SESSION 3A

HERBICIDAL ACTIVITY, SITES OF ACTION AND TARGETS FOR MANIPULATION. I

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INVITED PAPERS 3A-1 to 3A-8

THE SITE OF ACTION OF THE SULFONYLUREA HERBICIDES

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ABSTRACT

The sulfonylureas are a new class of highly active herbicides. Genetic and biochemical studies on the mode of action of these herbicides have demonstrated that they act in plants to block the production of the essential amino acids valine and isoleucine by inhibiting the enzyme acetolactate synthase (also known as acetohydroxy acid synthase), EC 4.1.3.18. This enzyme catalyzes the first step in the biosynthesis of valine and isoleucine. Acetolactate synthases from a wide variety of plant species were found to be equally sensitive to inhibition by the sulfonylurea herbicides with metabolic inactivation serving as the basis for selectivity.

INTRODUCTION

The sulfonylureas are a new and unique class of herbicides characterized by very low use rates, excellent crop selectivity, and low mammalian toxicity (Levitt et al., 1981). Use rates for these herbicides range from 4-20 grams per hectare. Several sulfonylureas have been developed for use on wheat and other small grain cereals, rice, soybeans, and for non-selective industrial weed control (Figure 1). Selectivity with the sulfonylureas is based on the ability of crop plants to metabolize the herbicides to non-phytotoxic products by crop plants (Sweetser et al., 1982).

The high herbicidal activity and remarkably low use rates suggest the sulfonylureas have a very specific mechanism of action. Previous studies on the mechanism-of-action of the sulfonylurea chlorsulfuron, the active ingredient in Glean®, demonstrated that this herbicide is a potent inhibitor of plant cell division (Ray, 1982a,b). However, processes such as RNA synthesis, protein synthesis, respiration, and photosynthesis are unaffected by sulfonylureas at times when cell division is significantly inhibited (Ray, 1982a). Recent studies with bacteria have shown that the sulfonylurea sulfometuron methyl, the active ingredient in Oust®, prevents the growth of bacteria by inhibiting the enzyme acetolactate synthase (ALS) (LaRossa and Schloss, 1984). This enzyme is required for the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine. This report outlines the biochemical basis for the mechanism of action of the sulfonylurea herbicides in higher plants.

Chlorsulfuron
(Glean®)

DPX-T6376
(Ally®)

Sulfometuron Methyl
(Oust®)

DPX-F5384
(Londax®)

DPX-F6025
(Classic®)

Fig. 1. Structures of sulfonylureas which are the active ingredients in the various Du Pont herbicides noted. Glean® and Ally® are used for weed control in wheat. Oust® is a non-selective herbicide. Londax® is used for weed control in rice and Classic® for soybeans.

MATERIALS AND METHODS

Pea Root Cultures

Aseptic cultures of excised pea roots (*Pisum sativum* L. var Alaska) were established and grown according to published procedures (Ray, 1984). Organic supplements such as amino acids and herbicides were filter sterilized and added to autoclaved culture medium. Root growth, measured as increases in root length, was determined 120 hrs after the start of the experiments.

Acetolactate Synthase Extractions and Assays

Acetolactate synthase was extracted from plant tissue as described previously (Ray, 1984). When green tissue was used as a source of ALS, 2% w/v polyvinylpolypyrrolidone was added to initial extraction buffer. Enzyme assays were carried out as described by Ray (1984). The I_{50}, defined as the concentration of sulfonylurea which inhibits ALS by 50% in a 30-min fixed time assay, was calculated by regression analysis as described previously (Ray, 1984).

RESULTS

The growth of excised pea roots in culture provide a sensitive and quantitative measure of the effects of sulfonylureas, such as chlorsulfuron, on plant growth. Initial experiments were conducted to evaluate the ability of undefined nitrogen sources, such as casein hydrolysate, to reverse the inhibitory effects of chlorsulfuron on pea root growth. Results from a typical experiment, reported in Table 1, show that at a concentration of 28 nM, chlorsulfuron almost totally inhibits root growth. However, when casein hydrolysate at a final concentration of 0.1% w/v is included in the culture medium a significant amount of protection against chlorsulfuron inhibition is observed. By adding different combinations of amino acids to chlorsulfuron treated roots, it was found that two amino acids, valine and isoleucine, each added at a 100 µM, provided complete protection against sulfonylurea induced growth inhibition (Table 1). These findings are consistent with the bacterial experiments of LaRossa and Schloss (1984). Both valine and isoleucine are required for complete reversal. Isoleucine alone is ineffective, while valine is only partially effective in restoring growth. These results strongly suggested that sulfonylureas inhibit the biosynthesis of valine and isoleucine in plants. The reversal of growth inhibition by the addition of intermediates of the valine-isoleucine pathway indicated that site of sulfonylurea action was at one of the early steps of the biosynthetic pathway of these amino acids (Ray, 1984).

TABLE 1

The effects of casein hydrolysate, valine, and isoleucine on chlorsulfuron induced growth inhibition in cultures of excised pea roots.

Supplement	Net Growth (mm)	
	Control	+ 28 nM Chlorsulfuron
No Addition	42.3	9.5
Casein Hydrolysate (0.1%)	42.4	31.0
Valine (100 µM)	47.6	20.9
Isoleucine (100 µM)	34.8	10.1
Valine + Isoleucine (100 µM ea)	44.8	42.0

Valine and isoleucine are synthesized in plant cells through a common series of enzymes, the first of which is ALS. This enzyme catalyzes the condensation of two molecules of pyruvate to yield acetolactate in valine formation, or the condensation of α-ketobutyric acid with pyruvate to yield acetohydroxybutyric acid in isoleucine biosynthesis. Acetolactate synthase has been extracted from pea shoots as well as a number of other plant species and has been found to be highly sensitive to inhibition

by chlorsulfuron (Ray, 1984). The effect of other sulfonylureas on acetolactate synthase is similar to that of chlorsulfuron. For example, Figure 2 shows progress curves for DPX-F6025 inhibition of ALS from pea shoots (see Figure 1 for chemical structure). The amount of inhibition is not constant but instead increases with time, and the rate of inactivation increases with increasing sulfonylurea concentration. Similar inhibition kinetics were found for sulfometuron methyl inhibition of bacterial

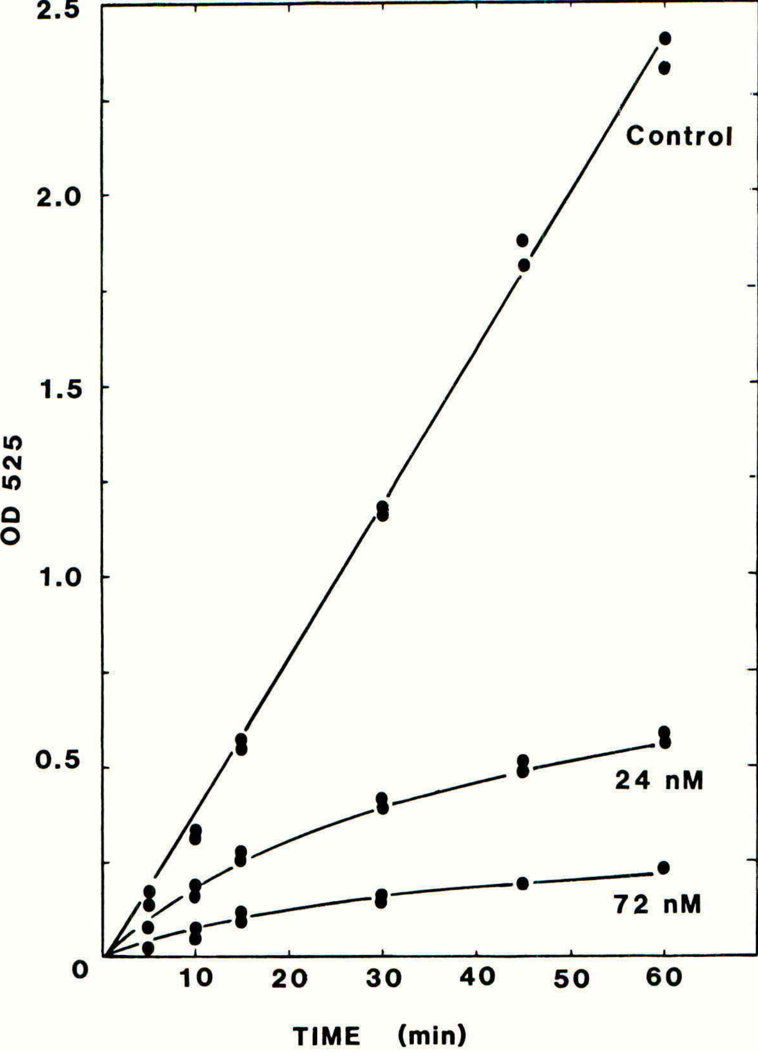

Fig. 2. Progress curves for DPX-F6025 inhibition of ALS from pea shoots. The structure for DPX-F6025 is given in Fig. 1.

ALS (LaRossa and Schloss, 1984) and for chlorsulfuron inhibition of pea ALS (Ray 1984). Detailed kinetic analyses of sulfonylurea inhibition of the bacterial enzyme suggest that the herbicides act as slow tight binding inhibitors of ALS and that they may compete with the binding of the second pyruvate molecule to the ALS active site (Schloss 1984).

In addition to the biochemical studies, detailed genetic studies with microorganisms (LaRossa and Schloss, 1984; Falco and Dumas, 1985), and higher plants (Chaleff and Ray, 1984; Chaleff and Mauvais, 1984) also demonstrate that ALS is the site of action of the sulfonylurea herbicides. With higher plants, tobacco mutants, isolated through cell culture selection techniques, were shown to possess a single, semi-dominant, nuclear gene mutation that confers chlorsulfuron and sulfometuron methyl resistance (Chaleff and Ray, 1984). The basis for this resistance is an altered form of ALS with reduced sensitivity to inhibition by sulfonylurea herbicides (Chaleff and Mauvais, 1984). This reduced sensitivity is evident in progress curves for chlorsulfuron inhibition of ALS from normal sulfonylurea sensitive tobacco and sulfonylurea resistant mutants (Figure 3a & 3b). As with ALS from pea shoots, sulfonylurea inhibition of

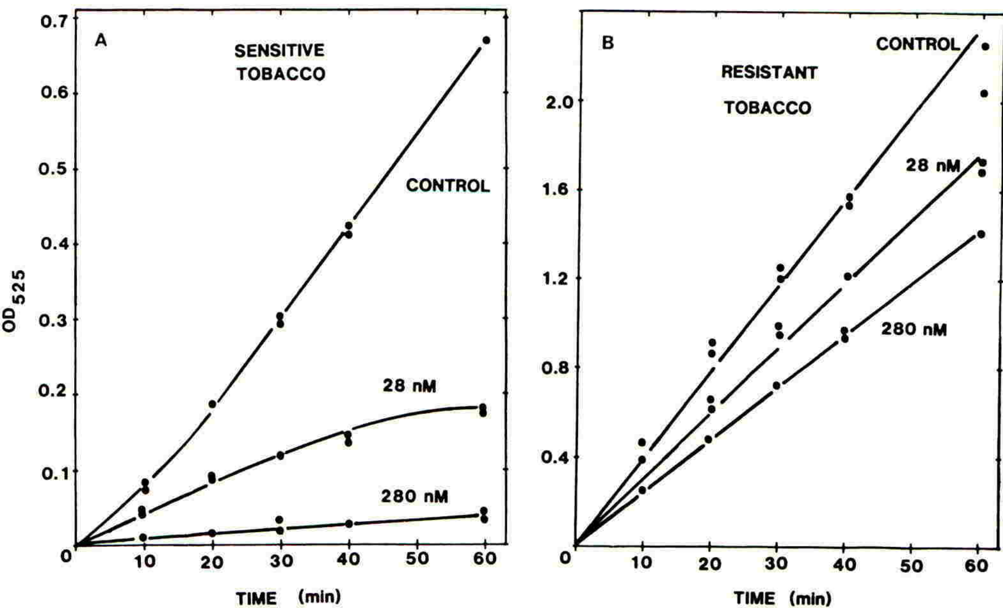

Fig. 3. Progress curves for chlorsulfuron inhibition of ALS activities from sulfonylurea sensitive (A) and resistant tobacco (B).

the enzyme from normal herbicide sensitive tobacco increases with time. However, the response of ALS from the resistant tobacco is quite different. Although the enzyme activity is reduced, inhibition does not increase with time, enabling it to function in the presence of the sulfonylurea.

ALS from higher plants is extremely sensitive to inhibition by chlorsulfuron (Ray, 1984). The sulfonylurea concentrations needed for plant ALS inhibition are similar to those required for inhibition of pea root growth. The I_{50} for ALS inhibition, defined as the concentration of sulfonylurea that inhibits ALS activity 50%, is 21 nM (7.5 ppb) for chlorsulfuron with ALS from pea shoots. The I_{50} values for inhibition of ALS from a variety of plants species have been determined with a number of different sulfonylurea herbicides (Table 2). In all cases these highly active herbicides proved to be potent inhibitors of ALS regardless of the source of the enzyme. Thus, ALS from wheat, which is tolerant to chlorsulfuron, is just as sensitive to inhibition by chlorsulfuron as the enzyme from sensitive species such as wild mustard. These results emphasize the role of plant metabolism in crop tolerance to sulfonylurea herbicides (Sweetser et al., 1982).

TABLE 2

Inhibition of ALS from crops and weeds by various sulfonylureas. Structures of the sulfonylureas are given in Figure 1.

	I_{50} (nM)				
ALS Source	Chlorsulfuron	Sulfometuron Methyl	DPX T6376	DPX F5384	DPX F6025
Pea	20.9	15.3	13.9	63.8	6.0
Wheat	21.8	12.9	30.1	13.6	5.1
Rice	24.0	18.1	29.9	16.3	5.8
Soybean	32.4	46.4	37.4	74.6	7.7
Morningglory	24.3	19.8	54.2	97.9	6.8
Wild Oats	15.9	7.1	14.7	7.8	3.1
Wild Mustard	10.9	9.3	12.6	9.0	3.1
Barnyardgrass	24.3	21.1	21.8	15.1	3.6

A good correlation exists between the herbicidal activity of sulfonylureas and their ability to inhibit ALS. For example removing the chloro-group from chlorsulfuron not only reduces the herbicidal activity of the molecule (Sauers and Levitt, 1984), but also greatly increases the I_{50} value for ALS inhibition indicating a less active ALS inhibitor than chlorsulfuron (Table 3). Substituting a carboxylic acid group in this ortho-position, results in a herbicidally inactive sulfonylurea (Sauers and Levitt, 1984). Consistent with this loss of herbicidal activity is the loss of inhibitory activity against ALS (Table 3).

TABLE 3

The I_{50} values for inhibition of ALS by analogs of chlorsulfuron.

Structure	I_{50} (nM)
Cl, O, SO_2NHCNH, N, N, N, CH_3, OCH_3	21
O, SO_2NHCNH, N, N, N, CH_3, OCH_3	684
COOH, O, SO_2NHCNH, N, N, N, CH_3, OCH_3	Inactive

DISCUSSION

Biochemical and genetic studies with plants and microorganisms provide convincing evidence that ALS is the site of action of the sulfonylurea herbicides (Chaleff and Mauvais, 1984; LaRossa and Schloss, 1984; Falco and Dumas, 1985; Ray, 1984). The biochemical evidence for this is the fact that ALS is very sensitive to inhibition by the sulfonylureas. By inhibiting ALS, sulfonylureas block the production of the essential amino acids valine and isoleucine which in turn leads to growth inhibition. Supplying sulfonylurea treated plant tissue with these two amino acids overcomes the herbicide induced growth inhibition.

Additional evidence for ALS as the target site of sulfonylurea herbicides in higher plants comes from genetic studies of sulfonylurea resistant tobacco mutants. These mutants possess an altered form of ALS which is less sensitive to inhibition by sulfonylureas than ALS from normal herbicide sensitive plants. The altered ALS has been shown to co-segregate with the resistance phenotype (Chaleff and Mauvais, 1984).

Sulfonylureas such as chlorsulfuron inhibit acetolactate synthase from a wide variety of species including those tolerant to the herbicide in field. The same is true for DPX-F6025, and the other herbicides listed in Table III. The general sensitivity of ALS enzymes to inhibition by sulfonylureas indicates that herbicide metabolism plays a key role in sulfonylurea crop tolerance.

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In addition to the sulfonylureas, other classes of herbicides have also been found to block essential amino acid production in plants. Glyphosate inhibits the formation of aromatic amino acids by inhibiting the enzyme 5-enolpyruvlyshikimate-3-phosphate synthase, a key enzyme in biosynthesis of phenylalanine and tyrosine (Steinrucken and Amrhein, 1980). In addition the imidazolinone herbicides, like the sulfonylureas, have been found to block valine and isoleucine biosynthesis by inhibiting ALS (Shaner et al., 1984). Thus it appears that essential amino acid biosynthesis is emerging as a new major target site for herbicides.

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COMPARISON OF THE MODE OF ACTION OF CHLORSULFURON BETWEEN HIGHER PLANTS AND ANIMALS

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ABSTRACT

Chlorsulfuron inhibited the growth of tobacco cell cultures at 0.001 ppm although cultured cells of hamster kidney (BHK cells) continued to grow in a medium containing 100 ppm chlorsulfuron. Half inhibition of tobacco cell culture growth was estimated to be 0.5 ppb. As shown by Ray (1984), acetolactate synthase from tobacco cell cultures was strongly inhibited by chlorsulfuron having an I_{50} value of 5 - 10 ppb (14 - 28 nM). No inhibition was found in the enzymes from hamster cells and chick liver at 100 ppb. The reaction products of plant and animal enzymes were surveyed with GC and HPLC, and these suggested that acetolactate was the product. pH dependence and co-factor requirement were compared between tobacco and animal enzymes.

