SESSION 4D

BIOCHEMICAL ASPECTS OF HERBICIDE ACTION AND SELECTIVITY

SESSION ORGANISER DR D. J. COLE

POSTERS 4D-1 to 4D-12

EFFECTS OF HERBICIDES ON THE LIPID COMPOSITION AND MAGNESIUM-DEPENDENT, POTASSIUM-STIMULATED ATPASE ACTIVITY OF OAT SHOOT PLASMA MEMBRANES

R.S. BURDEN, D.T. COOKE, C.S. JAMES

Department of Agricultural Sciences, University of Bristol, AFRC Institute of Arable Crops Research, Long Ashton Research Station, Bristol, BS18 9AF, UK.

ABSTRACT

Many agrochemicals inhibit or modify plant membrane lipid biosynthesis while others may induce membrane damage through oxidative mechanisms or by direct insertion. The operation of the K^+-stimulated, Mg^{2+}-dependent ATPase of the plasma membrane, essential for nutrient transport into the cell, is dependent on the integrity and lipid composition of the membrane. Following treatments of intact oat plants, the activity of the shoot plasma membrane (Mg^{2+}-K^+) ATPase was found to be stimulated by a γ-keto triazole herbicide and by the growth regulator tetcyclacis but inhibited by the herbicides alachlor and barban and by the fungicide fenpropimorph. The herbicides triallate, SAN 9785 and acifluorfen had no significant effect on the enzyme. Lipid analysis indicated that there may be a relationship between the amount of cholesterol in the oat plasma membrane and (Mg^{2+}-K^+) ATPase activity.

INTRODUCTION

The magnesium-dependent, potassium-stimulated ATPase of the plasma membrane of higher plants generates an electrochemical gradient across the membrane by extruding protons. As this provides the energy for the active transport of ions and nutrients into the cell, the controlled operation of the ATPase is thought to be critical for the maintenance of many cellular functions. Regulatory mechanisms operating during normal cellular growth, may include gene regulation, enzyme regulation via phosphorylation, allosteric regulation through the binding of small molecules including plant hormones, and control via alterations in the lipid content of the membrane (Brauer and Tu 1989, Cooke and Burden 1989).

Given the importance of the plasma membrane (Mg^{2+}-K^+) ATPase, inhibitors of the enzyme should have potential as herbicides (Böger 1987). Known inhibitors include vanadate (Brauer and Tu 1989), diethylstilbesterol (Balke and Hodges 1979), erythrosine B (Gimmler 1988), glyceollin (Giannini *et al.* 1988), alkyltrimethylammonium bromides (Kasamo 1982), local anesthetics (Kasamo 1988), 2,2,2-trichloroethyl-3,4-dichlorocarbanilate (Blein *et al.* 1986) and an unknown toxin from *Cerospora beticola* (Blein *et al.* 1988). However, it is unlikely that any of these compounds possess either the activity or the selectivity to be seriously considered for practical use or as a lead for analogue synthesis.

Studies of ATPase regulation are frequently performed using reconstituted enzyme preparations. However, there are dangers in interpreting these experiments. An alternative approach is to perturb the structure or composition of the plasma membrane *in vivo* using

xenobiotics and then determine consequent effects on ATPase activity. Clearly, the most useful chemical probes would be those having a well established and highly specific mode of action on the membrane or its constituents. It is also possible that xenobiotic/ATPase studies may help elucidate the modes of action of compounds which are at present obscure.

In the present investigation, we have selected a variety of substances, mostly herbicides, which are known or believed to alter lipid biosynthesis or else damage cellular membranes. Effects of these compounds (structures in Fig. 1) in sub-lethal doses on the growth and lipid composition of whole oat shoots are compared with the corresponding effects on the lipid composition and (Mg^{2+}-K^{+}) ATPase activity of highly purified plasma membrane fractions. The results are discussed in relation to the mode of action of the compounds and to the regulation of ATPase activity, particularly as this may be influenced by membrane lipid composition.

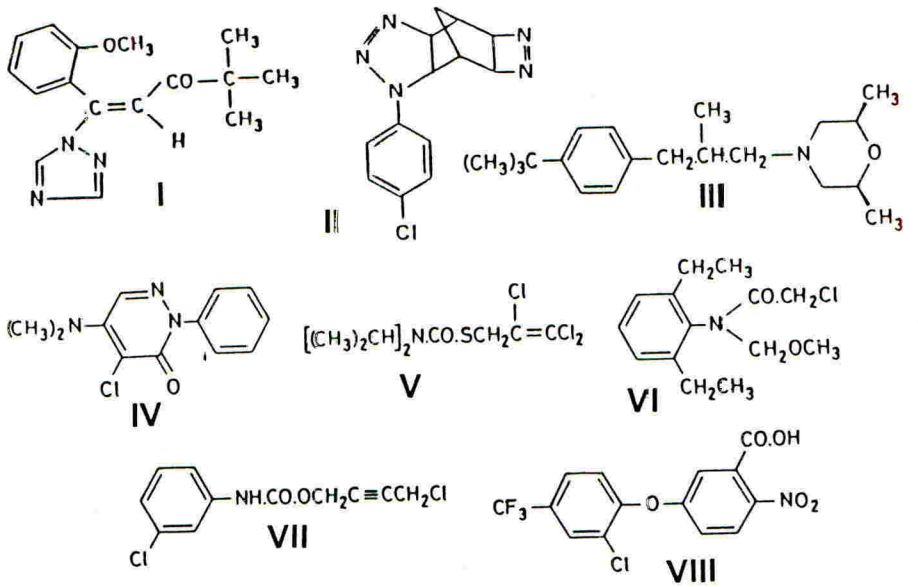

Fig. 1. Structures of γ-keto triazole (I), tetcyclacis (II), fenpropimorph (III), SAN 9785 (IV), triallate (V), alachlor (VI), barban (VII) and acifluorfen (VIII).

MATERIALS AND METHODS

Growth of plants and treatment with xenobiotics

Oat seedlings (cv. Apollo) were grown in pots containing moist sand/vermiculite in a greenhouse with conditions as previously described (Burden *et al.* 1987a). At emergence they were treated with a single dose of the technical grade xenobiotics (10 or 100 μM) in 1% ethanolic solution as a root drench (200 ml per pot). At 12 days after emergence, shoot growth was measured, samples of the shoots were harvested and the tissue lyophilised. For membrane preparations, oat seedlings (cv. Peniarth) were

grown in trays of vermiculite and supplied with a balanced nutrient regime. The plants were grown in a controlled environment; 16 h day at 20°C, 8 h night at 16°C, RH 70% and PAR 350 µmol m^{-2} s^{-1}. Plants were treated with the xenobiotics (5 µM, except tetcyclacis and γ-keto triazole 1µM) as a root drench at emergence and thereafter at two day intervals. They were harvested nine days after emergence and fresh tissue used for membrane preparations.

Preparation of oat shoot plasma membranes

The two-phase aqueous polymer technique for plasma membrane isolation and purification as developed by Larsson *et al.* (1987) was used as described previously (Cooke *et al.* 1989).

Analysis of sterols, phospholipids and fatty acids

Total sterols of shoot tissue were analysed by glc of the acetate derivatives on a bonded SE-52 capillary column using an internal standard of β-cholestanol as described before (Burden *et al.* 1987a). GC-Mass spectra were obtained on a Kratos MS-80 coupled to this column. Total fatty acids in the saponifiable fraction of the shoot lipid extract were analysed, following methylation with diazomethane, on a RSL 500 BP capillary column using helium carrier gas. Heptadecanoic acid was used as internal standard. The sterol and phospholipid composition of the purified plasma membrane fractions was determined as before (Cooke *et al.* 1989). Separation of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was by HPLC and the component fatty acids were analysed by glc after hydrolysis of the phospholipids.

Estimation of (Mg^{2+}-K^{+}) ATPase activity

This was done according to the method of Hodges and Leonard (1974) with some modifications (Cooke *et al.* 1989).

RESULTS

All of the xenobiotics, at the application rates used reduced the shoot growth of oat plants, sometimes with phytotoxic symptoms (Table 1).

TABLE 1. Growth and fatty acid composition of oat shoots following xenobiotic treatment.

Compound (µM)	Shoot height (cm)[a]	Fatty acids (mg/g dry wt)				
		16:0	18:0	18:1	18:2	18:3
Untreated	31.3	1.70	0.25	0.30	0.88	17.05
γ-Keto triazole (10)	15.2	0.92	0.25	0.15	0.65	6.85
Tetcyclacis (10)	8.2	1.95	0.32	0.25	1.15	19.33
Fenpropimorph (10)	23.2	1.30	0.25	0.23	1.10	11.44
SAN 9785 (100)	15.2	1.65	0.25	0.17	6.60	9.05
Triallate (100)	8.0	1.15	0.17	0.15	0.72	12.15
Alachlor (100)	8.8	0.97	0.15	0.13	0.67	8.80
Barban (100)	9.3	0.83	0.12	0.10	0.75	6.43
Acifluorfen (100)	12.8	1.37	0.15	0.15	1.00	11.65

[a] average of 16 plants per treatment

Effects on lipids and ATPase activity are given in the Tables, with the data presented in Tables 1 - 4 being the mean of two experiments. Because of the difficulties in determining absolute amounts of lipids in membrane preparations, the results in Tables 3 - 4 are expressed as a percentage composition. The abnormal sterols induced by γ-keto triazole and fenpropimorph were identified tentatively by their retention times relative to β-cholestanol, and by their mass spectra.

TABLE 2. 4-Demethyl sterol composition of oat shoots following xenobiotic treatment

Compound (μM)	Sterols (mg/g dry wt)						
	a	b	c	d	e	f	g
Untreated	0.16	0.05	0.25	0.31	-	-	-
γ-Keto triazole (10)	0.07	0.04	0.06	0.12	0.42		
Tetcyclacis (10)	0.99	0.04	0.09	0.08	-	-	-
Fenpropimorph (10)	0.03	0.02	0.09	0.04	-	0.17	0.36
SAN 9785 (100)	0.09	0.03	0.23	0.24	-	-	-
Triallate (100)	0.06	0.02	0.10	0.12	-	-	-
Alachlor (100)	0.06	0.03	0.11	0.13	-	-	-
Barban (100)	0.04	0.02	0.05	0.09	-	-	-
Acifluorfen (100)	0.04	0.02	0.11	0.13	-	-	-

Identity of sterols: a = cholesterol, b = campesterol, c = stigmasterol, d = sitosterol, e = 14α-methyl-Δ^8-cholestenol, f = Δ^8-cholestenol, g = pollinasterol

TABLE 3. Fatty acid composition of principal phospholipids of oat shoot plasma membranes following xenobiotic treatment

Compound	Phospholipid	Fatty acids (% of total)					
		16:0	16:1	18:0	18:1	18:2	18:3
Untreated	PE	31.7	7.1	2.6	5.4	32.2	21.0
	PC	31.8	9.2	2.5	7.0	24.7	24.7
γ-Keto triazole	PE	33.6	2.9	4.4	2.9	39.4	16.8
	PC	38.0	5.1	6.6	3.6	28.5	18.2
Tetcyclacis	PE	40.3	9.8	3.3	6.6	26.2	13.8
	PC	39.3	4.5	6.2	4.5	24.2	21.3
Fenpropimorph	PE	34.0	4.8	2.7	3.6	36.2	18.7
	PC	30.4	4.4	3.6	4.1	32.2	25.4
SAN 9784	PE	35.8	9.4	2.9	5.0	34.1	12.6
	PC	30.0	4.3	2.3	6.8	38.8	17.8
Triallate	PE	33.7	10.3	3.3	6.5	30.3	15.9
	PC	30.2	6.3	2.7	6.9	30.0	23.9
Alachlor	PE	41.2	7.6	5.1	4.9	27.0	14.1
	PC	38.0	3.6	3.7	5.2	26.5	23.0
Barban	PE	32.9	6.7	2.3	4.6	36.0	17.6
	PC	28.5	4.6	2.3	5.2	34.9	24.1
Acifluorfen	PE	36.3	6.7	3.8	5.3	28.8	19.1
	PC	31.8	5.2	3.0	5.7	26.2	28.1

TABLE 4. 4-Demethyl sterol composition of oat shoot plasma membranes following xenobiotic treatment

Compound	Sterols (% of total)						
	a	b	c	d	e	f	g
Untreated	22.8	8.0	41.8	27.4	-	-	-
γ-Keto triazole	27.4	5.3	15.4	6.8	45.0	-	-
Tetcyclacis	72.9	4.4	16.5	6.2	-	-	-
Fenpropimorph	2.8	-	2.9	2.4	-	18.2	73.7
SAN 9785	27.2	7.2	40.3	25.3	-	-	-
Triallate	20.4	11.4	39.7	28.5	-	-	-
Alachlor	24.2	9.0	43.3	23.5	-	-	-
Barban	18.9	6.8	45.6	28.7	-	-	-
Acifluorfen	20.9	8.6	43.9	26.6	-	-	-

Identity of sterols as in Table 2.

TABLE 5. Specific activity of H^+-ATPase of oat shoot plasma membranes isolated from plants treated with xenobiotics

Compound	ATPase specific activity	
	Mg^{2+}	Mg^{2+}/K^+
Untreated	0.18 (± 0.012) (n = 18)	0.87 (± 0.031) (n = 18)
γ-Keto triazole	0.43 (± 0.012)*	1.08 (± 0.017)*
Tetcyclacis	0.45 (± 0.019)*	1.34 (± 0.053)*
Fenpropimorph	0.16 (± 0.014)	0.63 (± 0.014)*
SAN 9785	0.18 (± 0.007)	0.87 (± 0.009)
Triallate	0.20 (± 0.034)	0.80 (± 0.145)
Alachlor	0.17 (± 0.010)	0.670 (± 0.009)*
Barban	0.22 (± 0.044)	0.62 (± 0.061)*
Acifluorfen	0.23 (± 0.030) (n = 6)	0.72 (± 0.064) (n = 6)

* Significant at the 99.8% level of confidence or better (unpaired 't' tests)

The mass spectra of the sterol acetates were as follows: 14α-methyl-Δ^8-cholestenol 442 (18%), 427 (100), 367 (32), 273 (10); Δ^8-cholestenol 428 (100%), 413 (21), 368 (11), 353 (14), 255 (15); pollinasterol (14α-methyl-9β,19-cyclo-5α-cholestanol) 442 (13%), 427 (10), 382 (100), 367 (61), 269 (39). The spectrum of the 14α-methyl-Δ^8-sterol is characterised by the exceptionally strong M-15 ion.

DISCUSSION

This work has appraised the performance of the plant plasma membrane (Mg^{2+}-K^+) ATPase following xenobiotic treatment *in vivo*. As mentioned before, the controlled operation of the enzyme must depend on membrane integrity with the nature of the membrane lipids a key factor in the modulation of activity. Lipids may control activity through changes in bulk fluidity or through alterations in the immediate microenvironment of the enzyme (Cooke and Burden 1989). In studies with comparable mammalian

ATPases, such as the (Ca^{2+}-Mg^{2+}) ATPase of the sarcoplasmic reticulum, fluidity was found to be relatively unimportant. Instead, the enzyme activity was found to be highly dependent on the composition of the immediate surrounding lipid with phospholipids, particularly phosphatidylcholine containing C-18 component fatty acids, increasing activity through binding at the 'annular' site and sterols, in this case cholesterol, enhancing activity through binding to the protein-protein interface of the enzyme sub-units (Simmonds *et al.* 1982). Thus, changes in membrane phospholipids and/or sterols arising from xenobiotic treatment might be expected to exert strong influences on ATPase activity.

The experimental herbicide, γ-keto triazole (Fig. 1) is a potent specific inhibitor of C-14α demethylation in plant sterol biosynthesis (Burden *et al.* 1987b). As in comparable cases of azole treated fungi (Thomas *et al.* 1983), the replacement of normal membrane functional sterols by 14α-methyl sterols should be deleterious to membrane function and to overall growth. In the present experiments, γ-keto triazole induced a large increase in 14α-methyl-Δ^8-cholestenol at the expense of the normal phytosterols, both in whole shoots (Table 2) and in the purified plasma membrane (Table 4). This was accompanied by a significant increase in ATPase activity (Table 5).

Tetcyclacis is a plant growth regulator (PGR) which substantially elevates the cholesterol content of oat shoots and membranes (Burden *et al.* 1987a). This was confirmed in the present experiments (Tables 2 and 4) and, of all the compounds tested here the PGR also induced the most marked increase in ATPase activity, both with and without K^+ ion stimulation (Table 5). A speculative explanation of this is that some of the extra cholesterol may bind to the non-annular site of the enzyme and thereby enhance activity (Simmonds *et al.* 1982). It is of interest to note that exogenous cholesterol, applied to pea stem tissue, led to an enhanced uptake of K^+ ions (Hendrix and Higinbotham 1973). This could well be the result of such a cholesterol-stimulated increase in ATPase activity, with potassium influx coupled to proton extrusion.

Fenpropimorph (Fig. 1), widely used in agriculture as a systemic fungicide, has the remarkable property of being able to replace, almost totally, normal membrane phytosterols by cyclopropyl and/or Δ^8 sterols (Costet-Corio and Benveniste 1988). In both whole shoots (Table 2) and in the purified plasma membrane (Table 4), the sterols found to accumulate were tentatively identified, from their mass spectra and relative retention times, as pollinasterol (14α-methyl-9β,19-cyclo-5α-cholestanol) and Δ^8-cholestenol. The ATPase activity following fenpropimorph treatment showed a significant decrease from that found in control, a result which contrasting with the increase in activity observed after administration of γ-keto triazole. This is surprising considering that, in *Saccharomyces cerevisiae*, the abnormal sterols induced by triazoles and morpholines have comparable effects on membrane fluidity and porosity to ions (Steel *et al.* 1989). However, as indicated in Table 4, the cholesterol content of the membrane was drastically reduced following fenpropimorph treatment but slightly enhanced with the γ-keto triazole. Therefore, as discussed above, the status of cholesterol in the enzyme lipid microenvironment rather than the contribution of sterols to bulk fluidity, could be a major factor in influencing ATPase activity.

In contrast to the above compounds, the herbicide SAN 9785 had little effect on sterol composition (Tables 2 and 4) but its application led to a build-up of linoleic (18:2) acid in the whole shoots (Table 1). This is

likely to be the result of an inhibition of the 18:2 → 18:3 conversion in the monogalactosyldiacylglycerol component of the thylakoid membrane (Wang and Hildebrand 1988). There was also evidence of a similar inhibition in the plasma membrane, for the linoleic/linolenic acid ratio was much increased in both PE and PC components (Table 4). SAN 9785 had no effect on ATPase activity.

The precise mode of action of the widely used chloroacetamide and thiocarbamate herbicides remains tantalisingly obscure but, in each case, lipid biosynthesis seems to be the most likely target site (Fedtke 1987). In the present work, total fatty acid levels, expressed on a mg/g dry weight basis, were depressed following triallate and alachlor treatment (Table 1) but this was also observed with other herbicides in these experiments. At high concentrations, alachlor has been reported to disrupt cellular membranes causing electrolyte leakage, a property also showed by the wild oat herbicide barban (Watson *et al.* 1980). Alachlor and barban also showed some inhibition of (Mg^{2+}-K^{+}) ATPase activity *in vitro* (Watson *et al.* 1980) and, in the present experiments, both significantly inhibited the enzyme after application *in vivo* (Table 5).

