SESSION 9B

HERBICIDAL ACTIVITY: SITES OF ACTION AND TARGETS FOR MANIPULATION. II

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SAFENING OF SULFONYLUREA HERBICIDES TO CEREAL CROPS: MODE OF HERBICIDE ANTIDOTE ACTION

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ABSTRACT

The safening action of a number of commercial antidotes with the sulfonylurea herbicides, chlorsulfuron and metsulfuron methyl, on cereal crops is related to the ability of the antidotes to increase the metabolism of the herbicide by the crop plant. For example, 2 to 5 fold increases in the metabolism rates of chlorsulfuron and metsulfuron methyl have been observed in wheat and corn. However, the rate of herbicide metabolism by sensitive broadleaf crops and weeds is not affected by the antidotes. The increase in sulfonylurea metabolism occurs within hours following antidote treatment. The protein synthesis inhibitor, cycloheximide, prevents the antidote metabolism effect.

INTRODUCTION

There has been considerable interest in the use of herbicide antidotes or safeners to enhance crop tolerance to a number of herbicides. The most successful safening reported to date is on corn and sorghum with thiocarbamate and acetanilide herbicides (Hatzios 1983, Parker 1983). The safening of chlorsulfuron on cereals has also been reported by these workers [Parker et al., (1980), Hatzios (1984)]. Mersie and Foy (1984) have reported the safening of corn against metsulfuron methyl injury.

In spite of the general interest in herbicide antidotes, little information exists on the mode of antidote action. Considerable research has been directed at understanding the safening action of the antidote, N,N-diallyl-2,2-dichloroacetamide (DDCA); however, there is still little agreement as to its mode of action. Proposals vary from competition for the site of herbicide action (Stephenson et al., 1979) and antagonism of the EPTC inhibition of fatty acid synthesis (Wilkinson & Smith 1975) to enhanced thiocarbamate detoxification via sulfoxidation and/or glutathione conjugation (Leavitt & Penner 1979, Lay & Casida 1976).

Metabolism of chlorsulfuron by tolerant crop plants has been shown to play a key role in the tolerance of cereal crops to Glean® (Sweetser et al., 1982). This paper shows that the safening action of commercial antidotes with the sulfonylurea herbicides chlorsulfuron and metsulfuron methyl is due to an increase in the rate of metabolic inactivation of these herbicides.

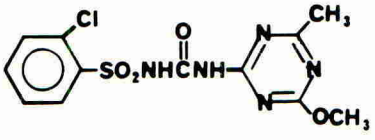
MATERIALS AND METHODS

All plant materials were grown in controlled-environment growth rooms in plastic pots containing the potting medium, Terra-Lite Metro-Mix®-350.

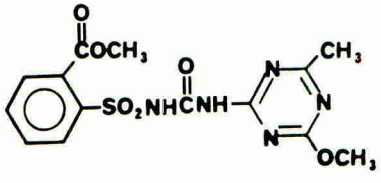
The [^{14}C] labeled sulfonylurea herbicides were synthesized by Du Pont NEN Products at the following levels of activity: [^{14}C-triazine] chlorsulfuron (15.2 μCi/mg), [phenyl-^{14}C(U)] metsulfuron methyl (24.8 μCi/mg), and [pyrimidine-2-^{14}C] sulfometuron methyl (153 μCi/mg).

Metabolism studies with the [^{14}C] sulfonylurea herbicides were made on excised leaves from plants 1 to 3 weeks old. The leaves were cut under water and the cut ends placed in 20 cc vials containing 2 cc of 2 ppm 14C herbicide. The leaves were removed from the vials after allowing a one hour uptake period, washed and the cut ends placed in 20 cc vials containing 5 cc of 1/4 strength Hoaglands solution. After one to eight hours, the leaves were washed with distilled H_2O, frozen in liquid N_2, and stored at -80°C until required for extraction. The frozen leaves were extracted with 80% acetone, and the level of unmetabolized herbicide determined by HPLC as described by Sweetser et al., (1982). The half-life ($T_{1/2}$) of the herbicide in the leaf was calculated from a semi-log plot of the percent unmetabolized herbicide vs. time.

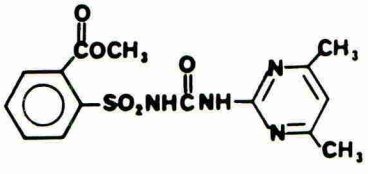
Chemical Structures



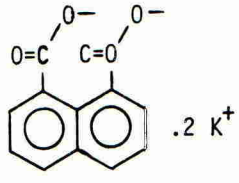
Chlorsulfuron



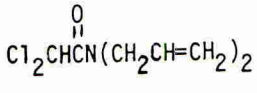
Metsulfuron Methyl



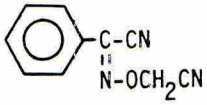
Sulfometuron Methyl



K^+ Na



DDCA



Cyoxmetrinil

RESULTS

Since metabolism has been shown to be the basis for crop tolerance to chlorsulfuron (Sweetser et al., 1982), this investigation was to establish whether the safening action of antidotes was due to their effect on herbicide metabolism.

The metabolism of the sulfonylureas in excised leaves follows pseudo first order kinetics (Figure 1). The metabolism of chlorsulfuron in wheat is rapid ($T_{1/2}$ <2 hours) while there is slow metabolism in Brassica kaber leaves.

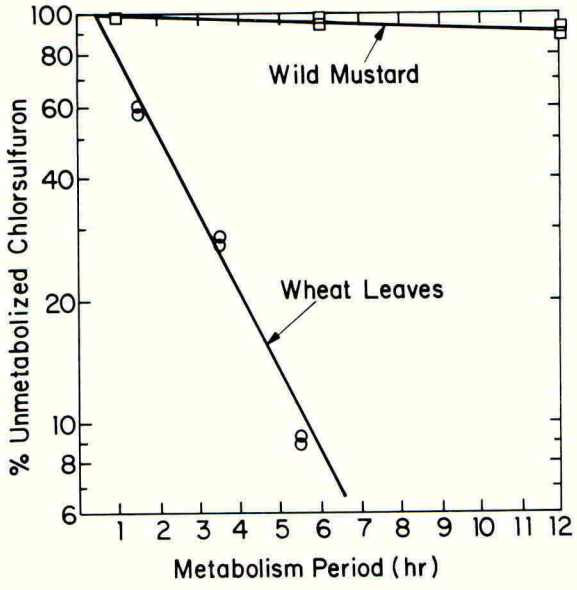


Fig. 1. Metabolism of Chlorsulfuron by Tolerant Wheat Leaves and Brassica kaber leaves.

The relationship of herbicide tolerance to its half-life for chlorsulfuron, metsulfuron methyl, and sulfometuron methyl is outlined in Table 1. The tolerant wheat, barley and wild oats show very rapid chlorsulfuron leaf metabolism with half-lives of 1 hour or less. Sensitive broadleaf crops and weeds show little or no chlorsulfuron metabolism ($T_{1/2}$ = 30 hours or greater). A similar pattern of metabolism is seen with metsulfuron methyl. The tolerance of wheat to metsulfuron methyl is less than that for chlorsulfuron and this is reflected in a slower rate of metsulfuron methyl metabolism by wheat leaves. Metabolism of sulfometuron methyl, a broad spectrum herbicide, is slow in both broadleaf plants and cereals.

TABLE 1

Metabolism of Sulfonylurea Herbicides by Isolated Leaves of Sensitive and Tolerant Plants

Plant	Herbicide	Metabolism Half-Life (hours)	Relative Tolerance
Wheat	Chlorsulfuron	0.8-2.2	T
Barley	"	0.8	T
Wild Oats	"	0.6-0.8	T
Corn	"	3.5-5-0	S-I
Soybeans	"	>30	S
Brassica kaber	"	>30	S
Convolvulus arvensis	"	>30	S
Wheat	Metsulfuron Methyl	2.0-3.9	T
Corn	"	5.5-7.6	S-I
Soybeans	"	>30	S
Brassica kaber	"	>30	S
Convolvulus arvensis	"	>30	S
Wheat	Sulfometuron Methyl	17.3	I
Corn	"	13.0	S-I
Soybeans	"	>30	S

* Relative Postemergent Tolerance
T = Tolerant
I = Intermediate
S = Sensitive

The tolerance of corn to chlorsulfuron and metsulfuron methyl is less than would be expected from their metabolism rates in corn leaves. This may be due to differences in the pathways of metabolism. Figure 2 illustrates the difference in the metabolic pathway for chlorsulfuron in wheat and corn. The metabolism of chlorsulfuron by wheat involves a hydroxylation reaction on the phenyl ring followed by rapid conjugation. Both metabolites are inactive as herbicides. This pathway is seen in corn, although there is also a second pathway which involves metabolism on the triazine portion of the molecule. Metabolite B-1 is not rapidly conjugated and still shows considerable herbicidal activity. Other factors such as uptake, translocation, and root metabolism of the herbicides may also play a role in the reduced tolerance of corn.

A postemergent treatment of corn with an antidote results in a dramatic increase in the rate of chlorsulfuron metabolism (Figure 3). The metabolic half-life has been reduced from 3.6 hours to 1.0 hour in corn leaves treated with K+ NA.

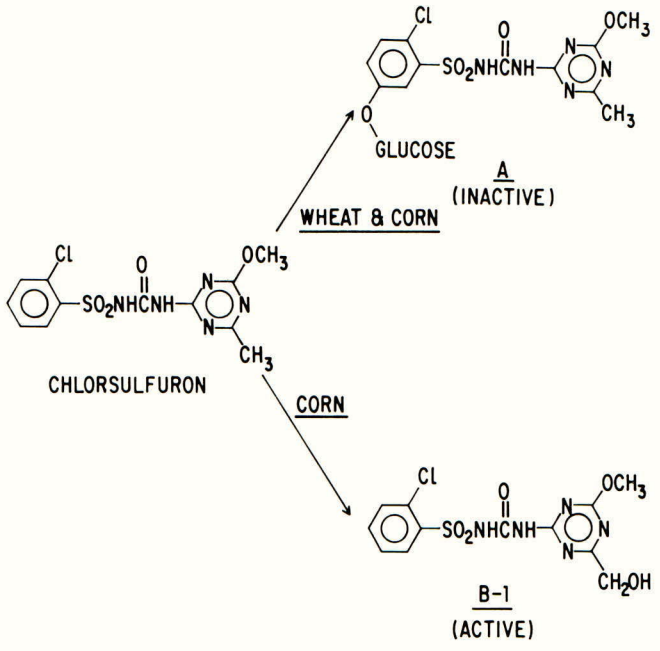


Fig. 2. Chlorsulfuron Metabolism by Wheat and Corn.

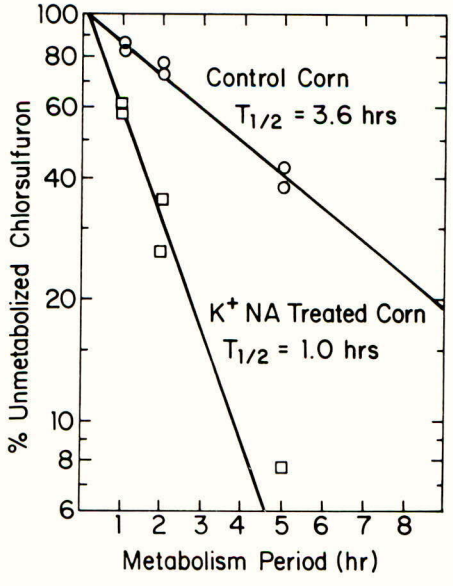


Fig. 3. Effect of K^+ NA on Chlorsulfuron Metabolism.

Similar increases in herbicide metabolism are seen with other commercial antidotes and other sulfonylurea herbicides (Table 2). The antidote treatments gave no significant increase in herbicide metabolism in sensitive broadleaf plants.

TABLE 2

Effect of Commercial Antidotes on Sulfonylurea Herbicide Metabolism by Isolated Leaves

Plant	Half-Life (Hours)			
	Control	K+ NA	DDCA	Cyoxmetrinil
	Chlorsulfuron			
Corn	3.5	1.0	1.1	1.6
Wheat	1.8	0.8	1.8	0.7
Soybeans	>50	>50		
Brassica kaber	>50	>50		
	Metsulfuron Methyl			
Corn	5.5	1.4	1.6	1.6
Wheat	2.0	1.0	1.8	0.8
	Sulfometuron Methyl			
Wheat	17.3	7.9	13.3	9.6

NOTE: Postemergent antidote treatment given 16 hours prior to the metabolism studies.

The increase in sulfonylurea metabolism following antidote treatment is very rapid and is over 80% complete in 3 hours following an antidote treatment via leaf uptake (Figure 4).

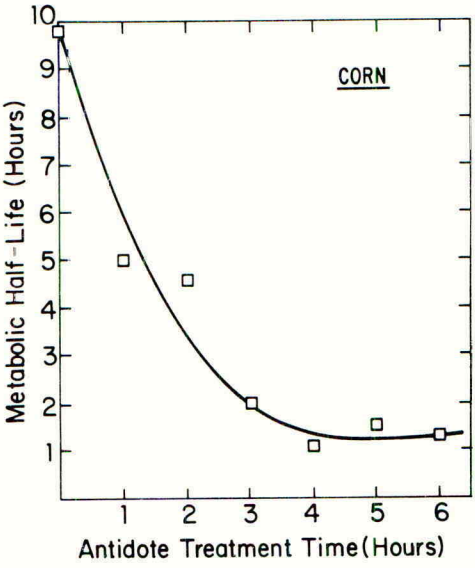


Fig. 4. Induction of Chlorsulfuron Metabolism with K^+ NA.

