these pores. The prolonged leaching of cells through the Cruden Bay soil is likely to be related to the lower rate of drainage of this soil. This results from its subsoil structure which contrasts with that of the other two soils. Cruden Bay subsoil was more massive, having a bulk density of 1.49 Mg m^{-3} compared to 1.24 and 1.13 Mg m^{-3} for Craibstone and Insch, respectively, and had fewer water-conducting channels. The impeded drainage through the Cruden Bay subsoil will have resulted in the formation of a temporary perched water-table in the topsoil. This probably facilitated the dispersion of cells out of non-conducting pores into the water-conducting channels. This process would be repeated during and immediately following each rainfall event, progressively releasing entrapped cells and producing the prolonged breakthrough observed for the Cruden Bay soil.

Figure 1. Leaching pattern of model biocontrol agent *P. fluorescens* from intact cores of Cruden Bay clay loam, Craibstone loamy sand and Insch sandy loam.

Each cluster of five bars represents results from one rainfall event for five replicate cores. Where a bar is not shown, there were no genetically modified *P. fluorescens* detected in the leachate.
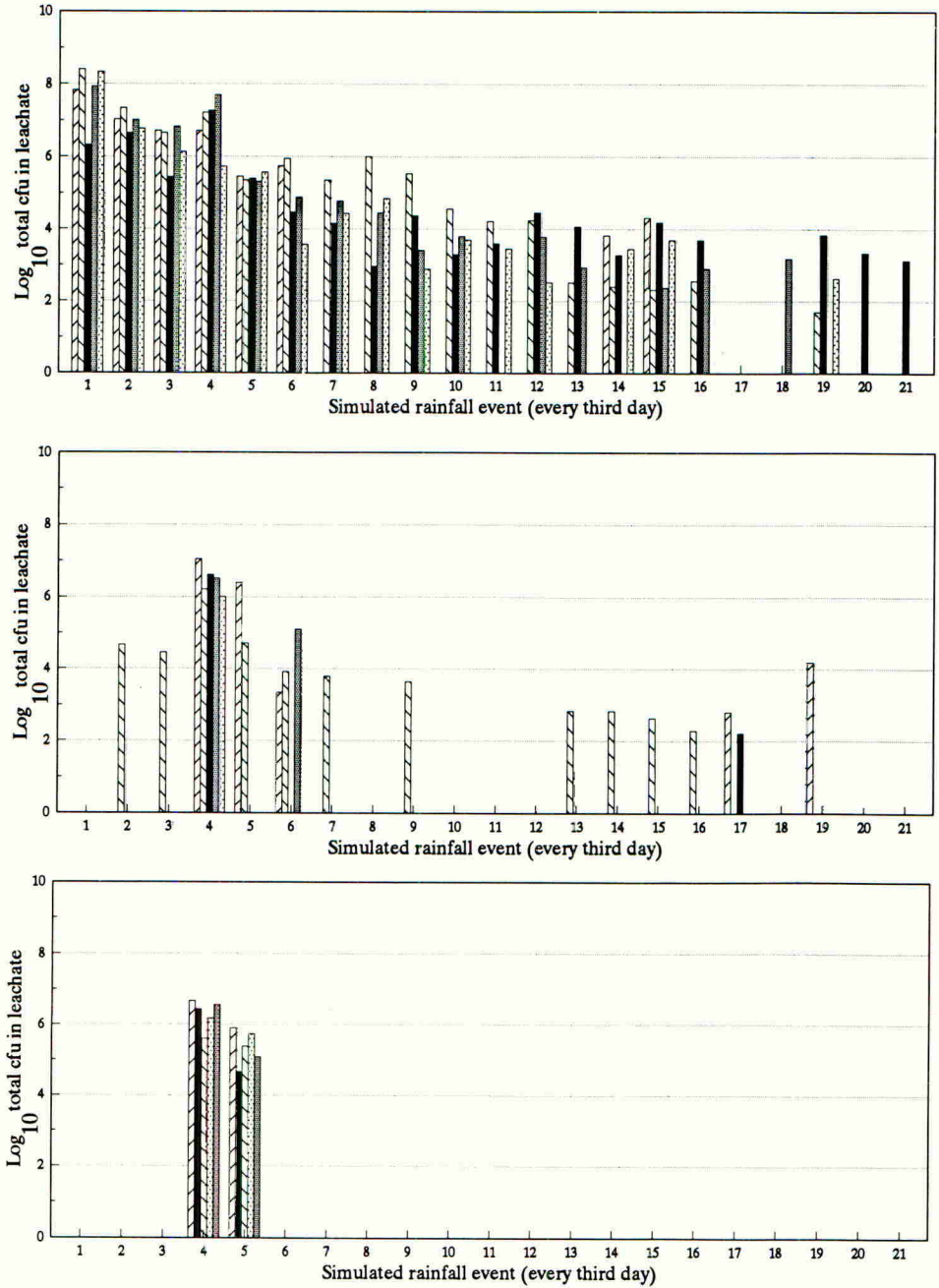
Figure 2 shows the leaching of *B. subtilis* through one of the three soils, Craibstone. Although the ecophysiology of *B, subtilis* and *P. fluorescens* differs markedly, the leaching patterns of the two organisms were broadly similar. This suggests that dispersal of biocontrol agents will be determined more by soil structural properties than by cell type. The total number of cells leached was far fewer for *B. subtilis* than for *P. fluorescens* (0.11% and 0.01% of the initial inoculum, respectively).