INTRODUCTION

Sulfonylurea type herbicides including chlorsulfuron have an extremely high herbicidal activity but their toxicity to animals is very low (Levitt *et al*. 1981). The mode of action of chlorsulfuron has been reported to be at the inhibition of acetolactate synthase, a key enzyme for the biosynthesis of valine or isoleucine (Ray 1984, Chaleff & Mauvais 1984). Recently a number of herbicides that inhibit amino acid synthesis have been developed. In the plant kingdom including microorganisms all amino acids are biosynthesized, but in the case of animals the so-called essential amino acids are not synthesized. Therefore some of the amino acid biosynthesis pathways are potentially good targets for herbicidal action because of the possibility of low animal toxicity.

In this report, the mode of action of chlorsulfuron, one of the sulfonyl urea herbicides, was compared using tobacco tissue and hamster kidney cell cultures. In the case of acetolactate synthase assay, chick liver was also used as an enzyme source.

MATERIALS AND METHODS

Plant and animal

Tobacco cell (*Nicotiana tabacum* L. cv. Samsun) suspension culture was maintained by weekly transfer to a MS medium (Murashige & Skoog 1962) containing 2,4-D at a final concentration of 1.5 uM. The culture was placed on a gyratory shaker at 120 rpm, and incubated at 27°C under fluorescent lamps.

A hamster kidney cell line (BHK) was maintained by weekly transfer to Eagle's medium containing calf serum. The cells were incubated at 37°C. The chick livers were prepared from 4 week-old chick, and stored at -85°C before usage.

Extraction and assay of acetolactate synthase

Acetolactate synthase was extracted from plant and animal cells, and animal tissues as described by Ray (1984). The materials were homogenized in 5 volumes of buffer 0.1 M K_2HPO_4 (pH 7.5), 1 mM sodium pyruvate, 0.5 mM

$MgCl_2$, 0.5 mM thiamine-pyrophosphate (TPP), 10 µM flavin adenine dinucleotide (FAD), and 10 % v/v glycerol. The homogenate was centrifuged at 12,000 rpm for 20 min. The enzyme was collected at 20 to 50 % saturation with ammonium sulphate by centrifugation at 10,000 rpm for 15 min, and the pellet was dissolved in buffer containing 0.1 M K_2HPO_4 (pH 7.5), 0.5 mM $MgCl_2$ and desalted on column of Sephadex G-25 equilibrated with the same buffer.

Acetolactate synthase assays were carried out on final volume of 0.5 ml at 30°C. The final reaction mixture contained 20 mM K_2HPO_4 (pH 7.0), 20 mM sodium pyruvate, 0.5 mM TPP, 0.5 mM $MgCl_2$ and 10 µM FAD. Assays were terminated by adding 50 µl of 6 N H_2SO_4. Acetolactate was determined as described by Westerfield (1953). The optical density of the solution was determined at 525 nm. Protein was determined by Bradford's method (Bradford 1976) using a Bio-Rad protein assay solution (Bio-Rad, Richmond U.S.A.).

Gas chromatograph

The acetolactate synthase assay reaction mixture was incubated at 30°C for 1 hr, and added to 2 volumes of ethanol, and centrifuged at 3,000 rpm for 10 min. To the supernatant, sodium sulphate (anhydrous) and methanol were added. The organic solvent was evaporated *in vacuo* at 40°C. The residue was treated by trimethylsilyl trifluoroacetamide and heated at 100°C for 30 min. The trimethylsilyl (TMS) derivatives were assayed with a Shimadzu GC-5A gas chromatograph equipped with a i.d. 3 mm x 1.5 m glass column, packed with 3 % SE-30 (Chromosorb WAW DMCS 60-80 mesh). The operating conditions were: N_2 carrier gas 50 ml/min, injection port 250°C, column 85°C, and detector 250°C.

High performance liquid chromatograph

Reaction mixtures of acetolactate synthase assay were incubated at 30°C for 1 hr, followed by the addition of 6 N H_2SO_4. The reaction mixture was heated at 60°C for 15 min, 2 volumes of ethanol were then added, and the mixture was cetrifuged at 3,000 rpm for 15 min. The supernatant was assayed with a Shimadzu LC3A high performance liquid chromatograph equipped with 4.6 mm i.d. x 15 cm column packed with Zorbax ODS. The operating conditions were: mobile phase 80 % methanol, flow rate 0.3 ml/min, and detector UV 300 nm.

RESULTS

The effects of chlorsulfuron on the growth of cell cultures of tobacco plants and hamster kidney

Seven days after the initiation of cell culture, chlorsulfuron was added to the medium. Another seven days after the treatment, the growth of the culture was measured. As shown in Figure 1, the growth of tobacco cells was inhibited almost totally at 1 ppb with half inhibition at 0.5 ppb. In the hamster kidney cells the growth was not inhibited at 100 ppm chlorsufuron, although a complete inhibition by 10 ppm actinomycine D was demonstrated (Figure 2).

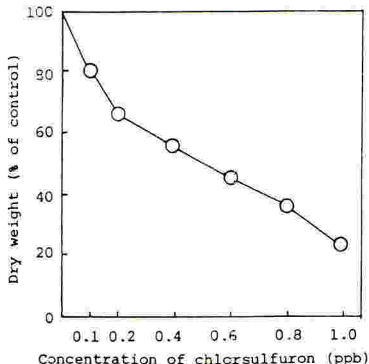

Fig.1. Effect of chlorsufuron on tobacco cell cultures

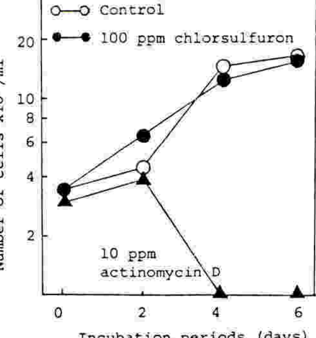

Fig.2. Effect of chemicals on growth of hamster kidney cell cultures (BHK)

Recovery of the growth of tobacco cell culture from chlorsulfuron-inhibition by the supply of amino acids

In the case of the growth of pea roots, chlorsulfuron-inhibition was overcome by the addition of valine and isoleucine (Ray 1984). The result of such a recovery in tobacco cell culture by the supply of valine, isoleucine and leucine is shown in Table 1. In the case of an additive concentration of over 1 mM, there was found to be a feedback-like inhibition of growth. Chlorsulfuron inhibition was also recovered by the supply of the three amino acids in tobacco cells. The recovery was best especially when 5 ppb chlorsulfuron was used together with a 400 μM amino acid concentration.

TABLE 1

Effect of three amino acids on chlorsulfuron inhibition of growth of tobacco cell culture

Three amino acids* added (uM)	Dry weight (mg)		
	Control	Chlorsulfuron 5 ppb	Chlorsulfuron 10 ppb
0	869	126 (14.5)**	97 (11.2)
100	839	264 (31.5)	261 (31.1)
200	786	450 (57.3)	415 (52.8)
400	740	613 (82.8)	533 (72.0)
600	710	438 (61.7)	576 (81.1)
800	643	431 (67.0)	559 (86.9)
1,000	450	471(104.7)	465(103.3)

* Three amino acids: valine, isoleucine, and leucine.
** Percent of control.

Effect of chlorsulfuron on acetolactate synthase

In the case of tobacco cell culture, the extracted enzyme was completely inhibited by 40 ppb chlorsulfuron showing half inhibition at 18 - 36 nM (Fig. 3). This corresponds to the results in pea of Ray (1984). On the other hand, the enzymes extracted from hamster kidney cell culture and chick liver

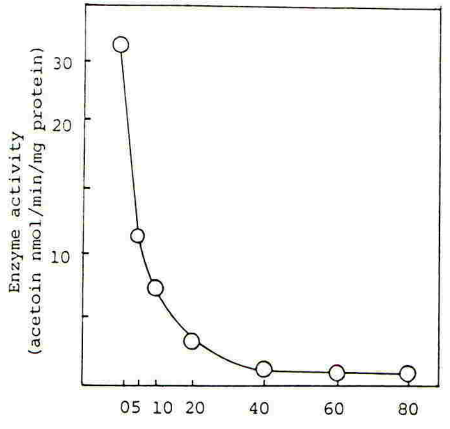

Fig. 3. Dose response for inhibition of acetolactate synthase of tobacco cell culture by chlorsulfuron.

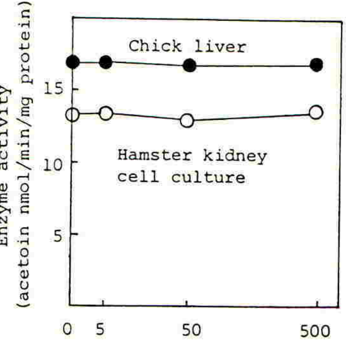

Fig. 4. Effects of chlorsulfuron on acetolactate synthase of chick liver and hamster kidney cells.

were not inhibited by 500 ppb chlorsulfuron (Fig. 4).

Reaction products in the acetolactate synthase reaction mixtures

The acetolactate synthase activity from animal sources was also assayed by the determination of acetoin content in reaction mixture after hydrolysis of reaction products. Valine and isoleucine are essential amino acids for animals, and there is a possibility that animals do not contain any acetolactate synthase. For these reasons the reaction products of the enzyme reactions with tobacco and hamster kidney cell cultures and chick liver were isolated and their characteristics were examined.

After a reaction of one hour, ethanol was added and the protein was discarded. The sample, which was evaporated and treated with trimethylsilyl trifluoroacetamide was injected into a gas chromatograph. As shown in Fig. 5, each product from the three reaction mixtures showed the same peaks at a retention time of 17 minutes, although before the reaction none of the sample showed any peak at this particular point.

After decarboxylation of the reaction product by H_2SO_4, the samples were analyzed by high performance liquid chromotography (HPLC). As shown in Fig. 6, the products from both tobacco cell culture and chick liver showed peaks identical to the authentic acetoin. Without the decarboxylation by H_2SO_4, we could not detect any peak at such point.

From these experiments, it was evident that all three reaction systems including plant and animal cells appeared to produce the same product, acetolactate.

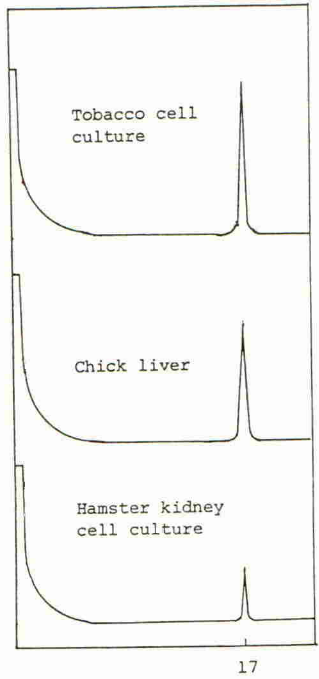

Fig. 5. Gas chromatograms of trimethylsilyl derivatives in reaction mixtures.

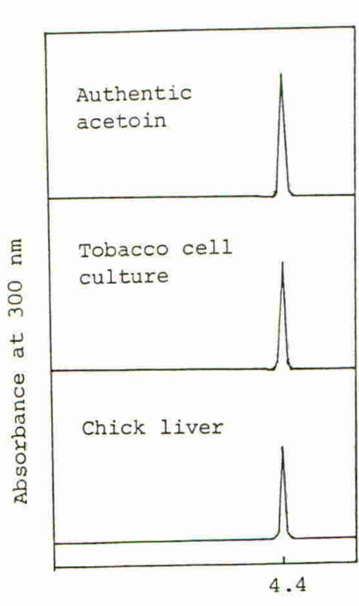

Fig. 6. Chromatograms of H_2SO_4 treated reaction products by HPLC. The reaction mixture was incubated for one hour and treated with 6N H_2SO_4 for 15 min at 60°C.

The optimum pH for the acetolactate synthases from plant and animal cell cultures

The effects of pH on the reaction are shown in Fig. 7. In tobacco cell culture enzyme, the optimum pH was found to be 8.0 with a broad peak. In the case of hamster kidney culture cells, the optimum pH was around 7.0 to 7.5 and the curve showed a sharper peak.

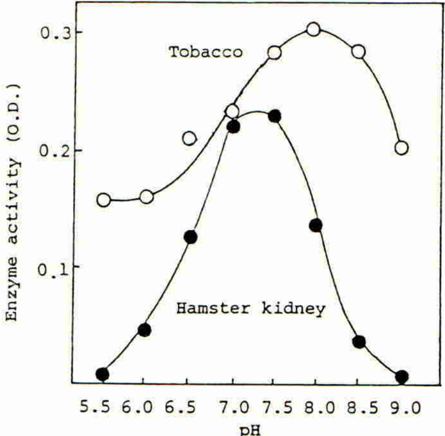

Fig. 7. Effect of pH on acetolactate synthase activity of cultured cells of tobacco and hamster kidney.

Effects of the co-factors on the acetolactate synthase activity

Usually in the assay system of acetolactate synthase, TPP, FAD and Mg^{++} are added as the co-factors. The effects of co-factor addition on each enzyme system from tobacco, chick and hamster were examined.

The results are shown in Table 2. In the case of tobacco cell eyzyme, there was only a small decrease in activity when TPP was excluded. The lack of FAD or Mg^{++} produced little decrease. On the other hand, in both animal enzyme systems, TPP was found to be essential, but a lack of FAD or Mg^{++} had almost no effect. In the hamster enzyme, higher activity was found when FAD was omitted.

TABLE 2. Co-factor requirement of acetolactate synthase of tobacco and hamster kidney cell cultures and chick liver.

Component	Acetoin n mol/hr/mg protein		
	Tobacco cells	Hamster cells	Chick liver
Complete	2,894 (100)*	592 (100)	1,208 (100)
-TPP	1,925 (67)	290 (49)	351 (29)
-FAD	2,637 (91)	516 (87)	1,527 (126)
$-Mg^{++}$	2,567 (89)	742 (125)	1,225 (101)
-TPP, FAD	1,936 (67)	167 (28)	355 (29)
-TPP, Mg^{++}	1,893 (65)	209 (35)	389 (32)
-FAD, Mg^{++}	2,289 (79)	845 (142)	1,425 (118)
-TPP, FAD, Mg^{++}	1,976 (68)	22 (4)	683 (57)

- : Omitted from complete reaction mixture.

* Percent of complete mixtures.

From these results, it was concluded that the tobacco enzyme was able to synthesize acetolactate from pyruvate without TPP, but the enzyme from animal sources require TPP as a co-factor, a feature of a typical allosteric enzyme.

Inhibitory properties of chlorsulfuron on acetolactate synthase from tobacco cell culture

As mentioned above, tobacco acetolactate synthase was detected without TPP addition. The Hanes-Woolf plot (Fig. 8) shows that under these experimental conditions, the inhibition was noncompetitive. With some lower concentration of chlorsulfuron, uncompetitive inhibition was observed. These phenomena should be investigated in more detail using purified enzyme preparations.

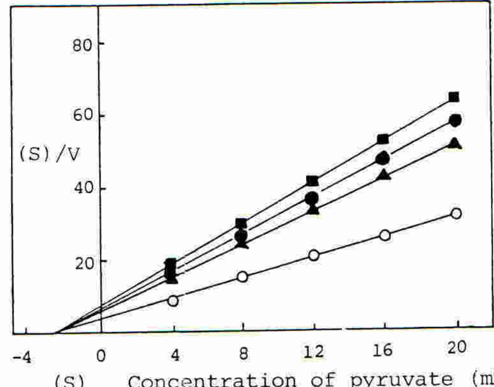

Fig. 8. Inhibitory properties of chlorsulfuron to acetolactate synthase from tobacco cell cultures (the Hanes-Woolf plot). Concentrations of chlorsulfuron were 0 (○), 4 ppb (▲), 6 ppb (●), and 8 ppb (■). TPP was omitted from the complete reaction mixture. (S)/V : Pyruvate concentration (mM)/ produced acetoin (µ mole)

DISCUSSION

In the simple biological system of cell culture, chlorsulfuron showed a severe inhibition in growth of plant cells (tobacco) but no effect on animal cells (hamster). The biochemical background for such difference between plant and animal cells was probably due to the fact that acetolactate synthase from tobacco was inhibited by chlorsulfuron at an extremely low concentration, but the herbicide had no effect on the enzyme from animal cells.

Although we could hardly detect acetolactate in the reaction products because of its instability, these experiments showed that both plant and animal enzyme systems showed its formation by identification as acetoin after decarboxylation by H_2SO_4. Without enzyme reaction or H_2SO_4 treatment, no acetoin was detected. These facts show that even in animal in which branched amino acids are essential there is acetolactate synthase having tolerance to chlorsulfuron.

Acetolactate synthases from tobacco and hamster kidney cell cultures or chick liver showed that there were differences in not only chlorsulfuron susceptibility but also pH dependence and co-factor requirement.

In Salmonella typhimurium and Escherichia coli, there were found to be two acetolactate synthases. One of them, ALS I, was susceptible to chlorsulfuron but others, ALS II in S. typhimurium and ALS III in E. coli, were

resistant to the herbicide (LaRossa *et al*. 1984). The latters should be compared with the animal enzymes.

The inhibitory properties of chlorsulfuron to acetolactate synthase from tobacco cell cultures were found to be noncompetitive. This suggests that the inhibitor binds to a point other than the active site. As mentioned above, such relationships should be clarified by using more purified enzyme prepartions. The inhibitory properties will give some suggestions concerning the design of new inhibitors or herbicides having the same mode of action.

ACKNOWLEDGEMENTS

The authors express their deep thanks to Du Pont Japan Co. Ltd., Dr. Manabu Takehara, Medical School of Kobe University and Dr. Chiharu Nakamura, Faculty of Agriculture of Kobe University for supplies of chlorsulfuron, hamster kidney cell culture and tobacco cell culture, respectively.