The increased herbicide metabolism following the antidote treatment is strongly inhibited by the protein synthesis inhibitor, cycloheximide (Table 3).

TABLE 3

Effect of Cycloheximide on Metabolism Induction by Antidotes

Leaf Treatment*	Metabolism Half-Life (Hours)
Control	11.8
Cycloheximide[1]	5.0
K+ NA[2]	0.7
Cycloheximide + K+ NA	4.5

* All leaf treatments made on excised leaves 5 hrs. prior to the one hour uptake of (^{14}C) chlorsulfuron.

1. Uptake concentration of cycloheximide = 20 ppm
2. Uptake concentration of K+ NA = 200 ppm

DISCUSSION

Metabolism of sulfonylurea herbicides to inactive metabolites by the crop plants is a key factor in crop tolerance. The data reported in this paper show that treatment of cereals with antidotes of widely different structures results in a rapid increase in the rate of this metabolism. Considerable differences are seen in the ability of various antidotes to safen different crops. For example, DDCA is a poor safener for chlorsulfuron and metsulfuron methyl on wheat and DDCA does not increase the metabolism of these herbicides in wheat. On the other hand, the rate of metabolism of sulfometuron methyl is increased by treatments with K+ NA, and cyoxmetrinil; although the metabolism is still not rapid enough to achieve significant safening.

The enzyme systems responsible for the metabolic inactivation of sulfonylurea herbicides have not been characterized; but in view of the metabolic pathways involved, it is probable that a cytochrome P-450 system is involved. The cytochrome P-450 in insect and mammalian systems is reported to be inducible by a number of chemicals. The fact that the protein synthesis inhibitor, cycloheximide, completely prevents the antidote induction of metabolism suggests that the induction of a P-450 system in the cereal plants may occur.

ACKNOWLEDGMENTS

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ROLE OF GLUTATHIONE-RELATED ENZYMES IN THE MODE OF ACTION OF HERBICIDE ANTIDOTES

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ABSTRACT

Dose-response, time-course and structure-activity studies show that maize (Zea mays L.) seedlings treated with antidotes (N,N-diallyl-2,2-dichloroacetamide and related compounds) against the phytotoxicity of chloroacetanilide and thiocarbamate herbicides contain increased levels of glutathione S-transferase. Our results, together with similar findings for glutathione synthetase and cytosolic glutathione S-transferase in other laboratories indicate that activities of the enzymes regulating the cellular levels of glutathione and the rate of its conjugation with the herbicide molecules are critical for the prevention of chloroacetanilide and thiocarbamate injury to maize plants.

INTRODUCTION

Chemical antidotes of the acetamide type known for their ability to counteract phytotoxicity of chloroacetanilide and thiocarbamate herbicides to maize (Zea mays L.) have also been found to cause a marked increase in the levels of glutathione (GSH) and cytosolic glutathione S-transferase (GST, EC 2.5.1.18) in this plant (Lay and Casida 1976, Mozer et al. 1983, Lay and Niland 1985, Adams et al. 1983, Kőmives et al. 1985, Ezra et al. 1985).

Since GST-mediated metabolism of thiocarbamates and chloroacetanilides in maize leads to the formation of inactive GSH-conjugates (Eq. 1 and 2, Lay and Casida 1976, Lay and Niland 1985, Mozer et al. 1983), antidote effects may be due to increased rate of herbicide detoxication in protected plants (Lay and Casida 1976).

$$R^1\text{-S-CO-N}R^2R^3 \longrightarrow R^1\text{-SO-CO-N}R^2R^3 \xrightarrow[\text{GST}]{\text{GSH}} \text{GS-CO-N}R^2R^3 \quad (1)$$

$$CH_2Cl\text{-CO-N}R^4R^5 \xrightarrow[\text{GST}]{\text{GST}} \text{GS-CO-N}R^4R^5 \quad (2)$$

In this paper we report data on the effects of acetamide antidotes I-IV on the activity of microsomal GST and glutathione reductase (GR, EC 1.6.4.2). Microsomal GST may be of importance in the GSH-conjugation reactions of lipid soluble electrophilic xenobiotics (Morgenstern and DePierre 1983),

$CH_3\text{-CO-N}(CH_2\text{-CH=CH}_2)_2$ (I) $CH_2Cl\text{-CO-N}(CH_2\text{-CH=CH}_2)_2$ (II)
$CHCl_2\text{-CO-N}(CH_2\text{-CH=CH})_2$ (III) $CCl_3\text{-CO-N}(CH_2\text{-CH=CH}_2)_2$ (IV)

while GR catalyzes the enzymatic reduction of glutathione disulfide (GSSG) to GSH, and plays an important role in maintaining a high GSH/GSSG ratio (Foster and Hess 1980). In bioassays, the antidotes showed superior (III, R-25788, commercial antidote), moderate (II and IV) and little or no biological activity (I) (Lay and Casida 1976). For comparison, antidote effects on GSH content and on the levels of cytosolic GST were also measured.

MATERIALS AND METHODS

Chemicals

EPTC and R-25788 were previous samples (Dutka and Kőmives 1983). N-Acetyl, -chloroacetyl, and -trichloroacetyl diallylamine were synthesized by known method (Stephenson et al. 1978).

Triton X-100, 1-chloro-2,4-dinitrobenzene, GSH, GSSG, and other compounds not indicated above were from commercial sources, and were used without further purification.

Plant material

Seeds of maize (Pioneer 3950 hybrid, kindly provided by Dr. E.Széll, Cereal Research Institute, Szeged, Hungary) were germinated at 28 ^{o}C in an incubator for 60 h. Chemicals were applied at given concentrations to 14x2 cm Petri dishes containing uniformly sized maize seedlings. At given intervals seedlings were removed and root and shoot tissues were separated for analysis.

Extraction and assay of GSH

GSH content of roots of maize seedlings was determined spectrophotometrically using 5',5-dithiobis-(2-nitrobenzoic acid)(Fedtke 1981).

Isolation of microsomes

The method of Dr. U.Enkhart (Technische Hochschule, Mühlhausen, GDR; private communication) was used to isolate microsomes from shoots of maize seedlings. Briefly, plant material was rinsed with distilled water, blotted dry and ground to a fine powder with liquid nitrogen in a mortar and pestle. The frozen tissue powders were slurried with 4 volume of 0.1 M potassium phosphate buffer (pH 7.6), containing 20 v/v % glycerine, 10 mM KCl, 10 mM $MgCl_2$, 10 mM EDTA, 10 mM sodium metabisulfite and 5 mM mercaptoethanol. After standing 15 min the slurry was squezzed through 4 layers of cheesecloth and differentially centrifuged. The microsomal fraction was sedimented at 105,000 g for 90 min, after removal of larger particulate matter by centrifugation at 1500 g for 10 min, and 10,000 g for 15 min. The microsomes were washed twice with 0.1 M Tris-HCl (pH 8.0), in order to remove cytosolic contamination (Morgenstern and DePierre 1983).

Assay of GST

Cytosolic and microsomal GST were assayed spectrophotometrically using 1-chloro-2,4-dinitrobenzene as second substrate (Mozer et al. 1983, Morgenstern and DePierre 1983). The 3 ml assay mixture contained 0.1 M potassium phosphate (pH 6.5), 0.2 v/v % Triton X-100 (only in case of microsomal GST), 5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene, and varying amounts of 105,000 g supernatant or microsomal suspension. The reaction was followed continuously in a thermostated (25±0.05 ^{o}C) chamber of a spectrophotometer at 340 nm.

Assay of GR

GR activity was assayed spectrophotometrically (Foster and Hess 1980) by monitoring GSSG dependent oxidation of NADPH at 340 nm in 0.15 mM NADPH, 3 mM $MgCl_2$, 0.5 mM GSSG, and 50 mM Tris-HCl (pH 7.5).

Protein estimation

Protein was estimated by the method of Lowry et al. (1952) using bovine serum albumin as standard.

RESULTS

Time-response relationships

Exposure of maize seedlings to 0.1 mM III detectably increases the root GSH content and the levels of the shoot enzymes studied. The observed changes occur within 12 h, and reach 239-284 % values relative to the controls (Table 1).

Dose-response relationships

Antidote III increases the root GSH content and the levels of the shoot enzymes studied in maize seedlings in a concentration dependent manner, from 10 µM to 0.1 mM. Higher doses of the antidote lead to larger responses (Table 2).

TABLE 1

Effect of treatment time of 2.5-day old maize seedlings with 0.1 mM III on the levels of root GSH, shoot cytosolic and microsomal GST, and shoot GR

Time (h)	Ratio[a] (treated/control)			
	GSH	Cytosolic GST	Microsomal GST	GR
0	0.97±0.08	1.15±0.12	1.02±0.06	0.90±0.21
6	0.91±0.10	1.05±0.08	1.19±0.11	0.92±0.23
12	1.27±0.09	1.30±0.12	1.28±0.12	0.99±0.18
24	1.98±0.15	2.06±0.24	2.38±0.18	1.78±0.22
48	2.39±0.20	2.76±0.31	2.84±0.53	2.55±0.61

[a]Control values for GSH, cytosolic and microsomal GST, and GR were 0.47±0.06 μmol/g fresh weight, 0.88±0.11 μmol/min.mg protein and 62±8 nmol/min.mg protein, and 0.15±0.03 nmol/min.mg protein, respectively

TABLE 2

Effect of 24-h exposure of 2.5-day old maize seedlings to different concentrations of III on the levels of root GST, shoot cytosolic and microsomal GST, and shoot GR

III concn (M)	Ratio[a] (treated/control)			
	GSH	Cytosolic GST	Microsomal GST	GR
10^{-7}	0.95±0.07	1.14±0.11	0.92±0.12	1.05±0.24
10^{-6}	1.29±0.11	1.64±0.20	1.18±0.13	1.53±0.19
10^{-5}	1.95±0.25	1.59±0.18	2.37±0.31	1.92±0.29
10^{-4}	1.98±0.15	2.06±0.24	2.38±0.18	1.78±0.22

[a]Values for control plants as in Table 1

Structure-activity relationships

The most effective compound at 0.1 mM concentration in increasing the root GSH content and the levels of the shoot enzymes studied was III. Less effective, although still active were II and IV. No significant effects were detected after treatments with I.

TABLE 3

Effect of 24 h exposure of 2.5-day old maize seedlings to 0.1 mM I-IV on the levels of root GSH, shoot cytosolic and microsomal GST, and shoot GR

Antidote	Ratio[a] treated/control			
	GSH	Cytosolic GST	Microsomal GST	GR
I	0.92±0.06	1.14±0.13	1.03±0.13	0.88±0.23
II	1.57±0.18	1.68±0.09	1.32±0.14	1.50±0.21
III	1.98±0.15	2.06±0.24	2.38±0.18	1.78±0.22
IV	1.32±0.10	1.28±0.10	1.75±0.22	1.45±0.14

[a]Values for control plants as in Table 1

DISCUSSION

In accord with literature findings (Lay and Casida 1976, Lay and Niland 1985, Mozer et al. 1983, Carringer et al. 1978, Ezra et al. 1985, Adams et al. 1983), treatments of maize seedlings with acetamide-type antidotes increased the root GSH content and the activity of cytosolic GST in the shoot (Table 1-3). Time course, dose-response, and structure-activity data (Table 1-3) indicate closely similar increases in the levels of GR and microsomal GST. From these results, it appears that activities of the enzymes that regulate the GSH levels in maize seedlings and the rate of its reaction with thiocarbamate sulfoxides and chloroacetanilides are regulated in a coordinated manner.

GSH may have multiple functions in plant cells which relate to protection against herbicide damage. In chemical (Lay and Casida 1976, Carringer et al. 1978, Leavitt and Penner 1979) and/or GST-catalyzed reactions (Lay and Casida 1976, Mozer et al. 1983, Ezra et al. 1985, Shimabukuro et al. 1978) GSH forms non-phytotoxic conjugation with herbicides. In addition, GSH can function indirectly, via an ascorbate-dehydroascorbate cycle, to maintain low endogenous levels of hydrogen peroxide (Foster and Hess 1980) that may be generated in herbicide-stressed plants (Dodge 1983).

Thus, elevated levels of GSH as a result of increased synthesis (Adams et al. 1983) and increased activity of GR (Table 1-3), as well as higher activity of cytosolic and microsomal GST indicate an enhanced ability of the plant to detoxify thiocarbamate sulfoxide metabolites and chloroacetanilide herbicides.

Cytosolic and microsomal GST enzymes in mammals may act as glutathione peroxidases catalyzing the reaction between GSH and lipophilic hydroperoxides (Morgenstern and DePierre 1983), thereby protecting cell membrane polyunsaturated fatty acid moieties against peroxidation. Though not reported in plants, this activity of the maize GST enzymes may explain partial protection by III against phytotoxicity of herbicides that are not detoxified by GSH-conjugation (e.g., linuron, Parker 1983, Hatzios 1984) and induce lipid peroxidation in plants (Dodge 1983).

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AMINOBENZOTRIAZOLE AS A SYNERGIST OF UREA HERBICIDES

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ABSTRACT

Phenylurea herbicides are primarily detoxified by hydroxylation and N-dealkylation. On the other hand, 1-aminobenzotriazole has been shown to act as a suicide substrate of animal and plant P-450 monooxygenases. Simultaneous administration of chlortoluron or isoproturon to wheat plants showed that (1) at appropriate concentrations, ABT alone was not phytotoxic , (2) it strongly inhibited the detoxication of the two herbicides, and (3) it exerted a synergistic effect on the phytotoxicity of both herbicides.