Acifluorfen disrupts cellular membranes through singlet-oxygen induced oxidation of the unsaturated fatty acids of the phospholipids (Matringe and Scalla 1988). Application of the compound to oat at 100 µM produced weak and twisted plants but few abnormal effects were observed in either fatty acids or sterols. The ATPase activity was not affected.

To conclude, xenobiotics clearly have potential to exert considerable influences on (Mg^{2+}-K^{+}) ATPase activity *in vivo*. The results require careful interpretation but alterations in activity are most likely to result from changes in membrane lipids. The role of cholesterol in the plasma membrane of oat shoot merits further investigation. This should be coupled with the development of techniques for appraising, more fully, the nature of the lipid microenvironment of the enzyme.

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PROPERTIES OF PROPANIL HYDROLASE IN SOIL BACTERIA

K. HIRASE S. MATSUNAKA

The Graduate School of Science and Technology, Kobe University, Nada-ku, Kobe 657, Japan.

ABSTRACT

Rice is resistant to propanil by virtue of aryl acylamidase I enzyme which carries out detoxification of the herbicide. In order to obtain propanil resistance in other crops a bacterial source of a similar enzyme is required. The enzyme may then be isolated, cloned and used to genetically transform plants, thus conferring herbicide resistance. Bacterial strains of the *Pseudomonas* and *Alcaligens* genera were found to degrade propanil. In all cases, propanil was metabolized to form 3,4-dichloroaniline. Additionally, one strain produced another unknown metabolite. The optimum pH and temperature, and Km values of the propanil hydrolases subsequently isolated from these strains were determined. The carbamate insecticide carbaryl acted as a competitive inhibitor.

INTRODUCTION

Propanil is an acylamide herbicide used predominantly in paddy fields. Microorganisms(Lanzilotta & Pramer, 1970a, 1970b; Hammond *et al.*, 1983; Pelsy *et al.*,1987), mammalian liver(Williams & Jacobson, 1966) and some plants (Adachi *et al.*,1966a, 1966b; Yih *et al.*,1968; Still & Kuzirian, 1967; Still, 1968) have aryl acylamidases which hydrolyse amide bonds. Because rice plants have this enzyme, they are resistant to propanil but many other plants lack such an enzyme and are susceptible to this herbicide. Many studies have revealed that this enzyme is capable of metabolizing propanil to 3,4-dichloroaniline(DCA) and propionic acid. Aryl acylamidase may play an important role in herbicide degradation in soil.

Recently breeding of herbicide resistant crops following the use of genetic recombination techniques has been carried out in many laboratories. There are some successful examples in creating plants resistant to 2,4-D (Perkins *et al.*,1987), glyphosate (Comai *et al.*,1985; Shah *et al.*,1986), bialaphos(De Block *et al.*,1987), and bromoxynil(Stalker *et al.*,1988). These examples have all been achieved by introducing a bacterial gene encoding a herbicide detoxifying enzyme excepting glyphosate resistance which was conferred by engineering over production of the target enzyme or inserting an enzyme less sensitive to glyphosate. Because the creation of resistant plants to broad spectrum herbicides may allow their selective use for crop protection, further progress can be expected in this field.

If other propanil resistant crops can be created in addition to rice, this herbicide could be used in upland fields as well as in paddy fields. Consequently, propanil will be awarded new worth. In our study, many microorganisms were tested for their ability to degrade propanil and the properties of their propanil degrading enzymes were investigated.

MATERIALS AND METHODS

Organisms and degradation of propanil in growing cultures

Four strains of bacteria (strain No. 0164, 2278, 3582 and 3585) which degrade propanil were isolated from soil after enrichment culture. The bacteria were grown with shaking at 25℃ in 0.5 % yeast extract medium, pH 7.0. Degradation of propanil was performed in test tubes (18 x 1.5 cm) containing 5 ml of medium on a shaker at 25 ℃. The final propanil concentration was 500 μM. Bacterial growth was determined by optical density at 660 nm.

The extraction and determination of propanil and its metabolite were conducted as follows; 5 ml of ethanol was added to the same volume of medium and shaked for ten min. The mixture was then centrifuged at 27,000 x g for 10 min. The supernatant was passed through a millipore filter(0.22 μm pore diameter) and the filtrate was used for HPLC measurement (LC-3A, Shimadzu). A C18 reverse phase column(0.5 x 15 cm; Cosmosil) was used. The mobile phase was acetonitrile : water (9:11 v/v). Flow rate was 0.8 ml/min and samples were analysed at 254 nm.

Preparation of crude enzyme

Bacteria were cultured for 48 hours in 0.5 % yeast extract (100 ml) at 25 ℃ on a rotary shaker, and harvested by centrifugation at 17,000 x g, for 10 min. Cells were washed with water and suspended in 10 ml of 100 mM Tris-HCl buffer (pH 8.8) containing 1 mM EDTA-Na_2 and 0.1 % 2-mercaptoethanol. Cells were homogenized by sonication in ice water and supernatants were used for enzyme assays after centrifugation at 17,000 x g at 0 ℃ for 10 min.

Enzyme assays

Propanil hydrolysing activity was determined by measuring the amount of 3,4-dichloroaniline (DCA) produced (Kodama & Akatsuka, 1975). Tests were usually started by adding 1 ml of 400 μM propanil to 1 ml of crude enzyme in a test tube. The mixture was kept at 40℃ for 40 min and 2 ml of 10 % trichloroacetic acid was added to terminate the reaction. The mixture was kept at 0℃ for 5 min and centrifuged at 2,000 rpm for 5 min. Two millilitres of 0.1 % *p*-dimethylaminocinnamaldehyde was added to 1 ml of the supernatant. After standing for 15 min, the optical density was measured at 540 nm. The amount of enzyme that produces 1 μmole DCA/min/mg total protein was defined as 1 unit. Protein concentration was measured by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Four strains of bacteria (strain No. 0164, 2278, 3582 and 3585) capable of degrading propanil were isolated from soil after enrichment culture. These bacteria were all Gram-negative aerobic rods and had motility. They were positive for oxidase and catalase. Strain No. 0164 and 2278 produced acids from glucose only under aerobic condition, and No. 3582 and 3585 did not under both aerobic and anaerobic conditions. These results revealed that strain No. 0164 and 2278 belong to *Pseudomonas* whereas strain No. 3582 and 3585 belong to *Alcaligens*.

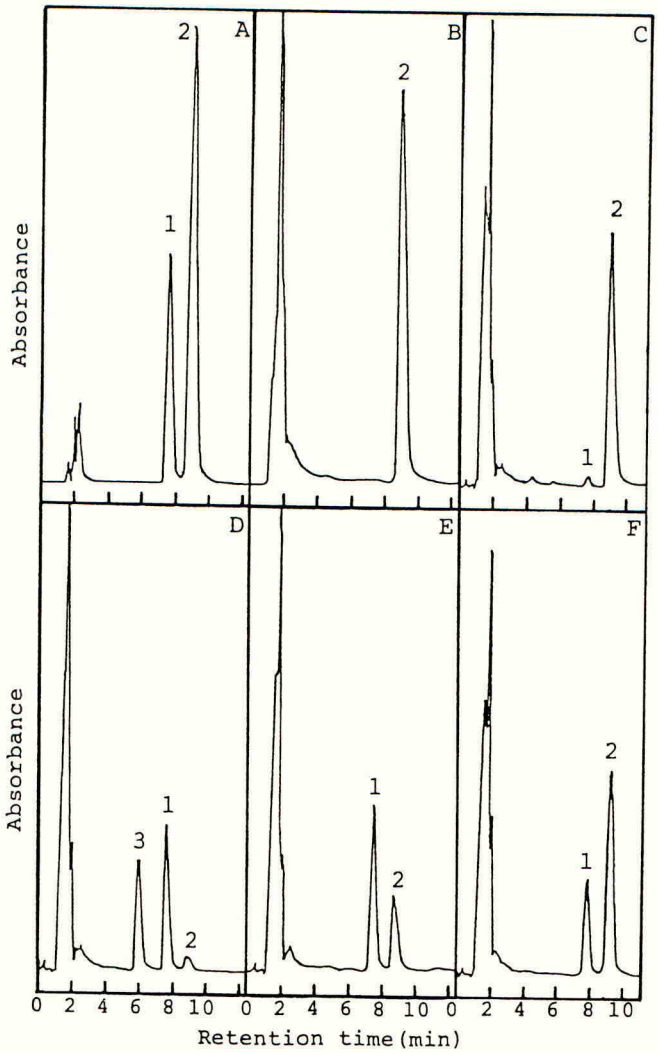

Figure 1. Degradation of propanil by four bacterial strains for 8 days in yeast extract medium as determined by HPLC. A: References; 250 μM DCA and 250 μM propanil, B: Blank (250 μM propanil incubated without bacteria), C: 0164, D: 2278, E: 3582, F: 3585. Peak 1: DCA, Peak 2: propanil, Peak 3: unknown.

Degradation of propanil in growing culture

These bacteria metabolize propanil, forming 3,4-dichloroaniline (DCA) in yeast extract medium. In addition 2278 produced not only DCA but also another compound shown by the appearance of a new peak (peak 3) on the HPLC chromatogram (Figure 1). This new peak has not been identified yet. These

bacteria are assumed to have the enzyme which may be called propanil hydrolase or aryl acylamidase which exists similarly in rice plants.

The time course of propanil degradation in yeast extract medium was measured by HPLC (Figure 2). Strain 2278, which could metabolize propanil most rapidly in the four strains, degraded the herbicide completely in 8 days with a half life of 2.8 days. The second most effective strain was 3582 for which the half life of propanil was 3.3 days. Strains 0164 and 3585 degraded propanil slowly in contrast to 2278 and 3582. The bacterial growth rate of 0164 and 2278 were higher than that of 3582 and 3585. The amount of DCA produced from propanil coincided roughly with the decrease in propanil in the case of 2278, 3582 and 3585. However 0164 produced only a small amount of DCA that did not balance the disappearance of propanil in the medium. These results shows that 0164 is capable of metabolizing DCA into other compounds and that there is limited or no ability to metabolize DCA in other strains.

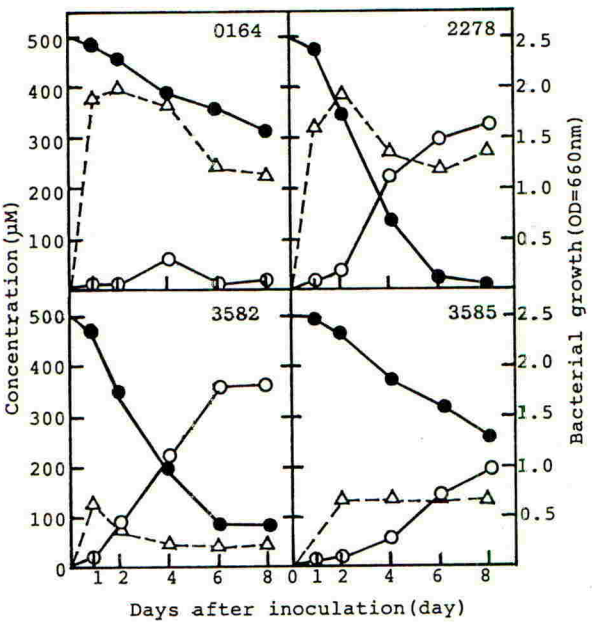

Figure 2. Degradation of propanil (●), formation of DCA (○) by the four bacterial strains, and bacterial growth (△) in yeast extract medium.

Factors influencing enzyme activity

The enzyme reaction proceeded linearly in proportion to the amount of crude enzyme and time for 40 min (data not shown). As a rule, these enzymes were active over a broad pH range of 8.0 to 11.0 (Figure 3). Furthermore the enzyme activity of 2278 was 74 % of that at optimum even at pH 12. The optimum pH of 0164, 2278, 3582 and 3585 were all alkaline,

being 9.0, 10.0, 9.0 and 8.0 respectively. These properties are similar to those of *Fusarium solani* (Lanzilotta & Pramer, 1970b) or *Aspergillus nidulans* (Pelsy *et al.*, 1987). The optimum temperatures of 0164, 2278 were both 40℃, and those of 3582 and 3585 were both 50 ℃ . These enzymes are active at relatively high temperature; even at 70 ℃, the enzyme from 2278 had significant activity(Figure 4).

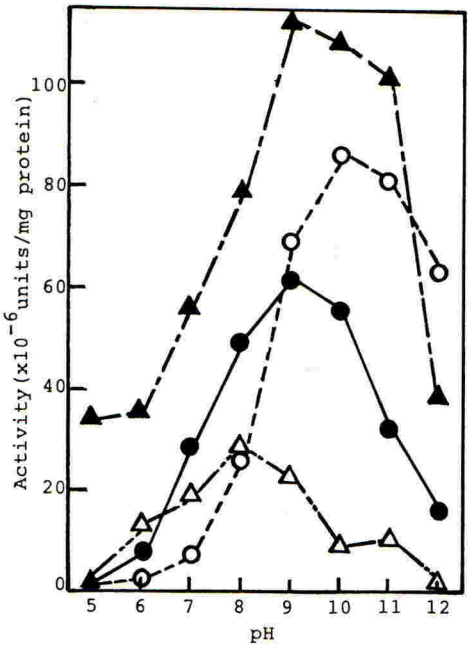

Figure 3. Influence of pH on propanil hydrolase of 0164 (●), 2278 (○), 3582 (▲) and 3585 (△).

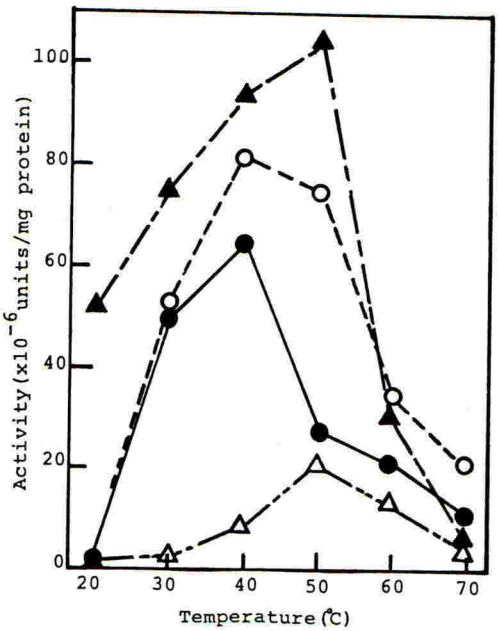

Figure 4. Influence of temperature on propanil hydrolase of 0164 (●), 2278 (○), 3582 (▲) and 3585 (△).

The effect of substrate concentration on enzyme activity was also examined (Figure 5). The Km values of these enzymes were calculated by the method of Lineweaver and Burk (Lineweaver & Burk, 1934). They were found to be 0.72, 0.35, 0.53 and 1.5 mM in the case of 0164, 2278, 3582 and 3585 respectively and they are slightly higher than propanil hydrolases from other sources. For instance, the Km values of *F. solani* (Lanzilotta & Pramer, 1970b) and *A. nidulans* (Pelsy *et al.*, 1987) enzymes are 0.195 mM and 0.13 mM respectively.

Inhibitors

Mixed or successive application of propanil with carbamate or organophosphorus insecticides causes injury to rice plants in paddy fields (Bowling & Hudgins, 1966). This is due to an ability of these insecticides to interfere with the detoxification of propanil(Matsunaka, 1968). In this study, propanil hydrolysis by the enzymes of 0164, 2278

and 3582 were inhibited by 10%-30% and that of 3585 was inhibited 60%-70% by 100 μM carbaryl or the same concentration of metolcarb which are carbamate insecticides (TABLE 1).

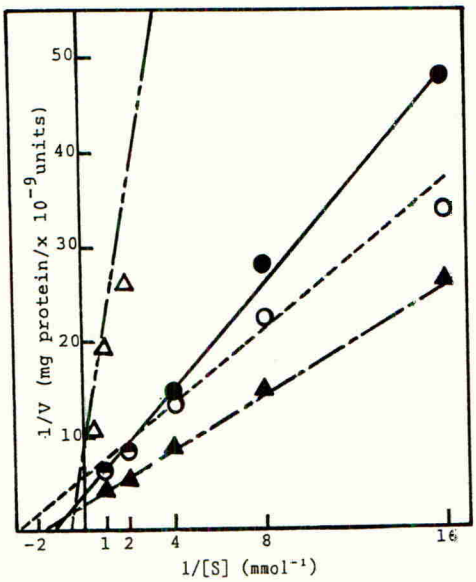

Figure 5. Influence of substrate concentration on propanil hydrolase of 0164 (●), 2278 (○), 3582(▲) and 3585 (△).

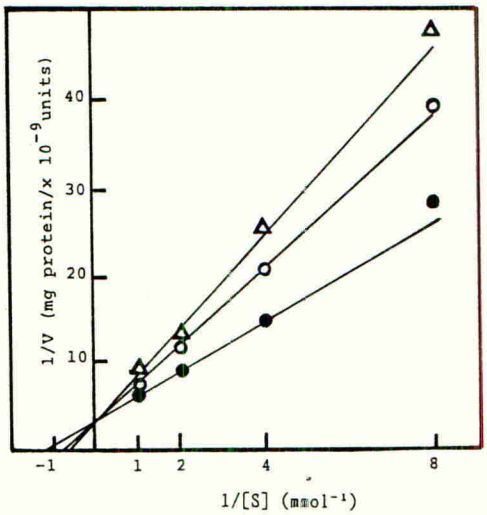

Figure 6. Inhibition by carbaryl of the propanil hydrolase from 0164. (●); Control, (○); 10 μM carbaryl, (△); 50 μM carbaryl.

TABLE 1. Effect of carbamate and organophosphorus insecticides on propanil hydrolase.

Insecticide (100 μM)	Inhibition %			
	0164	2278	3582	3585
control	0	0	0	0
carbaryl	27.9	28.4	28.2	72.7
metolcarb	19.5	13.0	21.4	58.0
fenitrothion	29.8	92.9	20.7	39.8
diazinon	17.8	23.7	3.7	45.7

These inhibition ratios are smaller than that of the rice enzyme which is inhibited by as little as 1 μM carbaryl. This result is favorable for producing propanil resistant crops because combined treatment of propanil and carbamate insecticides should not cause as much injury to the transgenic plants as to rice. The inhibiting ability of fenitrothion and diazinon which are organophosphorus insecticides is roughly similar to that of the two carbamate insecticides.

Lineweaver Burk plot in the presence of carbaryl showed that it is a competitive inhibitor of the enzyme from strain 0164 (Figure 6). Carbaryl is also a competitive inhibitor of fungal propanil hydrolases (Lanzilotta & Pramer, 1970b; Pelsy *et al.*, 1987).

TABLE 2. Preliminary classification of the four strains of propanil degrading bacteria and characteristics of their propanil hydrolase.

Strain No.	0164	2278	3582	3585
Genus	*Pseudomonas*		*Alcaligens*	
Enzyme Activity	++	+++	+++	+
Optimum pH	9.0	10.0	9.0	8.0
Optimum Temp. (℃)	40	40	50	50
Km (mM)	0.72	0.35	0.53	1.5
Inhibition effect of insecticides	weak	weak	weak	weak
Inhibitory mechanism of carbaryl	competitive	NT[a]	NT[a]	NT[a]
Cellular location	soluble	Membrane bound	Membrane bound	Membrane bound

a; Not tested.