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MODE OF ACTION OF THE IMIDAZOLINONES

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ABSTRACT

The imidazolinones are a new class of herbicides being developed by American Cyanamid. Symptoms of treated plants appear first in the meristematic regions, and then spread to the more mature parts of the plant. Measurements of various physiolgical processes in maize after treatment with imazapyr revealed that DNA synthesis was inhibited within 8 hours. There was also a decrease in the levels of soluble proteins and an increase in the level of free amino acids. When the levels of the individual amino acids were measured in maize leaves, most of the amino acid levels increased. However, two amino acids, valine and leucine, decreased. When maize seedlings were supplied exogenously with valine, leucine, and isoleucine, the deleterious effects of imazapyr on both growth and DNA synthesis were prevented. The imidazolinones were found to be potent inhibitors of acetohydroxyacid synthase (acetolactate synthase), the first enzyme in the biosynthetic pathway for valine, leucine, and isoleucine. It is proposed that the mechanism of action of the imidazolinones is the inhibition of acetohydroxyacid synthase which prevents the plant from synthesizing valine, leucine, and isoleucine.

INTRODUCTION

The imidazolinones are a new class of herbicidal chemicals discovered and being developed by American Cyanamid Company, Princeton, NJ. These herbicides kill monocotyledonous and dicotyledonous annuals and perennials. The symptoms first appear in the meristematic tissue where growth ceases soon after treatment, followed by chlorosis and then necrosis of the tissue. Die back spreads to the more mature parts of the plant. Death of the entire plant can take 3 to 4 weeks after treatment. In order to understand the unique herbicidal activity of the imidazolinones, studies were conducted on maize, a very susceptible species, to determine their mode of action.

MATERIALS AND METHODS

Plant material

For the physiological studies, maize seeds were planted in sand and watered with 0.2 mM $CaCl_2$ and grown in the dark at 28°C for 3 days. Plants were treated by drenching the sand with solutions containing an imidazolinone.

For measurements of the amino acid levels, maize was grown in a greenhouse (28 $\pm$ 5°C) under a photoperiod extended to 16 h by high intensity sodium lamps. Plants were grown for 8 to 14 days before treatment.

Plant growth measurements

The length of the youngest expanding leaf was measured periodically to the nearest mm. Each treament was replicated 3 times with 3 plants per replicate.

Respiration

Respiration of corn root tips was measured with a differential respirometer using the technique of Umbreit et al (1972).

Protein, lipid, RNA, and DNA synthesis

Measurements of the effects of the imidazolinones on protein, lipid, RNA and DNA synthesis was done by determining the incorporation of ^{14}C-labelled precursors into the appropriate metabolic product using the procedures of Gruenhagen and Moreland (1977) and Rost and Bayer (1976). All measurements were done on 2 cm root tips excised from maize roots grown in sand as described above.

Soluble protein and amino acids

The level of the soluble proteins in root tips was determined by extracting 100 mg of tissue in 6 ml of 10 mM phosphate buffer(pH 6). After centrifugation, the level of the protein in the supernatant was determined by the method of Bradford (1976). The level of the amino acids was determined by extracting the tissue by the method of Bieleski and Turner (1966) and determining the amount of total free amino acids in the extract by the method of Yemm and Cocking (1955). The levels of individual amino acids was determined by separating the amino acids on an ion exchange column by HPLC and detecting the amino acids by mixing the eluant with an o-phthaladehyde fluorescence reagent .

Acetohydroxyacid synthase

Acetohydroxyacid synthase activity from maize tissue was extracted and measured by the method of Miflin (1971).

Chemicals

Two imidazolinones were used, imazapyr (2- [4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl] nicotinic acid) and imazaquin (2- [4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl] -3-quinolinecarboxylic acid). These chemicals were of analytical grade and synthesized at the American Cyanamid Research Center, Princeton, NJ, USA.

RESULTS

Elongation of a rapidly growing maize leaf began to slow down within 3 h after application of imazaquin and essentially ceased by 8 h after treament (Figure 1). A similar phenomenon has been observed with maize roots.

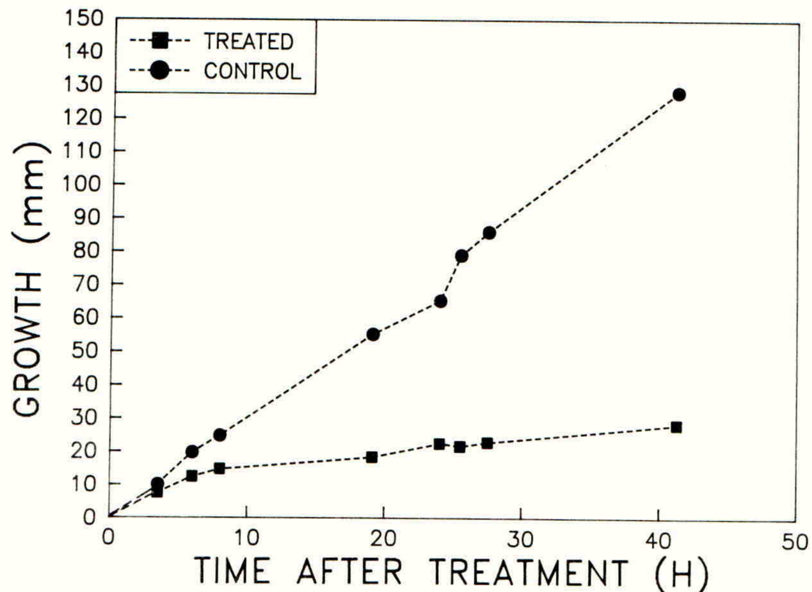

Fig 1. Effect of imazapyr on leaf elongation in maize.

Measurements of various physiological processes 24 h after treatment with imazapyr showed that there was no effect on the rate of lipid synthesis or protein synthesis (Figure 2). There was a 30% decrease in the rate of respiration, and a 15% decrease in the rate of RNA synthesis (Figure 2). The process most severely affected was DNA synthesis, which decreased 63% (Figure 2). A time course experiment showed that the inhibition of DNA synthesis did not begin until 5 to 7 hours after treatment (data not shown). This long time period suggested that inhibition of DNA synthesis was not the primary site of action of imazapyr.

The pool sizes of soluble proteins and free amino acid in maize root tips were also affected by imazapyr. There was a 37% decrease in the level of total soluble proteins and a 31% increase in the free amino acid levels (Table 1). Although there had been no apparent effect of imazapyr on the rate of protein synthesis in maize root tips, these changes in the levels of soluble proteins and free amino acids suggested that imazapyr was having some effect on protein synthesis.

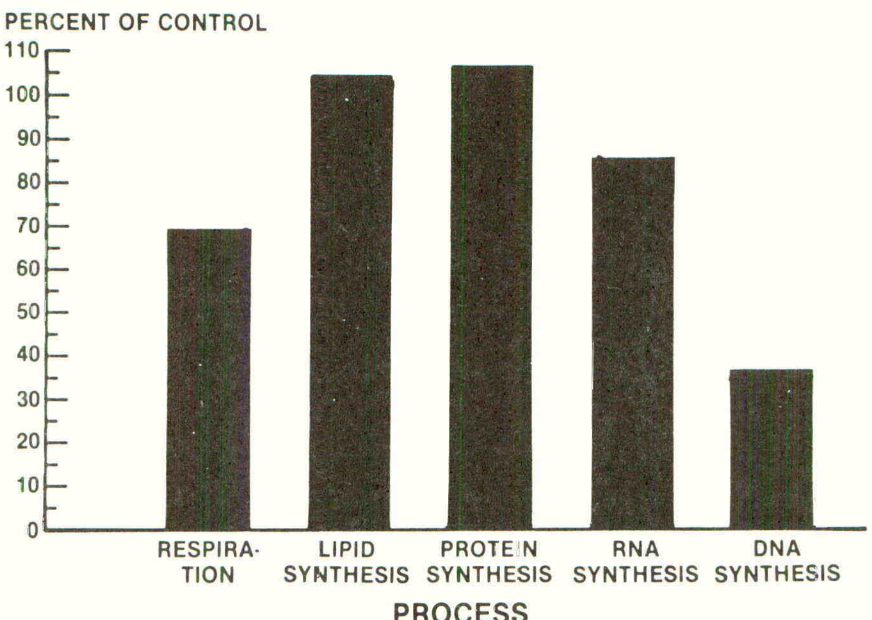

Fig. 2. Effect of imazapyr on physiological processes in excised maize root tips-24 hours after treatment.

TABLE 1

Effect of imazapyr on soluble protein and free amino acid levels in 3-day-old maize root tips.

Treatment	Protein (ug/gFW)	Amino Acid (mM Leu equivalent/gFW)
Control	640	126
Imazapyr (150 uM)a	405b	166b

a Seedlings were treated with 150 uM imazapyr for 24 hours prior to these measurements on 2cm excised root tips. b Significantly different from the control at P=0.05

Maize leaves treated with imazapyr also showed an increase in total free amino acid levels with time after treatment. Close examination of the effect of imazapyr on the individual amino acids revealed large increases in the levels of threonine, serine, glycine and alanine, but the levels of valine and leucine decreased dramatically (Table 2).

TABLE 2 Effect of imazapyr on amino acid levels in maize leaves 53 hours after treatment.

Amino Acid	A Control	B Imazapyr	B/A
	uM/gFW		
Aspartate	1.8	4.7	2.6
Threonine+Serine	3.2	29.1	9.1
Glutamate	5.6	13.5	2.4
Glycine	1.0	19.8	19.8
Alanine	9.7	42.3	4.4
Valine	1.1	0.4	0.4
Isoleucine	0.2	0.9	4.5
Leucine	0.2	nd	0.0
Tryrosine	0.5	1.8	3.6
Phenylalanine	0.1	0.7	7.0
Histidine	0.2	1.0	5.0
Lysine	0.3	0.6	2.0
Arginine	0.9	1.2	1.3

These two amino acids, plus isoleucine are synthesized via the same biosynthetic pathway. The decrease in the levels of valine and leucine may indicate that imazapyr is interfering with the synthesis of these amino acids. When maize seedlings were exogenously supplied with valine, leucine, and isoleucine, the inhibitory effects of imazapyr on growth and DNA synthesis could be largely prevented (Figure 3). In fact, the inhibitory effects of imazapyr on DNA synthesis could be reversed if the amino acids were supplied to the plants 24 hours after exposure to imazapyr (data not shown). Further work showed that all three amino acids were necessary for maximum protection. Similar results have also been found by Anderson and Hibberd (1985) in maize suspension culture.

The first enzyme in the branched chain amino acid pathway is acetohydroxyacid synthase. It was found that the imidazolinones are potent inhibitors of this enzyme (Figure 4).

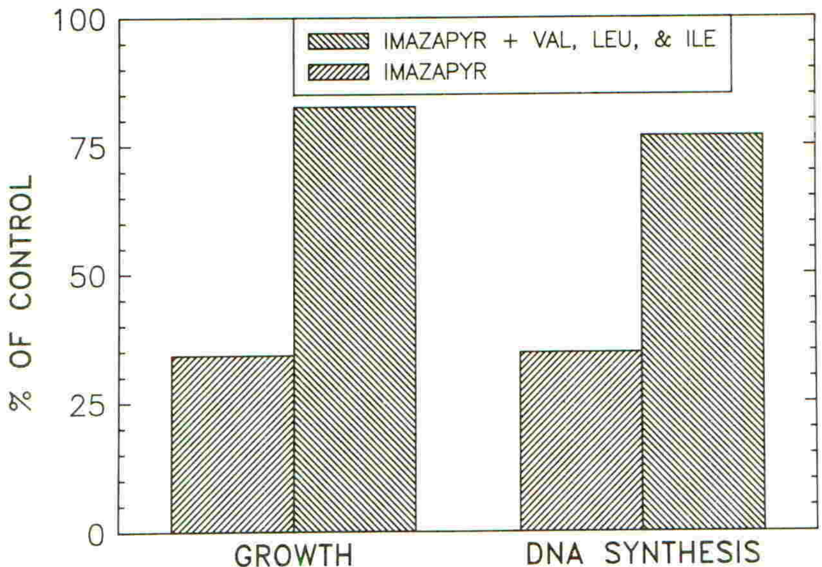

Fig. 3. Effect of exogenously supplying valine, leucine and isoleucine on the inhibitory action of imazapyr on growth and DNA synthesis. In the growth experiment plants were watered daily with 15 uM imazapyr plus or minus 1 mM each of the amino acids. After 14 days shoots were harvested and dry weights determined. In the DNA synthesis experiment 3-day-old maize seedlings were watered with 15 uM imazapyr plus or minus the amino acids. DNA synthesis was determined in 2 cm excised maize root tips 24 hours after treatment.

DISCUSSION

Based on these results the primary mode of action of the imidazolinones appears to be the inhbition of the synthesis of valine, leucine and isoleucine due to the inhibition of acetohydroxyacid synthase. Further support for this site of action is the recent isolation of a maize cell culture line which is resistant to the imidazolinones (Anderson et al, 1984). This resistant cell line contains an altered acetohyroxyacid synthase which is no longer inhibited by the imidazolinones. Resistance is expressed at the whole plant level and appears to be inherited as a single, co-dominant trait (Anderson et al, 1984). Acetohydroxyacid synthase is also the site of action of the sulfonylureas (Chaleff and Mauvis, 1985), another new class

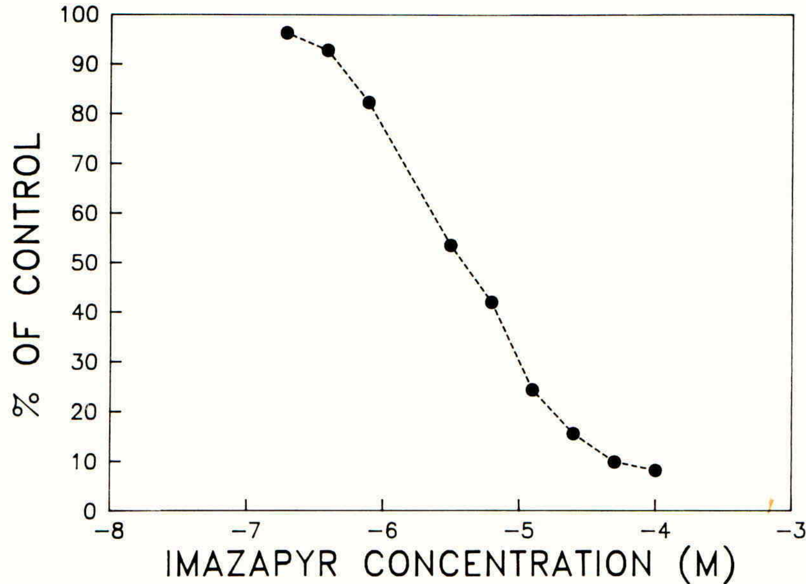

Fig. 4. Effect of imazapyr on in vitro activity of acetohydroxyadcid synthase activity extracted from 3-day-old maize roots.

of potent herbicides. Since both the imidazolinones and the sulfonyl ureas are potent, broad spectrum herbicides , acetohydroxyacid synthase must be an extremely important enzyme for proper plant function.

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THE METABOLIC ACTIVITY OF FLUAZIFOP ACID IN EXCISED APICAL MERISTEM SECTIONS

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ABSTRACT

The effect of fluazifop acid on macromolecular synthesis in excised apical meristem sections from Setaria viridis and Zea mays was monitored by incorporation of ^{14}C-precursors. DNA, RNA and protein synthesis were not inhibited by fluazifop acid, as determined by ^{14}C-thymidine, uridine and leucine incorporation over a 2 h incubation period. 0.1 μM fluazifop acid was inhibitory to ^{14}C-acetate incorporation into lipid in S.viridis apical sections and to ^{14}C-MVA incorporation in Z.mays, whereas ^{14}C-MVA incorporation in S.viridis and ^{14}C-acetate incorporation in Z.mays were insensitive to fluazifop acid concentrations up to 100 μM. Chromatographic analysis of the lipid components of Z.mays apical meristems treated with 1.0 μM fluazifop acid, revealed a reduced proportion of polar lipids and an increase in non-polar lipid fractions. A similar, but less pronounced effect was apparent with lipids extracted from Z.mays apical sections incubated with ^{14}C-MVA. It is concluded that lipid metabolism, possibly fatty acid and/or phospholipid synthesis, is a possible primary target for fluazifop acid. However, caution is needed in the interpretation of data from excised apical meristems and this is discussed in relation to other literature and experimental systems.

INTRODUCTION

Fluazifop-butyl (butyl 2-[4(5-trifluoromethyl-2-pyridyloxy)phenoxy] propionate) is a selective postemergence herbicide for the control of both annual and perennial grass weeds in broadleaf crops (Finney & Sutton 1980, Gibbard et al. 1982). In the susceptible annual grass Setaria viridis some necrotic lesions are apparent on older foliage, with abnormal morphological development in addition to chlorotic damage to younger tissue, 7 to 10 days after foliar application (Carr 1985). However, death of this species is not due to these foliar symptoms, but to necrosis of the apical meristematic region within 7 to 10 days of treatment (Carr 1985). Studies with ^{14}C-radiolabelled fluazifop-butyl have revealed a rapid translocation of the herbicide to the meristem (Carr 1985, Carr et al 1986). Metabolism studies have revealed that fluazifop-butyl, as with other phenoxy-propanoate herbicides, requires de-esterification in the foliage prior to translocation. The free acid, fluazifop, or a conjugate of it, is the mobile form of the herbicide and is the active form responsible for the primary mode of action of fluazifop-butyl in meristematic tissues (Carr et al. 1986, Hendley et al. 1985).

This paper presents initial experiments into the metabolic activity of fluazifop acid with excised S.viridis and Zea mays apical meristems. Sections of tissue containing the apical meristem were excised from young plants and incubated with ^{14}C-precursors of macromolecular synthesis in the presence or absence of fluazifop acid to establish the primary metabolic action of this major herbicide.

MATERIALS AND METHODS

Plant material

Seeds of S.viridis and Z.mays cv Aztec were sown in seed trays (355 x 215 mm) in J. Arthur Bowers potting compost and grown in a glasshouse at 26°C day and 21°C night temperatures with a 14 h photoperiod. Sections of tissue containing the apical meristem were obtained from 3-4 leaf S.viridis and 3 leaf Z.mays plants. In the former the 12 mm of shoot tissue immediately above the hypocotyl was excised. In Z.mays the 8 mm of tissue immediately above the basal node of the shoot was excised after the outer green tissue had been removed. Leaf sheath removal could not be carried out with S.viridis due to the small and delicate nature of the shoot tissue.