Figure 2. Leaching pattern of model biocontrol agent *B. subtilis* from intact cores of Craibstone loamy sand.
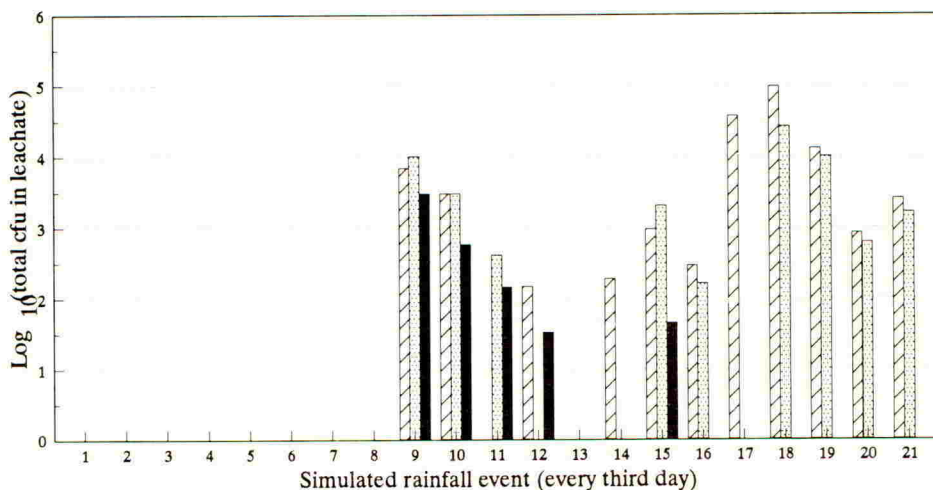

Interestingly, all the cells of *B. subtilis* appearing in the leachate were found to be spores. That spores are more readily leached than vegetative cells is probably because of the polysaccharidic capsule associated with the latter. This causes vegetative cells to aggregate and prevents their dispersal (Vandevivere & Bayveye, 1992) through all but the largest soil pores. Leaching of only spores of *Bacillus* highlights the link between persistence and dispersal of biocontrol agents, in a way analogous to chemical herbicides. The mobility of persistent spores will be an important consideration in risk assessment, particularly when the control agents are genetically modified.

The quantity of cells of both model biocontrol agents that were dispersed through the soil cores as leachate was determined, not only by the structural characteristics of the soil and the rainfall regime, but also by the antecedent (i.e. prior to inoculation) matric potential (data not shown). Significant dispersal only occurred when cells where added to rather wet soils (-30 kPa or wetter). Clearly, cells must initially enter large, water-conducting pores to be dispersed through the soil by percolating water.

The presence of plant roots and earthworms was found to have a very considerable affect on the dispersal of the model biocontrol agents (data not shown). Figure 3 indicates that the effect of plant roots is complicated, in some cases accelerating dispersal of biocontrol agents, but in others, retarding dispersal. The structure of the soil controls which of these predominates. Kemp et al. (1992) and Rattray et al. (1993) have recently investigated and reviewed the effects of roots on inoculum dispersal. The effects of earthworms on dispersal are two-fold. The earthworm gut can transport the inoculum through ingestion of plant litter or soil, or the tunnels formed by the earthworm may provide pathways of preferential flow (Gammack et al., 1991).