Table 2 summarises our major findings. The propanil hydrolase of 0164 is notable as a soluble enzyme, whereas those from the other strains are membrane bound (data not shown). Therefore purification of the 0164 enzyme may be easiest but its activity is lower than those of 2278 and 3582. Further investigation is necessary to decide which enzyme is most suitable as a candidate for purification and characterisation.

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DIFFERENTIAL AUXIN-SENSITIVITY AND MECOPROP-RESISTANCE IN CHICKWEED (*STELLARIA MEDIA*)

P. BARNWELL, C. EARLY, A.H. COBB

Herbicide Research Group, Department of Life Sciences, Nottingham Polytechnic, Clifton Lane, Nottingham NG11 8NS, UK

ABSTRACT

We have recently confirmed the initial observations of Lutman and colleagues (1985, 1987) that resistance to mecoprop is present in a biotype of *Stellaria media*, and have suggested the possibility of reduced mecoprop binding to an auxin site in resistant plants (Barnwell and Cobb, 1989). This hypothesis has now been tested by dose-response analysis of H^+-efflux induced by mecoprop in both resistant and susceptible biotypes of *S. media*. The resistant biotype (BAO) is over 170,000 times less receptive to mecoprop binding than a susceptible biotype (WRO). It is suggested that this may be a major component of mecoprop resistance in *S. media*.

INTRODUCTION

Chickweed (*Stellaria media*) is of global distribution, is regarded as the most widespread and abundant broadleaf weed in British cereals (Makepeace, 1982) and is found in most annual crops and grassland. The species exhibits somaclonal variation in both morphology and physiology, and is well adapted to agricultural practices, since it germinates and flowers almost the whole year round. Chickweed control is routinely achieved using auxin-type herbicides, and post-emergent treatment with mecoprop has proved particularly effective over the last 30 years. These herbicides are foliarly-applied and non-persistent, so the evolution of resistant plants is unlikely and seldom reported (LeBaron and Gressel, 1982). However, chickweed resistance to mecoprop has recently been documented by Lutman and colleagues (1985, 1987) in plants found predominantly in grassland on farms near Bath, Avon, UK. In glasshouse studies, Lutman and Snow (1987) noted at least a sixfold difference in sensitivity between resistant and susceptible plants and found the distribution of resistance to be irregular in eighteen *S. media* populations examined.

We have recently confirmed and extended these observations using seed from resistant (BAO) and susceptible (WRO) *S. media* biotypes, and begun an investigation into the physiological basis of this resistance (Barnwell and Cobb, 1989). We observed altered elongation growth in resistant seedlings and speculated that reduced mecoprop binding to an auxin site could be central to mecoprop resistance. However, it is acknowledged that mecoprop uptake, translocation and metabolism could be equally significant. In this paper, dose-response analyses of H^+-efflux induced by mecoprop is presented from both resistant and susceptible biotypes, and confirms a reduced binding affinity in resistant plants.

MATERIALS AND METHODS

Seeds of S. media were sown in trays lined with moist tissue paper, sealed in plastic bags to ensure high humidity and incubated in the dark at 24°C for 48 h. Seeds were then given a cold treatment (4°C for 48 h) to ensure maximum germination before a further incubation in the dark at 24°C for 150-170 h. Approximately 120 etiolated hypocotyls (40-50 mm in length) were carefully harvested for each experiment and tissue segments 15 mm long prepared by cutting 3-18 mm below the cotyledons and apex. Preparation time was approximately 40 min and performed in dim light. Segments were stored in 50 ml 0.175 mM MES-Tris buffer, pH 6.5 for 15 min and vigorously aerated (900 ml/min). After washing in fresh buffer, segments were transferred to 7 ml vials (20 segments per vial) containing 2.0 ml 0.175 mM MES-Tris buffer plus 1.0 mM KCl as incubation medium. Treatment vials were maintained at 25 ± 0.1°C in a water bath and individually aerated with humidified air via hypodermic needles (90 ml/min). After a 45 min preincubation or equilibration period mecoprop was added to the vials and changes in medium pH were accurately recorded for a further 90 min.

Changes in H^+-efflux were analysed using a curve fitting programme (Weyers et al, 1987; Fitzsimons et al, 1988; Fitzsimons et al, 1989) which allowed the objective determination of R_{AMP} (the amplitute of the response, ie. $R_{MAX} - R_{MIN}$) and H_{50} (the auxin concentration giving a half maximal response and hence a measure of receptor affinity).

Mecoprop (99%, British Greyhound, Birkenhead, Merseyside, UK) was initially dissolved in acetone, neutralised with KOH and diluted with distilled water such that the final acetone concentration was always less than 1.0% (v/v).

RESULTS

Dose-response analysis of mecoprop-induced H^+-efflux was based on previous studies on auxin action in this laboratory using etiolated segments from spring oat (Fitzsimons, 1989). The methodology employed, in this case with a dicotyledonous plant, was essentially similar to studies with spring oat, although the amplitude of the response was considerably less using S. media. Thus, mecoprop induced rates of H^+-efflux of 0.95 pH units/h in spring oat but only 0.21-0.29 pH units/h in S. media. Consequently, a minimum of 25 separate observations of H^+-efflux was considered necessary for each dose-response to yield reliable and reproducible data.

Figure 1 presents dose-response curves of mecoprop-induced H^+-efflux obtained from mecoprop-susceptible (WRO), mecoprop-resistant (BAO) and a commercially obtained biotype of S. media (Herbiseed, Berkshire, UK). Segments from susceptible plants exhibited considerable sensitivity to exogenous mecoprop over a wide concentration range, with as low as 10^{-8} mM causing significant rates of H^+-efflux. In contrast, segments from resistant and "commercial" populations yielded much shallower dose-response curves and required relatively high mecoprop concentrations to induce equivalent rates of H^+-efflux. The sensitivity parameters computed from this data are presented in Table 1.

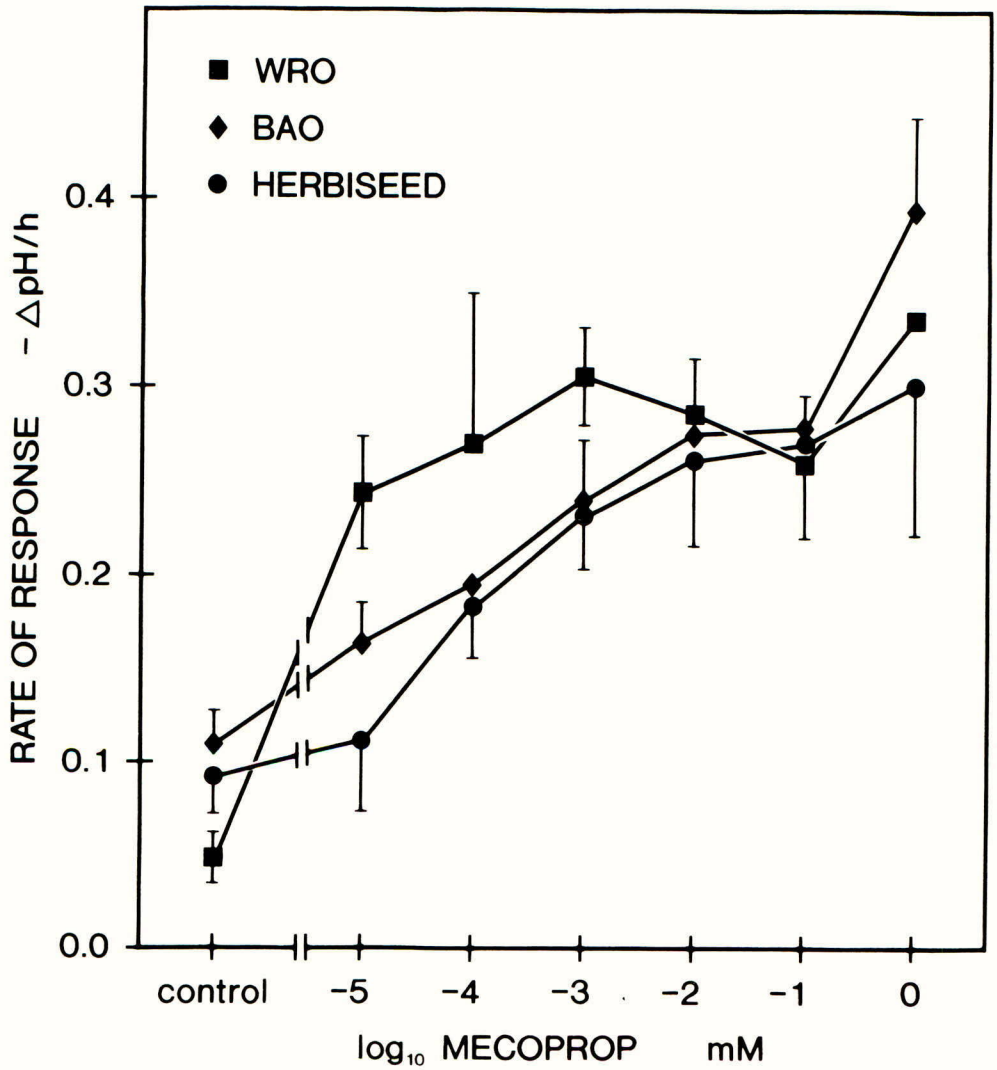

FIGURE 1: Dose-responses of mecoprop-induced H^+-efflux from mecoprop-susceptible (WRO), mecoprop-resistant (BAO) and a commercially obtained biotype of S. media (mean ± SE, n > 3).

TABLE 1 : Sensitivity parameters for mecoprop-induced H^+-efflux from three S. media biotypes.

Biotype	R_{AMP} (pH units/h)	H_{50} (mM)	n
Herbiseed	0.21	2.13×10^{-4}	56
WRO (susceptible)	0.29	1.46×10^{-8}	29
BAO (resistant)	0.29	2.49×10^{-3}	27

From these determinations two major observations may be made;

(1) Whilst seedlings from both resistant and susceptible populations presented an identical response amplitude, receptor affinity to mecoprop was 170,000 times less in resistant plants.

(2) Behaviour of the commercially obtained population was closer to that of the mecoprop-resistant (BAO) rather than the mecoprop-susceptible (WRO) biotype.

DISCUSSION

It is now well established that the binding of an auxin to a membranous or cytoplasmic receptor stimulates the activity of an ATPase located at the cell membrane that pumps H^+ into the cell wall. The resultant apoplast acidification initiates rapid cell elongation in young tissues (eg. Evans, 1985). Secondary messengers are thought to link auxin-binding to H^+-efflux (Brummell and Hall, 1987) and sustained cell elongation requires the involvement of selective genome expression, also induced by auxins (Guilfoyle, 1986). Thus, the many processes that result in cell elongation are triggered by the initial event of auxin-binding to a receptor. Mecoprop is a most effective synthetic auxin (Fitzsimons et al, 1988) but, unlike natural auxin, cannot be rapidly degraded in vivo. Consequently, susceptible plants, such as S. media, receive an auxin "overdose" from which they cannot recover and so die from uncontrolled and aberrant growth (Pillmoor and Gaunt, 1981).

The assay used has proved especially suitable for studies of both natural and synthetic auxins in both monocotyledonous and now dicotyledonous plants, since it is highly sensitive, very specific to auxins and is a particularly rapid response. However, it suffers a major disadvantage in that etiolated tissues are used which, although highly sensitive to auxins, may not be relevant to green seedlings growing in the field. Given this caveat, a consideration of the data in Figure 1 and Table 1 clearly demonstrates that mecoprop resistant plants show greatly reduced binding affinity to mecoprop, in that approximately 10^5 times more mecoprop is needed to give an equivalent H^+ yield as from mecoprop-susceptible seedlings. Furthermore, the finding that the commercial S. media population responded more like the resistant rather

than the mecoprop-susceptible population, implies further variations in nature and questions the agricultural origins of this particular population.

An apparent discrepancy exists between the magnitudes of the H_{50} values presented in Table 1 and the ED_{50} values recently published, suggesting only a 22 fold difference between the WRO and BAO populations (Barnwell and Cobb, 1989). However, the former value relates to an early molecular event at a receptor, whilst the latter is the net result of a herbicide treatment measured several days later as plant fresh weight. Perhaps a threshold response is therefore indicated such that H^+-efflux and hence sustained herbicidal symptoms are only initiated when sufficient receptors are occupied.

A further point of interest relates to natural auxin-binding in these S. media biotypes and this will be reported elsewhere.

ACKNOWLEDGEMENTS

Dr P.J.W. Lutman kindly donated S. media seed from resistant and susceptible biotypes. This study was funded by the National Advisory Body of the UK and we thank Sharon Miller for typing the manuscript.

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METABOLISM OF BENTAZONE IN SOYBEAN AND THE INFLUENCE OF TETCYCLASIS, BAS 110 AND BAS 111

J.M. LEAH, T.L. WORRALL, A.H. COBB

Herbicide Research Group, Faculty of Science, Nottingham Polytechnic, Clifton Lane, Nottingham, NG11 8NS, U.K.

ABSTRACT

The metabolism of ^{14}C-bentazone in intact leaves of resistant soybean and susceptible Capsella bursa-pastoris was investigated in the presence and absence of tetcyclasis, BAS 110 and BAS 111. In the light soybean leaves produced two major metabolites identified as 6-hydroxybentazone and a glycosyl conjugate. These were confirmed by co-chromatography of the former and β-glucosidase incubation respectively. Metabolism was more rapid in darkness. No metabolism of bentazone occurred in C. bursa-pastoris under the conditions used and leaf necrosis was observed after 48 h. Pre-treatment of leaves with tetcyclasis, BAS 110 and BAS 111 had no effect on uptake of ^{14}C-bentazone but consistently prevented metabolism such that leaf necrosis was observed in all cases.

INTRODUCTION

Bentazone (3-isopropyl-benzothiadiazin-4-one-2,2-dioxide) is currently the ninth best selling pesticide in the USA (Finney, 1988) and has long been established as a selective herbicide in major world crops including soybean, cotton and rice. Its mode of action is thought to be the selective inhibition of weed photosynthesis (Mine and Matsunaka, 1975).

Since its introduction in the early 1970s few detailed studies have been reported on bentazone metabolism. However, the literature suggests that differential metabolism may form the basis of selectivity in certain instances. Metabolism was faster in resistant than susceptible soybean cultivars (Hayes & Wax, 1975), and involved conversion to a 6-hydroxy, an 8-hydroxy and finally a glycosyl form in rice, (Mine et al, 1975). This has been confirmed more recently in soybean using cell suspension cultures (Sterling and Balke, 1989). Bentazone hydroxylation may possibly involve cytochrome P450 activity. This is thought to catalyse many detoxification reactions in higher plants (Higashi, 1985; Hendry, 1986). However, characterisation of plant cytochrome P450 is poor due to problems with extraction and purification, which include low specific activity (Markham et al, 1972), instability in vitro (Leah et al, unpublished observations) and its membrane bound nature (Gressel, 1988).

Many molecules are known which inhibit cytochrome P450 reactions. Investigations into the mode of action of the nitrogen heterocycle growth regulators, tetcyclasis, BAS 110 and BAS 111 have suggested that they bind to the protoheme iron of cytochrome P450 thereby inactivating the P450 oxidation reactions of gibberellin synthesis (Rademacher et al, 1984).

Inhibition of P450 activity by tetcyclasis, BAS 110 and BAS 111 is thought to be quite specific in plants (Rademacher et al, 1987) although a variety of P450 reactions are inhibited depending upon concentration.

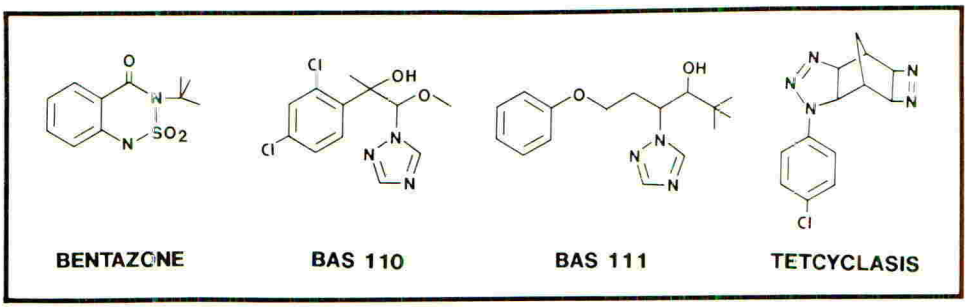

FIGURE 1: Structures of bentazone and potential inhibitors of bentazone metabolism.

Cell suspension cultures of maize and cotton have been used to demonstrate the inhibitory action of tetcyclasis pre-treatment upon the oxidative metabolism of chlorotoluron (Cole and Owen, 1987) and bentazone metabolism has also been studied after tetcyclasis pre-treatment of rice and soybean in cell suspensions (Sterling and Balke, 1988). In the latter study, it was suggested that inhibition of bentazone metabolism by tetcyclasis may occur as a consequence of cell damage in soybean. Clearly the commercial potential for interactions of tetcyclasis and many other triazoles with herbicides such as bentazone is of great academic importance and commercial potential. However, little biochemical evidence exists regarding such metabolic interactions in leaves. The aim of the present study was to investigate further the hydroxylation and glycosylation reactions of bentazone in resistant soybean and susceptible (C. bursa-pastoris) species and to study the effects of tetcyclasis, BAS 110 and BAS 111 upon bentazone metabolism.

MATERIALS AND METHODS

Growth conditions

Soybean (cv. Harcor) seedlings were grown in vermiculite, watered daily from above and supplemented twice weekly with 0.2% (v/v) Fisons "Liquinure". Seedlings of C. bursa-pastoris were grown in a peat based potting compost and watered every other day. Growth conditions for both species were 18 - 25°C and a 16 h photoperiod of 200-450 $\mu mol/m^2/s$ photosynthetic photon flux density. Plants were used after three weeks when the first trifoliate was fully expanded in soybean and the third leaf pair had expanded in C. bursa-pastoris.

Studies of ^{14}C-bentazone metabolism in intact leaves

Tetcyclasis ($10^{-5}M$), BAS 110 ($10^{-6}M$) and BAS 111 ($10^{-6}M$), kindly donated by BASF (F.R.G.), were prepared in 0.2% (v/v) Agral '90' (ICI) after initial solubilisation in a minimal volume of acetone.

Five µl aliquots were applied to the base of the mid-rib of at least ten leaves of each species and control plants treated with surfactant only. After 24 h, leaves were treated with bentazone (BZ) as follows: 0.2 µCi of phenyl-[U-^{14}C]-BZ (45.5 mCi/mmole) was formulated in a total volume of 5 µl of Basagran (BAS 351H, 480 g a.i./1, BASF) at a field-rate equivalent to 13 mM BZ and applied to the pre-treated leaf region. At intervals leaves were harvested and carefully washed in 100% (v/v) methanol to remove surface activity. Leaves were then extracted in 80% (v/v) acetone in a hand homogeniser and centrifuged at 7,500 x g for 5 min. Washings were retained to assess BZ uptake. The pellet was then re-extracted twice and supernatants combined and concentrated by evaporation. Recoveries of ^{14}C activity in supernatants were always greater than 95% of the total ^{14}C activity in the pellet. Pellets typically contained less than 5% of ^{14}C activity which was representative of supernatant metabolites upon TLC analysis.