Nucleic acid and protein metabolism

After excision, S.viridis apical sections were washed in buffer containing 1 mM tricine NaOH pH 6.5, 1% aq (wt/vol) sucrose and 0.001% aq (wt/vol) chloramphenicol. After washing, 90 sections were incubated for 2 h at 25°C in the dark with the above buffer containing 0, 0.1, 1.0, 10 or 100 µM fluazifop acid and 0.25 µCi/ml ^{14}C-thymidine (497 mCi/mmol, 184 G Bq/mMol) or 0.50 µCi/ml ^{14}C-uridine (529 mCi/mmol, 19.6 G Bq/mMol) or 0.25 µCi/ml ^{14}C-leucine (348 mCi/mmol, 12.9 G Bq/mMol). After this period the incubation medium was removed by filtration and the sections washed for 5 min with 25 ml buffer containing unlabelled precursor (1 mM thymidine, 1 mM uridine or 1 mM leucine) at 4°C. This washing medium was removed by filtration and the sections sub-divided into 6 replicates of fifteen, weighed and stored overnight in 2 ml 20% aq (wt/vol) TCA at -20°C. Each thawed replicate was homogenised using an Ultra-Turrax homogeniser for two 20 s periods. The precipitate was collected on a Whatman GF/C filter and washed with 5 ml 10% aq (wt/vol) ice cold TCA and 1 ml ice cold 80% aq (vol/vol) ethanol. Filtrates were collected and a 100 µl aliquot taken for scintillation counting. Filters were dried at 35°C and decolorised overnight in a scintillation vial with 100 µl H_2O_2 (30 vols) prior to scintillation counting. ^{14}C-activity in the filter corresponded to precursor incorporated into nucleic acid or protein, whereas ^{14}C-activity in both filter and filtrate corresponded to precursor uptake by the apical sections.

Lipid metabolism

Apical sections from S.viridis and Z.mays were used to study the effect of fluazifop acid on lipid metabolism. Incubation conditions were as above except that 1.0 µCi/ml ^{14}C-acetate Na salt (58 mCi/mmol, 2.14 G Bq/mmol) and 1.0 µCi/ml ^{14}C-mevalonic acid (MVA) lactone (51.4 mCi/mmol, 1.9 G Bq/mmol) were the precursors. After incubation apical sections were washed in cold precursor and sub-divided into 6 replicates of fifteen (S.viridis) or 5 replicates of five sections (Z.mays) prior to storage overnight in 2 ml 1 mM tricine-NaOH pH 6.5 at -20°C. Each thawed replicate was homogenised as above. The precipitate was collected on a Whatman GF/C filter and washed with 2 ml tricine buffer. These aqueous filtrates corresponded to non-lipid products derived from ^{14}C-acetate or ^{14}C-MVA and were discarded. The filter was then washed with 2 ml chloroform: methanol (2:1) and the filtrate was collected in a vial, evaporated to dryness and decolorised overnight with 100 µl H_2O_2 (if necessary) prior to scintillation counting. The filters were dried at 35°C prior to scintillation counting. ^{14}C-activity in the chloroform:methanol wash corresponded to incorporated precursor and ^{14}C-activity in both this organic wash and in the filter, was designated precursor uptake. For both filtrate aliquots and dried filters 5 ml of Fisofluor "2" scintillant (Fisons Ltd) was added prior to counting in an LKB Wallac Scintillation Counter, Model 81000.

Thin layer chromatography of lipid extracts

Fifty apical sections (approx 1 g) were excised from Z.mays and incubated with 10 ml buffer in the presence and absence of 1 μM fluazifop acid with either 1 μCi/ml ^{14}C-acetate or ^{14}C-MVA lactone for 2 h. After incubation the sections were ground to a fine powder in liquid nitrogen in a mortar, 15 ml methanol:chloroform (2:1) added and homogenised for 60 s with an Ultra-Turrax homogeniser and filtered. The residue was re-homogenised with 5 ml methanol-chloroform and 1.5 ml distilled water and filtered, and final residues washed by filtration with a further 5 ml methanol:chloroform. Combined filtrates were added to a separating funnel with 3 ml chloroform and 6 ml distilled water and thoroughly mixed. The chloroform layer was collected and cleared by centrifugation prior to concentration in a stream of N_2. Aliquots were applied to Kieselgel 60 (MERCK ART. 5553) 0.2 mm thick aluminium-backed tlc plates and developed in petroleum ether (b.p. 60-70°C):diethyl ether:acetic acid (90:10:1) until the solvent front had migrated 10 cm. After development, a 12 mm wide lane, within which the sample had migrated, was excised from the plate and sectioned into twenty one 0.5 cm portions. Each portion was added to 5 ml Fisofluor "2" scintillant for counting. Rf values for unlabelled standards, developed in the same system, were visualised using 0.012% (wt/vol) Rhodamine 6G.

RESULTS

Apical sections used in this study were metabolically competent for at least 4 h after excision, giving linear rates of ^{14}C-precursor incorporation throughout this period (Carr 1985). ^{14}C-thymidine and ^{14}C-leucine incorporation were inhibited by 25% with 100 μM fluazifop acid after 2 h incubation (Fig. 1A). However, concentrations which are likely to correspond more closely to a lethal concentration within apical meristems of sprayed S.viridis plants (ie 0.1-1.0 μM) caused much less inhibition. ^{14}C-uridine incorporation into RNA was insensitive to as high as 100 μM fluazifop acid and 10 μM caused a marginal stimulation (Fig. 1A). Lipid metabolism was more sensitive to inhibition in apical sections excised from S.viridis. All concentrations of fluazifop-acid caused 29-36% inhibition of ^{14}C-acetate incorporation, whereas no inhibition of ^{14}C-MVA incorporation was observed (Fig. 1B). However, the lower concentrations stimulated ^{14}C-MVA incorporation. Conversely, in Zea mays, all concentrations of fluazifop acid were inhibitory to ^{14}C-MVA incorporation but stimulated ^{14}C-acetate incorporation.

The rates of incorporation of ^{14}C-acetate and ^{14}C-MVA by both species are compared in Table 1. The rate of ^{14}C-acetate incorporation was similar in both apical systems and substantially greater than ^{14}C-MVA incorporation. However, there was eight times more ^{14}C-MVA incorporated by Z.mays.

TABLE 1

Rate of incorporation of ^{14}C-acetate and ^{14}C-MVA in apical sections

	pmoles precursor incorporated/min/g F. wt	
	^{14}C-acetate	^{14}C-MVA
S.viridis	10.95	0.42
Z.mays	14.69	3.55

Rates are calculated from 2 h incubation data for control treatments of Figs. 1 and 2.

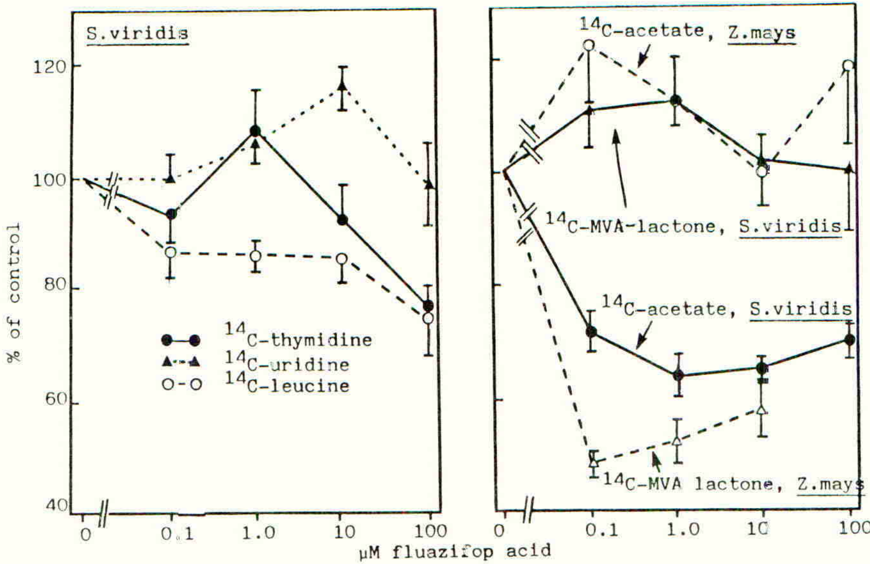

Fig. 1. Effect of fluazifop acid on the incorporation of ^{14}C-labelled precursors into macromolecular and lipid synthesis in excised apical sections. Data is expressed as % of control after 2 h incubation and bars represent S.E.'s from two to four experiments each containing six replicates. Uptake of precursor as % of control for 100 µM fluazifop acid was 84.8 ± 2.7% S.E. (^{14}C-thymidine), 91.8 ± 4.3% (^{14}C-uridine), 86.9 ± 5.6% (^{14}C-leucine), 87.4 ± 2.7% (^{14}C-acetate, S.viridis), 113.2 ± 6.9% (^{14}C-acetate, Z.mays), 125.4 ± 4.0% (^{14}C-MVA lactone, S.viridis) and 109.3 ± 14.3% (^{14}C-MVA lactone, Z.mays - 10 µM).

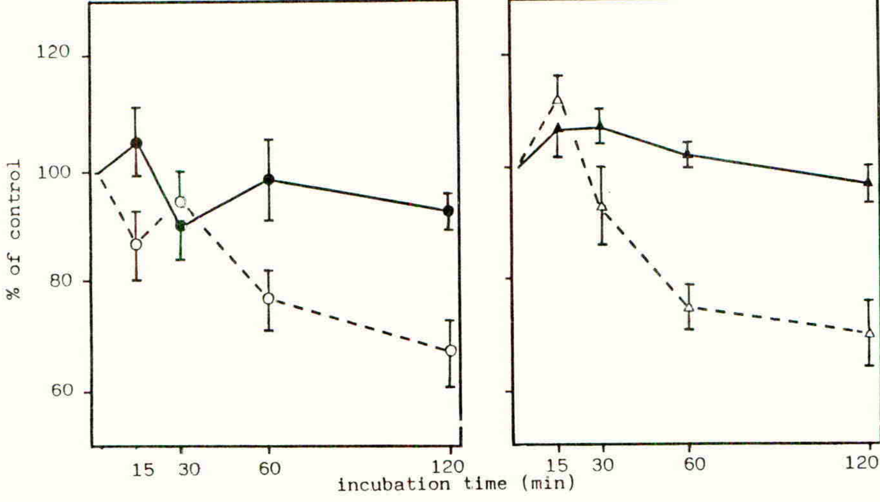

Fig. 2. A time-course of uptake (closed symbols) and incorporation (open symbols) of ^{14}C-acetate (circles) and ^{14}C-MVA lactone (triangles) with 1 µM fluazifop acid. Data are expressed as % of control and are a mean of two experiments each containing six replicates. Bars represent S.E.'s.

Figure 2 shows data from time-course experiments of ^{14}C-acetate uptake and incorporation with S.viridis apical sections and ^{14}C-MVA uptake and incorporation with Z.mays apical sections. These were carried out in the presence or absence of 1 μM fluazifop acid to ascertain whether the inhibition of incorporation was simply due to the reduced uptake of this precursor. Uptake of ^{14}C-acetate was reduced to 90% of the control value after 120 min incubation, whereas incorporation was reduced to 65% of control values after the same period. Incorporation of ^{14}C-MVA with Z.mays apical sections was stimulated after 15 min incubation but was inhibited by 7, 25 and 30% after 30, 50 and 120 min respectively. No inhibition of uptake of this precursor occurred throughout the period of study (Fig. 2).

In order to obtain an indication of the class of lipids affected by fluazifop acid, lipid extracts of Z.mays apical sections, incubated with the two lipid precursors in the presence or absence of 1 μM acid, were separated by tlc and analysed by scintillation counting. Figure 3A reveals that although 1 μM fluazifop acid did not reduce the total incorporation of ^{14}C-acetate it did alter the lipid composition. The proportions of ^{14}C-labelled polar lipids remaining near the origin (Rf 0-0.1) were reduced as a result of fluazifop acid incubation whereas non-polar hydrocarbons, which migrated near the solvent (Rf 0.9-1.0) were increased from c. 12 to 30% of total ^{14}C-activity recovered. The composition of lipid extract from sections incubated with ^{14}C-MVA was also altered by 1 μM fluazifop acid incubation. The majority of ^{14}C-labelled lipids remained at or near the origin in both control and treated meristem sections. However, an additional compound of Rf 0.8 was obtained after fluazifop acid treatment. Additionally, there appeared to be twice the ^{14}C-activity at the solvent front with treated sections (Fig. 3B).

DISCUSSION

In these studies a 2 h incubation time was chosen as excised apical sections were metabolically competent up to and beyond this period. Additionally, any inhibition by fluazifop acid observed within this time could be considered a primary effect and thus avoid monitoring secondary effects likely to occur with longer incubation times. In such experiments with excised tissues it is also considered important not only to monitor the incorporation of a precursor but also its uptake into the tissue. This is illustrated by data on ^{14}C-thymidine and ^{14}C-leucine where an inhibition of incorporation occurred with the higher concentrations of fluazifop acid (Fig. 1A). Similar inhibitions of uptake of these two precursors occurred, and it may be concluded that the observed reduction in DNA and protein synthesis was as a consequence of reduced uptake rather than a direct effect on synthesis. Thus, it seems likely that fluazifop acid has no primary effect on protein and nucleic acid synthesis and this is in agreement with data obtained using Z.mays coleoptiles (Peregoy and Glenn, 1984).

Diclofop-methyl and other phenoxy-propanoate herbicides induce similar symptoms to those of fluazifop-butyl in susceptible grass species (Hoerauf and Shimabukuro 1979, Hoppe 1981, Kocher et al. 1982, Kocher et al. 1984) and it is possible that these herbicides may have a common primary site of action owing to their structural similarities. Hoppe (1980, 1981) has shown that diclofop has little or no effect on the incorporation of radioactive precursors into protein and nucleic acids. However, incorporation of ^{14}C-acetate into lipids of Z.mays root tips was inhibited by 5 μM diclofop 12 h after pre-treatment of seedlings (Hoppe and Zacher 1982).

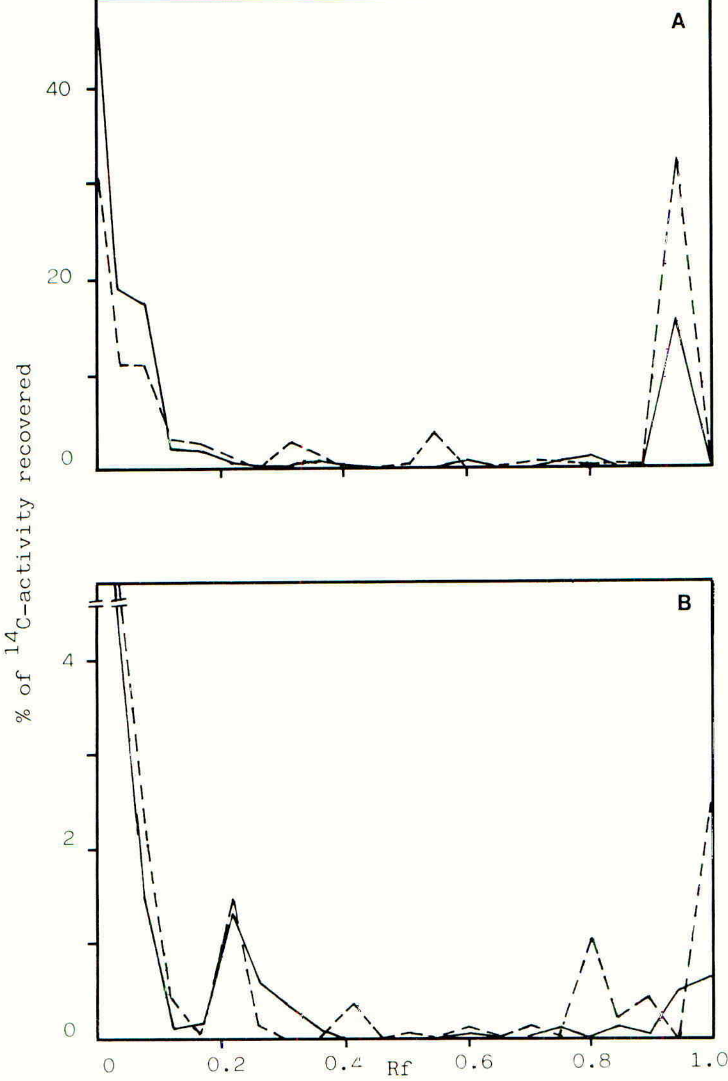

Fig. 3. The composition of ^{14}C-lipid extracts from Z.mays meristem sections incubated in the presence (broken line) or absence (solid line) of 1 μM fluazifop acid as separated by tlc. Sections were incubated with ^{14}C-acetate (A) or ^{14}C-MVA lactone (B) as described in the Methods.

^{14}C-acetate incorporation was inhibited in apical sections excised from S.viridis and as no significant reduction of the uptake of this precursor occurred (Fig. 1B), this reduced incorporation could be a primary target of fluazifop acid (Fig. 2). Acetate is a precursor of all lipid components whereas MVA, which is derived from acetate, is a precursor of the isoprenoid class of lipids, such as sterols, phytol and carotenoids. In S.viridis apical sections ^{14}C-MVA incorporation was insensitive to fluazifop acid (Fig. 1B) indicating that the synthesis of long-chain fatty acids, triglycerides and membrane lipids (ie phospholipids) may be a target of

fluazifop acid (Fig. 4, site (1)). This is in agreement with data of Hoppe and Zacher (1982) who showed a reduced phospholipid synthesis with diclofop in *Z.mays* root tips which could be partially overcome by the addition of fatty acids such as oleate.