Fig. 3. The possible roles of plant roots in mediating the dispersal through soil of microbial biocontrol agents.
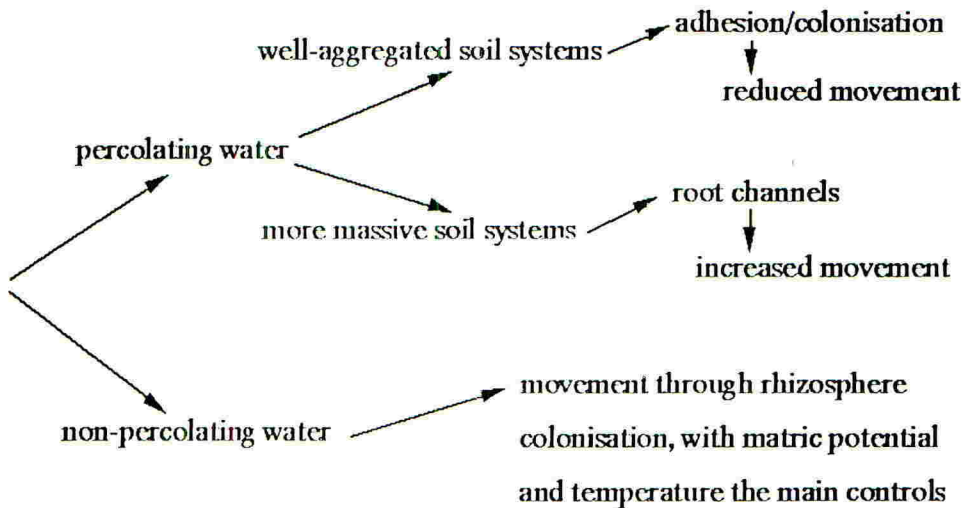
Persistence and dispersal are important considerations in assessing the risks associated with a potential biocontrol agent. They must, however, be considered along with and in the context of other considerations, some of which will depend on whether the control agent has been genetically modified. These additional considerations include gene transfer, undesirable target effects such as establishment of competitive but poorly performing strains, and non-target effects such as displacement of indigenous microbial populations, potential pathogenicity effects, and even effects on soil nutrient cycling and other microbial processes. The bioluminescence-based detection system used in this study offers great potential in contributing to the package of techniques needed to assess these important considerations of risk assessment. This report has emphasised the soil properties and water regime conditions which influence dispersal and, to some extent, persistence of biocontrol agents such as

bioherbicides. These aspects must be considered if an effective risk assessment of this form of biotechnology is to be made and its full potential realised.

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ENGINEERING CROPS FOR TOLERANCE TO SPECIFIC HERBICIDES :
A VALID ALTERNATIVE

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ABSTRACT

Tolerance to specific herbicides can now be obtained via a new set
of techniques. Especially the use of recombinant DNA technology
offers several benefits, both for crop protection and for the
environment.
Along with the first developments of such varieties, a number of
issues were raised addressing alleged risks of the technology.
Based on the case-by-case approach, several examples can now be
forwarded to illustrate that crops engineered for specific herbicide
tolerances are ready to enter the market without risk for the
environment.

INTRODUCTION : A VALID ALTERNATIVE

The development of a chemical product is focussed on the screening of new
compounds for a desired herbicidal action, accompanied by thorough
demonstration of toxicology and environmental safety.
As more safety requirements have been imposed, the search for suitable products
becomes more extensive and valid alternatives were explored.
In an alternative approach, one works from the other side of the product action,
i.e. the crop which has to be protected. Instead of screening for a product, an
"ideal" product is selected, preferably with a wide range of activity (e.g. table 1
summarizes the menu of such an ideal product). Crops - or varieties within crops -
will then be selected that will specifically tolerate the selected product.