Metabolite separation and analysis

Concentrated leaf extracts were dissolved in a minimum of 80% (v/v) acetone, applied to 10 x 10 cm silica gel TLC plates (Kieselgel 60, F 254, Merck) and separated using chloroform:methanol:ammonium hydroxide (13:7:1 v/v/v) as solvent. Positions of solvent front and any fluorescent bands visible under U.V. light were noted prior to sectioning the TLC plate into 1 cm squares. All TLC squares were counted for ^{14}C activity by liquid scintillation spectrometry using 10 ml of 'Hi-safe' (LKB) as scintillant.

Unlabelled BZ metabolites, corresponding to ^{14}C-labelled metabolites were obtained in higher yields to allow identification by co-chromatography with pure 6-hydroxybentazone (6-OHBZ) and by β-glucosidase treatment of the putative glycosyl-OHBZ metabolite. In this case soybean trifoliates were sprayed with Basagran (480 g a.i./1) at a rate of 1.44 Kg a.i./ha using a Mardrive laboratory sprayer (Marine Engineering Company Ltd., Stockport, UK) fitted with a single Lurmark 02-f80 nozzle, which delivered 300 l/ha spray volume at 3 bars pressure. After 10 days, trifoliate leaves were processed as previously described. Fluorescent bands visible upon TLC, corresponding to ^{14}C-labelled BZ metabolites, were scraped off and extracted in 80% (v/v) acetone and again with distilled water. Samples were centrifuged at 7,500 x g for 5 min and evaporated to dryness. Extracts were incubated with 0.5 ml of 0.1 M sodium acetate buffer pH 5.0 containing 1 mg/ml (5.3 units/mg) β-glucosidase (Sigma) at 35°C for 2 h in a shaking water bath as previously described (Hayes and Wax, 1975). Samples were evaporated to dryness and re-dissolved in methanol. Treated and untreated samples were concentrated and compared by TLC analysis.

RESULTS

Identification of two major BZ metabolites, namely 6-OHBZ and a glycosyl-OHBZ conjugate, is illustrated in Figure 2. The first metabolite produced in soybean was identified as 6-hydroxybentazone by co-chromatography with the known standard (Rf = 0.51-0.55). The second metabolite (Rf = 0.35-0.38), which was initially isolated from the TLC plate, was susceptible to β-glucosidase treatment resulting in the production of

6-OHBZ, again identified by co-chromatography with standards. This strongly suggests that at least one glucose molecule is being enzymatically cleaved from the BZ molecule. However, it does not demonstrate how many sugar residues are conjugated, since sites other than on the 6-hydroxy position of the BZ molecule may also permit glycosyl conjugation.

The effect of light (16 h photoperiod) and total darkness on BZ metabolism by soybean is shown in Figure 3. Metabolism of BZ to 6-OHBZ was rapid in both light and dark conditions with 20-30% of applied activity in this metabolite after 2-3 h. The glycosyl-OHBZ conjugate was only detected after 24 h and activity in this fraction then increased with time. After 7 d in light conditions, the concentration of BZ in the leaf was reduced to 42% of the original amount, with about 33% in the 6-hydroxy form and 25% in the conjugated form. After 7 d in darkness final BZ levels were reduced to about 22% and the major metabolite was the glycosyl-OHBZ conjugate, comprising about 52% of total ^{14}C activity in the leaf. Production of the conjugate from 6-OHBZ appears to be faster under dark conditions resulting in about twice the levels of the glycosyl form, when compared to metabolism in the light.

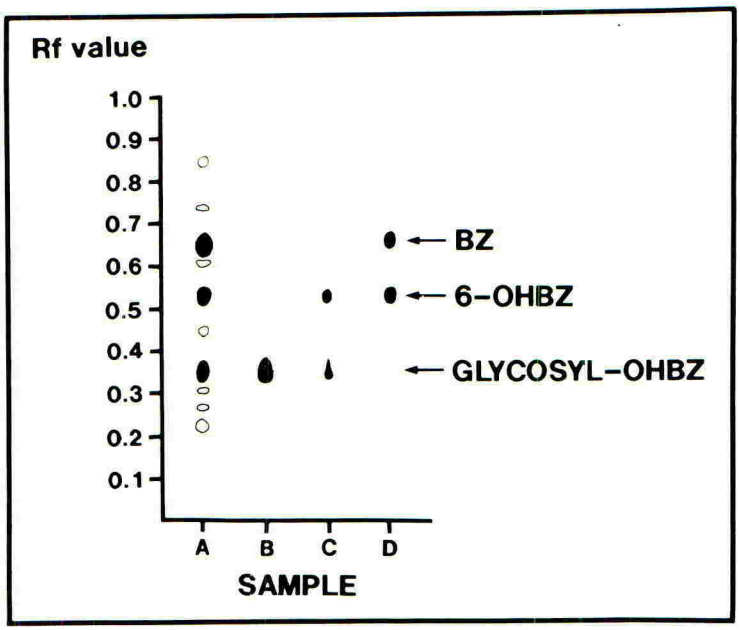

FIGURE 2: Separation of BZ metabolites by TLC visualised under U.V. light: Lane A, soybean leaf extract 10 days post-Basagran treatment showing all fluorescent components; Lane B, isolated glycosyl-OHBZ; Lane C, glycosyl-OHBZ metabolite after β-glucosidase treatment; Lane D, Authentic BZ and 6-OHBZ standards.

The relative rates of BZ uptake by soybean trifoliate leaves after various pre-treatments in light conditions are illustrated in Figure 4. Uptake of BZ by leaves pre-treated with surfactant alone (control) showed

no significant difference from pre-treatments with tetcyclasis, BAS 110 and BAS 111. Uptake reached a maximum after about 2 h resulting in a maximum of approximately 20% of the total ^{14}C-BZ being absorbed by the leaf. The percentage accumulation of total BZ metabolites in trifoliates

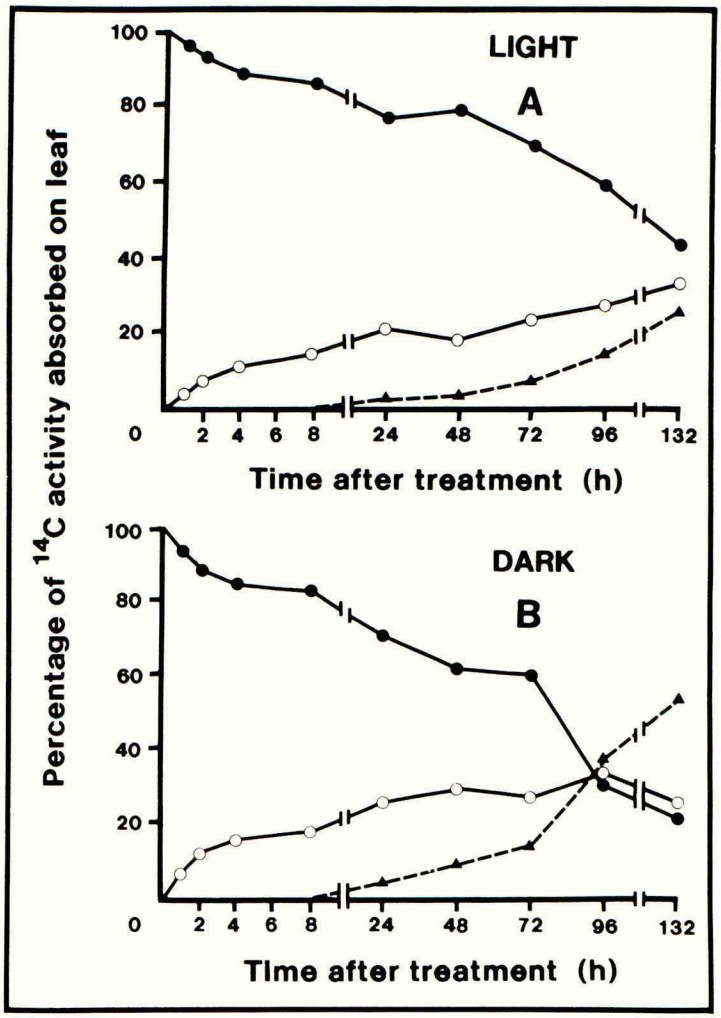

FIGURE 3: Time course of ^{14}C-BZ metabolism in trifoliate leaves of soybean light and darkness (see text). Values are a percentage of total ^{14}C activity absorbed onto leaf after washing.

Key

BZ: ●—●

6-OHBZ: ○—○

glycosyl-OHBZ: ▲—▲

with no triazole pre-treatment was rapid and may be initially limited by

the rate of uptake. Metabolite accumulation appeared to be biphasic as a result of glucosyl transferase activity after 24 h, as previously noted in Figure 3. In contrast to controls, pre-treatment with tetcyclasis (10^{-5} M), BAS 110 (10^{-6} M) and BAS 111 (10^{-6} M) under the conditions described dramatically inhibited metabolism. Indeed, leaf chlorosis and necrosis occurred about 48 h after BZ treatment in all cases of triazole pre-treatment. Levels remained below 10% after a week with no glycosyl-OHBZ being produced with any of the triazole pre-treatments. Experiments initially using BAS 110 and BAS 111 at 10^{-5} M produced no BZ metabolism under the conditions described and caused rapid leaf necrosis beyond 48 h.

BZ was not metabolised at all in C. bursa-pastoris under the conditions used. Leaves became chlorotic after 24 h and necrotic after 48 h. The rate of ^{14}C-BZ uptake was similar to that for soybean but maximum absorbed levels reached only 12% of total applied ^{14}C activity after 24 h. After this time leaf damage precluded further measurement of uptake.

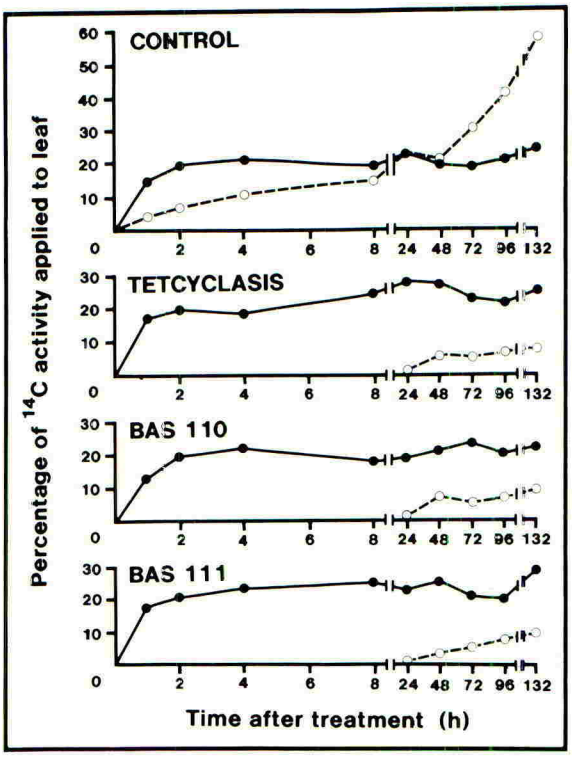

FIGURE 4: Time course of ^{14}C-BZ uptake in trifoliate soybean leaves after various treatments (see text). Values are a percentage of the total ^{14}C activity applied to leaf. Broken lines indicate abundance of total BZ metabolites expressed as a percentage of ^{14}C activity absorbed onto leaf after washing.

DISCUSSION

Metabolism of BZ to 6-hydroxy and 8-hydroxy derivatives and then to glycosyl conjugates has been shown previously in resistant plants such as soybean (Mine et al, 1975; Sterling and Balke, 1989). In this study, the 8-hydroxy metabolite could not be detected although it may have co-migrated with 6-OHBZ during TLC. Only a single ^{14}C-glycosylated metabolite was detected by TLC separation and it may be possible for further conjugation with glucose. The procedure for metabolite extraction cannot determine whether the glycosyl metabolite is bound, or has been extracted into its free form. However, the hydroxylation-glucosylation route of BZ metabolism has been clearly confirmed in the leaves of soybean in this study. No metabolism of BZ was detected in C. bursa-pastoris leaves and this supports previous studies where none was observed in susceptible soybean cultivar cell suspension cultures (Sterling and Balke, 1989). A difference in the pattern of metabolite accumulation was noted between soybean leaves grown in light and darkness. The triazoles used in this study, were very effective at inhibiting the hydroxylation of BZ in soybean whilst having no effect on uptake. In view of the inhibitory action of such compounds on P450 oxidations (Rademacher et al, 1987) the inhibition of BZ hydroxylation suggests that this may be carried out by a P450-type enzyme.

Hydroxylation of BZ by soybean is rapid and may have been limited by the rate of uptake. Since BZ hydroxylation commenced immediately, it is possible that the enzyme responsible was very rapidly induced or was already present in sufficient amounts in the plant to metabolise BZ. Only a small proportion of the ^{14}C-BZ absorbed onto the leaf reaches the site of toxicity/metabolism so that the apparent level of unmetabolised BZ, which remains as high as 80% in light conditions in soybean after 48 h (Figure 3A), is unlikely to reflect the amount of BZ at the site of action. Apparent rates of metabolism are therefore likely to depend on the rate of uptake through the leaf tissue, and at a molecular level, upon diffusion rates to the site of toxicity/metabolism. This rate is also affected by the formulation of the herbicide. Results also suggest that 6-OHBZ may be a poorer substrate for glycosylation than BZ is for hydroxylation, resulting in an initial accummulation of the intermediate.

In dark conditions, conversion of 6-OHBZ to glycosyl-OHBZ is more rapid after 3 days than in light conditions. This may be due to reduced availability of hexose for conjugation reactions inspite of increased carbohydrate synthesis in light. The overall metabolic rate of the leaf will be less in dark conditions but this does not appear to affect the rate of BZ metabolism. However, the significance of this observation requires further study.

While there is no direct evidence that BZ metabolism is prevented specifically by cytochrome P450 inhibition, it is well known that tetcyclasis, BAS 110 and BAS 111 are inhibitors of several cytochrome P450 reactions (Rademacher et al, 1987; Cole and Owen, 1987; Sterling and Balke, 1988). Furthermore, inhibition of cytochrome P450 enzymes by nitrogen heterocycles appears to be quite selective. The dramatic inhibition of 6-OHBZ production and the resultant leaf necrosis provides some in vivo evidence of cytochrome P450-dependant BZ metabolism in soybean since the hydroxylation reaction is inhibited by all 3 triazoles used.

ACKNOWLEDGEMENTS

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TRIASULFURON AND ITS SELECTIVE BEHAVIOUR IN WHEAT AND *LOLIUM PERENNE*

A.M. MEYER; F. MÜLLER

University of Hohenheim, Institute of Phytomedicine, Section Phytopharmacology, D-7000 Stuttgart 70, FRG

ABSTRACT

^{14}C-Triasulfuron was applied to wheat and *Lolium perenne* plants at the 3rd leaf stage. Uptake, translocation and metabolism were subsequently investigated. Translocation out of the treated leaf over 6 days was very low (< 2 %). When the herbicide was applied to the roots in nutrient solution 38.9 % of the radioactivity was found in the shoots of wheat and 72.3 % in the shoots of *L. perenne*. Extraction of the radioactive material from wheat showed triasulfuron to be metabolised rapidly. After one day only 14.0 % could be identified as the unchanged active substance. One metabolite was present as a sugar-conjugate. The same types of metabolites, but to a lesser extent, were found in *L. perenne*. Seventy percent of the radioactive material was identified as triasulfuron. These experiments have demonstrated that *L. perenne* absorbs more triasulfuron, translocates higher amounts of the compound to the shoots and metabolises the active substance more slowly than wheat.

INTRODUCTION

Triasulfuron (3 - (6-methoxy-4-methyl-1,3,5-triazin-2-yl)-1-[2-(2-chloroethoxy)-phenylsulfonyl]urea) is a sulfonylurea herbicide used for the control of broadleaf weeds in cereals. It shows activity also against some grass weeds, notably *Lolium perenne* which can be controlled selectively in wheat (Amrein & Gerber 1985; Buchholz *et al.* 1986; Madsen 1988). Triasulfuron is the active ingredient (20 %) in the commercial herbicides Logran® and Amber® (registered trademarks of CIBA-GEIGY Ltd., Basle, Switzerland).

MATERIALS AND METHODS

Radioactive substance

The trials were conducted with ^{14}C-triasulfuron labelled in the phenylring (specific activity 24.4 µCi/mg; 0.903 MBq/mg) supplied by CIBA-GEIGY Ltd., Basle, Switzerland. The radiochemical purity was 97 %.

Treatment of plants

Winter wheat (*Triticum aestivum* cv. Kanzler) and *Lolium perenne* (cv. Cropper) were grown in horticultural soil in the greenhouse. The plants were treated at the 3 leaf stage. Foliar treatment of each plant with ^{14}C-triasulfuron was carried out in a solution containing a blank commercial formulation and 0.025 % Citowett. Each plant was treated with a

Hamilton syringe, application being made to the second leaf (wheat 20 µl, *Lolium perenne* 10 µl). For root uptake studies the radiolabelled herbicide was administered in 2 ml of Hoagland nutrient solution per plant.

Uptake and translocation studies

After the exposure period the treated plant parts were rinsed 3 times with water to remove herbicide that had not penetrated into the tissues. These aqueous samples were subjected to liquid scintillation counting (Packard TRICARB 2000 CA, Packard, Downers Grove, Ill, USA) in a scintillation cocktail containing 1000 ml dioxane, 40 g naphthalene, 20 g ethyleneglycol, 4 g PPO and 0.2 g POPOP. The plant material was dried for 4 h at 90°C and combusted in a Packard TRICARB 306 sample oxidiser. The evolved $^{14}CO_2$ was absorbed in Carbosorb II® and subjected to liquid scintillation counting. This scintillation cocktail contained 1000 ml toluene, 10 g PPO and 0.2 g POPOP.

Metabolism studies

After an exposure of 1 day and 6 days in nutrient solution the roots were rinsed 3 times with water to remove the radiolabelled herbicide not taken up. The plants were separated into shoots and roots which were extracted 5 times with acetonitrile/water (4:1). Before the first three extractions the plant material was ultra-sonicated for 3 minutes. The supernatants were collected and afterwards reduced to the aqueous phase by rotary evaporation and finally extracted with toluene and then chloroform. The non extractable residues were combusted. Each extract was then radioassayed. The recovery of radioactive material was 95-98%. Extraction efficiency was between 93 - 99 % for shoots and 89 - 93% for roots.

The extracts were separated on 0.25 mm Kieselgel $60F_{254}$ (Merck, Darmstadt, FRG). The TLC plates were developed in chloroform/methanol/formic acid/water (150:42:2:6, v/v/v/v). The radioactivity on the plates was evaluated with a TLC Linear-Analyzer LB 2832 (Berthold, Wildbad, FRG).

Enzymatic hydrolysis

The aqueous phase remaining from the toluene and chloroform partitioning was mixed with 0.1 M sodium acetate and 6 U/ml β-glucosidase (Sigma, Deisenhofen, FRG). This mixture was incubated at 36°C for 20 h at pH 5.0. After hydrolysis the liberated ^{14}C-substances were extracted with chloroform and subjected to TLC analysis, where the amount of ^{14}C-radioactivity permitted.