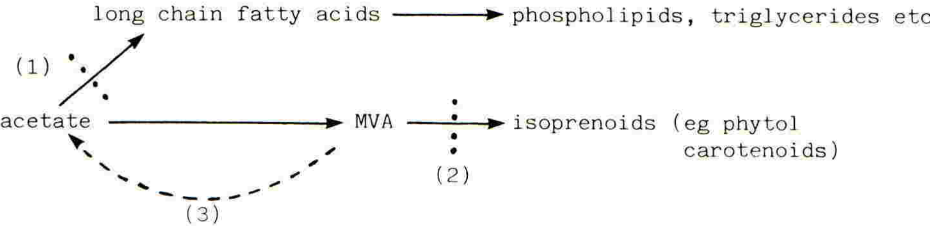

Fig. 4. A summary of lipid metabolism in excised apical sections suggesting possible sites of fluazifop acid inhibition.

In *Z.mays* apical sections, ^{14}C-acetate incorporation was insensitive to fluazifop acid, whereas ^{14}C-MVA incorporation was inhibited by fluazifop acid (Fig. 1B) indicating that isoprenoid synthesis was reduced (Fig. 4, site (2)). The composition of the lipid fractions with both precursors was altered by 1 µM fluazifop acid particularly those derived from ^{14}C-acetate. The proportions of polar lipids eg fatty acids, phospholipids were reduced whereas the non-polar lipids eg carotenoids, phytol, were increased (Fig. 3). Since no reduction of total ^{14}C-acetate incorporation occurred in *Z.mays*, the apparent increase in MVA products could be explained by inhibited fatty acid and phospholipid synthesis (Fig. 4, site (1)) with an increased channelling of ^{14}C-acetate through the MVA pathway. However, the inhibition of ^{14}C-MVA incorporation contradicts this explanation. The tlc data (Fig. 3B) reveals that the majority of ^{14}C-MVA was incorporated into polar lipid and that there was an increase in the proportion of non -polar lipids extracted from 1 µM fluazifop treated apical sections. Therefore, ^{14}C-MVA may not be incorporated via the MVA pathway into hydrocarbons, such as carotenoids and phytol, but into more polar lipids in *Z.mays* apical sections (route (3), Fig. 4) and it is this latter process that may be affected by fluazifop acid at site (1) (Fig. 4). This formation of polar lipid from ^{14}C-MVA may not occur in *S.viridis* meristem sections as there was 8 x more ^{14}C-MVA incorporated in untreated *Z.mays* compared to *S.viridis* (Table 1).

These data indicate that caution is necessary in the interpretation of results from these and other excised meristem experiments. The meristem system used must first be fully characterised and extrapolation avoided between apical and root tip meristems and between apical meristems from different species. In addition to factors such as metabolic competence and morphological development, parameters such as uptake and availability of precursors, compartmentalisation of herbicide and precursor within the tissue also needs to be considered.

In conclusion, this work has revealed that lipid synthesis is probably a primary target of fluazifop acid, possibly fatty acid and/or phospholipid synthesis. However, further detailed work is necessary to confirm these observations and pinpoint the precise point of action.

ACKNOWLEDGEMENTS

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THE SITE OF ACTION OF QUIZALOFOP-ETHYL, NCI-96683

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ABSTRACT

Quizalofop-ethyl* (NCI-96683) at 0.1 kg ha^{-1} a.i. gave excellent control of established Echinochloa crus-galli in pots and under field conditions. After the application of ^{14}C-Quizalofop-ethyl to leaf surfaces, good translocation was shown by the accumulation of radio active material in the meristematic tissue at the stem base. The rapid swelling of cells in the meristem just below the shoot apex were observed first, followed by the severe destruction of young cells including those in intercalary meristems. The shoot apical meristem, however, remained relatively undamaged despite the complete destruction of the surrounding meristematic tissue. Rapid suppression of growth and reduction in viability of Sorghum halepense rhizome was observed after application of Quizalfop-ethyl. The inhibition of plant protein synthesis, lipid synthesis and RNA synthesis was observed at 10^{-4}M, 10^{-5}M and 10^{-4}M herbicide concentration respectively. In addition, the leaking of electrolytes from the cells of Avena sativa coleoptile segments was observed at 10^{-5}M a short period after herbicide application.

INTRODUCTION

Quizalofop-ethyl, Ethyl 2-[4-(6-chloro-2-quinoxalinyloxy)phenoxy] propionate, (NCI-96683) is a new post emergence grasskilling herbicide which controls a wide range of annual and perennial graminaceous weeds (Sakata et al, 1983). It is being developed by Nissan Chemical Industries, Ltd.

The objective of this report is to review the present understanding of the mode of action or the action target of this herbicide against graminaceous weeds.

Fig.

Chemical structure of Quizalofop-ethyl

Cl, N, N, O, CH_3, $OCHCOOC_2H_5$

*^{14}C-labeled uniformly in chlorophenyl ring

METHOD AND MATERIALS

Growth and treatment of plant materials

The plant species used in the present study were Echinochloa crus-galli, Sorghum halepense, Sorghum bicolor and Avena sativa. Field trials to observe herbicidal efficacy were carried out on the experimental plots of Nissan Chemical Ind., Saitama, Japan. The trials involved the foliar application of Quizalofop-ethyl to Echinochloa crus-galli and to Sorghum halepense 70-80 cm in height at the leaf stage of 4.5-5.0, which had been grown from rhizomes planted 2 months before. Plants used for the growth suppression studies and for the microscopic observation study were grown in 10 cm pots in greenhouse conditions. Those used for the studies of biochemical and physiological effects were grown for 72 hrs in total darkness at 27°C.

Penetration and translocation study

Foliar penetration and the distribution of the translocated active ingredient with Echinochloa crus-galli was studied by applying 3 µl of 3,000 ppm ^{14}C-labeled Quizalofop-ethyl acetone solution, to the surface of the 4th leaf at the 5th leaf stage. At various time intervals the plants were divided into four parts. Radio active material which could not be removed from the treated leaf by washing with 80 % aqueous methanol was considered to have penetrated.

Microscopic study

The microscopic study was carried out using the 4th leaf stage of Echinochloa crus-galli. Quizalofop-ethyl was applied post-emergence at a rate of 0.1 kg ha^{-1} a.i. Plant tissue sections including the apical meristem of shoot of treated plants were obtained at 1, 2, 4 and 6 days after treatment. Sections from the treated plant were embedded on glass plates after fixing by FAA fluid or Carnovsky fixing. The tissue sections were then stained with toluidine blue or hematoxyline.

Systemic action of the herbicide

To investigate the characteristics of Quizalofop-ethyl as a systemic herbicide, the damage to underground rhizome systems of Sorghum halepense was observed by digging out the root system of this plant after herbicide application in the field. In the greenhouse, the growth suppression of rhizome was observed using Sorghum halepense at the 3-3.4 leaf stage. After the foliar application of Quizalofop-ethyl at 0.1 kg ha^{-1} a.i., the rhizomes from the treated plants were weighed and their viability estimated by measuring α-naphthylamine oxidative potential. This was performed after the reciprocal shaking of 20 ppm α-naphthylamine solution with segments of rhizome for 3 hrs. This was followed by the addition of 1 % sulfanilate and 100 ppm NaNO2 solutions, and the optical absorbance of the sample solutions was measured at 510 nm.

Biochemical and physiological studies

1 cm length sections from the coleoptiles of etiolated Avena seedlings were used in the following studies. The leakage of electrolytes from coleoptile cells was carried out by incubating 0.4 g of Avena sections in the herbicide solution containing sucrose and Tween 80. The conductivity of the sample solutions was measured to establish electrolyte leakage. The incorporation study of ^{14}C-lecine into the protein fraction was carried out according to the method of Moreland et al (1969). 1.2 g of Avena coleoptile sections from etiolated seedlings were incubated in 0.01 M phosphate buffer solution containing 1 % sucrose, 2 % ethanol, 0.5 µ Ci of ^{14}C-leucine, 40 g chloramphenicol and the herbicide for 8 hrs at 30°C.

After incubation, sample sections were collected and homogenized in 0.1 M tris-buffer (pH 7.5), followed by centifugation to separate the supernatent from the residual sedimentation. The diluted solution of TCA acid was added at final concentration of 5 % to sediment the protein. An 0.1 N alkaline soluble fraction from this sedimentation was obtained to detect radioactivity. An incorporation study of ^{14}C-orotic acid into plant RNA or that of ^{14}C-acetic acid into lipid fractions was also carried out. A similar volume of Avena segments was incubated in the herbicide solution, but ^{14}C-orotic acid or ^{14}C-acetic acid was added in place of ^{14}C-leucine. To obtain the RNA fraction from the segments, precipitation by perchloric acid was performed according to the method by Mizuno (1969). Lipid fractions were obtained by homogenating the segments with chloroform-methanol-water, followed by the addition of chloroform and water to separate the chloroform layer from methanol-water layer. The measurement of radioactivity in each fraction was carried out by Liquid scintillation counter.

RESULTS

Herbicidal symptoms

Newly developing leaves of susceptible grass weeds treated with Quizalofop-ethyl showed a complete suppression of growth within 24 hrs of the application of the herbicide onto the leaf surface. This was followed by the appearance of yellowing symptoms. Yellowing was followed by the appearance of necrotic symptoms throughout the leaf system 6 days after treatment. A similar development of symptoms was observed after the foliar application of the herbicide in the field. Complete death of plants in the field was observed within 6-8 days of treatment.

Microscopic study

From the microscopic study of the treated plant tissue sections, the swelling of the cells in the region of the meristems under the apical meristem was clearly observed. The stainability to the tissue decreased 2 days after application. Destruction of the cells of the meristem or in the intercalary meristems or in the bundle sheath, was observed 4 days after application of the herbicide. The complete death of cells in the intercalary meristems surrounding the apical meristem and in the basal part of younger leaf sheaths was observed 6 days after application. However, the shoot apical meristem remained unaffected until the rapid destruction of the surrounding tissues led to its death indirectly.

Penetration and translocation study

The result of the study concerned with the penetration and distribution of the translocated active ingredient is shown in Table 1. One day after the application of ^{14}C-Quizalofop-ethyl to the plants, about 31 % of the radioactive material had penetrated. And the amount of translocated radioactivity detected in the untreated leaves was 0.59, 0.14 % in the stem base and 0.11 % in the root system. By 6 days after treatment, 51 % of labeled herbicide had penetrated, however, there was not a significant increase in the amount translocated.

Systemic action of the herbicide

Figs. 1 and 2 show the result of the study concerned with the systemic characteristics of Quizalofop-ethyl. The growth of the rhizome system was suppressed 1 day after application, and this resulted from the rapid translocation of active ingredient to the rhizome system. The α-naphthylamine oxidative potential of the rhizomes from the treated plants was also reduced 1 day after treatment.

Biochemical and physiological studies

Table 2 shows the result of the studies concerned with the effect of Quizalofop-ethyl on the biosynthesis of protein, RNA and lipid in plants. An interference with lipid synthesis was observed at a concentration of 10^{-5}M. At 10^{-4}M, the incorporation of ^{14}C-leucine into the protein fraction and ^{14}C-orotic acid into the RNA fraction were inhibited significantly. The concentration of active ingredient in the incubating sample solution varied from 10^{-3} to 10^{-7} ppm, and the inhibitory activities were significant at the 5 % level in Duncan's Multiple Range Test. The leakage of electrolytes from the cells was observed after 14 hrs of incubation, particularly at herbicide concentration of 10^{-5}M.

DISCUSSION

The radiotracer study showed that Quizalofop-ethyl was both xylem and phloem mobile. However, the amount of active material in the untreated part of the plant (Echinochloa crus-galli) remained below 1 % of the total amount applied. On the other hand, the destruction of the target tissues was observed clearly at 4 days after treatment, following the symptoms of rapid swelling of the cells in that region at 2 days after treatment.

The results suggest that the rapid initial effect of this herbicide on the intercalary meristems seems to inhibit later translocation and accumulation of this herbicide in untreated plant parts. The amount of active ingredient required to affect the target site seems to be less than 1 % of the total amount applied.

In the case of S. halepense, sufficient of the active ingredient to both suppress rhizome growth and drastically reduce rhizome viability had been translocated from the leaves to the rhizome within a day of application.

The biosynthesis of protein, RNA and lipid were inhibited within 8 hrs of incubation. However, although these results do not yet show the primary target of this herbicide. The susceptibility of lipid synthesis and the results of the leaking study, both suggest that the effect on cell membranes is central to further elucidation of this problem.

TABLE 1

Penetration and distribution of ^{14}C-active material in treated plant

DAT	treated leaf		untreated parts			^{14}C
	remained	removed by washing	leaves	stem base	root	recovered
1	27.3 %	59.6 %	0.52 %	0.12 %	0.10 %	87.6 %
2	23.6	66.6	0.53	0.10	0.09	83.9
4	35.1	52.6	0.75	0.10	0.11	88.6
6	44.4	42.6	0.58	0.07	0.10	87.8

TABLE 2

Effect of Quizalofop-ethyl on protein, RNA and lipid synthesis in Avena coleoptole segments

	Protein	RNA	Lipid
Significant level	10^{-4}M	10^{-4}M	10^{-5}M
% inhibition at 5 % significant level	30 %	57 %	24 %

Fig. 1 The rhizome growth of the treated plant (Sorghum halepense)

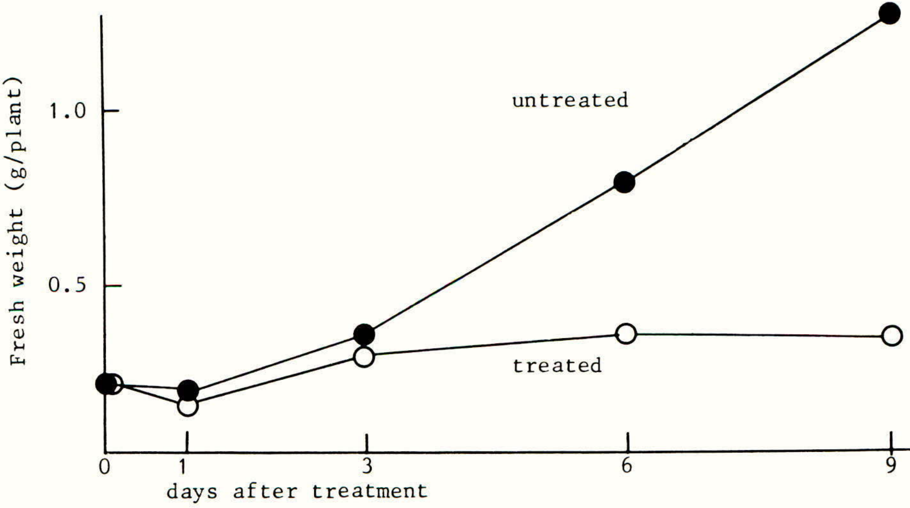


Fig. 2 2 α-naphthylamine potential of rhizomes from the treated plant (*Sorghum halepense*)

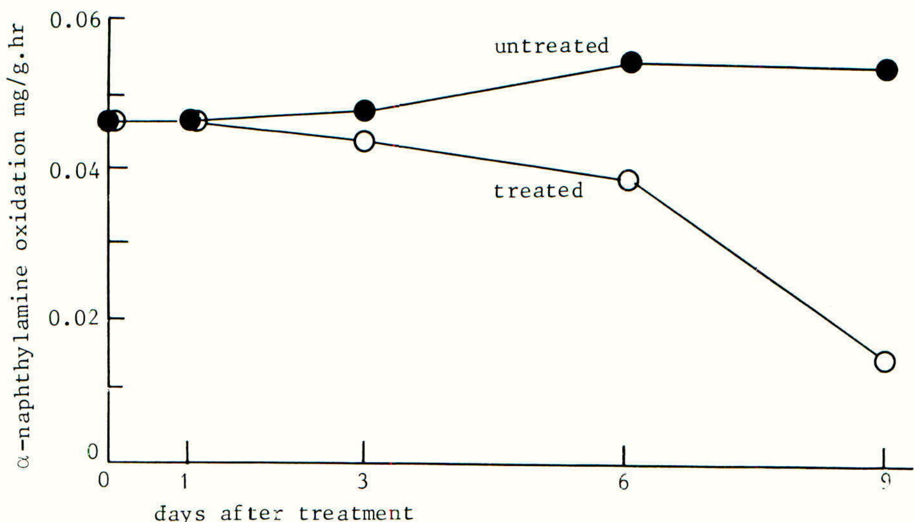
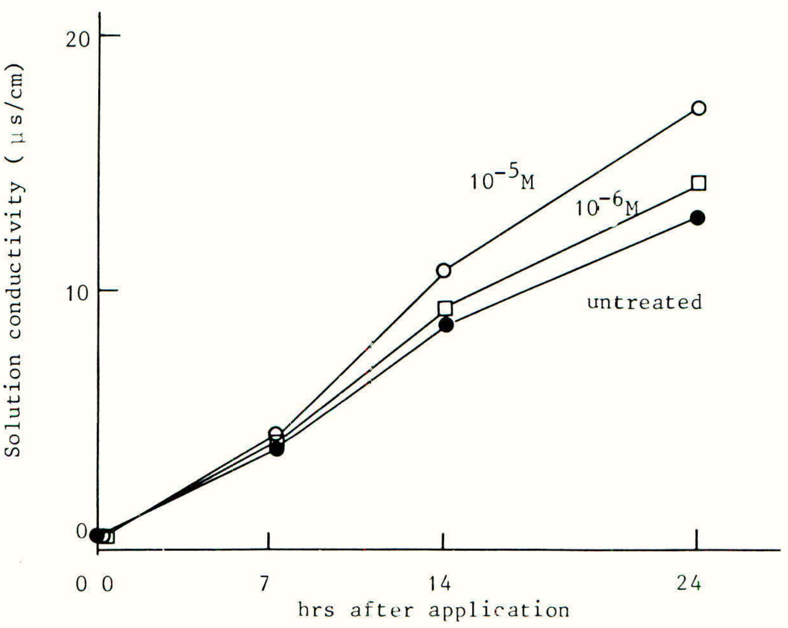


Fig. 3 Leaking of electrolytes from the cells of *Avena sativa*



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THE MODE OF ACTION AND BASIS OF SELECTIVITY OF DIFLUFENICAN IN WHEAT, BARLEY AND SELECTED WEED SPECIES

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ABSTRACT

Diflufenican [N-(2,4-difluorophenyl)-2-(3-trifluoro-methylphenoxy) -3-pyridine carboxamide] is a new selective herbicide which acts against a wide range of weeds, both pre- and early post-emergence, at rates of application of 50-250g/ha. Studies on the mode of action and selectivity of diflufenican have been carried out on _Triticum aestivum_ L. cv Flanders (resistant), _Hordeum vulgare_ L. cv Triumph (resistant), _Setaria viridis_ (susceptible), _Stellaria media_ (susceptible), _Viola arvensis_ (susceptible) and _Galium aparine_ (moderately susceptible). The uptake and movement of diflufenican to the site of action and its metabolism have been investigated together with its biochemical action. The primary mode of action of diflufenican appears to be on carotenogenesis. Carotenoid levels are decreased in treated plants and there appears to be a concomitant accumulation of carotenoid precursors. The resulting low levels of coloured carotenoids is insufficient to protect the plant from photo-oxidations which result in chlorophyll destruction and membrane disruption.