TABLE 1 : A short list of characteristics of an "ideal" chemical weed control
agent (Müllner *et al.*, 1993)

- fully selective (no phytotoxicity to crop)
- full control of weeds (dicots, monocots, annuals, perennials)
- high flexibility (e.g. timing of the application to extent post emergence)
- ease of use
- no evolution of resistance
- rapid degradation
- good toxicological and ecotoxicological profile
- fits into integrated crop management
- fits into "non-tillage" system
- cost effective

Today the three main strategies to develop herbicide tolerant plants can be summarized as (J. Howard and C. Barszczynski, 1992) :

- Germplasm screening : the selection of specific genotypes and introgression of the trait through breeding and/or fusion techniques.
- Induction and selection of mutations : the random mutagenic process followed by thorough screening.
- Recombinant DNA technology : the specific introduction of a function with an altered herbicide binding site, or a herbicide detoxifying enzyme or an overexpression of a target protein.

Products based on these approaches - with the exception of the Recombinant DNA technology - have been introduced into the market and have proven their usefulness in the weed control schemes.

Out of these categories the Recombinant DNA technology is definitely the most precise and direct, and yielding important benefits :

1) More emphasis on environmental and health safety of the chemical
   So far the choice of chemicals was limited largely to those to which a certain tolerance level would already exist in crops. Many products could stand the screening on specific phytotoxicity, but would fail on their ecotoxicology and toxicological profile. Yet, other products with an interesting toxicological profile, were designated as non-selective herbicides because of their overall phytotoxicity. In fact, engineering tolerance to such a product in one crop, would fit such a chemical into the list of "ideal" chemical weed control agents.

2) Full control of all weeds
   As the tolerance has been specifically engineered into the crop variety, the weed control can be complete. This also includes very closely related species or regrowth/volunteers, which proved to be a problem with traditional weed control.

3) Flexibility will result in lowering the number of applications
   Traditional weed control schemes involve careful balancing and anticipating weed problems. Treatments are done pre-emergence as well as post-emergence. The limitations of the weed control capacity of the individual products has a prominent effect on this.
   As in the new case only the crop varieties are tolerant (and in most cases this tolerance is independent of a specific developmental stage), the timing of application is flexible and can be postponed until a weed problem is identified (if any). Therefore, it is anticipated that many of the complicated preventive treatment schemes will be condensed to applications only addressing actual weed problems.

4) The amount of a.i. will be reduced
   Because of the high efficiency of the intended products, the amount of a.i. per application can be reduced considerably. In combination with the reduction of

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the number of applications and the positive toxicological profile, a significant improvement towards environmentally compatible crop protection will be achieved.

Based on this forecast of less a.i. of safer products applied in a flexible way to control weeds, it has been argued that genetical engineered herbicide resistant crops are "a moral imperative for world food production" (J. Gressel; 1992).

OBSERVING THE DEBATE

Within the framework of the heated debate on environmental impacts of chemicals, these alternatives have also been debated (e.g. as summarized by CAST (Duke *et al*, 1991). In table 2, some arguments of both opponents and propagators are listed on key issues in environmental and socio/economic factors.

TABLE 2 : Key environmental and social arguments in the debate of genetically engineered herbicide tolerant plants	
Issue 1 : Weediness	
The genetically modified tolerant plant will be uncontrollable, it will proliferate beyond boundaries and invade important natural niches.	The effect of the gene is well known and specific. The modified plant is identical to the original plant within all but the introduced aspects.
Genetically modified plants need to be compared with the introduction of an exotic species. It's behavior is completely unpredictable.	Single gene changes are not comparable to the introduction of an entire genome. Therefore, the familiarity criteria is essential.
A single gene can change the performance of a plant substantially, changing a beneficial crop into a noxious weed.	A gene for herbicide tolerance confers no selective advantage, except when the plants are grown in the presence of the herbicide.
Genes will "escape". They will invade wild sexually compatible weeds and create even more problems.	Genes will become part of the natural "gene pool". In specific crops this may extend into wild species. As no competitive advantage is transferred, this has no further meaning for the environment.
Issue 2 : Herbicides	
Chemical herbicides are bad. They can contaminate soil and water, and some are associated with negative effects on health.	New developments focus on product with a favorable toxicological and environmental profile.