RESULTS

Uptake and translocation

Leaf treatment

After 6 days, wheat and *Lolium perenne* had taken up 28.7 and 22.6 % respectively of the foliarly applied ^{14}C-triasulfuron (Table 1). The main portion of radiolabelled compound remained in the shoot. There was little translocation out of the treated leaf. After 6 days 0.4 % could be found in the non treated parts of wheat and 1.5 % of the radioactive material taken up was exported out of the treated leaf of *L. perenne*.

TABLE 1. Rate of triasulfuron uptake after leaf treatment (1.1 μM).

Plant species	Applied radioactivity	Exposure time 1 d	6 d
Wheat	100	14.0	28.7
L. perenne	100	13.6	22.6

Data are percent of mean values of 3 replicates of 5 plants.

Root treatment

The radiolabelled compound was taken up from nutrient solution. In wheat 21.9 % and 46.6 % of the applied radioactivity was found in the roots 1 day and 6 days respectively after application. Uptake by *L. perenne* was less efficient; 3.3 % and 14.4 % were taken up after 1 day and 6 days respectively (Table 2). In both plant species the same amounts were translocated within one day into the shoots (Figure 1). The fraction of translocated compound was similar after 1 day and 6 days exposure in wheat but increased to 72.3 % after 6 days in *L. perenne*. On a dry weight basis the amount of radioactivity was always higher in leaves of *L. perenne* than in leaves of wheat. The highest amount of radioactivity was found in older leaves with higher transpiration rates (Table 3).

TABLE 2. Rate of uptake after root treatment with triasulfuron in both species (0.25 μCi/plant).

Plant species	Applied radioactivity	Exposure time 1 d	6 d
Wheat	100	21.9	46.6
L. perenne	100	3.3	14.4

Data are percent of mean values of 3 replicates of 5 plants.

Metabolism

Radiolabelled triasulfuron was applied via the roots. Plant extracts were obtained 1 day and 6 days after application. In wheat at least 11 separate bands of radioactivity could be distinguished by TLC in shoot extracts and 9 bands in root extracts. In *L. perenne* tissue extracts 6 bands were found for the shoot and 9 for the roots. Most of the substances were present in very low levels. These compounds are referred to as 'other' in Figure 2 which shows the relative amounts of the 3 principal bands in root and shoot extracts.

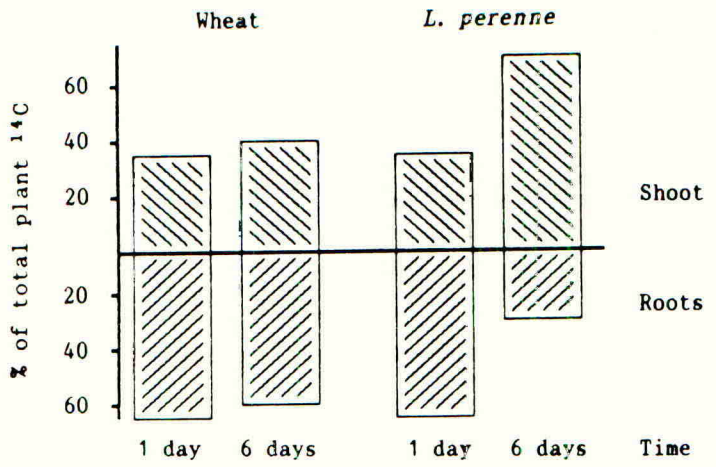

FIGURE 1. Distribution of radiolabelled compounds in shoot and roots after uptake of triasulfuron via roots (0.25 µCi/plant). Total radioactivity in the plants was 100 %.

TABLE 3. Distribution of radioactivity/dry weight in dpm/mg after root uptake (0.25 µCi/plant).

Plant species	Exposure time	^{14}C-Concentration (dpm/mg dry weight)			
		Roots	Oldest leaf	2nd leaf	Youngest leaf
Wheat	1 day	2 814	1 091	80	64
	6 days	2 075	2 075	128	69
L. perenne	1 day	4 019	1 411	224	153
	6 days	6 811	14 042	635	891

Data are percent of mean values of 3 replicates of 5 plants.

In wheat one dominant metabolite (Rf 0.28) comprising 70.4 % of the total radioactivity was found one day after application. This metabolite was found predominantly in the roots. Fourteen percent of the radioactivity remained as unchanged triasulfuron and 15.7 % was present as 'other' compounds. Six days after application, the pattern was very similar: 65.5 % of the total radioactivity was located in the band at Rf 0.28, 8.2 % as the parent compound and 26.3 % as 'other' compounds.

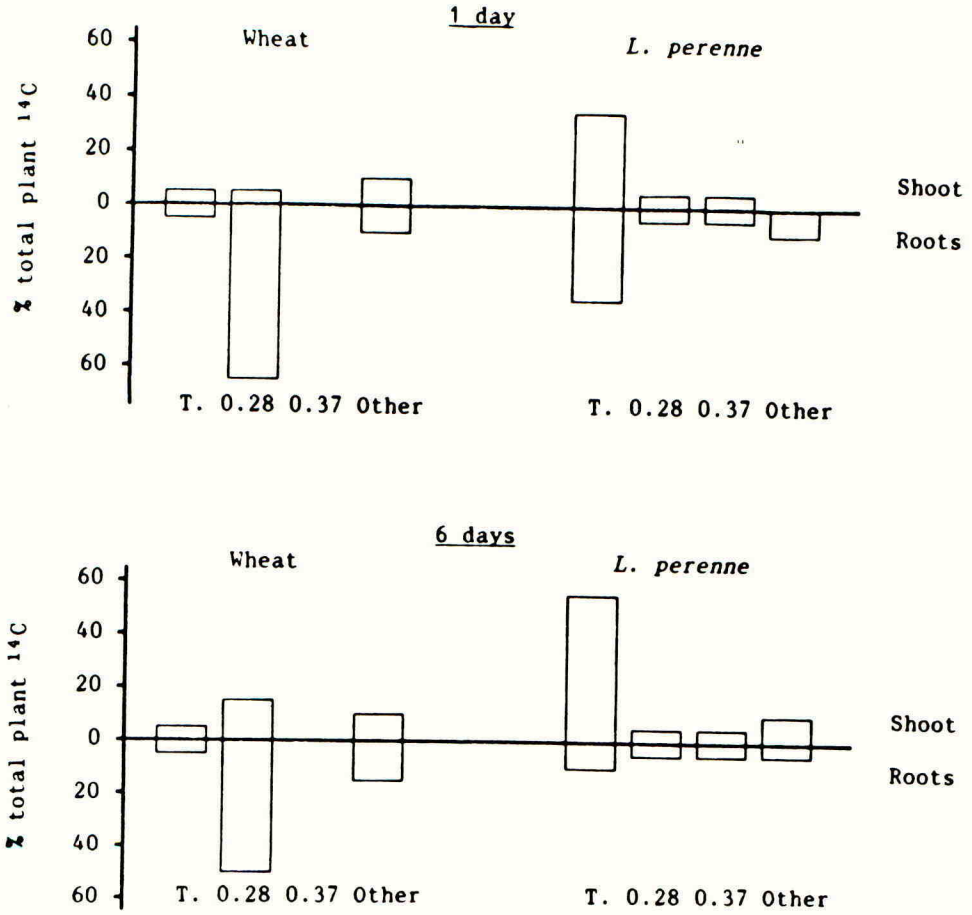

FIGURE 2. Relative amounts of the main ^{14}C-substances in shoots and roots after root treatment with triasulfuron (0.25 µCi/plant). Total radioactivity in the plants was 100 %. T. = ^{14}C-triasulfuron, 0.28, 0.37 = metabolites with Rf-values 0.28 and 0.37, Other = other substances.

In *L. perenne* 70.3 % of the radioactivity was found as unchanged triasulfuron one day after application. The amounts in roots and shoots were comparable. Only 10 % of the radioactivity was found as the Rf 0.28 metabolite whilst 13.1 % of the radioactivity separated as 'other' metabolites. Six days after application 67.1 % of the radioactivity was present as triasulfuron located predominantly in the shoot. The compound at Rf 0.28 amounted to 10.7 % and the 'other' metabolites to 15.9%. In *L. perenne* a band at Rf 0.37 was detected in small quantities (3.6 % after 1 day, 6.3 % after 6 days) which was not observed in wheat.

Enzymatic hydrolysis

An enzymatic hydrolysis using β-glucosidase was carried out with the aqueous fractions in order to discover whether the polar metabolites were conjugates. Parallel assays were run without β-glycosidase to determine the effects of temperature and Na-acetate. The aqueous fractions of wheat and *L. perenne* extracts contained predominantly the compounds at Rf 0.28 and 0.37. After enzymatic hydrolysis chloroform-soluble compounds were detected (Figure 3).

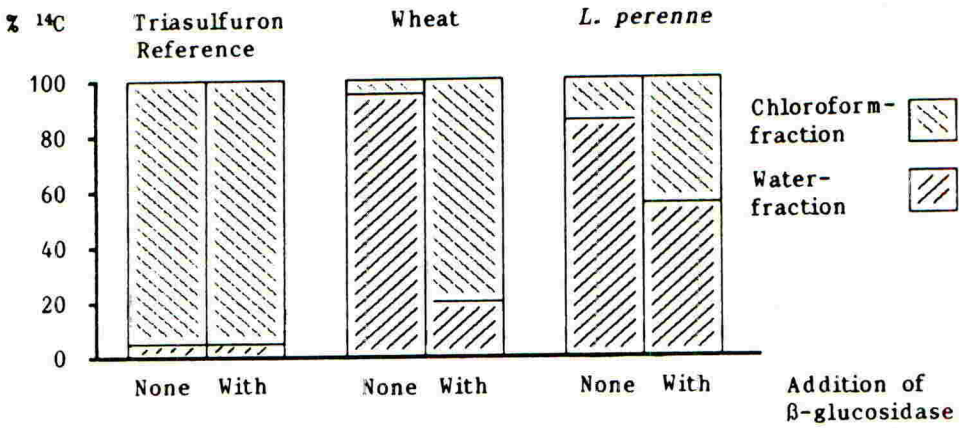

FIGURE 3. Partitioning of the radiolabelled compounds after hydrolysis of aqueous fractions (100 %) of wheat, *L. perenne* extracts, and reference solution of triasulfuron in chloroform and water fractions.

Triasulfuron itself was not changed under the conditions of hydrolysis. With and without enzyme 96 % of the radioactivity was found in the chloroform phase. In wheat extracts only 3.5 % separated into the chloroform phase in the absence of the enzyme but 84.5 % in its presence. In *L. perenne* extracts 16.5 % chloroform-soluble compounds occurred without enzyme. With enzyme 58.7 % of the radioactivity was found in the chloroform phase.

The hydrolysed extracts of wheat were separated by TLC (Figure 4). The aqueous fraction contained mainly the compound at Rf 0.28. Little change was observed in the absence of the enzyme. In the presence of β-glucosidase radioactivity appeared in a band at Rf 0.74 whereas that at Rf 0.28 disappeared. The TLC behaviour of the aglycone corresponds to authentic OH-phenyl triasulfuron.

DISCUSSION

The herbicide triasulfuron is used pre- and post-emergence, having both foliar and soil activity. After leaf application of 20 g a.i./ha or more only small quantities were translocated to other parts of the plant. Triasulfuron was taken up well by roots in wheat and *Lolium perenne*. Growth of *L. perenne* ceased after triasulfuron treatment whereas wheat continued to grow.

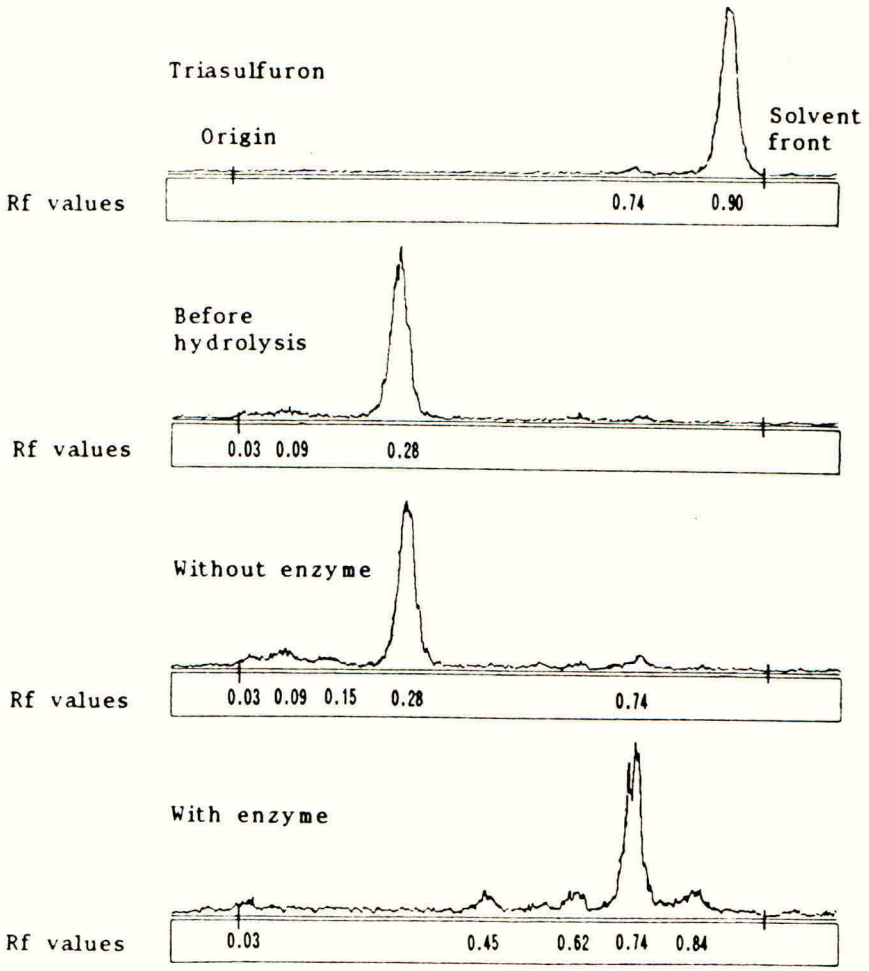

FIGURE 4. TLC-separation of the aqueous fraction of wheat extracts before and after addition of β-glucosidase or with addition of buffer solution only.
Solvent: chloroform/methanol/formic acid/water (150:42:2:6, v/v/v/v).

One day after treatment only small amounts of the parent compound could be found in wheat plants. A compound was formed which could be enzymatically decomposed. This compound could be the 5-*O*-phenyl-glucose conjugate analogous to that described by Beyer *et al.* (1988) for chlorsulfuron. After enzymatical hydrolysis probably phenyl ring hydroxylated triasulfuron is formed on the basis of similar TLC behaviour to that of the known reference compound. This compound is found only in small quantities in the aqueous fraction of wheat extracts as well as in the organic solvent fraction. In *L. perenne* it seems that more than one metabolite occurs which can be decomposed by β-glucosidase. After enzymatic hydrolysis

greater amounts of radiolabelled compounds are found in the chloroform fraction than expected from the amount of the metabolite Rf 0.28. In both plant species the same metabolites are found. Even in wheat extracts traces of a compound with an Rf value of 0.37 were present.

Compared to wheat, *L. perenne* takes up relatively more triasulfuron which is translocated in higher quantities into the shoot. The transformation of triasulfuron into putatively inactive compounds is slower in *L. perenne*. Thus the uptake, translocation and the rate of metabolism seem to be the reasons for the selective activity of triasulfuron.

ACKNOWLEDGEMENTS

We thank CIBA-GEIGY Ltd., Basle for support and providing radiolabelled triasulfuron and reference compounds.

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QUINMERAC - STUDIES TO INVESTIGATE SELECTIVITY IN WHEAT

R. BERGHAUS, G.RETZLAFF

BASF Aktiengesellschaft, Landwirtschaftliche Versuchsstation
D-6703 Limburgerhof, FR-Germany

ABSTRACT

Quinmerac (BAS 518 H, 7-chloro-3-methyl-8-quinoline carboxylic acid) is being developed as a herbicide to control cleavers (Galium aparine) in cereals, oilseed rape and sugar beet. Earlier mode of action studies indicated that quinmerac has auxin activity.
Recent experiments have shown a primary influence of quinmerac on root physiology. Even low concentrations of quinmerac in the nutrient solution led to a rapid inhibition of growth, transpiration and CO_2-assimilation in cleavers. Plants with their roots removed suffered less injury when incubated with quinmerac than those with intact roots, although similar amounts were actually taken up. In contrast, wheat roots were almost completely insensitive to quinmerac.
Further studies have revealed that differences in root and leaf uptake of quinmerac could not be responsible for its selectivity but that its metabolism in wheat is much faster than that in cleavers.
These observations have led to the conclusion that the extreme sensitivity of cleaver roots to quinmerac, accompanied by its slower rate of metabolism, may explain its susceptibility in contrast to the tolerance of wheat.

INTRODUCTION

During the last few years, cleavers (Galium aparine) has become one of the major weeds in many crops of northern Europe. In 1985 quinmerac (BAS 518 H, 7-chloro-3-methyl-8-quinoline carboxylic acid) was presented as a new experimental herbicide to control cleavers and other weeds in cereals, sugar beet and oilseed rape (Wuerzer et al. 1985, Nuyken et al. 1985). Quinmerac was also shown to be effective against Veronica hederaefolia and Lamium purpureum.

Earlier studies of the mode of action revealed that quinmerac has auxin activity (Berghaus & Wuerzer, 1987). The compound is taken up mainly by the roots and is translocated acro- and basipetally. In this paper, additional experiments dealing with the mode of action are presented to explain the selectivity of quinmerac in wheat.

MATERIALS AND METHODS

Plant material

Cleavers (Galium aparine) and wheat (Triticum aestivum cv. Caribo) were grown in vermiculite in growth chambers at 22 °C and a 14 h photoperiod of 400 $\mu E/m^2S$ photosynthetic photon flux density. For the actual experiments, plants were transferred to a hydroponic nutrient solution (Rademacher & Jung 1983) when wheat plants had developed three leaves and cleavers the first whorl.

Leaf uptake

For uptake studies, 20 µl formulated ^{14}C-quinmerac (sp. act. 549.5 MBq/mM, labeled at C-atom 2 in the heterocyclus), in combination with an adjuvant, was applied onto the second leaf of wheat or the first whorl of cleavers with the aid of a micro syringe. After different time intervals, treated leaf samples were washed with a 0.05 % aqueous solution of nekanil to remove residues. Samples were than combusted in an oxidizer (Zinser, Oxymat, OX 300) and the evolved $^{14}CO_2$ was absorbed in a LS cocktail and radioassayed in a scintillation counter (Packard, Tricarb, 460 CD).

Root uptake

Root uptake was studied by cultivating the test plants in a hydroponic solution to which quinmerac has been added at a final concentration of 10 µM. At different time intervals, plants were removed from the culture medium and their roots were washed. Root and shoot radioactivity were measured as described above. The influence of the roots was ascertained by comparing the results obtained in parallel experiments in which plants with intact or with removed roots were used. In both cases, the plants were grown in a similar nutrient solution and radioactivity determination was carried out as in the other studies.

Transpiration and herbicidal activity

Transpiration was determined by cultivating plants in a hydroponic solution to which different concentrations of quinmerac had been added. Water loss was measured by substituting the consumed water with nutrient solution. After 11 days the herbicidal damage was evaluated visually. Parallel to this, plants were cultivated and treated in a similar way after their roots had been cut off.