INTRODUCTION

Diflufenican [N-(2,4-difluorophenyl)-2-(3-trifluoro-methylphenoxy) -3-pyridine carboxamide] is a recently developed selective herbicide for both pre- and post-em use. It is effective against a wide range of herbaceous weeds at doses of 50-250g a.i./ha. It produces chlorosis and bleaching in susceptible species. The bleaching action of diflufenican suggests that a major mode of action of this herbicide is on the photosynthetic pigments.

Many herbicides are now known which cause bleaching by inhibition of carotenoid biosynthesis (e.g. norflurazon). Carotenoids perform several important functions in photosynthesis. They act as accessory light harvesting pigments, but their most important function is in protecting the photosynthetic apparatus from harmful photo-oxidations which occur under conditions of high light intensity. Under these conditions excessive amounts of excited chlorophyll are generated, some of which may become the longer lived triplet chlorophyll which can transfer its excess energy to oxygen to yield the highly destructive oxidising agent, singlet oxygen, $^1O_2$. Carotenoids protect against this photo-oxidation by quenching the excess energy of triplet chlorophyll or singlet oxygen (Britton, 1982).

In the present investigation the uptake, translocation, metabolism and mode of action of diflufenican was examined in order to establish the basis for selectivity.

MATERIALS AND METHODS

General

Diflufenican was supplied by May and Baker Ltd. as a 50% w/v aqueous suspension concentrate.

The species used were:- Winter wheat (Triticum aestivum L. cv Flanders) (resistant), barley (Hordeum vulgare L. cv Triumph) (resistant), Setaria viridis (susceptible), Stellaria media (susceptible), Viola arvensis (susceptible) and Galium aparine (moderately susceptible). Unless otherwise stated seeds were planted in 12.5cm pots at a depth of 1cm in John Innes No.2 compost.

Uptake and Translocation of [^{14}C]-diflufenican

Soil applied

A concentration of diflufenican equivalent to a field dose of 200g/ha was applied to the top layer of soil (15g dry weight) along with 15µCi of the ^{14}C-labelled compound and evenly mixed. The pots were placed in a growth cabinet under standard conditions (14h day; day temp. 20°C $\pm$ 0.5°, night temp. 18°C $\pm$ 0.5°; humidity 90% $\pm$ 5%, light intensity 27,000 lux). After a period of 7 days the shoots were harvested, fresh (FW) and dry weights (DW) were taken and the samples combusted using a Packard Oxidiser (model B 306) and the levels of ^{14}C determined by liquid scintillation counting, (Packard model 300c).

Root applied

Uptake and translocation were also investigated using a hydroponic system. Seeds were grown in vermiculite, watered with ¼ strength Hoaglands solution and placed in a growth cabinet under standard conditions for 7 days. The roots were washed to remove the vermiculite and three plants placed in each 120ml coloured glass bottle containing 105ml of the ¼ strength Hoaglands solution. The shoot was supported by a sponge and the roots aerated by bubbling air through the solution. 1.5µCi of ^{14}C-labelled diflufenican was applied to all bottles.

Six plants of each species were harvested after 1, 3, 7 and 14 days, 3 plants being freeze dried and autoradiographed; the remainder were separated into the root, mature tissue and new growth. FW and DW were taken prior to oxidation and the ^{14}C determined by liquid scintillation counting.

Leaf uptake of [^{14}C]-diflufenican

^{14}C-labelled diflufenican (0.1µCi) in a solution containing the surfactant Tween 20 was applied to the first leaf of a monocotyledonous species or to a cotyledon of a dicotyledonous species as 6 x 0.5µl droplets. Six plants were harvested at each harvest date (1, 3, 7 and 14 days). The treated leaf of all six plants was washed in 5ml of distilled water for 60s. Three plants were then freeze dried and autoradiographed; the remaining three were separated into roots, applied leaf, seed(monocotyledons), other cotyledon(dicotyledons) and new growth. FW and DW were obtained and the ^{14}C content determined.

Metabolism

Using the same system as the study of uptake from the soil, shoots were harvested 7 days after emergence. FW's were taken and the plant tissue was homogenised thoroughly in dried distilled acetone; after vacuum filtration, the homogenate was retained. The fibrous residue was dried at 50°C, pelletised, oxidised and radioassayed by liquid scintillation counting. The extract was reduced to dryness under vacuum at 30°C and resolubilised in a known volume of acetone. A white/pink deposit, thought to be anthocyanin pigments, was dissolved in 80% ethanol. The acetone soluble fraction was passed through Sep-pak silica cartridges (Waters

Associates). This was then reduced in volume (0.5ml) and applied as 2μl droplets to precoated tlc plates (250μm silica gel F254) run in a series of solvent systems:

a) Benzene: Ethylacetate 4:1
b) Chloroform: Methanol: Ammonia 80:19:1
c) n Hexane: Acetone 7:1

The plates were then autoradiographed, the spots scraped from them, and the ^{14}C content of the silica determined.

Herbicide Action

Unless stated otherwise, diflufenican was applied pre-em to the soil surface using a laboratory chromatography sprayer at a volume rate equivalent to 200l/ha. The sprayed pots were kept in a greenhouse at 20°C ($\pm$ 5°) with daylight supplemented by mercury vapour lamps (14h in 24h), and watered as necessary. After 7 days the effect of herbicide treatment on growth was evaluated by effects on shoot length, FW, pigment synthesis and membrane permeability.

Measurements of chlorophyll and total carotenoid content

Chlorophyll content was measured by the method of Arnon (1949). The plant pigments were extracted in 80% acetone, transferred to peroxide-free diethyl ether in a separating funnel and extracted according to the method of Britton and Goodwin (1971). Total carotenoid values were calculated from the absorbance at 450nm. Scanning absorption spectra of the carotenoid solutions in petroleum ether were also recorded.

Membrane permeability

This was determined using a modification of the method of Fletcher and Drexler (1980).

The primary site of action was determined by assessment of the above parameters over an 8 day period after early post-em application to S.viridis (susceptible).

The influence of light/dark treatments on the activity of diflufenican (2.8×10^{-5}M) applied pre-em to S.viridis was determined. The plants were maintained in growth cabinets as before. Initially the plants were kept in either normal light conditions or in darkness for 48h. Subsequently the plants were placed either in light or darkness until harvested, thus giving four regimes:- light-light, light-dark, dark-light, and dark-dark.

Analysis of variance and Duncan's Multiple Range Test (Snedecor, 1968) were carried out where appropriate.

RESULTS

Uptake and Translocation

Initial studies, using ^{14}C diflufenican applied to the soil were aimed at reproducing the field situation as accurately as possible. Shoot uptake from this system was related to species susceptibility (Table 1). When diflufenican was freely available to the roots there were no appreciable differences in levels of uptake between the species after 7 days, and no inter-species diversity in the rate of uptake (Table 2). The autoradiographs show uniform xylem transport to all parts of the plant in each species, with an accumulation of ^{14}C at the leaf tips (not presented).

The uptake of leaf-applied ^{14}C diflufenican was most rapid in V.arvensis followed by S.viridis and S.media, uptake by G.aparine was

delayed, while wheat and barley absorbed very little. Uptake was again related to species susceptibility (Table 3). The autoradiographs showed that for all species the level of ^{14}C was highest in the treated leaf. Transport of the herbicide to the remainder of the plant was relatively small and took place in an erratic fashion. Discrete accumulations of radioactivity were evident in the untreated leaves and sometimes the roots. The mode of transport is uncertain but will be investigated further.

Metabolism

All studies show that if metabolism of the applied compound does take place it is very slow. The extractable fraction accounted for about 90% of the label, the remainder was contained in the fibre fraction. This extracted fraction had at least 94% of the ^{14}C label dissolved in acetone while the other 6% was soluble in 80% ethanol. tlc of the extracts from the test species showed that at least 95% of the radioactivity on the plate was present as the parent compound.

Activity of Diflufenican

Wheat and barley (resistant) were relatively unaffected by diflufenican, whereas S.viridis, S.media and V.arvensis (susceptible) showed considerable reductions in FW, shoot length and chlorophyll content, as well as increased membrane leakage (Table 4). Following post-em application of diflufenican, a reduction in carotenoids occurred almost immediately, closely followed by a reduction in chlorophyll content (Table 5). FW reduction and increased membrane leakage became apparent a few days later.

Plants maintained totally in darkness (dark-dark) regime) showed little difference between control and diflufenican treatments (Table 6). Plants kept in bright light throughout demonstrated large reductions in FW, shoot length, chlorophyll and carotenoid levels while membrane leakage markedly increased. Effects on plants from the dark-light and light-dark regimes were intermediate, the severity of symptoms being slightly greater in the former. The mortality rates of diflufenican-treated plants closely mirror the severity of herbicide symptoms; light-light and dark-light plants died rapidly while dark-dark and light-dark plants survived as long as the controls.

DISCUSSION

Diflufenican produced varying degrees of damage on a range of selected plant species when applied pre- or early post-emergence. The symptoms included chlorosis and bleaching of the susceptible weed species S.viridis, S.media and V.arvensis; the resistant crop species wheat and barley were relatively unaffected while G.aparine was intermediate.

Differential metabolism did not account for the selectivity but evidence has been presented which shows that differential uptake is of major importance. The results of the hydroponic experiments appeared to show that there was very little difference in uptake between the species when the ^{14}C-diflufenican was equally available to the roots. However, the percentage of absorbed herbicide in the new tissue was generally greater in the more susceptible species presumably enhancing the level of inhibition.

When applied pre-em, diflufenican is absorbed by shoots, to a greater extent, by the shallow-rooted weed species than the deeper rooted crop species which remain unaffected. When applied to the leaf, uptake was related to species susceptibility. It would appear that post-em application

results in greater uptake by the susceptible species with transportation to growing tissue, while the reduced uptake by the resistant species coincides with the absence of severe effects.

In the conditions and duration of these experiments, diflufenican was metabolised to only a small degree, but there appeared to be some evidence of binding to lignin and cellulose fibres, possibly reflecting the relatively lipophilic nature of the compound.

Diflufenican action resulted in reduced fresh weight, shoot length, chlorophyll and total carotenoid content, while membrane leakage increased with herbicide concentration. These results correlate well with visual observations. A time-course study was performed to follow the sequence of effects on fresh weight, chlorophyll and carotenoid contents and membrane leakage. It appeared that diflufenican primarily affected the carotenoids, the other symptoms developing as an indirect result of the decrease in protective carotenoid levels. Reduction in chlorophyll levels rapidly followed the decline in carotenoid levels. It was evident that chlorophyll was not destroyed directly by diflufenican since susceptible plants grown in dim light accumulated significantly more chlorophyll following diflufenican treatment compared to similar plants grown under normal bright light (not presented).

The Hill reaction was not appreciably affected by diflufenican (*in vitro*) (Thompson, private communication) but *in vivo* treatment resulted in a reduction in the Hill reaction rate. Thus the Hill reaction *per se* was unaffected by diflufenican, although reductions of the photosynthetic pigments in plants treated *in vivo* resulted indirectly in a lowering of the photosynthetic capability. Preliminary experiments in which plants were grown solely in light or dark conditions revealed that symptoms of action of diflufenican were observed only when plants were grown in light conditions. Dark-grown plants showed no apparent difference between control and diflufenican treatments. The effect of varying light and dark treatments was studied in order to confirm that the herbicidal effects are a result of photo-oxidations in light due to lack or absence of protective carotenoids and not due to an activation of diflufenican in the plant by light. If diflufenican only exerted its toxicity in light because of activation in the plant, then plants in the light-dark regime should be badly affected and die. However, this is not the case as these plants survive as long as the controls. This suggests that in susceptible species, diflufenican acts by reducing carotenoid levels, which in turn, leads to photo-oxidative destruction of chlorophyll, membranes and other cell components in light, thus resulting in plant death.

Lack of carotenoids in herbicide-treated susceptible species could be due to either a) destruction of already-formed carotenoids, or b) interference in carotenoid biosynthesis. The evidence so far suggests that the latter is more probable. Preliminary work with tlc and scanning absorption spectra suggests that there may be an accumulation of phytoene and possibly other carotenoid precursors. If this is true then diflufenican may be inhibiting carotenoid biosynthesis at some point(s) in the desaturation sequence between phytoene and lycopene, in a similar manner to the action of other bleaching herbicides (Bramley *et al*, 1984). Further work is taking place which may support this theory.

TABLE 1

Uptake of soil applied ^{14}C diflufenican by selected species after 7 days (mean of 3 replicates)

Species	Uptake µCi/g DW	Rating*
Wheat (R)	0.0010	1
Barley (R)	0.0016	1.5
Galium aparine (MS)	0.0100	9.7
Setaria viridis (S)	0.0643	62.4
Stellaria media (S)	0.0760	73.8
Viola arvensis(S)	0.1080	104.9

TABLE 2

Uptake of ^{14}C diflufenican 7 days following root application to selected species (mean of 3 replicates)

Species	Uptake µCi/g DW	Rating*	Translocation‡
Wheat (R)	0.601	1	38.0
Barley (R)	0.578	0.96	13.5
Galium aparine (MS)	0.849	1.41	9.2
Setaria viridis (S)	0.476	0.79	12.0
Stellaria media (S)	0.227	0.38	29.4
Viola arvensis (S)	0.602	1.00	36.5

TABLE 3

Uptake of ^{14}C diflufenican 7 days following leaf application to selected species (mean of 3 replicates)

Species	Uptake µCi/g DW	Rating*	Translocation‡
Wheat (R)	0.428	1	19.19
Barley (R)	1.078	2.52	17.28
Galium aparine (MS)	1.753	4.10	29.95
Setaria viridis (S)	6.046	14.13	28.94
Stellaria media (S)	13.500	31.54	19.20
Viola arvensis (S)	43.380	101.36	39.60

* in relation to wheat ‡as a % of uptake

TABLE 4

Effect of a range of concentrations of diflufenican on shoot length(SL), fresh weight(FW), chlorophyll content(CHL) and membrane leakage(ML)

Species	Concn(M)	Herbicide activity (% of controls) 3.5×10^{-4}	1.4×10^{-3}	2.8×10^{-3}	7.0×10^{-3}	1.4×10^{-2}
Wheat	FW	105	83	73	91	79
	SL	105	96	85	97	92
	CHL	90	98	98	100	107
	ML	100	75	100	100	100
Barley	FW	109	102	94	90	102
	SL	107	103	101	97	102
	CHL	104	97	78	104	84
	ML	100	80	100	100	100
Setaria viridis	FW	84	61	33	13	23
	SL	90	82	69	56	60
	CHL	74	32	20	0	0
	ML	135	239	339	409	426
Galium aparine	FW	110	97	83	124	103
	SL	91	86	65	71	76
	CHL	99	96	98	99	72
	ML	61	132	150	118	132
Viola arvensis	FW	67	29	24	18	11
	SL	63	43	40	38	30
	CHL	92	53	53	56	24
	ML	130	170	320	680	690
Stellaria media	FW	94	53	35	8	4
	SL	98	71	69	43	37
	CHL	73	46	63	0	0
	ML	173	400	591	827	855

TABLE 5

The effect of diflufenican on S.viridis over an 8 day treatment period

Effect (% of control)	Time (days) 1	2	3	4	5	6	8
Fresh weight	109	112	88	67	51	31	21
Chlorophyll	100	90	54	43	46	33	25
Carotenoids	85	74	42	32	60	40	23
Membrane leakage	96	118	151	158	198	291	295

TABLE 6

The effect of varying light/dark growth conditions on the action of diflufenican applied to S.viridis

Treatment	light-light		light-dark		dark-light		dark-dark	
Assay	*C	‡D	C	D	C	D	C	D
Fresh weight(g)	0.661	0.336	0.405	0.326	0.931	0.426	0.430	0.445
Shoot length(cm)	2.85	1.96	2.89	2.59	8.62	7.92	8.88	10.05
Membrane leakage(%)	19	84	65	72	67	90	81	87
Chlorophyll (mg/g)	1.6	0.0	0.5	0.0	0.6	0.1	0.0	0.0
Carotenoids (µg/g)	119	2	43	6	45	4	2	2

*Control ‡Diflufenican

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STUDIES ON THE MODE OF ACTION OF THE HERBICIDE FLUOROCHLORIDONE

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ABSTRACT

The herbicide Fluorochloridone, which has been reported to interfere with carotenoid biosynthesis, has also been found to cause up to a 10-fold stimulation of ethylene release by soybean leaf disks. This increase was not due to lipid peroxidation, but rather to a substantial increase in the amount of 1-aminocyclopro-pane-1-carboxylic acid (ACC) in the soybean leaf tissue. The increase in ACC levels caused by Fluorochloridone can be blocked by inhibitors of de novo protein bio-synthesis, suggesting that the herbicide may act to control the level of ACC synthase, the key enzyme in the regulation of ethylene formation in plants. The significance of Fluorochloridone's effect on ethylene and ACC levels is discussed.