INTRODUCTION

Various physiological and biochemical factors can affect the responses of plants to herbicides. Among these factors, the capacity to metabolize foreign compounds enables the plant to control its intracellular content of herbicide. This important parameter, which affects both the efficiency and the selectivity of the herbicides, is itself governed by the structure of the foreign molecule and the metabolic equipment of the plant.

Although it is possible to control to some extent the metabolic processes within crops to achieve this selectivity, this is seldom possible with weeds. Another possible strategy consists in making use of substances that act specifically on the metabolism of herbicides, and this concept has been put into practice through the use of safeners and synergists.

Several safeners can be used in association with herbicides (Stephenson & Ezra, 1982). However, it must be stressed that a mechanism of action has been established only in the case of R-25788, which appears to enhance the rate of detoxication of EPTC in maize (Lay & Casida, 1978).

If one excludes the cases where combinations of herbicides by themselves lead to increased effectiveness and widened host range, the occurrence of herbicide synergists is less well documented. A well known example is ammonium thiocyanate, which increases the action of aminotriazole by lowering the formation of the less active and less mobile alanine derivative (Smith et al., 1969; Carter, 1965). In a somewhat similar way, it has been shown that carbamate and organophosphate insecticides potentiate the action of propanil by slowing down its hydrolysis ; more precisely, these insecticides inhibit the aryl acylamidase responsible for this hydrolysis (Frear & Still, 1968 ; Matsunaka, 1968).

Although examples of synergism are few, they indicate that this concept can be put to practical use, and in this paper, we show that the

activity of herbicides belonging to the substituted urea family can be enhanced in this way, at least in laboratory experiments.

According to Gross et al. (1979), chlortoluron (1-(3-chloro-4-methylphenyl)3,3-dimethylurea) is first metabolized in wheat (Triticum aestivum) through hydroxylation and N-demethylation reactions, then the resulting metabolites undergo conjugation reactions with sugar molecules (Fig. 1). Besides the extent of degradation of the parent herbicide, the nature of the intermediate metabolites is of great interest, since the hydroxymethyl-chlortoluron is inactive, while the mono-methyl metabolite retains some phytotoxicity (Ryan et al., 1981). In wheat, detoxication of the herbicide is mostly achieved through hydroxylation, only the first N-demethylation being of some importance in this case. Another substituted urea, isoproturon (1-(4-isopropylphenyl)3,3-dimethylurea) is metabolized through the same type of reactions, but here the bis-demethylated metabolite is formed in a greater amount,as will be shown below.

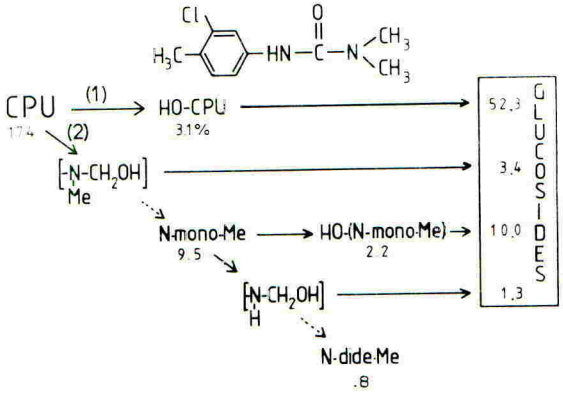

Fig. 1. Metabolism of chlortoluron (CPU) in wheat.

It is generally admitted that both hydroxylation and N-demethylation reactions are mediated by microsomal mixed-function oxidases (Hatzios & Penner, 1982). Such microsomal systems have been partially isolated and characterized from cotton, (Frear et al.,1969) and cucumber and pea (Makeev et al., 1977), and shown to catalyze respectively the N-demethylation of phenylureas, and the ring hydroxylation of 2,4-D.

As we have seen above, it is possible to decrease the rate of pesticide detoxication by means of compounds able to inhibit the relevant

Fig. 2. 1-Aminobenzotriazole.

reactions. Such a strategy is commonly used to potentiate the pyrethroid insecticides with 3,1-benzodioxole derivatives (e.g. piperonylbutoxide), which inhibit the insecticide degradation catalyzed by P-450 mixed-function oxidases (see for example Corbett, Wright & Baillie, 1984).

In this paper we examine the effect of another inhibitor of mixed function oxidases, 1-aminobenzotriazole (ABT) on the metabolism and phytotoxicity of two urea herbicides in wheat. ABT (Fig. 2) is a suicide substrate of cytochrome P-450, which transforms it in a reaction intermediate able to covalently bind with 1 or 2 haem nitrogens. The reaction leads to the irreversible inactivation of P-450 cytochromes (Ortiz de Montellano, 1981). This action of ABT has been demonstrated in vitro on a plant system : the cinnamate 4-hydroxylase of Jerusalem Artichoke tuber microsomes was inhibited by 90% after 30 min incubation (Reichhart et al., 1982).

MATERIALS AND METHODS

Metabolic studies

Wheat plants at the two leaf stage absorbed ^{14}C-labeled chlortoluron (CPU) or isoproturon (IPU) dissolved in the nutrient solution through the roots, during a 24 h period. The plants were then transferred to nutrient solution without herbicide for a further period of 4 d. When applied, ABT was also added to the nutrient solution for the whole period of the experiment (Fig. 3).

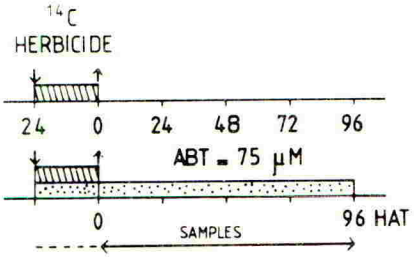

Fig. 3. Organization of the metabolic experiments.

Growth measurements

Pre-germinated wheat seeds were sown in river sand and watered with mineral nutrient solution containing appropriate concentrations of herbicides and ABT. Treatments were repeated three times in a completely randomized factorial design. After three weeks, the aerial parts were sampled, and their dry weight determined.

RESULTS

Effects of ABT on the metabolism of CPU and IPU

The metabolism of herbicides was followed in the aerial parts of the plants. These contained at least 90% of the absorbed radioactivity at the end of the treatment.

It was first established that ABT did not change the absorption of the herbicide : the total radioactivity taken up was the same in plants treated by the herbicides alone, or by the herbicides plus ABT.

ABT strongly inhibited the metabolism of CPU and IPU. In control plants, the degradation of herbicides followed first

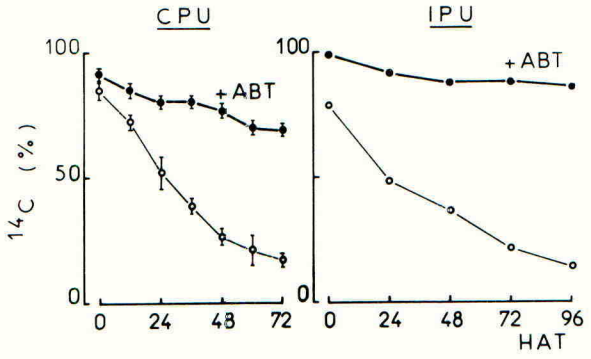

Fig. 4. Time course of chlortoluron (CPU) and isoproturon (IPU) degradation.

order kinetics, with half-lives of 29 h for CPU and 39 h for IPU. Fig. 4 shows that these degradation rates were so strongly reduced in ABT-treated plants, that it was impossible to estimate half-lives. At the end of the 24 h treatment, the CPU concentration was 14,1 µg/g f.w.; 4-d later, that concentration was lowered to 2,4 µg/g f.w. in control plants, but only to 10,2 µg/g f.w. in ABT-treated plants. Similar results were found for IPU.

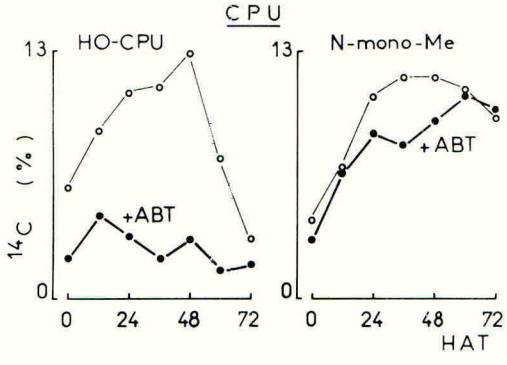

Fig. 5. Time course of the formation of OH-chlortoluron (OH-CPU) and N-monomethyl chlortoluron (N-mono-Me).

Chromatographic analysis revealed the existence of four intermediate metabolites of CPU, namely OH-CPU, N-monomethyl-CPU, N-monomethyl-OH-CPU, and bis-demethylated-CPU. All these intermediates were also found in ABT-treated plants, but in reduced amounts. That was especially true for OH-CPU, the formation of which was very strongly reduced by ABT treatment.

The various metabolic reactions were inhibited to different extents by ABT. The formation of N-monomethyl-CPU was less affected, so that it represented a predominant metabolite in ABT-treated plants (Fig. 5). These findings were consistent with a preferential inhibition of hydroxylation reactions (Fig. 1).

The situation was slightly different with IPU. A relatively large accumulation of N-monomethyl derivative was also observed, but in this case it could be explained partly by the strong inhibition of the formation of the bis-N-demethylated metabolite (Fig. 6).

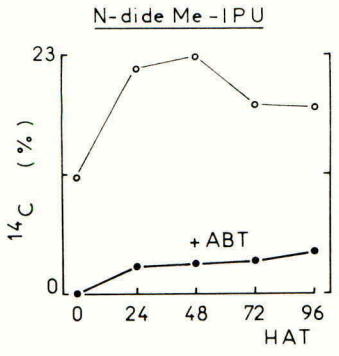

Fig. 6. Formation of bis N-demethylated isoproturon (N-dide-IPU).

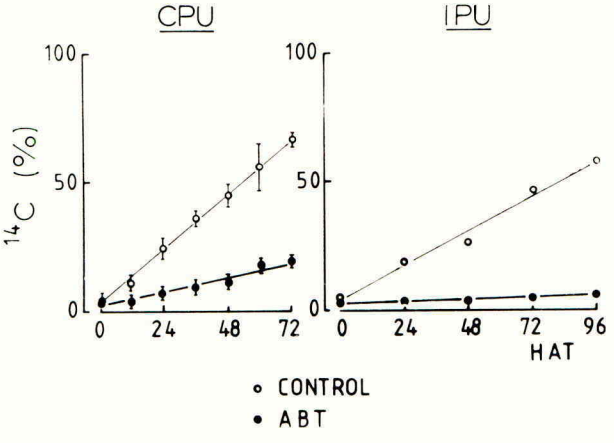

Fig. 7. Formation of conjugate metabolites of chlortoluron (CPU) and isoproturon (IPU).

Finally, the formation of the terminal metabolites, i.e. the sugar conjugates, was also strongly reduced with both herbicides (Fig. 7).

Synergistic effect of ABT on the phytotoxicity of phenylureas :

In the experiments reported here, wheat plants were treated with non- toxic or slightly toxic doses of herbicides, supplemented with various concentrations of ABT, which in no case were toxic by themselves. Fig. 8 shows that the effect of subtoxic or slightly toxic concentrations of CPU or IPU were strongly enhanced by addition of ABT, and from statistical analysis it was concluded that the effects were significant. For example, at the subtoxic concentration of 2.4 µM, the action of CPU was linearly enhanced by increasing concentrations of ABT (Fig. 8). A similar effect was found with a 1.2 µM concentration of IPU. These clear-cut increases of phytotoxicity thus indicated a synergistic effect.

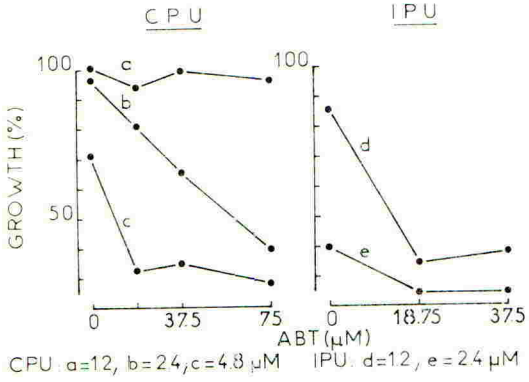

Fig. 8. Synergistic effect of ABT on the phytotoxicity of chlortoluron (CPU) and isoproturon (IPU).

DISCUSSION

The above results thus provide a coherent picture of the influence of ABT on the activity of phenylureas, since the observed inhibition of the herbicide detoxication actually resulted in an increase of the phytotoxic effects. However, it should be noted that our experimental system did not provide ideal conditions, since it involved some accumulation of a metabolite not devoid of phytotoxicity (the N-monomethyl derivative). It now remains to be shown that such a strategy can enhance the effect of herbicidal compounds on weeds.

A second conclusion is that mixed-function oxidases are probably responsible for the first steps of the detoxication of phenylureas. ABT, which does not affects peroxidase, is a powerful inhibitor of cinnamate 4-hydroxylase, which is a P-450 system specific to plants (Reichhart *et al.*, 1982). Indeed, it has been found that *in vitro*, CPU can be demethylated by Jerusalem Artichoke microsomes. This reaction was found to be sensitive to CO, the action of which could be reversed by light (Fonné *et al.*, 1984). However, it must be kept in mind that ABT is not entirely specific for P-450 systems : it can also inactivate chloroperoxidase (Ortiz de Montellano *et al.*, 1984), as well as pea peroxygenase (Durst, unpublished results).