High tolerance levels will encourage greater use of herbicides.	The use of effective herbicides in a flexible post-emergence scheme will introduce overall decrease in the use of herbicides.
Weeds will become resistant so that even more products and/or higher dosages will be needed.	The natural development of resistance to herbicides has been reported, yet this does not necessarily mean increase of application rates. As some of the tolerance strategies are new to the species, the development of resistance should take much longer.
Volunteer weeds (regrowth of previous crops) - in some cases ending up with different herbicide tolerances - will become completely unmanageable.	Volunteer weeds can be handled by different products as today. Careful combinations of herbicide type tolerance/crop should be developed considering follow-up crops. Management rules will be proposed.

Issue 3 : Social/Economic	
Farmers will have less options to produce in an environmental sound way.	Herbicide tolerant plants will broaden the possibilities of the farmers to choose the appropriate protection against weeds.
Farmers will be sold a package of seed and a chemical rendering them dependant on big multinational chemical companies.	Individuals choose based on efficiency, relative yield and economic and environmental factors.
Expensive developments - such as herbicide tolerant crops - can only be supported by big markets. Major crops will become even more standardized leading to further erosion into monocultures and loss of biodiversity.	The feasibility of the herbicide tolerance strategies being proven, the traits are introduced by different breeders in a wide range of elite germplasm. Efficient weed control is a prerequisite of agriculture at any scale.
All efforts should be directed to achieve sustainable agriculture. The only real type coherent with this is organic farming, prohibiting the use of all herbicides and other pesticides.	There is a clear endeavor towards a more sustainable agriculture. The input of products can be limited and more environmental compatible products can be used because of genetic engineering.

Clearly the arguments listed are extremely condensed and only capture the spirit of the opposing views. Yet they are so opposing that one can hardly imagine -in spite of all the public debate- that a reasonable reconciliation can be expected.

A knowledgeable observe can recognize different types of alleged risks which blur the picture :

1) Perceived risks :
   Big terms are casted and spelled out as risks although they are merely biological facts. E.g. the "gene escape" call seems to imply that some form of containment -from which the gene can escape- is intended. Quite the opposite is true. The gene -if judged to be without any hazard- will be allowed to become part of the natural exchange system (gene pool).

2) Hypothetical risks :
   As it is hard to identify impacts, worst-case scenarios have been used to address any potential event. However, it has to be emphasized repeatedly that such worst-case-scenarios have a value in identifying critical factors, but do not represent reality as such. Many of the ideas about invasiveness fall in this categories.

3) Generic risks :
   Although the review of genetically modified plants (and herbicides) is clearly a case-by-case approach, many generic claims influence the positions : chemicals are bad, genetically modified organisms are dangerous...
   Whereas it is recognized that the original value of such debates is identifying the key elements for a risk/benefit analysis, the arguments have to be honestly put in perspective instead of turning them into a political debate.

A FRAMEWORK FOR DECISIONS

1) <u>Selective herbicide tolerance introduced through genetic engineering has been studied extensively with success in the field.</u>

   Herbicide tolerance trials are the most advanced in terms of total trials and the number of years particular crop-by-crop trait combinations have been tested. (E. Chasseray and J. Duesing, 1992).
   Among the most common traits tested in the field in the period '86-'91., tolerance to glufosinate, glyphosate and sulfonylurea rank amongst the highest. There has been no observed adverse effect or unexpected behavior of the experimental plants under such very carefully monitored conditions.

2) <u>Large biosafety programs and initiatives have addressed particular issues : there is no indication of generic hazards associated with genetically engineered herbicide tolerance</u>

   At the European level, within the Biotechnology Action Program, several pan-

European collaborations have been addressing assessment methodology, establishment ability and competitiveness and gene dispersal from genetically modified plants. This research is now being completed in the Biotechnology Research for Innovation, Development and Growth in Europe (BRIDGE) program, Safety Assessment of the Deliberate Release of Two Model Transgenic Crop Plants, Oilseed Rape and Sugarbeet, which explicitly takes up the design of protocols and predictive modelling. Table 3 summarizes the research topics integrated in this program.