INTRODUCTION

Ethylene is a natural plant hormone involved with many aspects of plant growth, development and senescence (Abeles 1973). Under normal conditions growing plants produce ethylene only in relatively low amounts.

However, certain herbicides such as paraquat (1,1'-dimethyl-4,4'-bibyridylium dichloride) and diquat stimulate the production of propane, ethane and ethylene in the xanthophycean microalga Bumilleriopsis filiformis (Boehler-Kohler et al 1982). The herbicides chlorsulfuron (2-chloro-N-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl]benzenesulfonamide) and metalochlor (2-chloro-N-[2-ethyl-6-methylphenyl]-N-[2-methyl-methylethyl]-acetamide), also increase ethylene production in sunflower (Suttle, 1983) and soybean seedlings (Paradies et al 1981).

Fluorochloridone, 1-(m-trifluoromethylphenyl)-3-chloro-4-chloromethyl-2-pyrrolidinone, is a selective herbicide which has been reported by Lay and Niland (1983) and by Delvin et al (1981) to interfere with carotenoid and polar membrane lipid (St. John, 1985) biosynthesis in susceptible plant species. In the present study, we report that Fluorochloridone causes substantial ethylene production in soybean leaf disk tissues and that this increase of ethylene production is due to Fluorochloridone - induced synthesis of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid).

MATERIALS AND METHODS

Chemicals

($3-^{14}C$) Fluorochloridone Sp. Act 8.5 mCi/mM, with radiopurity 99.8% was synthesized by J. Kalbfeld, Stauffer Chemical Co. ACC was purchased from Cal Biochem. AVG (aminoethoxy vinylglycine) was a gift from Hoffman LaRoche, Nutley, N.J. The technical 100% trans-Fluorochloridone was 97.6% pure.

Ethylene and ACC Determination

Leaf disks 1.5 cm in diameter were cut from the primary leaves of 2 week old soybean seedling (Glycine max L. CV. Bragg). Three ml of a solution containing 5 x 10^{-6}M 6-benzylaminopurine, 0.05% Tween 20 and 10 x 10^{-7}M gibberellic acid with or without test chemical was placed into a 25 ml glass vial fitted with a septum cap. Two leaf disks were immersed in the solution

for 10 minutes and then were pressed against the vial wall with the lower leaf surface facing out. The vials were then incubated for 18 hr at 28°C under fluorescent light at 150 ft. c. After 18 hr, 1 c.c. of the head space volume of the vial was sampled by using a Tuberculin Syringe (Becton, Dichinson and Company, Rutherford, N. J.). The sample was then injected into a gas chromatograph (G.C.) (Bendix 2600) equipped with a 1/4 inch by 5 feet long glass column containing aluminum oxide. The conditions for the G.C. were: Detector 120°C; Injector 120°C; Oven 100°C. Ethylene production was expressed as nl $g^{-1}hr^{-1}$. Standard ethylene at 1.6 ppm was used to compare peak heights for quantitation of evolved ethylene. The quantitative determination of ACC was based on the liberation of ethylene from ACC with NaOCl in the presence of Hg^{+2}. Ethylene was again assayed by G.C. (Lizada and Yang, 1979).

RESULTS

Figure 1 shows that Fluorochloridone substantially increased ethylene production in soybean leaf tissues at concentrations ranging from 10 to 200 ppm. At 200 ppm, ethylene production from treated tissues reached the highest level which was nearly 10-fold higher than the control. Beyond 200 ppm ethylene production started to decline. Significant increases in ethylene production were noticed as soon as 10 hours after treatment. The amount of ethylene produced in treated tissues reached the maximum at 30 hour after treatment. Control tissues (without Fluorochloridone), did not change significantly during the 48 hr observation period (Figure 2).

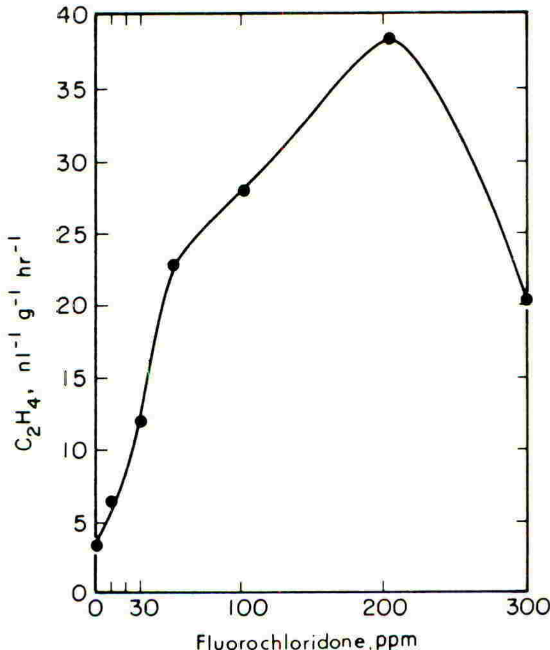

FIGURE 1: Effect of Fluorochloridone on ethylene production in soybean leaf disc tissues 18 hr after herbicide exposure. Data were the average of 8 replicates from two experiments.

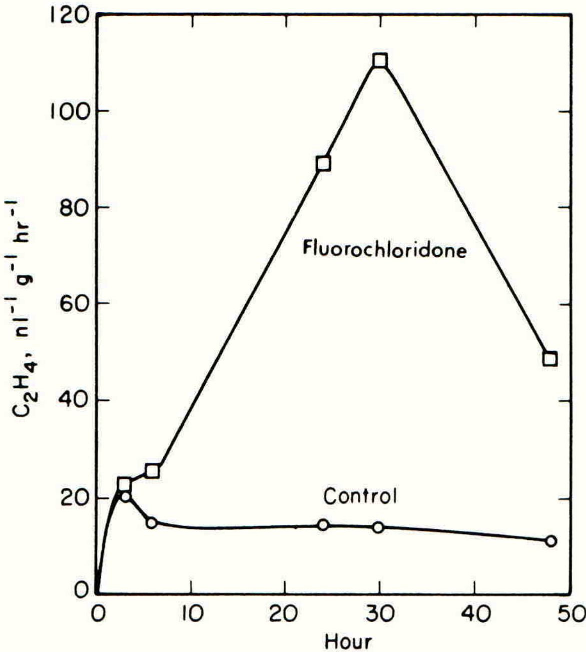

FIGURE 2: Effect of Fluorochloridone (100 ppm) on ethylene production in soybean leaf discs. Each time interval is the average of 6 replicates.

In order to determine how much Fluorochloridone taken up by leaf disks during the 10 min. presoaking period, (3-^{14}C)-Fluorochloridone was used. Table 1 indicates that the absorption of the herbicide by leaf disks ranged from 5.8 to 9.2% when the chemical concentrations in the solution ranged from 10 to 200 ppm. The amount of Fluorochloridone absorbed by two-leaf disks within 18 hr was approximately 9 to 14 ppm when the initial herbicidal concentration was between 100 and 200 ppm.

TABLE 1

Absorption of Fluorochloridone by Soybean Leaf Disks

Fluorochloridone ppm	μg Fluorochloridone Added to 3 ml Solution	μg Fluorochloridone as ^{14}C Recovered in Two Leaf Disks	% Recovery*
10	30	1.73 ± 0.44	5.8
30	90	4.79 ± 1.0	5.3
100	300	27.64 ± 11.4	9.2
200	600	41.85 ± 25.9	7.0

* Data were the average of two separate experiments with three replicates for each experiment. ^{14}C-Fluorochloridone was applied at 55,030 CPM in 1 μl ethanol. Incubation was done at 28°C for 18 hr. Leaf disks were then rinsed with water and homogenized with acetone/methanol (1:1, v/v) for LSC.

Ethylene production under various environmental conditions are summarized in Table 2. Under aerobic conditions Fluorochloridone treated tissues induced higher levels of ethylene. Anaerobic conditions dramatically reduced (by 92%) ethylene production by treated tissues.

TABLE 2.

The Effect of Fluorochloridone on Soybean Leaf Disk Tissues Ethylene Production under Various Conditions

Fluorochloridone Treated Leaf Disks Under	C_2H_4, $nl g^{-1} hr^{-1}$ [a] Fluorochloridone, ppm	
	0	200
light-air	22	260
light-N_2	3	20
dark-air	6	237
dark-N_2	3	53

(a) Data were the average of 2 to 3 replicates.

It is known that ACC is the precursor of ethylene in many plant tissues (Adams and Yang, 1979). Table 3 demonstrates that 100 ppm of exogeneous ACC stimulates the liberation of ethylene from soybean leaf disks to about the same extent as does 200 ppm of Fluorochloridone. AVG is a specific inhibitor of ACC synthase (Adams and Yang, 1979), at 2 ppm, it inhibited ethylene production in Fluorochloridone treated tissues. However, it did not interfere with the conversion of ACC into ethylene (Table 3).

TABLE 3

AVG Inhibition of the Fluorochloridone Induced Ethylene Production in Soybean Leaf Disk Tissues

Chemical	ppm	C_2H_4, $nl g^{-1} hr^{-1}$ [a]
ACC	100	54.4 ± 4.6
Fluorochloridone	200	48.4 ± 9.7
AVG	2	1.2 ± 0.2
Fluorochloridone + AVG	200 + 2	10.2 ± 1.5
ACC + AVG	100 + 2	57.1 ± 5.2
Control	0	2.5 ± 0.4

(a) Data were the average of 4 replicates.

Under anaerobic conditions, conversion of ACC into ethylene is known to be inhibited (Yang, 1980). Quantitative analysis for ACC in Fluorochloridone treated leaf disk tissues are presented in Table 4. The ACC content was found to be 36-fold greater in treated than in control tissues under anaerobic conditions. Under aerobic conditions, however, there was little or no difference in the amounts of ACC in treated or control leaf tissues. The reduction in ACC under aerobic conditions is due to the rapid conversion of newly formed ACC into ethylene by ethylene forming enzyme (EFE).

TABLE 4

Effect of Fluorochloridone on ACC Content in Soybean Leaf Disks After 18 Hr. at 26°C

Treatment	Condition	ACC nmole/g[a]
Control	aerobic	not detectable
Fluorochloridone	aerobic	1.38 ± 0.13
Control	anaerobic[b]	1.28 ± 0.17
Fluorochloridone	anaerobic[b]	35.95 ± 4.25

(a) Data were average of at least 12 replicates.

Cycloheximide was added to the herbicide treated tissues to determine whether inhibition of de novo protein synthesis would affect the stimulation of ethylene release by Fluorochloridone. Cycloheximide at 1.5 ppm reduced the stimulation of ethylene release by the herbicide by 84% and prevented it completely at 50 ppm (Table 5). This strongly suggests that Fluorochloridone increased the ACC content in plant tissues by the de novo synthesis of the enzymes in the ethylene biosynthetic pathway.

TABLE 5

Affect of Cycloheximide on Fluorochloridone Induced Ethylene Formation in Leaf Disks Tissues

Chemical	ppm	C_2H_4, $nlg^{-1}hr^{-1}$[a]
Control	0	3.1 ± 0.6
Fluorochloridone	200	72.3 ± 9.1
Fluorochloridone + Cycloheximide	200 + 1.5	11.3 ± 3.5
	200 + 5.0	6.6 ± 1.0
	200 + 10	4.2 ± 0.4
	200 + 50	1.9 ± 0.2

(a) Data were the average of 3 replicates.

Fluorochloridone induced ethylene production was also observed in whole plants. Table 6 shows that ethylene levels were 6-fold higher in the treated excised shoots of soybean seedlings than those of untreated seedlings during an 18 hr observation.

In addition to biosynthesis of ACC, ethylene may also be derived from peroxidative decomposition of lipids. The production of malondialdehyde is a result of lipid peroxidation. However, when herbicide treated leaf disks were analyzed for malondialdehyde content (Dhindsa et al 1981), it was found that no malondialdehyde was present in the treated tissue (data not shown). This suggests that lipid peroxidation was not involved in ethylene production by the treatment of Fluorochloridone.

TABLE 6

Production of Ethylene from Excised Shoot Soybean Seedlings Treated with Fluorochloridone

Fluorochloridone, ppm	C_2H_4, $nl g^{-1} hr^{-1}$
0	0.34 ± 0.1
200	2.08 ± 0.6

Two weeks old soybean seedlings were cut above cotyledons. The excised plants were 6 inch in height. The plant was placed in 2.3 x 20 cm test tube with 15 ml nutrient solution containing 200 ppm Fluorochloridone for 4 hr in growth chamber. After 4 hr, test tube was sealed with latex strip and continue incubation for another 18 hr. At the end of 18 hr incubation, 1 cc sample was removed for ethylene determination.

DISCUSSION

Fluorochloridone at less than 10 ppm in soybean leaf cells was found to cause a rapid evolution of the plant hormone ethylene. This stimulation of ethylene release was not light dependent but was significantly greater under aerobic than under anaerobic conditions. It was possible that this ethylene might have arisen as a result of membrane lipid peroxidation caused by the herbicide (Dhindsa et al, 1981). However, lipid peroxidation as a source for the evolved ethylene has been eliminated on the basis that no ethane and no malondialdehyde, other known products of plant lipid per-oxidation, could be detected after treatment of soybean with the herbicide.

In the normal biosynthetic pathway for ethylene in plants, Adams and Yang (1979) have shown that 1-aminocyclopropane-1-carboxylic acid (ACC) is the immediate precursor to ethylene and that ACC is rapidly converted to ethylene by plant cells under aerobic conditions. In the absence of oxygen ACC is not converted to ethylene and is found to accumulate. Fluorochloridone treatment of soybean leaf disks under anaerobic conditions caused an increase in the ACC level in the plant cells which was of approximately the same magnitude as the increase in ethylene under aerobic conditions. In addi-tion, AVG, a specific inhibitor of ACC synthase (Adams and Yang, 1979), substantially reduced stimulation of ethylene release by Fluorochloridone. The evidence demonstrates that the effect of Fluorochloridone on ethylene results from an effect on the amount of ACC in the cell rather than from an increase in the rate of conversion of ACC to ethylene.

Fluorochloridone could cause an increase in the rate of ACC biosynthesis in soybean leaf cells in a variety of ways: stimulation of the activity of the enzyme ACC synthase, increase in the amount of the synthase in the cell, or increase in the amount of S-adenosylmethionine, the precursor of ACC. In the presence of cycloheximide, which blocks the de novo biosynthesis of enzymes in the ethylene biosynthetic pathway, the stimulation of ethylene release by Fluorochloridone was substantially reduced. Therefore, the her-bicide probably acts by increasing the synthesis of one or more of the enzymes involved in ethylene biosynthesis. Although the particular enzyme which is so increased has not yet been identified conclusively, ACC syn-thase, the key enzyme in this pathway, is the most likely target. If ACC synthase is the target for Fluorochloridone, an interesting connection between Fluorochloridone and the plant hormone indole acetic acid (IAA)

becomes apparent (Figure 3). Fluorochloridone could be acting like IAA in stimulating biosynthesis of ACC synthase, or it could be acting to increase the level of IAA in the cell so that IAA is the proximate cause of the increase in ACC synthase. These questions are currently being investigated in our laboratory.

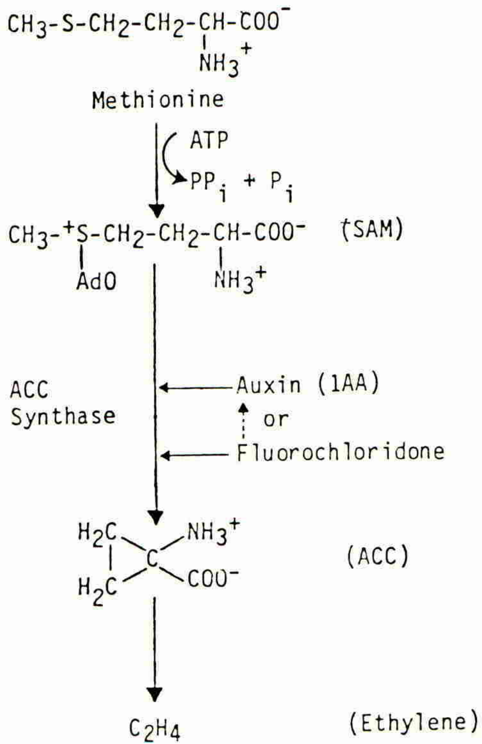

FIGURE 3: The proposed site of stimulating action of Fluorochloridone in the ethylene formation from soybean leaf disc tissues.

Finally, it has been found that Fluorochloridone can stimulate ethylene production from excised soybean seedling shoots as well as from soybean leaf disks. This observation eliminates the possibility that the ethylene observed in this work arose only from cells at the periphery of leaf disks in response to wounding of the tissue in preparing the leaf disks for assay.

There is no question that Fluorochloridone exerts its primary herbicidal effect by the inhibition of carotenoid biosynthesis leading to bleaching of susceptible plant tissues (Devlin et al, 1981 and Lay and Niland, 1983). The relationship, if any, between bleaching and the effect of Fluorochloridone on ethylene is not clear, but it is specific for Fluorochloridone. Norflurazone, (4-chloro-5-(methylamino)-2- , , -trifluoro-m-toly)-3-(2H)-pyridazinone) another bleaching herbicide, does not cause such a large stimulation of ethylene from soybean leaf disks and neither do any of another 20 commercial herbicides that have been examined. Considerable further work will be required to determine whether Fluorochloridone is herbicidal by two modes of action, but the effect of the herbicide on ethylene is certainly of interest.