Photosynthetic measurement

Cleavers were cultivated in a hydroponic solution. Quinmerac was applied either to the nutrient solution or as formulated material together with an adjuvant onto the leaves. CO_2-assimilation was measured as described by Retzlaff & Hamm (1976).

Metabolism

Radioactively labelled quinmerac was applied as in the root uptake study. Twenty four hours and 72 h after the addition of quinmerac the plants were divided into root and shoot components and homogenized in methanol with an Ultra-Turrax. The methanol extract was evaporated, redissolved in water, acidified with HCl to a pH of 1-2, and initially extracted with n-hexane. The aqueous phase was then adjusted to a pH of 9-10 with NaOH and extracted with ethyl acetate. This extract was analysed by TLC with the aid of a TLC Linear Analyser (Berthold).

RESULTS

Cleavers treated hydroponically with quinmerac stopped growing. Besides revealing auxin type morphological aberrations, the plants often developed a red colour. These symptoms were accompanied by a significant inhibition of transpiration (Fig. 1A); it was thus possible to detect the effect of quinmerac on the water consumption at a concentration as low as 10^{-9} M. In contrast, an influence of quinmerac on the transpiration of wheat plants could not be measured.

Photosynthetic assimilation of cleavers was decreased markedly 10 h after the addition of quinmerac to the nutrient solution (Fig. 1B). Leaf treatment with 500 g/ha a.i. decreased photoassimilation much more slowly and to a lesser degree. The photosynthesis of wheat plants was also not affected by quinmerac.

Both experiments suggested the importance of the root system of cleavers on the physiological activity of quinmerac. These results were confirmed by the herbicidal efficacy of quinmerac on cleavers with and without roots, after an addition to the nutrient solution (Fig. 1C). Plants with roots suffered more injury than plants without roots, although measurements with radioactive material had revealed that similar amounts of quinmerac were taken up.

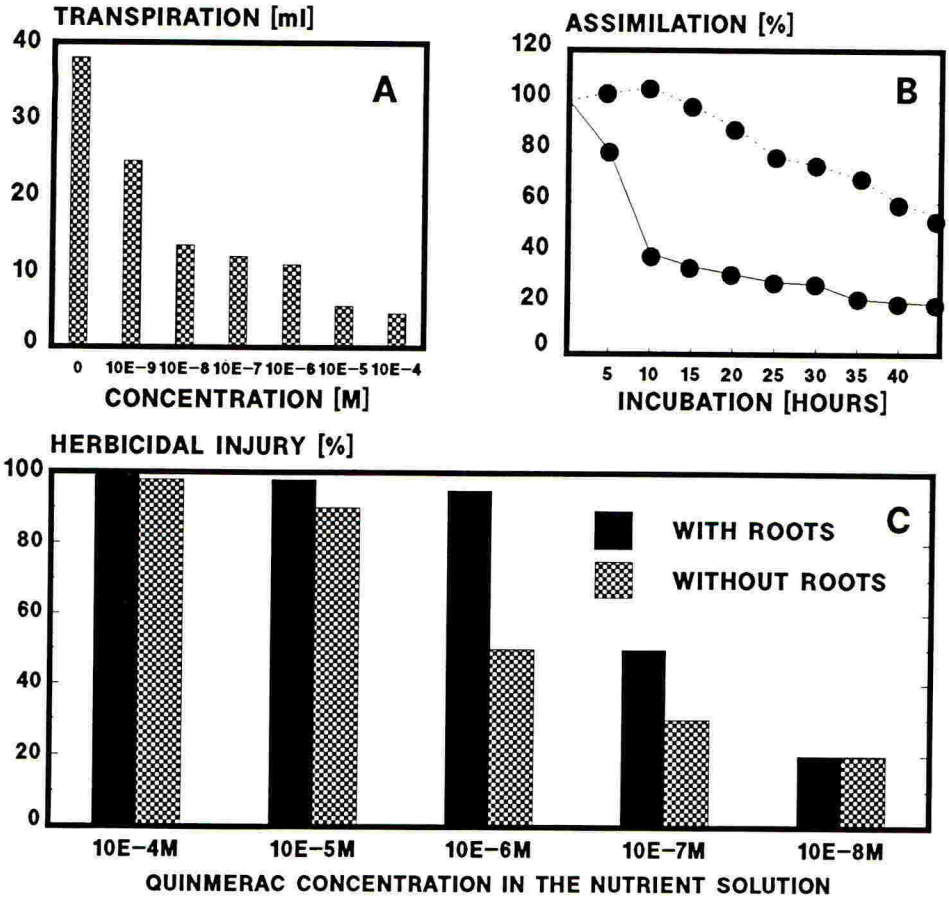

Fig. 1. A: Effect of different concentrations of quinmerac in the nutrient solution on the transpiration of cleavers. B: Effect of root application (10^{-5} M) (—●—) and leaf application (500 g/ha a.i.) (---●---) on photosynthesis of cleavers related to untreated control plants. C: Herbicidal injury to cleavers with and without roots after an addition of quinmerac to the nutrient solution.

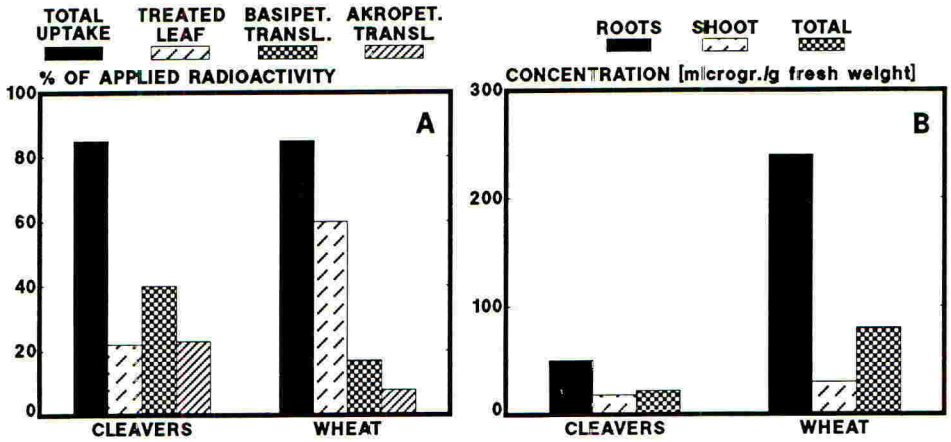

Fig. 2. A: Leaf uptake of formulated quinmerac by cleavers and wheat 5 days after application. B: Root uptake of quinmerac (10^{-5} M) by cleavers and wheat after an incubation period of 5 days.

Studies of leaf uptake (Fig. 2A) revealed that quinmerac penetrated the cuticles of wheat and cleavers at the same rate. However, translocation was better in cleavers, and this resulted in a significant accumulation of quinmerac in the basal parts of the plants. In contrast, translocation of quinmerac in wheat was less evident. On the other hand, a larger amount was taken up by the roots of wheat than by cleavers (Fig. 2B), which resulted in a distinct accumulation of quinmerac in the plants in relation to the herbicide concentration in the nutrient solution.

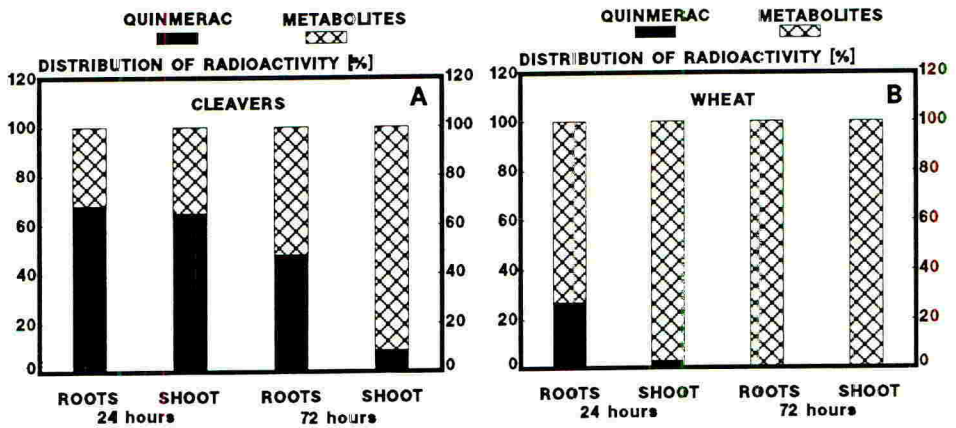

Fig. 3. Metabolism of quinmerac in cleavers (A) and wheat (B) after root incubation in a nutrient solution with 10^{-5} M quinmerac.

Differences were found in the metabolism of quinmerac in cleavers and wheat (Fig. 3) . After a root incubation of 24 h only about 40 % of the quinmerac was metabolised in cleavers but 73 % in the wheat roots and more than 90 % in the wheat shoot. Two days later, nearly all quinmerac was metabolised in wheat whereas considerable amounts could be detected in cleaver roots.

DISCUSSION

In earlier studies, the auxin properties of quinmerac were demonstrated in several test systems.The extreme sensitivity of cleaver roots to quinmerac in comparison to wheat was also shown (Berghaus & Wuerzer 1987). The strong inhibition of water consumption recorded when cleaver roots were incubated with quinmerac indicated a severe disturbance of the root physiology. Chang & Foy (1971) found comparable results with picloram and different plant species. They assumed an influence of picloram on root growth, phloem and xylem physiology and a disturbance of the stomata apertures. These explanations may also be true for quinmerac.

These results were complemented by an inhibition of photosynthesis in cleavers after root application of quinmerac. Water imbalance due to a disturbance of the root physiology leads to a rapid decrease of CO_2 fixation. A direct influence of quinmerac on photosynthesis is rather unlikely, because spray application to cleaver leaves did not decrease photosynthesis during the first 24 h and after this the reduction was significantly weaker. It is known from leaf uptake studies (Berghaus & Retzlaff 1988) that during the initial 24 h quinmerac may be translocated to the roots. In addition, the Hill reaction was only negligibly inhibited by quinmerac (not published). Similar findings were described for picloram (Sharma & Vanden Born 1971).

Herbicidal injury was significantly more severe when cleavers with intact roots were exposed to quinmerac rather than without roots, even though equal amounts of quinmerac were taken up. This confirmed our view of the herbicidal mode of action which appears to be linked to a disturbance of the root physiology, thus resulting in an inhibition of water and nutrient uptake. This in turn would lead to the observed stunting. Morphological aberrations in the shoot may not be a primary action of the herbicid. In contrast, wheat roots were insensitive to quinmerac, which explains the high selectivity of quinmerac in this crop. The reason for this may be different kinds of auxin receptors as described for naphtyl acetic acid (NAA) in mono- and dicotyledonous plants by Patel et al.(1986). Additional tests are necessary to clarify this point.

Leaf uptake studies showed no differences in the rate of quinmerac uptake between wheat and cleavers. Translocation of quinmerac was slower in wheat than in cleavers but this should not be important for selectivity, because roots are the dominant site of uptake for quinmerac (Berghaus & Retzlaff 1988). Here wheat plants accumulated significantly more quinmerac than cleavers, which proved that uptake differences can not be responsible for selectivity.

Metabolism of quinmerac was clearly lower in cleavers than in wheat. Although the experimental conditions allowed a continuous root uptake of quinmerac, only metabolites could be detected in the root and leaf tissues of wheat after 72 h whereas considerable amounts of quinmerac were found in the root tissues of cleavers. This could explain the selectivity in wheat.

CONCLUSIONS

The herbicidal activity of quinmerac resulted in a rapid and marked disturbance of cleavers root physiology. This led to an inhibition of water and nutrient consumption and a decreased photoassimilation. Auxin-type aberrations of the shoot may play a minor role. Wheat roots are almost insensitive to quinmerac, which results in a high degree of tolerance. The fast rate of quinmerac metabolism in wheat may be the reason for this tolerance.

ACKNOWLEDGEMENTS

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STRUCTURE, ALKYLATING REACTIVITY AND PHYTOTOXICITY RELATIONSHIPS OF CHLOROACETAMIDES

I. JABLONKAI, F. DUTKA

Central Research Institute for Chemistry, Hungarian Academy of Sciences, H-1525 Budapest, Hungary

ABSTRACT

The N- and SH-alkylating reactivities of chloroacetamide herbicides and other potential alkylating agents were investigated by reaction with 4-(4-nitrobenzyl)pyridine and *in vitro* glutathione conjugation. Reactivity relationships with molecular structure and phytotoxicity in tolerant and sensitive plant species are discussed. It is suggested that phytotoxicity is related primarily to reduced conjugation ability with glutathione *in vitro*.

INTRODUCTION

In spite of substantial studies on the mode of action of chloroacetamides over the past two decades the exact site(s) of action and the reaction(s) responsible for herbicidal activity are still unknown. It is thought that chloroacetamides act by alkylating biological nucleophiles. In one model a nucleophilic displacement may occur between the reactive *alpha*-halogen of chloroacetamides and the amino group of initiating methionyl-tRNA thus blocking protein biosynthesis (Jaworski, 1969). On the other hand, reversal by sulfhydryl reagents of inhibition of respiration by chloroacetamides suggested the importance of sulfhydryl groups in selective herbicide action (Jaworski, 1956). Metabolism studies have shown that chloroacetamide detoxication takes place *via* glutathione (GSH) conjugation (Lamoureux *et al.*, 1971; Jablonkai and Dutka, 1985; LeBaron *et al.*, 1988). Since numerous enzyme systems contain sulfhydryl group vital to the activity of the enzyme it was conceivable that a large variety of enzymes might be inhibited by chloroacetamides. However, nonspecific alkylation of enzyme sulfhydryl groups apparently is not important in the action of these compounds (Marsh *et al.*, 1975). Other potential sites (having SH group) of alkylation may include lipoic acid, the acyl carrier protein and coenzyme A (Jaworski, 1956; Fuerst, 1987).

These findings prompted us to study the relationships between chemical structure and N- and SH-alkylating reactivity as well as the phytotoxicity of chloroacetamide herbicides and other alkylating agents of this type.

fitted with an alkali flame ionization detector. The glass column (1 m x 2.7 mm I.D.) was packed with 80-100 mesh Gaschrom Q coated with 3% OV-17. Column temperature was programmed from 180°C to 250°C at 10°C min^{-1}. Ethyl chloroacetate was analysed isothermally at 50°C. Injector and detector temperatures were 220°C and 250°C, respectively. Nitrogen carrier gas was supplied at 30 ml min^{-1}. Ethyl chloroacetate and chlorfenprop-methyl were analysed by using flame ionization detection.

RESULTS AND DISCUSSION

N-Alkylating reactivity of the chemicals in Table 1 was determined spectrophotometrically following reaction with NBP reagent. Because of the large excess of the reagent alkylation can be described by first order kinetics. Slopes of calibration curves (Table 2) are proportional to the rates of reactions.

TABLE 2 **N- and SH-alkylation reactivity of haloacetamide derivatives**

No	N-alkylation[a]	SH-alkylation[b]
1	86	100±2
2	4162	-
3	50	-
4	9	100±3
5	218	100±2
6	8047	100±0
7	91	100±4
8	67	95±4
9	119	78±5
10	0.3	-
11	336	100±2
12	483	100±1
13	100	100±3
14	124	100±4
15	151	78±2
16	19	61±5
17	142	53±4
18	47	85±4
19	95	76±1
20	129	34±7
21	1	85±4

[a]Slopes of calibration curves
[b]Per cent of GSH conjugates at GSH/substrate=25

The high reactivity of iodoacetamide (**2**) and N,N-diallyl-bromoacetamide (**6**) is clearly shown. One N-allyl group (**4**) in the chloroacetamide (**3**) reduced activity but two N-allyl substituents (**5**) increased alkylating ability. *Ortho* substitution in the aromatic ring of a secondary chloroacetanilide (**8**) lowered activity while substitution in both *ortho* positions (**9**) enhanced reactivity. Replacement of a chlorine atom by a hydroxy group (**10**) led to loss of reactivity. The methyl derivative (**12**) of chloroacetyl indoline (**11**) was more reactive than the parent molecule. In the series of chloroacetanilide herbicides the alkylating reactivities of propachlor (**13**) and prynachlor (**14**) were close to that of secondary anilides (**7, 9**). The reactivity sequence of alkoxyalkyl (**15, 16, 17, 19**) and pyrazolylmethyl (**18**) chloroacetanilides was:

alachlor > acetochlor > metazachlor > dimetachlor > metolachlor

Steric and polarity factors are of prime importance in determining alkylating reactivity of chloroacetamides (Chupp and Olin, 1967). Low alkylation ability of compounds **3** and **4** compared to that of **5** may be due to their non-reactive, nonpolar iminol structure (Robin *et al.*, 1970):

Dialkyl substitution of the N atom prevents iminol formation. The higher reactivity of **12** compared to **11** may be due to the bulk effect of the 7-methyl group, enforcing a rotamer which is more active in alkylation processes (Chupp and Olin, 1967). The nature of the N-substituent may influence the reactivity of acetanilides in two ways:

- decrease in its electron withdrawing capability reduces reactivity,
- vicinity of its ether oxygen to the halomethylene group by anchimeric assistance of displacement of chlorine enhances alkylating activity.

For SH-alkylating ability only the compounds were investigated which could be analysed by g l c. Since the pKa value of the sulfhydryl group of GSH is 8.66 in the buffer used GSH exists in anionic form (GS^-) posessing high nucleophilicity in alkylation reactions. Differences in the SH-alkylation capacity of compounds determined by GSH conjugation *in vitro* were generally lesser than for N-alkylation (Table 2). With the exception of 2,6-dialkyl substituted anilides all compounds reacted readily with GSH at the ratio of GSH/substrate applied. The relatively high reactivity of molecules can be attributed to nucleophilic catalysis by the amino group of the Tris buffer (Frear and Swanson, 1970).