Finally, these results raise the problem of the specificity of the metabolic reactions undergone by herbicides in plants. As illustrated in Table 1, this specificity depends on the plant species and the nature of

TABLE 1

Metabolism of chlortoluron (CPU) and isoproturon (IPU) by various plant species and enzyme preparations.

CPU hydroxylation	in vivo	Lolium perenne	low
		Triticum aestivum	very active
	in vitro	Helianthus tuberosus	negligible
CPU demethylation	in vivo	Lolium perenne	very active
		Triticum aestivum	low
	in vitro	Helianthus tuberosus	very active
		Helianthus annuus	very low
CPU bis-demethylation	in vivo	Triticum aestivum	low
IPU bis-demethylation	in vivo	Triticum aestivum	very active

the absorbed molecule. For example, CPU is actively hydroxylated in vivo by wheat (T. aestivum), as we have seen, but only weakly by Lolium perenne (Ryan et al., 1981).This molecule is, however, actively demethylated by L. perenne (Ryan et al., 1981), but only weakly by T. aestivum. In vitro activities also differ according to the source of the microsomal preparation : Helianthus tuberosus microsomes possess a strong demethylase activity, while this reaction proceeds only feebly with H. annuus preparations (Durst, unpublished results). The difference in susceptibility according to the nature of the substrate molecule is illustrated by the comparison between the second N-demethylation carried out by one species (T.aestivum), respectively on CPU (slow reaction) and IPU (fast reaction). Even the first N-demethylation is more actively achieved by T. aestivum on IPU than on CPU. The diversity of the enzymatic equipment of various plant species and the apparently narrow specificity of the enzymatic systems involved thus suggest the possibility of modulating, with the use of enzyme inhibitors and inducers, not only the level of activity, but also the selectivity of herbicides.

ACKNOWLEGMENTS

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TRIAZINE RESISTANT GRASS WEEDS: CROSS RESISTANCE WITH WHEAT HERBICIDE, A POSSIBLE THREAT TO CEREAL CROPS

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ABSTRACT

Triazine resistant biotypes of two grass weeds: awned canary-grass (Phalaris paradoxa) and black-grass (Alopecurus myosuroides), exhibited resistance to methabenzthiazuron, a urea herbicide, both in whole plant and isolated chloroplasts. Both triazine resistant (R) and susceptible (S) biotypes were equally sensitive to chlortoluron. An increased tolerance to diclofop-methyl was also observed. Competition and fitness studies have shown that the R biotype of P. paradoxa was not inferior to the S biotype in any of the growth parameters examined. The R biotype was significantly superior to the S biotype in emergence rate, plant height and main spike weight under non-competitive conditions. These findings indicate a potential threat of an R biotype infestation of cereal fields and must be considered when designing weed control programs.

INTRODUCTION

The appearance of triazine resistant weeds is now a well documented phenomenon (Le Baron & Gressel 1982; Gressel 1985). Resistant weeds are generally found in places which were successively treated for many years with a single herbicide without rotation. Most of the reports deal with broad leaf weeds but triazine resistance was also described for several grass weeds such as Poa annua (Darmency & Gasquez 1981), Brachypodium distachyon (Gressel et al. 1983), and Setaria viridis (Gasquez & Compoint 1981). We have recently discovered in Israel along triazines treated roadsides three triazine resistant biotypes of the following grass weeds: P. paradoxa, A. myosuroides, and rye-grass (Lolium rigidum), which exhibited a plastidic mode of resistance to triazines (Yaacoby et al. 1985). These R biotypes were also resistant to triazinone herbicides.

Little information is available at present on cross resistance to herbicides other than triazines. Oettmeier et al. (1982) found in chloroplasts isolated from a triazine resistant biotype of Amaranthus retroflexus cross resistance to various uracil and urea herbicides. An increased tolerance to monolinuron and methabenzthiazuron was also reported for atrazine resistant Brassica campestris and Chenopodium album (Ducruet & De Prado 1982). A 2-to-3 fold increased tolerance to methabenzthiazuron and chlortoluron was recently reported in two different A. myosuroides stocks without cross resistance between stocks (Niemann & Pestemer 1984).

The degree of incidence of R biotypes in cultivated fields is dependent on their fitness i.e., their ability to compete with the S biotype (Warwick & Black 1981). Studies comparing the growth, physiological characteristics and fitness of R and S biotypes of several broad leaf weeds, have indicated that in the absence of herbicide the R biotypes were inferior at all levels of interbiotype competition

investigated (Conard & Radosevich 1979; Warwick & Black 1981; Ahrens & Stoller 1983; Holt & Radosevich 1983; Putwain et al. 1984; Gressel & Ben-Sinai 1985) One notable exception is the case of Chenopodium strictum where the R and S biotypes appear to be equally competitive (Warwick & Black 1981).

P. paradoxa and A. myosuroides are of major agronomic importance worldwide (Holm et al. 1979), infesting mainly winter and spring grown cereals, and causing severe yield losses (Hollies 1982). Methabenzthiazuron, chlortoluron and diclofop-methyl are among the commonly used herbicides for their control in cereals.

The purposes of this study are: (a) to examine the response of atrazine resistant biotype of P. paradoxa and A. myosuroides to wheat herbicides and (b) to compare the relative fitness of R and S biotypes under competitive and non-competitive conditions.

MATERIALS AND METHODS

Whole plant studies:

Seeds of P. paradoxa and A. myosuroides from separate populations of triazine-resistant and triazine-susceptible biotypes were collected from roadsides and adjacent fields in Israel as described previously (Yaacoby et al. 1985). The seeds (c. 30-50) were sown in separate rows 1-to-2 cm deep, in 20x20x6 cm plastic pots containing loamy sand soil. Pre-emergence treatments with formulated herbicide were applied immediately after sowing, using a chain driven laboratory sprayer at a spray volume of 300 litre/ha. Post-emergence treatments were similarly applied 35-to-40 days after sowing when seedlings were at the 3-to-4 leaf stage. The plants were grown in a screen-house under the ambient environmental conditions prevailing in the Israeli winter. The above-ground parts (shoots) were harvested 38 and 15 days after treatment for pre- and post-emergence treatments, respectively. Both fresh and dry weight were determined.

Isolation of chloroplasts and electron transport assay:

S and R plants of P. paradoxa and A. myosuroides were grown separately in 90 litre containers in soil in the screen house. Plants were fertilized weekly with a soluble 20-20-20 (N-P-K) fertilizer and irrigated as required. Leaf blades (5-to-20 g) were taken from the second fully expanded upper leaf when plants were 20-to-50 cm tall. Chloroplasts were isolated and electron transport was estimated by measuring oxygen evolution as previously described (Yaacoby et al. 1985). Analytical grade herbicides were added to assay mixtures as aliquots of stock solutions in 1% ethanol. All control assays contained an equal volume of 1% ethanol, which did not affect electron transport.

Fitness studies:

Fitness was estimated using several parameters. Seeds of R and S biotypes of P. paradoxa were separately sown in polystyrene trays divided into 96 chambers (35 ml each), filled with a mixture of loamy sand soil and peat (1:1 v/v). A single seed was sown in each chamber. The containers were transferred to a growth chamber (18/10°C day/night for 12 h photoperiod) for germination. Percent seedling emergence was recorded 2 weeks after sowing. The data were analysed as a completely randomized design with four replicates.

Uniform seedlings at the 1-to-2 leaf stage were removed with the sowing mixture and transplanted in polystyrene pots (60x50x30 cm) containing loamy sand soil. The seedlings were planted 6.7 cm apart in rows that were spaced 8.3 cm apart. There were 8 seedlings/row and 5 rows/pot, giving 40 seedlings/pot. There were five treatments corresponding to the different proportions of R and S seedlings in a given pot as follows: 100% R; 75% R, 25% S; 50% R, 50% S; 25%R, 75% S; 100% S. The plant arrangement within the grid was such that the two biotypes were evenly distributed throughout the pot. The pots were transferred to a screen house and were fertilized weekly and irrigated as described above. At harvest, 150 days after sowing, plant height, main spike dry weight and total shoot dry weight were determined. The data were analysed as a completely randomized design with three replications.

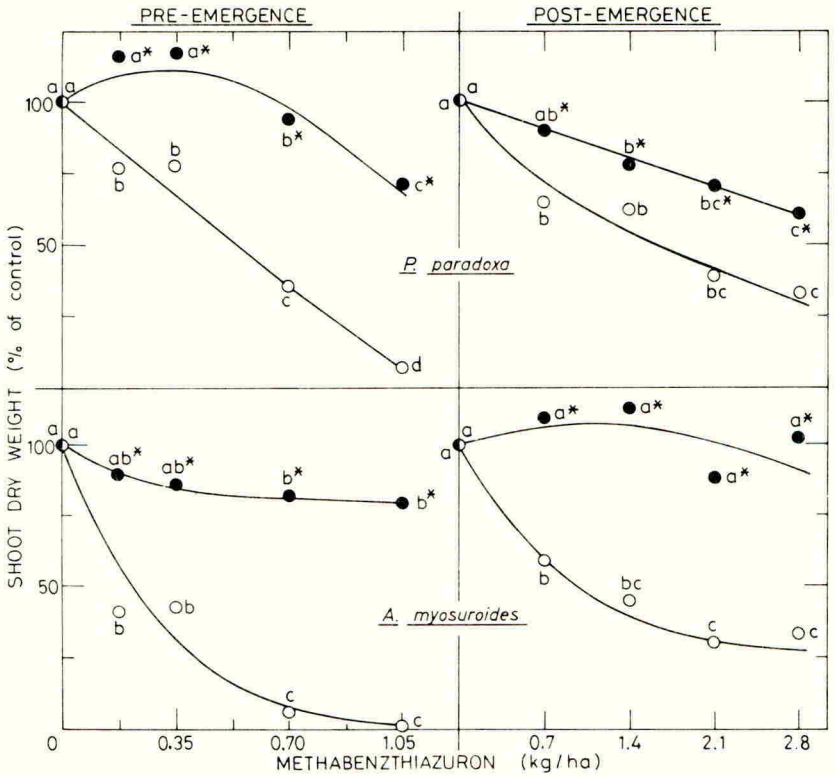

Fig. 1. Response of S (o--o) and R (•--•) biotypes of P. paradoxa and A. myosuroides to pre- and post-emergence applied methabenzthiazuron. Means in each biotype followed by the same letter are not significantly difference at the 5% level. One star (*) denotes a significant different (5%) between biotypes at the designated herbicide rate.

When each biotype was grown separately under non-competitive conditions, the R biotype of P. paradoxa was superior to the S biotype in its emergence rate. The R plants were taller and developed larger spikes on the main stem (Table 1). However, when the two biotypes were grown together at constant density but in varying proportions (Figure 4), each contributed to total shoot dry weight in proportion to its part in the population.

TABLE 1

Growth parameters for triazine-susceptible (S) and -resistant (R) biotypes of Phalaris paradoxa as determined under non-competitive conditions.

Biotype	Emergence (%)	Plant height (cm)	Main spike dry wt. (mg)
S	42.5 b	66 b	654 b
R	76.7 a	79 a	766 a

Means followed by the same letter in each column are not significantly different at the 5% level of Student's, Neuman Keuls multiple range test.

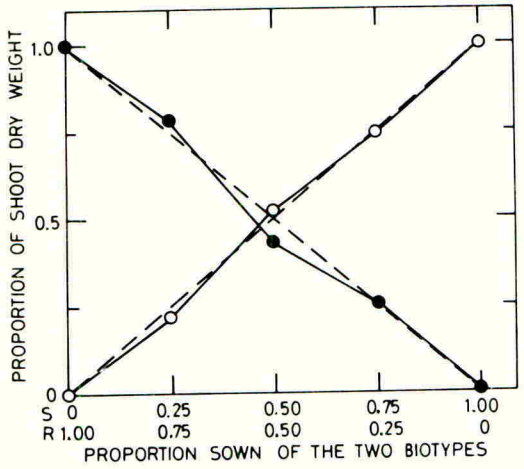

Fig. 4. Fitness differences between S (o--o) and R (•--•) biotypes of P. paradoxa grown together in varying proportions but at a constant overall density. Differences between biotypes were not statistically significant.

DISCUSSION

The cross resistance of triazine resistant biotypes of P. paradoxa and A. myosuroides to methabenzthiazuron is due to reduced inhibition of PSII electron transport, indicating resemblence between the modes of resistance to atrazine and methabenzthiazuron. This is not the case with

chlortoluron, another urea herbicide, which acts like diuron, inhibiting growth and PSII equally in R and S biotypes. These results are in agreement with those reported by Ducruet & De Prado (1982), showing cross resistance to methabenzthiazuron and monolinuron in the chloroplasts of triazine resistant biotypes of B. campestris and C. album, but not to chlortoluron. Conversely, Oettmeier et al. (1982) found that triazine resistant A. retroflexus, was cross resistant to both chlortoluron and methabenzthiazuron among various other herbicides. No cross resistance was found between two different stocks of A. myosuroides plants with a 2-to-3 fold increase in tolerance to either methabenzthiazuron or chlortoluron (Niemann & Pestemer 1984). These discrepencies among studies reporting cross resistance are probably due to secondary pleiotropic effects such as alteration in chloroplast lipid composition, or to allosteric effects of the mutation on the herbicide binding site.