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HERBICIDE RESISTANCE OF HORSEWEED (CONYZA CANADENSIS) IN HUNGARIAN VINEYARDS

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ABSTRACT

Insensitivity to atrazine and to some urea herbicides was found in fully developed plants of horseweed, Conyza canadensis, collected from vineyards where atrazine, and for three years, chlorbromuron had been applied extensively. Isolated chloroplast membranes from resistant plants exhibited a resistance factor of 1000 to atrazine, of 83 to chlorbromuron and of 40 to metobromuron. By studying the herbicide resistance of C. canadensis in the rosette stage, differences were found between resistant and susceptible biotypes in the composition of lipids and fatty acids in the thylakoid membranes of chloroplasts. The membrane lipids of resistant chloroplasts contained considerably more unsaturated fatty acids and exhibited a higher MGDG/DGDG ratio. The lipid matrix of thylakoid membranes of the resistant biotype were more fluid than those of susceptible ones.

INTRODUCTION

The resistance of Conyza canadensis to atrazine was observed first in Switzerland (Gressel et al 1982), but in the past few years triazine-resistant population of C.canadensis have been found in Hungarian vineyards where atrazine had been applied extensively (Hartman 1981; Mikulás and Pölös 1983). We attempted to clarify the physiological nature of this resistance (Lehoczki et al 1984.

During this year (1985), we have found in field experiments that when chlorbromuron was applied to whole plants of the triazine-resistant C. canadensis biotype there were some cases in which the herbicide was ineffective. In one particular vineyard chlorbromuron had been used to control triazine-resistant C.candensis since 1982.

In the present study, experiments were designed to establish the mode of resistance to photosystem II inhibitor herbicides and, in particular to gain more detailed information about the role of the thylakoid membrane lipids in herbicide resistance of C.canadensis.

MATERIALS AND METHODS

Hill Reaction Measurements in Presence of Herbicides

Chloroplasts were isolated from resistant and susceptible fully developed plants and measured as in Lehoczki et al (1984).

Plant Material for Lipid Analysis

Resistant and susceptible biotypes of C. canadensis plants were collected near Kecskemét in Hungary during the winter, when they existed in the rosette stage. The plants were then grown under laboratory conditions with a 16 h

and 8h dark regime, illuminated with white light of $10 w.m^2$ at 25°C for an additional two months.

In some experiments the harvested seeds of *C.canadensis* were germinated and seedlings were grown under the conditions mentioned above. Five to six month old plants in the rosette stage were used in the experiments.

Lipid Extraction and Separation

Lipids were extracted from the isolated chloroplasts with chloroform: methanol 2:1 by the procedure of Folch *et al* (1957). Lipid classes were separated by TLC on silica gel G Merck plates and developed with an acetone: benzene: water 91:30:8 or a petroleum: diethylether: acetic acid 85:15:1 solvent system (Khan *et al* 1977; Rouser *et al* 1970). Spots were identified by using authentic standards.

Fatty Acid Analysis

Fatty acid methyl esters were prepared from aliquots of total or separated individual lipids containing 17:0 as internal standard by esterification in the presence of 5% HCl in methanol at 80°C in ampoules sealed under CO_2. GLC analyses were carried out using a JEOL JGC 1100 instrument equipped with a dual flame ionization detector. The 1.6m long stainless steel columns were filled with 10% DEGS on Gaschrom P (Applied Sci. Lab). Quantification was achieved by a triangulation technique or with a Packard 603 electronic integrator. Quantities of lipids were determined from fatty acid methyl ester assays by GLC with references to 17:0 internal standard. To calculate the amounts of total and individual lipids, it was considered that 1 mol lipid contained 2 mol fatty acids.

Preparation of Liposomes

Total lipid extracts of the freshly isolated chloroplasts were prepared by the procedure of Folch *et al*.(1957). Neutral lipids and pigments were removed from the extracts by column chromatography on silicic acid (Rouser *et al*, 1967). The total polar lipid fraction was dried in a rotary evaporator and stored in chloroform under nitrogen. An aliquot of the lipid extract equivalent to 100-150 μg was dried in a test-tube and was dispersed in 4ml 0.1 M Tris-HCl buffer of pH 7.2 in a vortex mixer until the lipid dispersion became clear. In this way a multi-layered liposome solution was obtained.

Fluorescence Polarization Measurements

The fluorescence probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), was added to the liposome preparation in a few microliters of tetrahydrofuran and incubated for thirty minutes at 30°C with occasional mixing. The final amount of DPH was 1 umole per 100-150 μg lipids. The steady-state DPH fluorescence was measured with a Perkin-Elmer MPF-44A fluorescence spectrophtometer equipped for polarization and fitted with a temperature regulation unit. The samples were heated or cooled at a rate of 1°C/min. Fluorescence polarization values were determined as described previosly (Lehoczki *et al* 1984). Lipid dispersion without the fluorescence probe was used to correct for the light scattering of the preparations.

RESULTS

Resistance Factors in the Chloroplasts of Herbicide-Resistant *C. canadensis*.

To determine whether the photosynthetic electron transport of the chloroplasts of susceptible and resistant plants exhibited different sen-

sitivities to different herbicides, the Hill-inhibiting capacities of the herbicides were investigated and the herbicide concentrations required for 50% inhibition (I50 Values) were determined.

The activity of untreated chloroplast was 130$\pm$ 10 umole O_2mg chlorophyll $^{-1}$. h $^{-1}$ and 110$\pm$5 umole O_2 . mg cholorphyll $^{-1}$.h $^{-1}$ for samples isolated from susceptible and resistant leaf tissues, respectively.

Table 1 shows that chloroplasts from resistant plants were 1000 times less sensitive to inhibition by atrazine than those from susceptible plants. This indicates extreme herbicide resistance residing by the thylakoids. A degree of insensitivity to inhibition (i.e. resistance) was found with several urea herbicides investigated. The R/S values of chlorbromuron and metobromuron were 83 and 40 which indicates medium resistance.

Resistance factor value of linuron (R/S = 8.8) were not as large as those of chlorbromuron, but higher than with diuron (R/S = 1.8)and flumeturon (R/S = 1.8). Chloroplast from resistant plants were similarly sensitive to fenuron (R/S = 4.0) and uracil herbicides (lenacil R/S = 6.0; terbacil R/S = 3.3).

TABLE 1

I_{50} values for inhibition of the Hill reaction of isolated chloroplasts from fully developed plants of herbicide-resistant and herbicide-susceptible *C. canadensis* by several herbicides.

Herbicide	I_{50} concentration / M /		Resistant/Susceptible Ratio
	Resistant	Susceptible	
Atrazine	2×10^{-4}	2×10^{-7}	1000.0
Chlorbromuron	6×10^{-6}	5×10^{-8}	83.0
Metobromuron	4×10^{-6}	1×10^{-7}	40.0
Linuron	7×10^{-7}	8×10^{-8}	8.8
Fenuron	4×10^{-6}	1×10^{-6}	4.0
Fluometuron	7×10^{-7}	4×10^{-7}	1.8
Diuron	9×10^{-8}	5×10^{-8}	1.8
Lenacil	3×10^{-6}	5×10^{-7}	6.0
Terbacil	1×10^{-6}	3×10^{-7}	3.3

Chemical Composition and Physical States of Chloroplast Lipids in Resistant and Susceptible *C. Canadensis*

Content and Composition of Polar Lipids

The polar lipid content and relative amounts of lipid classes are given in Table 2. Chloroplast isolated from herbicide R *C. canadensis* in the rosette stage had a lower polar lipid content relative to chlorophylls than that from S plants. In chloroplasts of both biotypes, the glycolipids (MGDG, DGDG and SQDG) comprised about 80 mol percent of the total polar lipids whereas the remaining 20 mol percent was attributed to phospholipids. The chloroplast from the R biotype contained higher levels of MGDG and lower

levels of DGDG and PG than that from the S biotype. The relatively high amounts of PE may have resulted from contamination by mitochondrial and other extra-chloroplastic membrane fragments present in our chloroplast preparation. It should be noted, however, that a similarly high PE content was observed in highly purified chloroplasts of three other weed species, the R biotypes of which containing higher PE level (7-11%) than the S ones (Pillai and St. John 1981).

TABLE 2

Polar lipid content and composition of chloroplast membranes from herbicide-resistant(R) and herbicide-susceptible (S) biotypes of *C. canadensis*.

Lipid Class	Biotype	
	Resistant	Susceptible
Total Polar Lipids (nmol FA/mg Chl)	1894 ± 192	2682 ± 68
	mol % in total	
MGDG	54.0 ± 3.2	45.1 ± 2.7
DGDG	15.2 ± 2.8	25.2 ± 2.4
SQDQ	9.6 ± 1.0	8.6 ± 0.8
PG	4.8 ± 0.5	7.0 ± 1.0
PC	7.5 ± 0.8	8.6 ± 0.5
PE	8.9 ± 0.6	5.5 ± 0.8

Lipid content expressed as mol FA/mg chlorophyll and then converted to percentage of total polar lipids. Average values with SE are given for five separate experiments.

Fatty Acid Compositions of Total and Polar Lipids

Differences were observed between R and S plants in the fatty acid compositions of extracted total chloroplast membrane lipids (Table 3). The level of linolenic acid was higher and the levels of palmitic and linoleic acids slightly lower in chloroplasts from R *C. canadensis*. The R chloroplasts contained higher levels of unsaturated fatty acids than the S chloroplast.

TABLE 3

Comparison of fatty acid composition of total lipids of chloroplast membranes isolated from herbicide-resistant(R) and herbicide-susceptible (S) biotypes of *C. canadensis*.

Biotypes	Fatty Acid Composition/mol%/				
	16 : 0	18 : 1	18 : 2	18 : 3	18 : 3 / 18 : 2
Resistant	15.6	2 .0	8 .9	71.9	7.9
Susceptible	18. 7	2 .4	12. 8	62.6	4.8

The fatty acid compositions of the spearated individual lipid classes from R and S *C. canadensis* are given in Table 4. The glycolipids of chloroplasts from R *C. canadensis* had a higher level of linolenic acid and higher ratio of linolenic to linoleic acid than the S biotype. The chloroplasts from R *C. canadensis* also had a higher level of t-3-hexadecenoic acid and a lower palmitic acid concentration in PG. The R biotype contained a higher proportion of unsaturated fatty acids in glycolipids and PG. The ratio of total saturated to total unsturated fatty acids, inversely related to the membrane fluidity, was lower in MGDG, PG, DGDG and SQDG, but the same in PC and PE.

TABLE 4

Comparison of fatty acid composition of polar lipids of chloroplast membranes isolated from herbicide-resistant (R) and herbicide-susceptible (S) biotypes of *C. canadensis*.

Lipids	biotypes	Fatty Acids Compostion /mol%/						Ratio of Sat/Unsat Fatty Acids
		16:0	16:1	18:0	18:1	18:2	18:3	
MGDG	R	1.9	n.d	0.7	0.6	5.5	92.7	0.027
	S	3.9	n.d	1.0	2.7	7.7	84.7	0.051
DGDG	R	10.3	n.d	3.4	1.6	3.4	81.3	0.17
	S	13.3	n.d	4.7	2.4	6.2	73.4	0.21
SQDG	R	32.3	0.5	1.3	1.1	16.9	47.9	0.51
	S	36.9	1.2	1.0	2.4	17.6	40.9	0.61
PG	R	27.2	12.9	0.8	7.1	20.6	31.4	0.39
	S	35.8	8.6	0.8	6.7	16.4	31.7	0.58
PC	R	30.5	n.d	2.5	7.9	26.8	32.3	0.49
	S	31.9	n.d.	2.5	9.4	25.0	31.2	0.52
PE	R	41.2	n.d	2.5	6.0	26.4	23.9	0.78
	S	42.2	n.d	2.0	8.2	25.1	22.5	0.79

Fatty acids denoted by number of carbon atoms: number of double bonds.

Fluorescence Polarization of DPH in Total Lipid Vesicles

Figure 1 presents Arrhenius plots of the fluorescence polarization of DPH embedded in the total lipid liposomes. There are marked differences in the P values, and thus in the fluidity, of the R and S liposomes. The total polar lipids from the R chloroplast exhibited lower P values of DPH, and hence a more fluid lipid matrix, over the entire temperature range investigated. In the liposomes from S chloroplasts, the phase separation temperature was clearly detectable at around 27°C, as an inflexion point in Arrhensius plot. The thermotropic transition occurred in the temperature range 15-22.5°C in liposomes prepared from polar lipids of R chloroplasts. It is unlikely that a higher level of PE in R biotypes would be responsible for this phenomenon. PE, due to its polar head group, tends to increase the rigidity of the membranes instead of fluidizing them as observed here.

DISCUSSIONS

The target site of triazine, urea and uracil herbicides has been identified as the chloroplast thylakoid polypeptide herbicide binding Q_B - protein of 32 K Da in the photosystem II complex (Renger et al 1976) which is localised within the thylakoid membranes. Recent studies have shown that triazine-resistant plants normally synthesize this herbicide binding protein (Mattoo et al 1982) but in resistant chloroplasts it has a greatly reduced affinity to atrazine (Pfister and Arntzen 1979; Arntzen et al 1980).

Our results suggest that additional alteration of the structure and conformation in the herbicide-receptor region of the Q_B- protein may result in alteration of the binding affinity of some urea herbicides to this component. This change may contribute to a decrease of the herbicidal action. Further research is needed to clarify the nature of this herbicide resistance.

It is now generally agreed that a minor mutagenic modification of the Q_B- protein is responsible for the altered binding affinity of herbicides. The lipid environment is known to affect the lateral mobility and conformational flexibility of integral membrane proteins and that lipids may, therefore, control the accessibility of the herbicide binding sites of the Q_B- protein. In addition to a change in the primary structure of Q_B, the thylakoid lipid micro-environment may play a profound role in developing resistance to triazines.

The present results confirm and extend earlier observations showing that the lipid and fatty acid composition of the individual lipid species (Blein 1980; Pillai and St. John 1981; Burke et al 1982; Lehoczki et al 1984) of the R chloroplasts are different from those of the S chloroplasts. A greater degree of unsaturation was observed in MGDG, PG, DGDG and SQDG. As a result of these changes, the physical properties of the lipid matrix in the thylakoid membrane are also expected to be different in the R chloroplasts. From the saturated to unsaturated fatty acid ratios in the glycolipids, major polar lipid components making up 80% of the total polar lipid composition, a higher fluidity in the R C. canadensis may be predicted. The data presented in Fig 4 clearly show that the R biotype had a higher lipid matrix fluidity than the S chloroplasts. The P values were consistently lower for the former in the entire temperature range scanned. Further, the vesicles

of the R biotype exhibited two breaks in the temperature profile of P, while only one was present in the S profile. Assuming that the break at the higher temperature represents the onset, and that the lower temperature the completion of the phase separation, it can be concluded that in the R chloroplasts the thylakoid membranes are in a mixed fluid-gel state in only a limited temperature range.

The thylakoid lipids in the R chloroplasts differing in amount, in composition and in unsaturation of the fatty acids, and this may cause a deformation in the bilayer structure of the thylakoids. The major thylakoid lipids, MGDG, with a high proportion of unsaturated fatty acid constituents, particularly linolenic acid, have been assigned a functional role in maintaining the membrane fluidity and in regulating the balance between lamellar and non-lamellar lipid structures in the thylakoid membrane. Cone-shaped molecules like MGDG may serve conditions of special packing of large-molecule protein complexes in the thylakoid membranes. Thus, one might suggest that an increased amount of MGDG, with a higher proportion of unsaturated fatty acids, and a higher ratio of MGDG to DGDG in the thylakoids, will alter the assembly of protein complexes and the conformation of the Q_B-protein in the membranes. From the above results and those accumulated by others (Blein 1980; Pillai and St. John 1981; Burke *et al* 1982; Lehoczki *et al* 1984; Mattoo *et al* 1984), we have reached the conclusion that the lipid matrix is directly or indirectly involved in the normal function of herbicide binding protein, and formulated the hypothesis that resistance to herbicides may result form a change in the hervicide binding protein itself and in the lipid micro-environment of the Q_B-protein.

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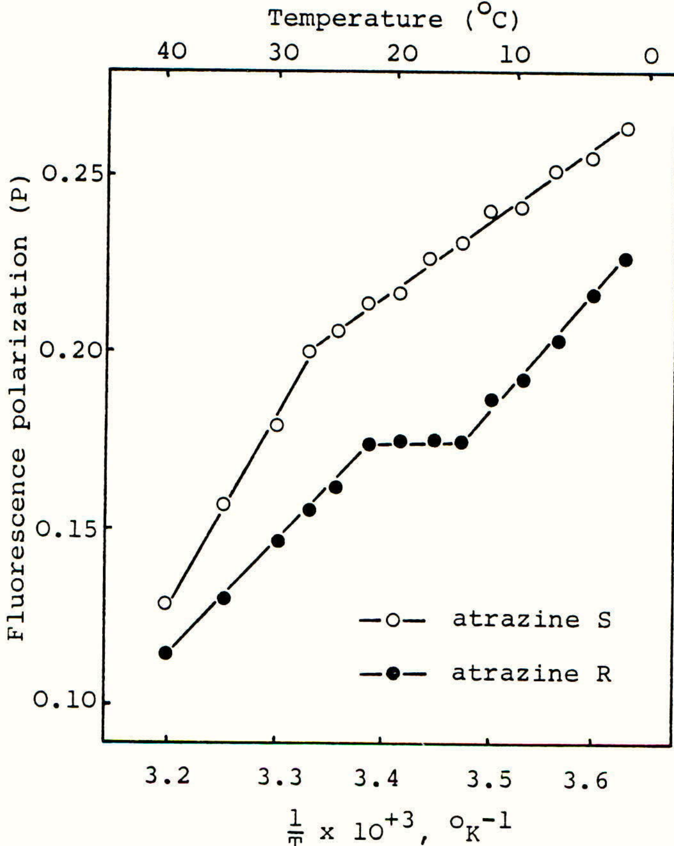

Fig. 1.
Arrhenius plots of the fluoresence polarization (P) of DPH embedded in the total lipid liposomes extracted from chloroplasts of C. canadensis.