TABLE 3 **Phytotoxicity of alkylating agents**

No	Applicaton rate (kg/ha)	Shoot fresh weight, % of control				
		Maize	Mustard	Oat	Ryegrass	Cleavers
1	5	112	125	104	83	100
	10	104	120	90	96	70
2	5	16	20	32	91	83
	10	8	5	22	53	27
3	5	108	105	88	98	71
	10	104	30	78	98	68
4	5	112	90	79	32	111
	10	96	85	41	0	114
5	5	100	95	41	19	74
	10	96	45	0	0	30
6	5	96	95	69	70	78
	10	120	35	79	26	69
7	5	112	110	77	94	108
	10	104	55	77	28	59
8	5	128	70	44	32	96
	10	96	5	19	0	29
9	5	96	60	17	30	20
	10	88	35	7	0	0
10	5	112	140	108	77	139
	10	120	50	100	77	81
11	5	120	120	92	60	53
	10	104	80	93	49	64
12	5	112	105	78	64	70
	10	104	20	64	36	26
13	1.25	105	72	58	8	40
	2.5	100	56	37	8	20
14	1.25	90	56	47	15	20
	2.5	95	24	32	0	0
15	1.25	79	20	0	0	40
	2.5	63	8	0	0	20
16	1.25	90	20	5	0	20
	2.5	68	0	0	0	10
17	1.25	53	8	0	0	20
	2.5	58	0	0	0	0
18	1.25	21	0	0	0	0
	2.5	18	0	0	0	0
19	1.25	18	0	0	0	30
	2.5	11	0	0	0	0
20	1.25	105	0	0	0	40
	2.5	95	0	10	0	20
21	1.25	79	80	89	54	50
	2.5	95	64	84	54	53

In agreement with previous observations (Hamm, 1974) we found (Table 3) that the less SH-reactive tertiary (especially 2,6-dialkyl substituted) anilides had greater herbicidal activity than that of secondary anilide, aliphatic and heteroaromatic amide structures. Contrary to anilides, in the aliphatic system phytotoxicity of compounds **3**, **4**, **5** (Table 3) increased with the number of allyl groups and could not be correlated with their N-alkylating activity (Table 2). Replacement of the chlorine atom of **5** by bromine (**6**) resulted in a significant reduction of bioactivity. This is probably due to the rapid decomposition of the molecule in the plant because of its high alkylating activity. Iodoacetamide, an inhibitor of numerous enzymes (Webb, 1966) showed non-selective herbicidal activity. Dialkyl substitution of secondary anilides in the 2,6-positions yielded greater biological activity and gave a more reactive molecule (**9**) in N-alkylation as compared to mono- (**8**) and non-substituted (**7**) anilides. The methyl group substituted at the 7-position of the indoline ring (**12**) enhanced both N-alkylating activity and phytotoxicity when compared to molecule **11**. For tertiary anilides the 2,6-dialkyl substituted derivatives (from **15** to **19**) showed higher efficacy than the molecules (**13**, **14**) without *ortho* disubstitution.

In conclusion our results demonstrate that:

- a relationship can be shown between molecular structure and N-alkylating ability of chloroacetamides,
- the phytotoxicity of these compounds is characterised primarily by their reduced capability to conjugate with GSH (Leavitt and Penner, 1979),
- to establish in more detail the relationship between chemical reactivity and phytotoxicity the alkylating ability of these compounds must be considered in conjunction with their lipophilic character. Evidence has previously been published showing a relationship between lipophilicity and herbicidal activity of chloroacetamides (Sirois, 1972).

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A NEW TYPE OF GRAMINICIDE, PP 600, WHICH INHIBITS FATTY ACID SYNTHESIS DE NOVO

K.A.WALKER, J.L.HARWOOD

Department of Biochemistry, University of Wales College of Cardiff, Cardiff, CF1 1ST.

T.LEWIS, S.M.RIDLEY

I.C.I. Agrochemicals p.l.c., Jealott's Hill Exptl. Station, Bracknell, Berks, RG12 6EY.

ABSTRACT

PP 600 (3-isopropyl-6-(N-[2,2-dimethylpropyl]-acetamido 1,3,5-triazine-2,4(1H,3H) dione) graminicide which, although differing in structure from the cyclohexanediones and aryloxyphenoxy propionates, produces similar herbicidal symptoms. To determine whether fatty acid synthesis was a target site for this compound, we have carried out a number of experiments using barley as the sensitive species and pea as the resistant species. Radiolabelling experiments using [1-^{14}C]acetate or [2-^{14}C]malonate as precursors showed that fatty acid synthesis de novo was highly sensitive to PP 600 (I_{50} = 1-2 μM) in barley leaves but was not affected in pea. Different results with the two precursors indicated that acetyl-CoA carboxylase was the target site and this enzyme was inhibited by PP 600 in direct assays. However, acetyl-CoA carboxylase activity measured *in vitro* was much less sensitive to PP 600 than tissue sections, suggesting that the herbicide is metabolised to a more active compound *in vivo*.

INTRODUCTION

Two classes of herbicides have been recently shown to inhibit lipid (and membrane) synthesis in sensitive plant species by acting at the level of acetyl-CoA carboxylase. The two classes are the cyclohexanediones and the aryloxyphenoxy propionates (Harwood, 1988a). The first visible symptoms which these herbicides cause are in newly formed leaves which become chlorotic within 2 to 4 days. A progressive necrosis of the meristematic tissue in the nodes and buds follows while the oldest leaves show senescent

pigment changes. In addition, the growth of roots is strongly inhibited with most of the damage visible in the meristematic zone of the root tip (see Walker et al., 1989).

PP 600 (3-isopropyl-6-N-[2,2-dimethylpropyl]-acetamido)-1,3,5-triazine-2,4(1H,3H)-dione) is another grass - selective herbicide which produces very similar symptoms in sensitive species as those caused by the aryloxyphenoxypropionates. Because PP 600 has a different chemical structure to the aryloxyphenoxy propionates or cyclohexanediones, we have carried out experiments to see if fatty acid synthesis and acetyl-CoA carboxylase are sensitive to this compound also.

MATERIALS AND METHODS

Materials

Pea (cv. Feltham First) seeds were obtained from Asmer Seeds, Leicester, U.K. Barley (cv. Maris Otter) seeds were kindly provided by Dr.T.Galliard, RHM Research Ltd., High Wycombe, Bucks., U.K. Seeds were germinated in soil-less compost and grown at 20°C with a 12 h photoperiod for 12-14 days.

Radiolabelled compounds were purchased from Amersham International, Bucks., U.K. Phospholipids and other complex lipid standards were from Sigma, Poole, Dorset, U.K. and were checked for purity by t.l.c. before use. Fatty acid standards were obtained from Nu-Check Prep., Elysian, MN 56028, U.S.A.

Chloroplast isolation

Chloroplasts were isolated from barley or pea leaves by the method of Mills and Joy (1980) as modified by Walker et al.(1988 b). Intactness for both types of chloroplasts was 80-90%.

Incubation

Incubations with leaf sections and chloroplasts were carried out as described by Walker et al.(1988 b) and assay of acetyl-CoA carboxylase as described by Walker et al.(1988 a).

For the analysis of total lipids, incubations were stopped by the addition of propan-2-ol and heated at 70°C for 30 min to inactivate endogenous lipases. Further extraction and separation by t.l.c. into lipid classes is described in Walker et al.(1988 b). Radiolabelled bands were detected using a spark chamber (Birchover Instruments, Hitchin, Herts, U.K.) and quench corrections for scintillation counting of radioactive samples made by the

external-standard method. The scintillant used was Opti-Fluor (Packard, Downers Groove, IL 60515, U.S.A.) and efficiencies for aqueous or non-aqueous samples were never less than 80%.

Acetyl-CoA carboxylase was assayed in a desalted Sephadex G-25 fraction of barley or pea leaf tissue prepared as described by Nikolau et al.(1984). Activity was measured as described by Walker et al.(1988 a) and zero time blanks contained less than 300 d.p.m.

RESULTS

PP 600 could be dissolved in aqueous solution up to a concentration of at least 100μM the highest concentration that was used in biochemical experiments. Barley leaves or pea leaf discs were incubated with [1-^{14}C]acetate or with [2-^{14}C]malonate in the presence of different concentrations of PP 600 and the incorporation of radioactivity into lipids was followed. Results from some of the experiments are shown in Table 1 and these showed that the lipid labelling in barley, but not in pea, was sensitive to the herbicide. Since about 90% of the radioactivity taken up by the tissue samples in the [1-^{14}C]acetate experiments was incorporated into lipids, it is not surprising that total uptake was also affected at high herbicide concentrations. With [2-^{14}C]malonate as precursor, inhibition of lipid labelling was also seen but this was much less than with [1-^{14}C]acetate as precursor. Although the presence of malonate decarboxylase in plant tissues (see Walker et al., 1988 a) means that some radiolabelled malonate can be converted to [2-^{14}C]acetate which would then be used as precursor, the results imply that PP 600 has its major inhibitory effect on fatty acid synthesis at the level of the conversion of acetate to malonate i.e. at the level of acetyl-CoA carboxylase. The I_{50} value for the effect on barley leaves is of the order of 1-2μM.

In contrast, PP 600 had no effect on the incorporation of radiolabel from [1-^{14}C]acetate or [2-^{14}C]malonate into acyl lipids in pea discs. Therefore, there is a similar selectivity with relation to lipid synthesis as to physiological activity. In this regard, PP 600 was similar to cyclohexanediones and aryloxyphenoxy propionates.

TABLE 1. Effect of PP 600 on the uptake of radioactivity from [1-^{14}C]acetate or [2-^{14}C]malonate and its incorporation into lipids of barley or pea leaves.

Conc. of PP 600 (μM)	Barley ^{14}C-Acetate		Barley ^{14}C-Malonate	Pea ^{14}C-Acetate	
	Total uptake	Lipids	Lipids	Total uptake	Lipids
0	100	100	100	100	100
2	79	36	-	99	98
5	72	22	92	99	100
10	64	18	85	98	98
20	67	10	74	102	105
50	68	8	61	105	105
100	34	3	55	104	106

Results are expressed as % control values. Incubations were carried out for 6h at 20°C with illumination (Walker et al.1988 b).

Analysis of the fatty acid methyl esters revealed some interesting changes in the pattern of radioactive products which were produced in the presence or absence of PP 600. With [1-^{14}C]acetate as precursor the major radioactive fatty acids labelled by barley leaves during the period of incubation were palmitate, oleate and linolenate with smaller amounts of linoleate (Table 2). In the presence of increasing concentrations of PP 600 a progressive reduction in the percentage of 18-carbon acids was seen. In addition, shorter chain products such as [^{14}C]laurate and [^{14}C]myristate were detected. As argued during the discussion of experiments with the aryloxyphenoxy propionate, fluazifop, such a result would be expected if the ratio of primer (acetyl-CoA) to addition unit (malonyl-CoA) was increasing (Walker et al.1988 a, b). Such a change in ratio would be caused by inhibition of acetyl-CoA carboxylase.

TABLE 2. Alterations to the labelling of fatty acids from [1-^{14}C]acetate and [2-^{14}C]malonate in barley leaf pieces in the presence of PP 600.

Precursor	Herbicide (μM)	Pattern of labelling (% total ^{14}C-fatty acids)						
		<16C	16:0	18:0	18:1	18:2	18:3	>18C
[1-^{14}C]-Acetate	0	0.1	12.7	1.4	24.6	54.8	5.7	-
	10	6.4	36.5	4.9	11.3	37.2	tr	-
[2-^{14}C]-Malonate	0	-	5.8	4.5	20.4	29.4	5.3	34.6
	10	-	0.4	6.3	-	-	-	92.3

When [2-^{14}C]malonate was used as a precursor, the products were enriched in very long chain (> than 18C) fatty acids. This was in keeping with the role of malonyl-CoA in elongation reactions (Harwood, 1988 b). The labelling of very long chain fatty acids was little affected by PP 600. At most concentrations (up to 50μM) the labelling of palmitate, oleate and linoleate was inhibited selectively. Some labelling of stearate was seen, in addition to very long chain fatty acids. Although the majority of stearate is made de novo, there is a small amount of this acid synthesised by elongation in some plant tissues (e.g. Bolton and Harwood, 1977). We presume that this explains the small amount of labelling of this acid in the presence of 10μM PP 600. The results in Table 2 show clearly that PP 600 has little effect on fatty acid elongation and that its actions are limited to de novo synthesis.

Fatty acid formation de novo is concentrated in plastids (see Harwood, 1988 b). Therefore, the effects of PP 600 on the synthesis of fatty acids from [1-^{14}C]acetate by isolated chloroplasts were investigated. Table 3 shows the results of an experiment with barley chloroplasts. Again, PP 600 inhibited fatty acid formation. However, it was noticable that the I_{50} value for such inhibition was markedly higher than for intact tissue (about 25μM). This suggested that PP 600 might have to be metabolised to a derivative in order to exert its full effect. To increase its effect, PP 600 was pre-incubated at 10μM with a barley leaf homogenate. After 20 mins at 4°C, chloroplasts were isolated and tested for fatty acid synthesis. Previous incubation of the homogenate with PP 600 made no difference to the ability of the subsequently isolated chloroplasts to make fatty acids although this pre-treatment did seem to

enhance the inhibitory effect of PP 600 when it was added to the incubation system.

TABLE 3. Inhibition of fatty acid synthesis in isolated barley chloroplasts caused by PP 600.

Herbicide conc (μM)	Fatty acid synthesis (% control) Expt.1	Expt.2
0	100	100
1	99	92
2	95	90
5	85	77
10	70	60
50	41	26
100	38	18

Incubations were carried out as described in Materials and Methods using [1-^{14}C] acetate as precursor for 1 hr. at 25^{o}C with illumination. Results are means of duplicate determinations.

In keeping with an action on acetyl-CoA carboxylase, PP 600 caused no inhibition of fatty acid synthesis from [2-^{14}C]malonyl-CoA by barley stromal fractions. In fact, an increase in total labelling was seen - atributable to prevention of dilution of the specific radioactivity of the substrate by acetyl-CoA carboxylase activity. Furthermore, the reduction in incorporation caused by the addition of unlabelled acetyl-CoA was essentially prevented by a simultaneous addition of PP 600 (data not shown).

To test for a direct effect of PP 600 on acetyl-CoA carboxylase, the herbicide was added to the enzyme incubation system. As shown in Table 4, increasing concentrations of PP 600 caused a progressive inhibition of activity. Again, the I_{50} value for such inhibition was rather high, being about 65μM. As discussed above we assume that the much greater sensitivity of barley tissue to PP 600 is due to metabolic activation. To see whether any evidence for such metabolism could be seen in the soluble preparations assayed for carboxylase, we carried out a time-course experiment with 50 M PP 600. The amount of inhibition of acetyl-CoA carboxylase was constant throughout the incubation period (10 min) and, thus, the experiment provided no direct evidence for herbicide activation.

TABLE 4. Inhibition of acetyl-CoA carboxylase by the direct addition of PP 600 to its assay mixture.

PP 600 Conc. (μM)	Total c.p.m. in ^{14}C-Malonyl-CoA (X10-3)	%Control
0	20.9	100
1	21.2	99
2	19.3	92
5	19.0	90
10	17.1	80
25	13.5	61
50	12.1	54
100	9.5	41

Assays were carried out using 50μg of protein and a 10 min incubation at 30°C under the conditions described under Materials and Methods. Results are means of duplicate determinations.

In a further test plants were sprayed with a 1M solution of PP 600 in 0.1% Triton X-100. After three days, Sephadex G-25 filtrates were prepared from treated and control barley leaves and assayed for acetyl-CoA carboxylase. No effect of spraying was seen on the activity of acetyl-CoA carboxylase. Moreover, preparations from untreated and treated leaves were equally sensitive to compound PP 600 in the assay.

DISCUSSION

Our experiments have shown that PP 600 inhibits lipid synthesis in sensitive but not the resistant plant species. In addition, this inhibition appears to be at the level of acetyl-CoA carboxylase. Therefore, PP 600 represents another herbicide structure which has this enzyme as a target site. The physiological and herbicidal effects seen subsequently after treating plants with PP 600 can be attributed ultimately to a lack of membrane lipid synthesis. However, unlike the cyclohexanediones and aryloxyphenoxy propionates, which also appear to act by inhibiting acetyl-CoA carboxylase in sensitive species, PP 600 is not very effective when tested directly with this carboxylase. We presume that PP 600 is metabolised to a more active derivative(s) in leaves and this accounts for the much greater sensitivity of whole tissue samples compared to chloroplasts or enzyme preparations.

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FATTY-ACID BIOSYNTHESIS IN ISOLATED ETIOPLASTS AND ITS INHIBITION BY HERBICIDES

K. KOBEK, H.K. LICHTENTHALER

Botanical Institute II, University of Karlsruhe, 7500 Karlsruhe, Kaiserstr.12, F.R.G.

ABSTRACT

Using isolated intact oat etioplasts, an *in vitro* assay was developed to measure the incorporation of [1-^{14}C]acetate into the total fatty acid fraction i.e. the complete sequence of plant *de novo* fatty-acid biosynthesis which is found in the plastid. After optimising this etioplast test-system the effect of the cyclohexane-1,3-dione derivative cycloxydim and the phenoxyphenoxypropionic acid herbicide diclofop on fatty-acid biosynthesis of isolated etioplasts of sensitive oat and tolerant pea plants was investigated. Variation of acetate and herbicide concentration revealed that the inhibition of fatty-acid biosynthesis by cycloxydim and D,L-diclofop is noncompetitive with respect to the substrate acetate. Isolated oat etioplasts as a test-system possess the advantage that also inhibitors of the photosynthetic electron transport (e.g. diuron, atrazine) can be screened for activity against fatty-acid biosynthesis. This is in contrast to oat chloroplasts, where fatty-acid biosynthesis, which is dependent upon the photosynthetic regeneration of ATP and NADPH, is inhibited by diuron and atrazine as a consequence of photosynthetic inhibition.

INTRODUCTION

Cyclohexane-1,3-dione derivatives like cycloxydim and aryloxyphenoxypropionic acid herbicides like D,L-diclofop inhibit fatty-acid biosynthesis of grasses, whereas other monocotyledonous and dicotyledonous plants are tolerant. The target of both herbicide groups is acetyl-CoA carboxylase, one of the initial enzymes of *de novo* fatty-acid biosynthesis (Burton et al., 1987; Focke & Lichtenthaler, 1987; Rendina & Felts, 1988; Secor & Cseke, 1988). Since *de novo* fatty-acid biosynthesis in higher plants seems to occur only in plastids (Ohlrogge et al. 1979), isolated intact chloroplasts were hitherto used by us and other research groups as an *in vitro* test-system to detect fatty-acid biosynthesis inhibitors (Hoppe & Zacher, 1985; Burton et al., 1987; Lichtenthaler et al., 1987; Kobek et al. , 1988a).

The formation of fatty acids by isolated oat chloroplasts is strongly dependent on light and photosynthesis, which provides the cofactors ATP and NADPH needed for fatty-acid synthesis. Therefore an indirect inhibition of fatty-acid biosynthesis by inhibitors of photosynthesis is to be expected. In order to

establish a test-system for inhibitors of fatty-acid biosynthesis using isolated plastids, which is independent of light, we studied the capacity of etioplasts isolated from oat and pea seedlings to synthesize fatty acids from ^{14}C-acetate. This new etioplast test-system for screening inhibitors of fatty-acid biosynthesis is described here and contrasted with the chloroplast test-system.

MATERIALS AND METHODS

Seedlings of oat cv. Flämingnova and pea cv. Lisa were cultivated on peat with nutrients (TKS II, Floratorf) at 25°C in a 14/10 h day/night cycle at a light intensity of 1500 µmol/m^2 /s or in the dark. Spinach was cultivated in the Botanical Garden of the University of Karlsruhe. Chloroplasts of these plants were isolated and incubated with [1-^{14}C]acetate as previously described (Kobek et al., 1988a). A similar system was applied for etioplasts. The latter were isolated from primary leaves of 6d old dark grown oat and pea seedlings. The isolation medium contained 330 mM sorbitol, 50 mM phosphate buffer (pH 8.0), 2 mM $MgCl_2$ and 0.2% BSA. The leaves (ca. 40g in 150 ml ice cold buffer) were homogenised in a mixer with replaceable razor blades within 10 s. The brei was filtered through 10 layers of cheesecloth and centrifuged for 30 s at 1000 x g to remove cell debris. After centrifugation of the supernatant for 2 min at 4000 x g at 4°C, the resulting pellet was resuspended in chilled isolation medium without BSA and filtered through 2 layers of miracloth. These plastid suspensions were used for the incorporation studies.