Diclofop-methyl is not a photosynthesis inhibitor, thus the increase in tolerance observed in the R biotypes might also be a result of some pleiotropic effect(s) accompanying the triazine resistance in these grasses.

The results presented here for the competitive ability of P. paradoxa differ notably from those reported for triazine resistant broad leaf weeds (see recent review - Gressel 1985). The R biotype was superior to the S at stages of development as early as seedling emergence. This advantage should affect their subsequent biomass productivity. Under such non-competitive conditions R plants were also taller than the S plants and produced bigger main spikes. In a competition study starting with seedlings, R plants seem to be at least as fit as the S plants. Higher emergence rate and plant height were also observed in R biotype of A. myosuroides seedlings grown under non-competitive condition (unpublished data).

Low fitness of R biotypes should result in their gradual elimination, once the selector (i.e. the herbicide) is removed (Gressel & Segel 1982). However, our results indicate an overall better fitness of the R biotype of P. paradoxa as compared to the S biotype. Thus, one may expect a population shift, increasing the proportion of R to S plants.

The better fitness combined with the observed cross resistance and wide geographical distribution of these triazine resistant weeds should alert scientists and farmers to the serious threat these weeds may pose by infesting cereal crops.

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THE INFLUENCE OF PLANT AGE ON TOMATO TOLERANCE TO METRIBUZIN

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ABSTRACT

The response of tomato of different ages to metribuzin was studied. Plant tolerance increased with plant age when all ages were treated with 0.1 mg/100 ml in vermiculite nutrient culture. However, when plants of different ages were given an amount of chemical adjusted for an estimate of average shoot or leaf dry weight on day of treatment, tolerance declined with plant age. Chlorophyll fluorescence measurements from different ages of leaf tissue vacuum infiltrated with metribuzin indicated that photochemical tolerance may also decline with tissue age. Tlc was performed on composite samples of vacuum infiltrated leaf tissue from different ages of plants and from different ages of tissue taken from plants of the same age. The production of radiolabelled metribuzin metabolites decreased with increasing plant and tissue age in these studies. It is suggested that chemical dilution with increasing plant size may play a major role in the apparent increase in tolerance in the field, rather than increasing enzymatic detoxification as previously suggested.

INTRODUCTION

In Ontario, chemical weed control for field seeded tomatoes is limited by the lack of herbicidal treatments which provide both adequate broad spectrum weed control and crop tolerance. Metribuzin [4-amino-6-tert-butyl-3-(methylthio)-as-triazin-5 (4H)-one] is effective against many problem weed species but crop injury is substantial until plants reach approximately one month in age (Stephenson et al. 1976). It has been established that variations in tolerance among different tomato cultivars is due to differences in rates of metribuzin metabolism (Stephenson et al. 1976). Thus investigators have suggested that increased tolerance with age is also due to an increase in enzymatic detoxification of metribuzin (Stephenson et al. 1976, Frear et al. 1983). However this problem was not directly approached in the above studies.

Machado and Ditto (1982) demonstrated that equal concentrations of chloroplasts isolated from different ages of tomato seedlings were equally sensitive to metribuzin. Thus, their study also suggests that mechanisms which act to increasingly prevent toxic accumulation at this site of action render tomatoes more tolerant with age. The objective of this work was to define the mechanism(s) which lead to the increased tomato tolerance to metribuzin with increasing age which has been observed in both laboratory and field situations.

MATERIALS AND METHODS

Tomato plants (cv Fireball) were grown in a controlled environment room with a light period of 16 hours, a day/night temperature of 26°C/21°C, a relative humidity of 65%, and a light intensity of 380 uE $m^{-2}s^{-1}$. Seed was sown in vermiculite contained in 360 ml styrofoam cups. The cups were doubled and the inner cup was perforated at the bottom to allow drainage. Nutrients were supplied by additions of half strength Hoagland's solution (Hoagland and Arnon, 1950). Technical grade metribuzin (99% pure) was used for all treatments. This was dissolved in acetone (maximum final volume 0.8%) and brought to required concentration with nutrient solution (whole plant treatments) or 0.35 M mannitol (leaf disc vacuum infiltration treatments). In some studies, technical metribuzin was augmented with ^{14}C-metribuzin labelled at the carbonyl carbon (5.5 u Ci/ml, specific activity 21.9 u Ci/uM).

Whole Plant Treatments

The dry weight gain (% of control) during a 7 day treatment period was determined for plants treated 15, 18, 21, 24 and 27 days from sowing. The average dry weight of shoots or leaf tissue on treatment days was determined 3 days earlier from a set of plants sown 3 days prior to the plants receiving herbicide treatments. Plants received 0.1 mg/plant, 1.68 mg/g dry weight of shoot on treatment day or 1.95 mg/g dry weight of leaf tissue on treatment day. All of these treatments were supplied in a nutrient solution volume of 100 ml. These latter two treatments were equivalent to a 0.1 mg/100 ml treatment for plants grown 15 days from sowing, but increased as shoot or leaf dry weight increased with age.

Chlorophyll fluorescence studies

Leaf discs, 0.5 cm in diameter, were taken from the terminal leaflet of the first true leaf of plants grown 15, 18, 21, 24 or 27 days from sowing, within one-half hour of the beginning of the light period for each day. Discs were vacuum infiltrated with 28 ppm metribuzin (determined to cause a 50% increase in relative fluorescence above control in leaf discs from plants 15 days from sowing), blotted dry and floated on distilled water in a glass crystallizing dish. The dishes were secured in a shaking water bath under lights (200 uE $m^{-2}s^{-1}$) for one hour before fluorescence measurements were made. Air and water temperature were maintained at 22°C and 21°C respectively, for the duration of the incubation period. Chlorophyll fluorescence was determined with a model SF20 Productivity Fluorometer (Richard Brancker Research Ltd., Ottawa, Canada) according to the method of Richards et al. (1983), except that terminal fluorescence levels (F_T) were determined at 200 seconds rather than 150 seconds. Chlorophyll a and b were determined for treatment days 15, 21, 24 and 27, for control discs before and after the incubation period and for treated discs after the incubation period, according to the methods of Arnon (1949).

Metabolism study

Two sampling techniques were employed to investigate metabolism of metribuzin in vacuum infiltrated leaf discs. For the first method, samples of leaf tissue (20 leaf discs, 1 cm in diameter) were taken from plants grown 12, 15, 18 and 24 days from sowing. The number of discs taken from each age of tissue within a plant was determined by the proportion of fresh weight a given age of tissue contributed to the total fresh weight of leaf tissue. For the second sampling method, plants were grown to 38 days from sowing, and composite samples of 20 discs each were taken from different

ages of tissue. Discs were vacuum infiltrated with radiolabelled metribuzin (2 mg/L in 0.35M mannitol) and incubated under lights for 8 hours. Thin layer chromatography and ^{14}C determinations were done as described by Stephenson et al. (1976). Tlc plates were developed in butanol:acetic acid:water in the ratio 4:1:5.

RESULTS

The response of different ages of tomato plants to the three different treatment regimes is illustrated in Fig. 1. Plants given the constant regime (0.1 mg/100 ml for all ages) responded in a manner similar to that reported by Stephenson et al. (1976), indicating that tomato tolerance to metribuzin increases with age.

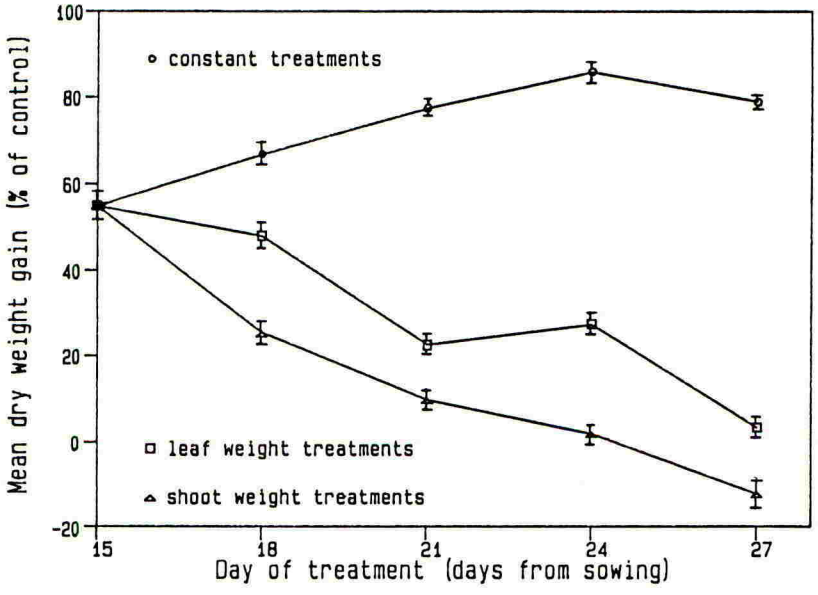

Fig. 1. Mean dry weight gain (% of control) following treatment at different ages with 0.1 mg (constant treatments), 1.68 mg/g dry weight of shoot on treatment day (shoot weight treatments) and 1.95 mg/g dry weight of leaf tissue (leaf weight treatments). Final treatment volume was 100 ml/plant. Vertical bars represent standard error of means.

The studies employing treatments based on tissue dry weights were done in an attempt to examine differences in response at different ages that might be due to dilution of herbicide in the larger plants. Indeed when treatment rates were based on a shoot weight or leaf weight basis severe injury was observed at most ages during the 7 day treatment period. In fact, sensitivity to metribuzin increased with plant age (Figure 1). Because metribuzin is translocated apoplastically (Fortino and Splittstoesser, 1974) most of the compound would be expected to accumulate

tissue cannot be ruled out.

The results of the metabolism study show that metabolite production in Fireball seedlings declines with both plant (Fig. 2) and tissue age (Fig. 3).

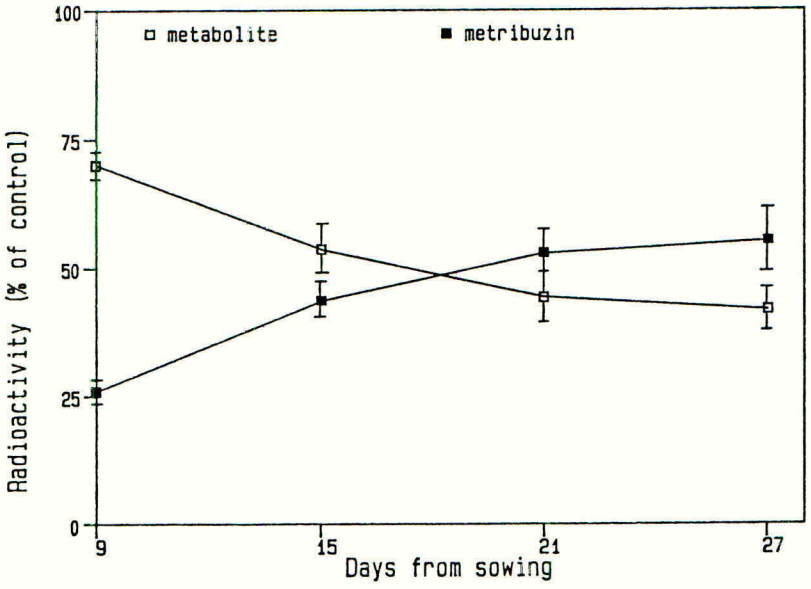

Fig. 2. Percent of total radioactivity on chromatograms as metribuzin and metabolite following vacuum infiltration of composite tissue samples taken from different plant ages. Vertical bars represent standard errors of treatment means.

The reduction seen in plant metabolite production with age (Fig. 2: 12% from day 15 to 27) is much less than the decline in tolerance seen with the leaf weight treatments (Fig. 1: 47% from day 15 to 27). This indicates that factors other than declining metabolism such as increasing translocation rates or increasing photochemical sensitivity were contributing to the response seen with the leaf weight treatments.

in foliar tissues. Thus treatments based on mass of leaf tissue should give a better indication of the response of different plant ages to the herbicide while controlling dilution effects. In additional studies (as yet unpublished) autoradiograms of different aged plants (15 and 24 days) treated with equal concentrations and volumes of ^{14}C-metribuzin solution (1 ppm) showed that distribution of label was similar for different plant ages. However, dilution of the label was evident in the older, larger plants.

In chlorophyll fluorescences studies, control discs, vacuum infiltrated with 0.35 M mannitol had similar chlorophyll a and b levels and showed similar F_T (terminal fluorescence) values as those obtained from discs of the same tissue age not subjected to the infiltration procedure. F_T values for both controls and treatments increased with age as did F_T values of treatments expressed as a % of controls (Table 1). Chlorophyll a, b and leaf disc dry weights were similar before and after incubation for each age, however dry weights increased with tissue age. Uptake of radiolabelled compound following infiltration was checked for discs taken from 15 and 24 day old plants, and was not significantly different at a 5% level of confidence, when expressed as dpm/mg dry weight of tissue. Because of the short incubation time, and because all ages of tissue should have been relatively depleted of carbohydrate reserves at the time of the assay, the effects of differences in metabolism between the treatments was expected to be minimal in these studies. Thus, the increase in chlorophyll fluorescence with age suggests that photochemical sensitivity increases with tissue age.

TABLE 1

Average terminal fluorescence levels (FT) in control discs (relative units), average % of control FT levels in treated discs for different ages of leaf tissue, average chlorophyll a + b/disc and average dry weight/disc. Values within rows followed by the same letter are not significantly different at the 5% level of confidence according to Duncan's New Multiple Range Test.