TABLE 3 : Research topics of the BRIDGE Program : Safety Assessment of the Deliberate Release of Two Model Transgenic Crop Plants, Oilseed Rape and Sugarbeet

Research topics Oilseed Rape	Research topics Sugarbeet

* Dispersal of introduced genes
    Large scale pollen dispersal
    Evaluation of outcrossing
    Monitoring seed dispersal
 	
* Outcrossing to relatives
    Behavior of introduced gene
    Analysis of position effect
 	
* Behavior of plant/populations
    Fate of hybrids
    Evolution of mixed populations
 	
* Stability of a GM plant
    Population and generation
 	
* Computer modelling
	* Evaluation physiology
	    Reference systems
	    Evaluation systems
	
	* Dispersal of genes
	    Spread of pollen
	    Spread to wild relatives
	
	* Behavior of plants/populations
	    Fate of hybrids
	    Evolution of mixed populations

Most of these studies are based on lines with herbicide tolerance obtained through genetic engineering. In fact, the oilseed plants contain the "bar" gene, coding for a resistant glufosinate (PGS), the sugarbeet plants are either tolerant to glufosinate (PGS) and glyphosate (MONSANTO).

These particular lines were chosen because of the ease of the herbicide marker system, the economic importance of the particular trait/crop combination and the environmental questions that were previously raised.

Complimentary initiatives have been taken at state national level. A prominent one, joining the authorities, industry and research institutes in a multi-year evaluation in the UK, is known by the acronym PROSAMO (Planned Release Of Selected And Modified Organisms).

Dr. Crawley's group at the Imperial College performed within this framework an ecological study in natural habitats and concluded for a number of oilseed rape lines that there were "no indications that genetic engineering for kanamycin tolerance or herbicide tolerance increased the invasiveness potential of a seed crop" (Crawley *et al.*, 1993)

These larger safety evolution programs served a dual role in providing factual data demonstrating safety and validating new tools and designs in order to streamline new developments.

3. Responsible choices on a case-by-case basis : products, crops, tolerance mechanisms

The fact that a certain tolerance mechanism is not genotype bound could give rise to the possibility of introducing that mechanism in virtually all important crops.
Yet, quite the opposite is true as industry will clearly weigh the new potential use against what the impact will be on existing and other potential uses. A typical example is the introduction of sulfonylurea tolerant oilseed rape, which then could jeopardize the major sulfonylurea application on the monocot-rotation cycle. This seemed important enough to cause a delay in the development of the sulfonylurea tolerant oilseed rape.

4. The regulatory system is in place

During the recent years, the involvement of different agencies has become more prominent on the assessment of products from genetic engineering. The necessary legal frameworks have been - or are being - installed. Because of the many field releases, many authorities have been involved in the assessment of the issues and were updated on the positive results. Thereby, capacity has been build to make proper assessment in view of any impact on the environment, which in most cases of herbicide tolerance will be virtually none.

5. The benefits are documented

We have briefly reviewed the potential benefits of these alternative approaches. For the first developments, sufficient data are now available to confirm the reality of this forecast.
Müllner *et al* (1993) present a case on the application of glufosinate tolerant crops. In the first instance, they demonstrate that the herbicide glufosinate fits the criteria of an "ideal" chemical weed control agent, with the main emphasis on the combination of broad toxicity combined with a good toxicological profile and the rapid degradation and inactivation.
Once tolerance to glufosinate was integrated into corn, oilseed rape and sugarbeet, it was demonstrated that an effective reduction of totally applied herbicide is achievable while guaranteeing good weed control. The glufosinate tolerant crops would furthermore enable the design of no tillage or intercroping systems.

Similar argumentation has been proposed for glyphosinate (Waters, 1991). A recent study performed by a prominent group of German institutes confirms the position of Recombinant DNA technology as a logical alternative in the development of herbicide tolerant crops (Antje Dietz *et al.*, 1993). An EPA funded report goes further in concluding that herbicide-tolerant crops "have the potential to reduce pollution and mitigate the environmental impact of pesticides" (R. Hoyle, 1993).

CONCLUSION

In a continuing strive for a better agriculture - better in terms of relative yield, lower impact on the environment, more sustainable growth - there is an even bigger need for flexible weed control systems.

The development of new tools was parallelled by the expression of additional concerns on issues related to weediness of the engineered plants, use of the herbicides and socio/economic factors. Industry has identified the aspects, including volunteer weed management, determining the choice of strategies.

The experience with the first developments confirm that engineering a crop for tolerance to a specific herbicide is a valid alternative in weed control, yielding important benefits.

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