	Days from Sowing				
	15	18	21	24	27
FT: Control discs	90.3a	93.7b	96.3b	97.8bc	100.1c
FT (% of control); treated discs	133a	132a	147b	151b	161c
Mean chl.(a+b) (ug/disc)	28.42a	ND[1]	31.35a	28.41a	30.11a
Mean dry weight (mg/disc)	1.67a	ND	2.52b	2.94c	3.24c

[1]Values not determined.

Machado and Ditto (1982) did not obtain differences in DCPIP reduction in metribuzin treated chloroplasts isolated from 18 and 31 day old Fireball seedlings. However, differences due to chloroplast age may have been masked in their studies because samples were taken from total plant foliar tissues rather than from a distinct age of tissue, and in the present study the possibility that more metribuzin reached the site of action in older

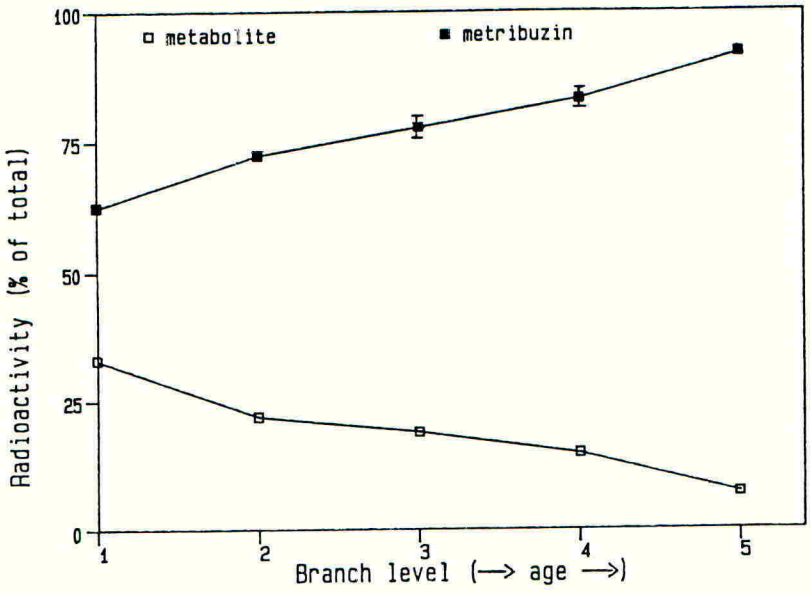

Fig. 3. Percent of total radioactivity on chromatograms as metribuzin and metabolite following vacuum infiltration of different tissue ages taken from 38 day old seedlings. Vertical bars represent standard errors of treatment means (where bars are absent, standard error of means were less than 1%).

In summary, these studies show that tomato tolerance declines with plant age when plants receive amounts of metribuzin adjusted for plant size. Declining plant metabolism and increasing photochemical sensitivity may contribute to this decline in tolerance. The apparent increase in tolerance of tomatoes to a constant amount of herbicide given at different ages can be attributed to an effect of dilution of the chemical with increasing plant size.

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THE CELL MEMBRANE AS A SITE FOR BENTAZONE ACTION

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ABSTRACT

Bentazone can induce rapid, concentration-dependent turgor changes in both light and darkness in isolated protoplasts (from Glycine max mesophyll cells and Commelina communis guard cells), abaxial epidermes (from C.communis) and etiolated coleoptiles (from Avena sativa). Hence, bentazone can alter protoplast size, stomatal apertures and coleoptile elongation, and each experimental system is sensitive to specific cations and may be inactivated by inhibitors of cytoplasmic ATP synthesis. A unifying scheme is proposed that interprets this data in terms of the cell membrane as a site of bentazone action, that is independent of any role in photosynthesis.

INTRODUCTION

Bentazone (3-isopropyl-1H-2,1,3-benzothiadiazine-4(3H)-one 2,2-dioxide) is a selective herbicide for the postemergence control of broadleaved weeds in several important crops including soybean, rice, peanut, pea, maize and beans. Studies conducted during the 1970s by several workers showed that this major herbicide inhibited electron flow at photosystem II, using isolated thylakoids (Pfister et al. 1975), chloroplasts (Mine & Matsunaka 1975, Böger et al. 1977) and cells (Retzlaff et al. 1979), and also inhibited photosynthesis in intact leaves (Penner 1975, Retzlaff & Hamm 1976, Potter 1977). However, subsequent studies in this laboratory clearly demonstrated that bentazone efficacy was particularly sensitive to environmental conditions both before and after application (Taylor et al. 1981), and this led to the hypothesis that bentazone could alter stomatal movement in Chenopodium album leaves (Dunleavy et al. 1982, Cobb et al. 1983) and isolated abaxial epidermes (Dunleavy & Cobb 1984 a and b). More recent observations (Nichols unpublished) have demonstrated that this response is not confined to C.album but is more widespread, suggesting that a feature of the bentazone molecule is an ability to alter stomatal movement, presumably by changing the permeability of the stomatal guard cell membranes.

The present study was conducted to examine further this membrane response in isolated protoplasts, epidermes and coleoptiles, in both light and darkness and to gain further insight into the role of the cell membrane as a site of bentazone action.

MATERIALS AND METHODS

Glycine max mesophyll cell protoplasts

Protoplasts were enzymatically prepared from mesophyll cells of first trifoliate leaves of Glycine max L. Merrill cv Fiskeby V using the method of Rees et al. (1985). Protoplasts were highly intact, c. 90% pure and capable of photosynthetic rates of up to 90 μmoles O_2 evolved.(mg.chlorophyll)$^{-1}$.hr^{-1}. For volume determinations protoplasts (2 x 10^6 ml^{-1}) were first incubated for 10 min at 25°C at a photon flux density (PFD) of 100 μmol.m^{-2}.s^{-1} (photosynthetically active radiation, PAR). An appropriate concentration of bentazone was then added to the reaction mixture (final concentration 0, 1, 10, 100 and 1000 μM) which was stirred (80 rpm), illuminated at 500 μmol.m^{-2}.s^{-1} and maintained at 25°C pH 7.6 for up to

90 min. Aliquots were withdrawn at regular intervals and protoplast diameters determined microscopically and, assuming the protoplasts to be spherical, converted to volumes using the formula $4/3\ \pi r^3$. Each bentazone treatment was replicated 4 times and mean protoplast volumes calculated ± S.E. (n = 40).

Commelina communis epidermes and guard cell protoplasts

Fully expanded leaves of Commelina communis L. were pretreated for 70 min on 25 ml 0.1 mM $CaCl_2$ and exposed to a photon flux density of 50 µmol. $m^{-2}.s^{-1}$ (PAR). Abaxial epidermes were carefully prepared, all visible contamination removed and the peels floated on pretreatment solutions for 60 min to induce either stomatal opening or closure. Opening was induced by the presence of 50 µmol.$m^{-2}.s^{-1}$ (PAR), 25°C, 100 mM KCl and < 50 µl/l CO_2, whereas closure was obtained by darkness, 25°C, > 500 µl/l CO_2 and the absence of KCl. Epidermal peels were then transferred to treatment solutions of varying KCl concentration ± 100 µM bentazone for 2 h and the environmental conditions maintained as described above. All solutions were maintained at pH 6.0 by the addition of KOH and pH remained constant throughout the period of study. Stomatal apertures were directly measured microscopically, each treatment was replicated 3 times and mean apertures calculated ± S.E. (n = 60).

Guard cell protoplasts were enzymatically isolated from abaxial epidermes according to the method of Fitzsimons and Weyers (1983). Protoplasts were incubated in buffer (pH 5.5) containing 10 mM KCl and 100 µM bentazone for up to 60 min at 25°C in darkness. Protoplast diameters were measured microscopically and mean volumes calculated ± S.E. (n = 30).

Coleoptile elongation in etiolated Avena sativa

Avena sativa L. cv Maris Tabard was grown in moist vermiculite for 3 d in total darkness at 25°C. Coleoptiles were removed in dim light, the apical 0.5 cm discarded and 1 cm segments preincubated on 25 ml 5 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.0) for 45 min at 25°C in darkness. Segments were then transferred to 25 ml of incubation medium (buffer plus bentazone or IAA) and maintained in darkness at 25°C for up to 60 min. Fifteen segments were carefully removed at predetermined intervals and their lengths precisely determined using an overhead projector. Each treatment was replicated at least 3 times, mean lengths calculated ± S.E. (n = 45) and regression values determined where appropriate. In Fig. 3A, r = 0.69, control; r = 0.71, 1 µM; r = 0.71, 10 µM and r = 0.72, 100 µM bentazone.

RESULTS

The data presented in Figures 1, 2 and 3 clearly indicate an action of bentazone at the cell membrane of mesophyll and guard cell protoplasts, abaxial epidermes and etiolated coleoptiles. In each case the bentazone-induced responses were rapid and suggested large turgor changes within the treated cells.

Physiologically competent protoplasts isolated from G.max mesophyll cells showed a rapid increase in volume in the presence of bentazone (Fig. 1A). This response was concentration-dependent, in that 10 µM bentazone induced a near doubling in protoplast volume (197% of control values) after 30 min of incubation in the light (Fig. 1B). No strict light-dependency was evident in this response, since 10 µM bentazone was also found to cause protoplast swelling in total darkness (161% of control values after 30 min incubation). 100 µM bentazone induced a similar volume increase in protoplasts isolated from C.communis abaxial epidermes (Fig. 2A). This protoplast swelling in darkness was equally rapid but more transient, since

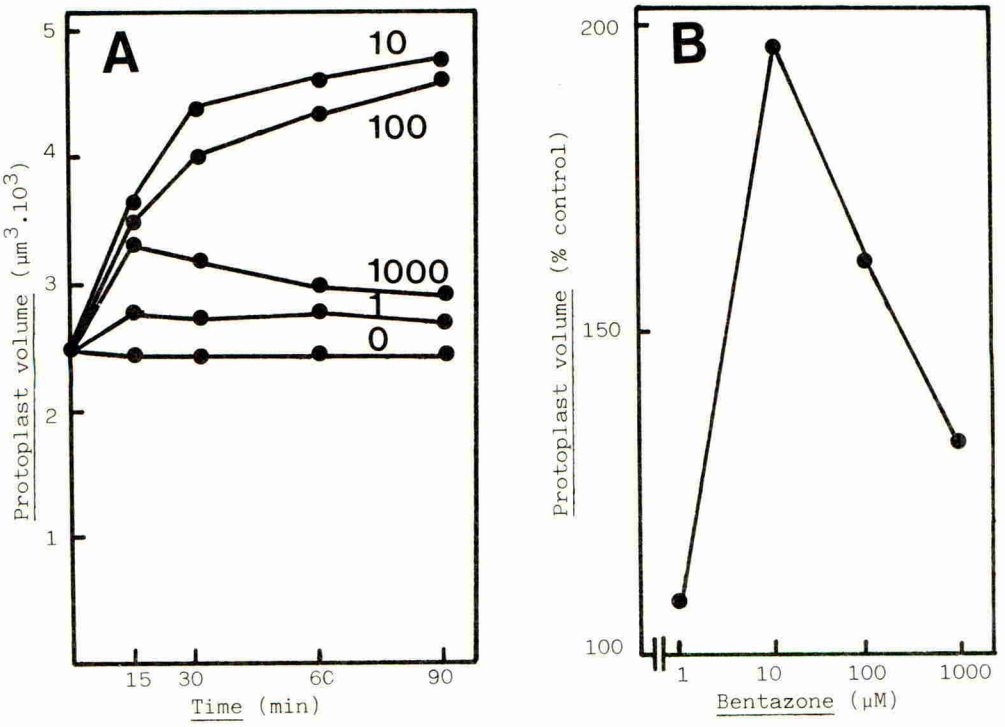

Fig. 1. Bentazone-induced protoplast swelling in isolated mesophyll cells from Glycine max incubated in the light, 25°C, pH 7.6 and in the presence of 0-1000 µM bentazone. Fig. 1A illustrates the time-course of protoplast swelling over a 90 min period, whereas Fig. 1B indicates a concentration-dependent response, expressed as % of control values after 30 min incubation.

volumes were observed to return to control values after 40 min of incubation, possibly implying a reversible response in this cell type. In both experimental systems little or no protoplast rupture was observed as a result of bentazone treatment. Furthermore, staining with neutral red, Evan's blue or fluorescein diacetate indicated a high degree of plasmalemma integrity suggesting fully functional cell membranes.

Guard cells function in vivo to control stomatal apertures by changes in their turgor. Hence, increased guard cell turgor, caused by potassium ion and water influx, induces stomatal opening, whilst decreased turgor, potassium ion and water efflux, results in stomatal closure. 100 µM bentazone was therefore added to functional C.communis abaxial epidermes for 2 h to examine the effect of turgor changes induced by bentazone in the incubation medium (Fig. 2B). In the light, where experimental conditions favoured stomatal opening, 100 µM bentazone induced a potassium concentration-dependent response. Below c. 80 mM potassium, bentazone caused stomatal opening relative to the control, whereas above c. 80 mM potassium, apertures were less than control values. On the other hand, 100 µM bentazone produced wider stomatal apertures than the control throughout the potassium concentration range when incubated in darkness. Thus, the

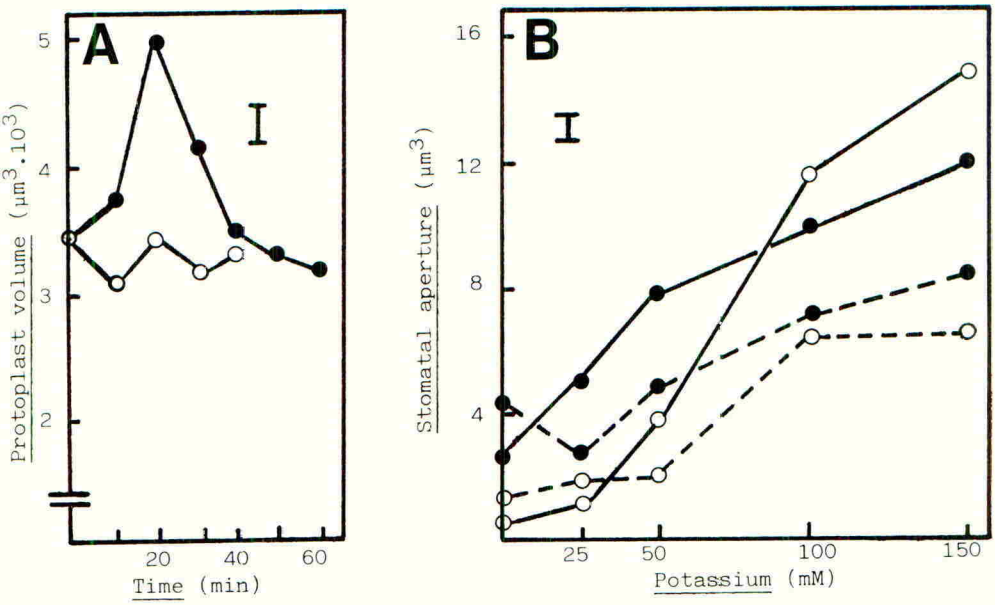

Fig. 2A. Bentazone-induced protoplast swelling in isolated guard cell protoplasts from Commelina communis incubated in darkness, 25°C and pH 5.5 for up to 60 min in the presence (●-●) or absence (O-O) of 100 μM bentazone. Fig. 2B. Effect of 100 μM bentazone on stomatal movement in abaxial epidermes of C.communis following 2 h incubation in either opening or closing conditions, at 0-150 mM KCl. Solid lines, opening conditions; broken lines, closing conditions; open circles, bentazone absent; closed circles, bentazone present.

bentazone-induced turgor changes observed with guard cell protoplasts in darkness (Fig. 2A) are reflected in wider stomatal apertures in dark-incubated epiderms (Fig. 2B).

Another well documented, rapid turgor response of plant cells is the elongation of etiolated Avena coleoptiles, particularly in the presence of indole acetic acid (IAA). Proton excretion from the coleoptile cells is thought to loosen the cell wall polysaccharides and the resultant influx of water causes an increase in cell length. Figure 3A clearly demonstrates that bentazone is able to induce coleoptile elongation in darkness in a concentration-dependent fashion. Indeed, 10 μM bentazone induced a three -fold increase in the rate of coleoptile elongation when compared to the control values, although it was not as active as IAA (Fig. 3B).

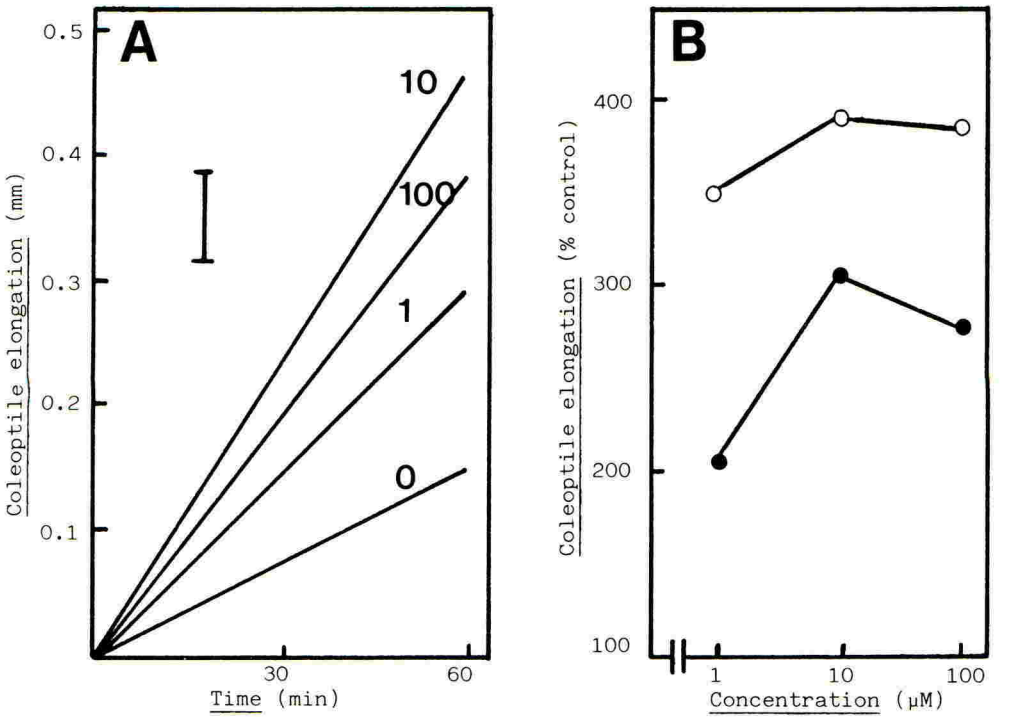

Fig. 3. Bentazone-induced coleoptile elongation in etiolated Avena sativa. Fig. 3A. Time-course of the response in the presence of 0-100 µM bentazone. Fig. 3B. Concentration-dependence of the response in the presence of bentazone (●-●) and auxin (O-O), expressed as % of control values after 60 min incubation.

DISCUSSION

Although there is abundant evidence in the literature designating bentazone as an inhibitor of photosynthetic electron flow at photosystem II (Moreland 1980), these findings clearly indicate a further action of bentazone at the plant cell membrane. Figures 1, 2 and 3 suggest that bentazone can induce rapid changes in the turgor of protoplasts, epidermes and coleoptiles in both light and darkness, and may therefore be considered to be independent of photosynthesis. Indeed, bentazone-induced swelling of G.max mesophyll cell protoplasts in darkness is particularly sensitive to inhibition by 10 µM rotenone and antimycin A (Rees, unpublished), from which it may be inferred that protoplast swelling has a requirement for ATP derived from mitochondria.

Protoplasts, epidermes and coleoptiles are all capable of rapid changes in cell turgor, mediated by the influx of cations and water into the cell in exchange for proton efflux, each possibly involving the action of membrane bound ATPases. Rees (unpublished) has demonstrated that G.max protoplast swelling is particularly sensitive to the magnesium ion concentration of the bathing medium and may be inhibited by compounds that prevent electron flow in mitochondria. Similarly, in stomatal movement potassium is the osmotically active cation (Fischer 1968) whose transport

into the guard cell may be prevented by several metabolic inhibitors (Outlaw 1983). Furthermore, coleoptile elongation in darkness has a requirement for calcium ions (Cohen & Nadler 1976) and is inactivated by inhibitors (see Davies 1973). Thus, there is good evidence in the literature for active cation transport at the cell membrane in each of the three experimental systems used in the study, although how bentazone may interact in each system remains to be established.

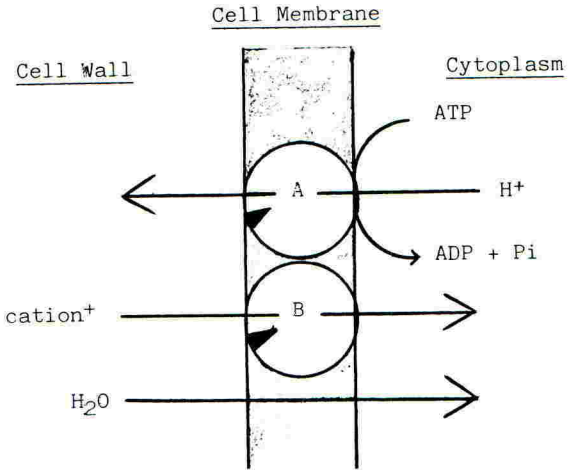

Fig. 4. A hypothetical scheme for bentazone action at the plant cell membrane. An ATPase proton pump (A) is envisaged to drive the efflux of protons in exchange for cations at a cation-specific transport site (B). One or both transport molecules may contain regulatory sites (◥) that are sensitive to bentazone and/or other regulatory molecules, such as IAA.

Since each bentazone-induced response is both rapid and concentration-dependent it is proposed that this herbicide may bind to active site(s) on the cell membrane altering cation and water flux, as represented in Fig. 4. In this scheme an ATPase proton pump is envisaged to drive the efflux of protons in exchange for cations at a cation-specific transport molecule. Bentazone could conceivably bind at one, both or at other sites on the membrane to alter cation influx and hence cell turgor. Evidence does exist, particularly with regard to coleoptile elongation, that the endogenous plant growth regulator IAA may induce increased cell turgor by binding to the cell membrane ATPase (eg Davies 1973). Furthermore, since bentazone can interact with auxin in this experimental system (Miller unpublished), an action of bentazone at a regulatory site on the cell membrane is conceivable. Indeed, the stoichiometry of cation transport in the presence or absence of bentazone, and other membrane active herbicides and regulators, is an active area of study in this laboratory, which may yield a greater understanding of bentazone and other herbicide actions at the cell membrane.

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OXYFLUORFEN ACTIVATION BY PHOTOSYNTHETIC ELECTRON TRANSPORT

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ABSTRACT

The effect of the p-nitro diphenyl ether herbicide oxyfluorfen on pigment bleaching and ethane generation from pea leaf discs and its effect on chloroplast photosynthetic reactions has been studied. Light dependent bleaching and lipid peroxidation induced by oxyfluorfen was lessened by pretreating leaf discs with monuron. Oxyfluorfen had no effect on electron flow through photosystems 1 and 11, but inhibited ferredoxin dependent $NADP^+$ reduction. Oxyfluorfen was also shown to promote ferredoxin dependent NADPH oxidation in a model system. These results suggest that oxyfluorfen is activated by photosynthetic electron transport via ferredoxin.

INTRODUCTION

The p-nitro diphenyl ether (DPE) herbicides induce pigment bleaching and lipid peroxidation of chloroplast membranes (Kunert and Böger 1981). Although light is required for their activation, the mechanism remains unclear. Orr and Hess (1982) proposed that DPE herbicides were activated by carotenoid pigments. Herbicidal activity was detected in etiolated plants and yellow mutants, and could be reduced by carotenoid synthesis inhibitors (Matsunaka 1969, Orr and Hess 1982). Alternatively, Kunert and Böger (1981) proposed that DPE herbicides were activated by photosynthetic electron transport. Herbicidal activity was reduced if electron flow was inhibited prior to DPE treatment. Diuron was also shown to prevent the formation of radical species induced by DPE herbicides in illuminated chloroplasts (Lambert et al 1984). Additionally p-nitro DPE herbicides inhibit CO_2 dependent O_2 evolution (Alscher and Strick 1984), non-cyclic photosynthetic electron transport (Moreland et al 1971) and energy transfer (Ridley 1983) in chloroplasts.

In this paper we report on some effects of the DPE herbicide oxyfluorfen on pea leaf material and isolated chloroplast membranes. Evidence is presented for activation of this herbicide by photosynthetic electron transport.

MATERIALS AND METHODS

Growth and treatment of plant material

Pea (Pisum sativum cv Meteor) plants were grown in moist Levington Universal Compost under glasshouse conditions and a mean air temperature of 22°C for 14-21 days. Leaf discs (13mm diameter) were cut from sub-apical leaves and floated on test solutions in glass petri dishes. These were transfered to a growth cabinet at 24°C under continuous illumination (350

μ mol $m^{-2}s^{-1}$ photon flux density). For ethane determinations, leaf discs were incubated as above in glass vials with an air tight screw cap containing a rubber septum.

Isolation of chloroplast membranes, and measurement of electron transport reactions

Chloroplast membranes were prepared by grinding pea leaves in a cold mortar in 50mM Tricine-NaOH, pH 7.6, containing 0.3M NaCl and 5mM $MgCl_2$. After squeezing through 4 layers of muslin and centrifugation at 5000g for 10 min, the pellet was resuspended in 50mM Tricine-NaOH, pH 7.6, containing 30mM NaCl and 5mM $MgCl_2$.

Light dependent oxygen uptake or evolution from the thylakoids was determined in a Hansatech oxygen electrode in a 3.0ml reaction volume containing 30mM Tricine-NaOH, pH 7.6, 5mM $MgCl_2$, 1.7mM NH_4Cl and chloroplast membranes containing 50-100μg chlorophyll. Illumination was provided by a 500W tungsten lamp giving 500μmol $m^{-2}s^{-1}$ photon flux density at the reaction chamber. Electron acceptors were 1.7mM potassium ferricyanide (FeCN) for photosystem 11, and 10μM paraquat for measuring electron flow through photosystem 1 and 11 in a Mehler reaction. For $NADP^+$ reduction, 13.3μM $NADP^+$ and 100μg ferredoxin were added to the basic mixture. Following illumination the absorbance change at 340nm was monitored.

NADPH oxidation

Oxidation of NADPH in darkness was followed in a model system in a 2.0ml reaction volume containing 0.33M Tris-HCl, pH 8.0 80μM NADPH, 150μg ferredoxin, 0.5 units ferredoxin $NADP^+$ reductase and chloroplast membranes containing 30μg chlorophyll. The reaction was followed as the loss of absorbance at 340nm.

Ethane determination

Ethane emmanation from leaf discs maintained in air tight flasks was determined on an alumina column at 125°C in a Pye Unicam GCD chromatograph. Ethane in the flask headspace was identified and quantified by comparison with authentic samples.

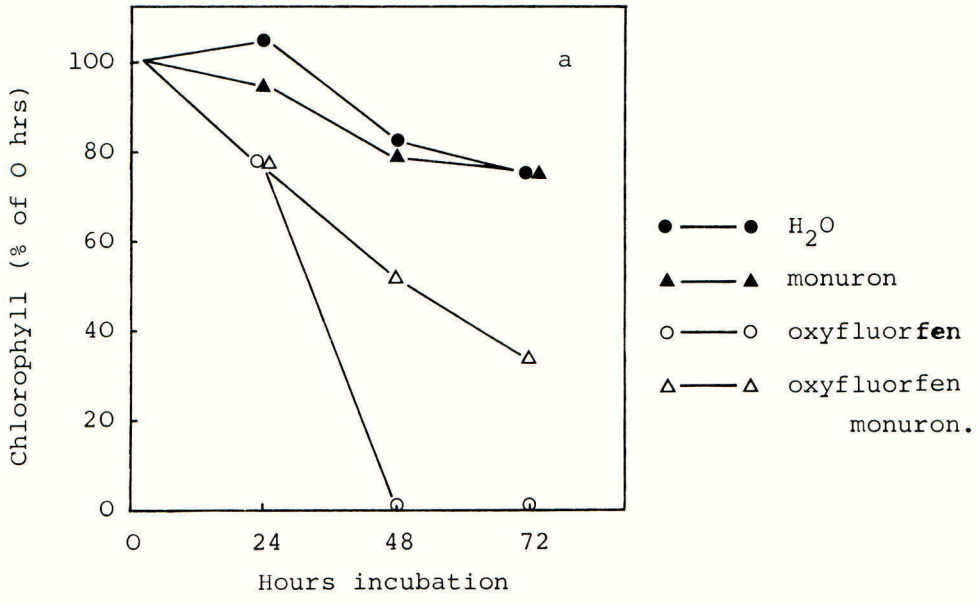
Chlorophyll

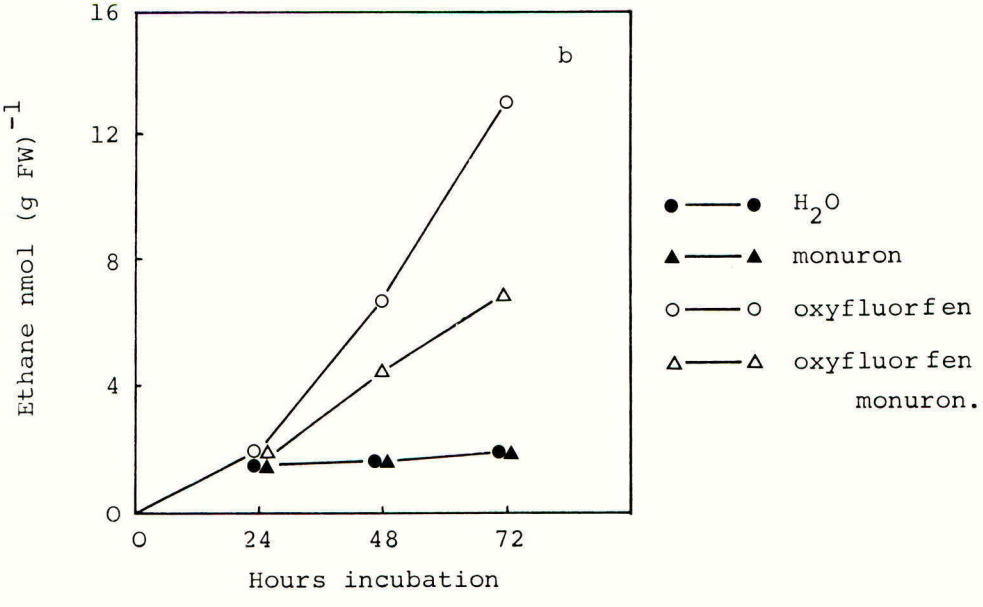
Chlorophyll in treated leaf discs was extracted with 80% acetone and quantified according to Arnon (1949).

RESULTS

Oxyfluorfen induced chlorophyll bleaching and ethane generation from pea leaf discs (figure 1). Incubation of leaf discs on monuron (0.1mM) for 24h in darkness prior to transfer to oxyfluorfen and illumination reduced the damage. This indicated that a functional photosynthetic electron flow system was necessary for oxyfluorfen activation.

Figure 1. The effect of oxyfluorfen on chlorophyll breakdown(a) and ethane evolution (b) from pea leaf discs. Discs were incubated for 24 hours prior to oxyfluorfen treatment on either H_2O (○, ●) or 0.1mM monuron (△, ▲).





The effect of oxyfluorfen on electron flow reactions of isolated pea thylakoids was compared to the effect induced by paraquat and monuron (table 1). Neither oxyfluorfen or paraquat affected photosystem 11, although this was strongly inhibited by monuron. Electron flow through both photosystem 1 and 11, from H_2O to paraquat was also unaffected by oxyfluorfen. The ability of these herbicides to accept electrons from photosystem 1 was examined. Paraquat promoted oxygen uptake in a Mehler type reaction, monuron was inhibitory to electron flow, but oxyfluorfen was without effect. These data indicate that at high concentrations (50μM), oxyfluorfen had no effect on electron flow through photosystem 1 and 11.

TABLE 1

The effect of oxyfluorfen, paraquat and monuron on electron transport reactions of pea chloroplasts. Results are expressed as the percentage change compared to the control rate.

	Photosystem 1 $H_2O \rightarrow$ FeCN	Photosystem 1 & 11 $H_2O \rightarrow$ paraquat	Ability to accept e^- from photosystem 1.
H_2O control	100%	100%	100%
Paraquat (10μM)	103.9%	--	422.4%
Monuron (10μM)	6.5%	2.7%	9.4%
Oxyfluorfen (1μM)	107.1%	98.3%	98.6%
Oxyfluorfen (10μM)	105.8%	98.7%	93.6%
Oxyfluorfen (50μM)	94.7%	94.5%	89.1%

The effect of oxyfluorfen (10μM) on $NADP^+$ reduction by illuminated chloroplasts is shown in figure 2. This herbicide caused a significant inhibition of ferredoxin dependent $NADP^+$ reduction. The involvement of ferredoxin in DPE herbicide activation was investigated further in a model system. Ferredoxin can be reduced by NADPH in the presence of ferredoxin $NADP^+$ reductase. This reaction was followed as oxidation of NADPH (figure 3). NADPH oxidation only occured if both ferredoxin and ferredoxin $NADP^+$ reductase were present in the reaction mixture. Addition of oxyfluorfen was found to enhance the rate of NADPH oxidation (figure 3), The reaction was again dependent on ferredoxin and ferredoxin $NADP^+$ reductase present in the reaction mixture.

DISCUSSION

Results presented in this paper show that photosynthetic electron flow is necessary for oxyfluorfen activation. Furthermore, data in figures 2 and 3 suggest that oxyfluorfen can accept electrons from ferredoxin. Illuminated chloroplasts in the presence of ferredoxin are also known to reduce a variety of other substances including myoglobin, cytochrome C (Davenport and Hill 1963) and dinitrophenols (Wessels 1965).

Figure 2. $NADP^+$ reduction by illuminated chloroplasts.

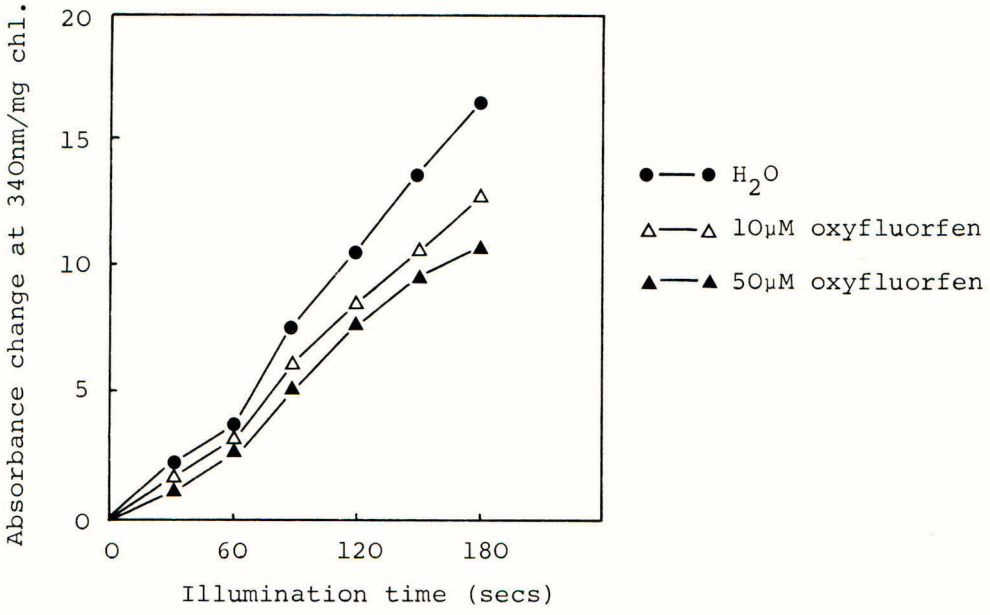
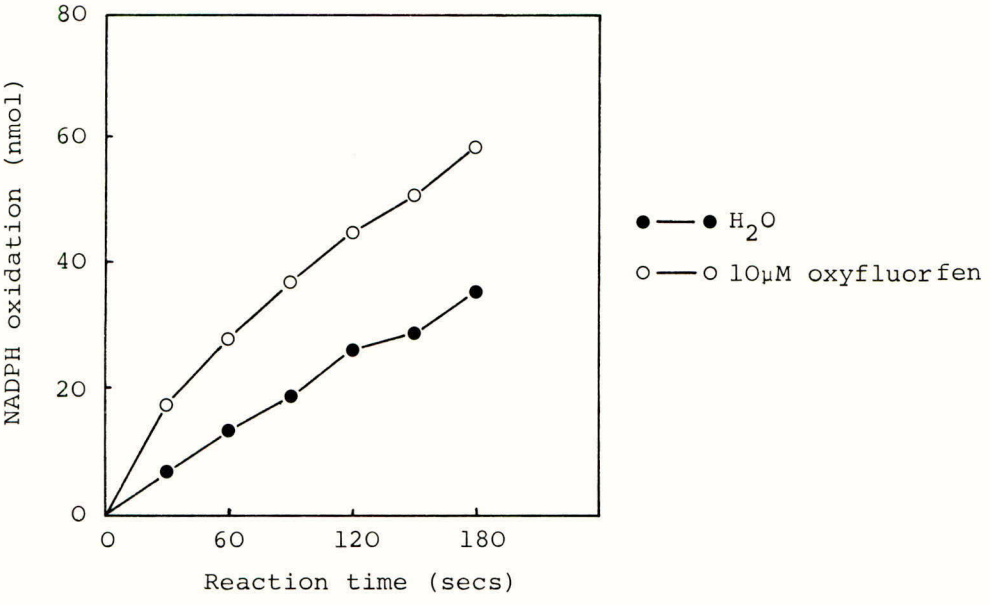
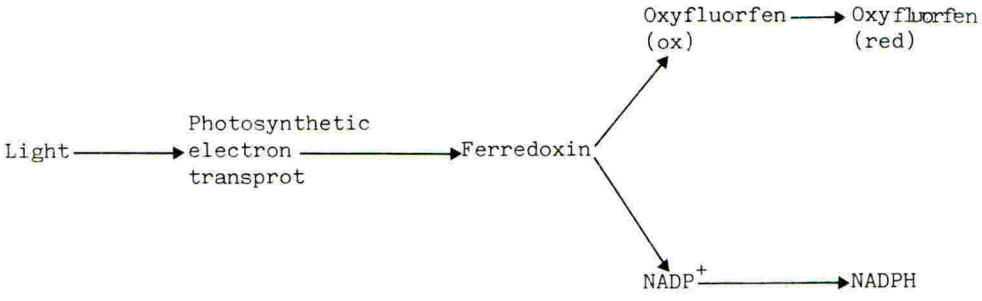


Figure 3. The effect of oxyfluorfen on ferredoxin and ferredoxin $NADP^+$ reductase catalysed oxidation of NADPH.



This study and previous investigations have demonstrated that DPE herbicide activity was lessened if electron transport was inhibited (Pritchard et al 1980, Kunert and Böger 1981). Reduction of p-nitro DPE herbicides by ferredoxin may form a radical species capable of initiating lipid peroxidation. Draper and Casida (1985) observed reduction of p-nitro DPE herbicides to p-nitroso derivatives, and the binding of these derivatives to unsaturated lipids. This generated nitroxide radicals which were sufficiently reactive to initiate lipid peroxidation. Nitroxide radicals were also detected in the lipid fraction of bean leaves which had been treated with nitrofen and illuminated (Draper and Casida 1985). The requirement of a lipid environment for herbicide activity could explain the relationship betwen DPE lipophilicity and herbicide activity (van de Burg and Tipker 1982) and the action of certain antioxidants such as α-tocopherol in reducing DPR damage (Kunnert and Böger 1984).

Figure 4



The data presented in this paper supports the hypothesis that p-nitro DPE herbicides are activated by photosynthesis electron transport at a site on the chloroplast thylakoid in the vicinity of ferredoxin and compete with endogenous electron flow to ferredoxin $NADP^+$ reductase. This is summarised in figure 4.

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AUTHOR INDEX