Incubation of isolated etioplasts with [1-^{14}C]acetate was carried out at 20 °C in 0.5 ml plastid suspensions in the dark. The incubation medium contained 300 mM sorbitol, 50 mM tricine, 50 mM potassium phosphate (pH 8.1), 10 mM $NaHCO_3$, 4 mM ATP, 2 mM $MgCl_2$ and 0.3 mM Coenzyme A. Each assay mixture contained 37 kBq [1-^{14}C]acetate (35.7 µM), of which 2-3% was incorporated into fatty acids (incubation time was 20 min). The plastid concentration amounted to ca. 0.5 µg (pea) or 1.5-2.0 µg (oat) carotenoids per sample. The reaction was started by addition of the freshly isolated plastids to the incubation medium, already containing the labelled acetate and the herbicides. Saponification of lipids, extraction of total fatty acids and scintillation counting was as described before (Kobek et al., 1988a).

RESULTS AND DISCUSSION

Oat etioplasts incorporated [1-^{14}C]acetate into the total fatty acid fraction at high rates: 600-700 nmol acetate per mg carotenoids per hour. This incorporation rate was dependent on the age of the plastids. It was highest in the youngest etioplasts (isolated from 4 d old seedlings) and then decreased with increasing age. A sharp decline of fatty-acid synthesis capacity was observed in etioplasts from oat seedlings older than 7 days. Such an age-dependent decrease in fatty-acid biosynthesis capacity was also shown for oat chloroplasts (Kobek & Lichtenthaler,

1988b). In this investigation we used etioplasts isolated from 6d seedlings, which were found to incorporate ^{14}C-acetate into fatty acids with rates almost as high as etioplasts from 4d seedlings. The 6 d seedlings had larger primary leaves, giving a higher yield of etioplasts.

The composition of the incubation medium for the fatty-acid biosynthesis assay with etioplasts differed from that used for isolated chloroplasts (Kobek et al., 1988a). Addition of ATP strongly enhanced *de novo* fatty-acid biosynthesis capacity, the optimum ATP concentration of 4 mM being higher than in the chloroplast assay (2 mM). This was expected, since chloroplasts, by contrast to etioplasts, produce ATP by photosynthesis. Low concentrations of coenzyme A (0.3 mM was optimum) in the etioplast medium stimulated fatty-acid formation, whereas dithioerythritol (DTE) or dithiothreitol (DTT) had no stimulatory effect. DTE Concentrations higher than 2 mM were inhibitory. Sauer and Heise (1983) detected in spinach chloroplasts an inhibition of fatty-acid biosynthesis in the dark by DTT. Addition of NADPH to the incubation medium had no influence on the rate of fatty-acid formation. This ineffectiveness of exogenously applied NADPH to etioplasts with high fatty-acid biosynthesis capacity indicates that etioplasts must possess mechanisms to produce NADPH, e.g. the oxidative pentose phosphate cycle. In fact, this pathway was demonstrated in plastids (Simcox et al., 1977). It is also assumed that the glycolytic pathway proceeds in plastids (Liedvogel & Bäuerle, 1986). That both pathways exist in etioplasts must be anticipated to explain the high *de novo* fatty-acid biosynthesis capacity of isolated etioplasts demonstrated here.

The optimum pH-value of the incubation medium for *de novo* fatty-acid biosynthesis of isolated oat etioplasts (pH 8.1) was found to be slightly higher than in the chloroplast assay (pH 7.9). This may be due to the fact that the pH in the chloroplast stroma increases during illumination, whereas etioplasts exhibit no light-driven endogenous alkalisation.

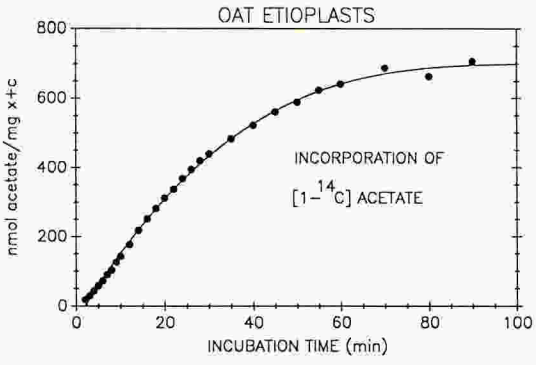

FIGURE 1. Incorporation of [1-^{14}C]acetate into the total fatty-acid fraction of oat etioplasts during dark incubation. The concentration of applied [1-^{14}C] acetate was 35.7 µM. The carotenoid content (x+c) was 1.5 µg per assay. The values shown are from one of three experiments with similar kinetics; the two other experiments only differed in the absolute rate of ^{14}C-acetate incorporation (range: 470-630 nmol acetate per mg x+c per hour) but not in the kinetics. Five per cent of the ^{14}C-acetate was incorporated into the total fatty-acid fraction during 1 h of incubation (total incorporation rate after 60 min: 615 nmol acetate per mg x+c).

With the optimum incubation-medium described above, the incorporation of ^{14}C-acetate into the fatty acids of isolated oat etioplasts was linear for about 30 min and, in contrast to isolated chloroplasts, continued with only slightly reduced rates for more than 1 hour (Figure 1). Isolated oat etioplasts incorporated in the dark 650 (±140) nmol [1-^{14}C]acetate/ mg carotenoids/ hour as calculated from the 20 min incubation period (pea etioplasts: 340 ± 40 nmol). The capacity of isolated oat etioplasts to synthesise fatty acids was ca.40% higher in darkness than in light, which may be due to light-induced photooxidative damage or competition for NADPH by fatty-acid biosynthesis and chlorophyll synthesis (light-induced reduction of protochlorophyllide). For this reason the etioplast test-system should be used only in the dark in order to obtain maximum rates.

Cycloxydim and D,L-diclofop inhibited the fatty-acid formation of isolated oat etioplasts in a concentration-dependent manner (Figure 2), as was previously described for oat chloroplasts (Kobek et al., 1988a). The I_{50}-values for this inhibition of fatty-acid biosynthesis were 0.3 µM for both herbicides and are only slightly higher than the I_{50}-values found in oat chloroplasts (0.1 µM for diclofop and 0.15 µM for cycloxydim). The inhibition by both herbicides was reversible as was determined by washing with herbicide-free buffer. For this purpose the etioplasts were incubated in 100 µM herbicide solutions for 5 min, were centrifuged and the pellet washed three times with herbicide-free assay medium. These etioplasts then exhibited fatty-acid biosynthesis capacity, which was equal to a control etioplast preparation without herbicides.

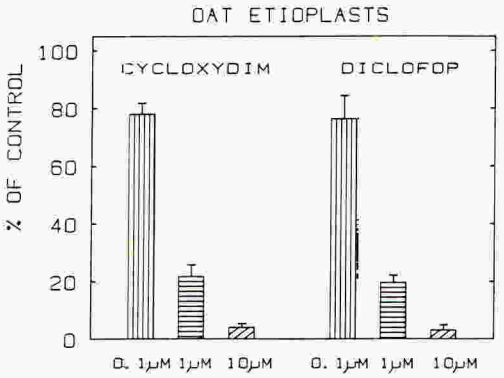

FIGURE 2. Inhibition of *de novo* fatty-acid biosynthesis (^{14}C-acetate incorporation into the total fatty acid fraction) of isolated oat etioplasts by the herbicides cycloxydim and D,L-diclofop. The incorporation rate of ^{14}C-acetate in the controls during the 20 min incubation period was 490 ± 45 nmol acetate per mg x+c.

The percentage inhibition by cycloxydim and diclofop varied with the pH value of the suspension medium and was highest at a pH-value of 7.8 (Figure 3). At higher pH-values the relative inhibition of fatty-acid formation decreased. This decrease in percentage inhibition may be due to differences in the herbicide uptake into the plastid compartment at different pH-values. Cyclohexanediones as well as aryloxyphenoxypropionic acids are weak acids. At low pH the herbicides are less dissociated resulting in a better uptake into the plastids than at a more

alkaline pH. Such a pH-dependent uptake has also been described for other herbicides, e.g. for bentazone (Retzlaff et al.,1979).

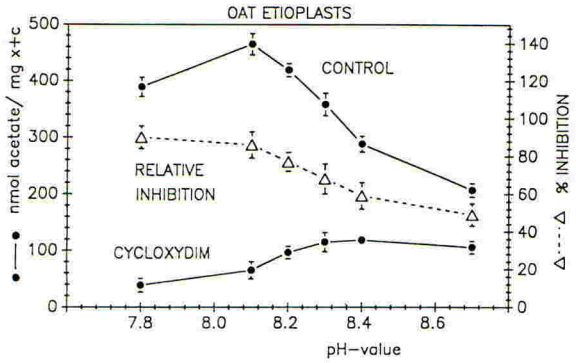

FIGURE 3. Dependence of ^{14}C-acetate incorporation into fatty acids of oat etioplasts upon pH and inhibition by 1 µM cycloxydim.

In etioplasts from tolerant pea, cycloxydim up to 100 µM did not affect fatty-acid biosynthesis. D,L-Diclofop in concentrations of 1 µM and 10 µM had no effect, but 100 µM diclofop inhibited the incorporation of ^{14}C-acetate into the fatty acids by ca. 20 % (Figure 4). This is in good agreement with results obtained with chloroplasts isolated from pea and spinach, where cycloxydim had no effect, whereas diclofop and other aryloxyphenoxypropionic acids in concentrations of 100 µM partially inhibited the synthesis of fatty acids (Kobek et al. 1988c).

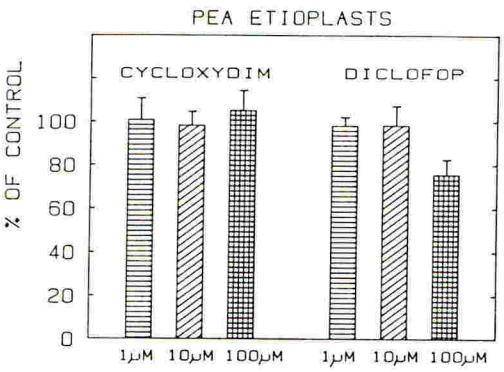

FIGURE 4. Incorporation of ^{14}C-acetate into fatty acids of isolated pea etioplasts in the presence of cycloxydim and D,L-diclofop. ^{14}C-Acetate incorporation rate of controls (20 min incubation period) was 265 ± 50 nmol acetate/ mg x+c/ h. Etioplasts were isolated from the primary leaves of 6 d old seedlings.

Variations of the substrate concentration (^{14}C-acetate) at different concentrations of cycloxydim or diclofop in the oat etioplast assay revealed that the inhibition of fatty acid-biosynthesis is noncompetitive with respect to acetate (Figure 5). There are limitations in applying the principles of enzyme kinetics to the biosynthetic sequence of a whole organelle, yet this can be done with good results. The apparent Km-value for acetate was calculated to be about 110 µM. This Km-value was also

measured with isolated oat chloroplasts at light incubation. The apparent K_i-values in the oat etioplast system were 0.5 (±0.2) µM for cycloxydim and 0.4 (±0.15) µM for diclofop. These apparent K_i-values, determined at the plastid level with enzyme kinetic methods are in the same range as the detected I_{50}-values. This is to be expected since in the case of noncompetitive inhibitors K_i and I_{50} should be of the same range and be independent of substrate concentration (Brandt et al., 1987).

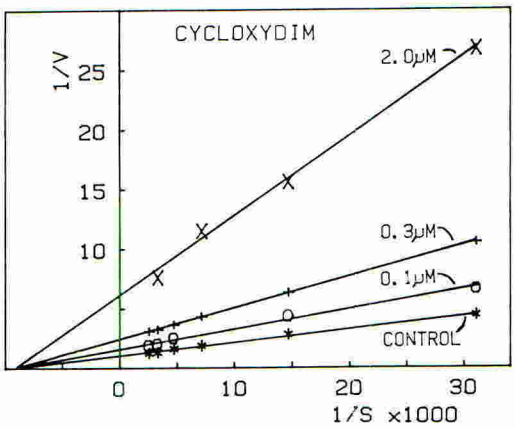

FIGURE 5. Determination of the mechanism of inhibition by cycloxydim of acetate incorporation into fatty acids of isolated oat etioplasts.

In illuminated oat chloroplasts inhibitors of photosynthetic electron transport such as diuron and atrazine not only blocked photosynthesis, but also affected *de novo* fatty-acid biosynthesis. The I_{50}-values for inhibition of fatty-acid biosynthesis in oat chloroplasts were 4 µM for diuron and 8 µM for atrazine (Figure 6). In contrast, in isolated oat etioplasts even 100 µM diuron or atrazine did not affect *de novo* fatty-acid biosynthesis (Figure 6). The I_{50}-values for photosynthesis inhibition (photometrically measured via reduction of DCPIP by broken oat chloroplasts) were 0.6 µM for diuron and 3 µM for atrazine and both herbicides fully inhibited photosynthesis at a concentration of 100 µM. Under these conditions the *de novo* fatty-acid biosynthesis activity of illuminated chloroplasts was reduced to ca. 8 to 12 % of the control rate. This remaining activity corresponded to the rate of fatty-acid biosynthesis, found in herbicide-free oat chloroplasts incubated in the dark. This low rate of *de novo* fatty-acid biosynthesis of isolated chloroplasts in the dark is independent of the light-stimulated regeneration of ATP and NADPH and can therefore not be blocked by the photosynthesis herbicides diuron and atrazine. Since *de novo* fatty-acid biosynthesis in chloroplasts mainly depends upon the photosynthetic regeneration of ATP and NADPH, diuron and atrazine inhibit the fatty-acid biosynthetic pathway in an indirect way by blocking the photosynthetic light reactions. In etioplasts, which lack thylakoids, neither diuron nor atrazine had any effect on fatty-acid biosynthesis, even at high concentrations (Figure 6).

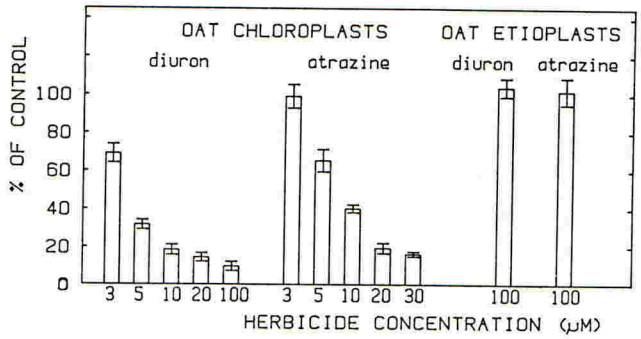

FIGURE 6. Effect of different concentrations of the photosynthetic inhibitor herbicides diuron and atrazine on *de novo* fatty-acid biosynthesis of chloroplasts and etioplasts of 6 d old oat seedlings. Incubation (20 min) with ^{14}C-acetate and herbicides was carried out either in the light (chloroplasts) or in the dark (etioplasts). Mean values of 6 determinations from two plastid isolations.

CONCLUSIONS

Our experiments indicate that isolated etioplasts represent a very suitable *in vitro* test-system for the study of inhibitors of *de novo* fatty-acid biosynthesis such as the acetyl-CoA carboxylase inhibitors cycloxydim and diclofop, tested here. In this test-system inhibitors of the fatty acid synthetase e.g. cerulenin and thiolactomycin can also be investigated (Feld et al., 1989). The capacity of oat etioplasts to synthesise fatty acids is high and the inhibitory effect of cycloxydim and diclofop on fatty-acid biosynthesis is comparable to that found in the chloroplast test-system. In contrast to chloroplasts, *de novo* fatty-acid biosynthesis in etioplasts is independent of the photosynthetic ATP and NADPH formation and therefore insensitive to inhibitors of photosynthetic electron transport. This permits to study direct effects of photosynthesis-inhibitors on *de novo* fatty-acid biosynthesis. Furthermore, in screening for new inhibitors of fatty-acid synthesis, artefactual results due to photosynthetic inhibition can be avoided.

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INHIBITION OF FATTY-ACID BIOSYNTHESIS IN ISOLATED CHLOROPLASTS BY THE ANTIBIOTICS CERULENIN AND THIOLACTOMYCIN

A. FELD, K. KOBEK, H.K. LICHTENTHALER

Botanical Institute II, University of Karlsruhe, Kaiserstr. 12,
D-7500 Karlsruhe 1, FRG

ABSTRACT

The inhibition by the two natural antibiotics, thiolactomycin and cerulenin, of de novo fatty acid biosynthesis in isolated chloroplasts was compared with that of the graminicides cycloxydim and diclofop. The antibiotics affect the fatty acid synthetase, whereas the graminicides inhibit acetyl-CoA-carboxylase. The two antibiotics displayed a strong concentration-dependent inhibition of incorporation of 1-[14C]-acetate into the fatty-acid fraction of oat and spinach chloroplasts. The I_{50}-values for the inhibition of acetate incorporation into fatty acids were about 50µM for cerulenin and about 4 µM for thiolactomycin in both mono- and dicotyledonous plants. The selective grass herbicides of the cyclohexanedione-type and the aryloxyphenoxypropionic acid-type inhibited de novo fatty acid biosynthesis with much lower I_{50}-values of 0.1 to 0.3 µM.

INTRODUCTION

The complete sequence of fatty acid biosynthesis in higher plants starting from acetate is found in chloroplasts (Ohlrogge et al., 1979; Sauer & Heise, 1983; Lichtenthaler et al., 1987) and also other forms of plastids such as etioplasts (Kobek & Lichtenthaler, 1989c). The biosynthetic sequence in chloroplasts comprises acetyl-CoA synthetase (Kuhn et al., 1980), acetyl-CoA carboxylase (Nikolau et al., 1984) as well as the multienzyme complex-fatty acid synthetase (Ohlrogge et al., 1979).

Two antibiotics, thiolactmycin and cerulenin, are known to be inhibitors of the-fatty acid synthetase. Cerulenin was isolated from the culture filtrate of the fungus Cephalosporium cerulens (D'Agnolo et al., 1973) and thiolactomycin from Norcardia sp. (Oishi et al., 1982). Thiolactomycin is thought to block ACP-acetyltransferase as well as 3-oxoacyl-ACP-synthase in Escherichia coli (Nishida et al., 1986) but has little effect on the fatty-acid synthetases of yeast and rat liver (Hayashi et al., 1983). It also blocks ^{14}C-acetate and ^{14}C-malonyl-CoA incorporation in spinach, castor bean, oat and avocado (Nishida et al., 1984; Yamada et al., 1984, Kato et al., 1987). Thiolactomycin thus only blocks the Type II fatty-acid synthetase which occurs in plants (chloroplasts) and in most bacteria, whereas the Type I fatty-acid synthetase of animal tissue and yeasts is not affected. However recently it has been doubted that thiolactomycin affects the ACP-acetyl transferase in E. coli (Lowe & Rhodes, 1988), or in higher plants (Focke et al., unpublished). In contrast, cerulenin affects the fatty-acid synthetases of all organisms (Type I and Typ II) as concluded from the work with bacteria, Euglena, yeast, rat liver and two higher plants (oat and spinach) (Vance et al., 1972; Ohno et al., 1974; Packter & Stumpf, 1975). From the two 3-oxoacyl-ACP synthases (= ketoacylsynthases) present in the plant fatty acid synthetase complex the 3-oxoacyl-ACP-synthase I (responsible for de novo fatty acid synthesis) is affected by cerulenin, whereas the enzyme II which

catalyses elongation from 16:0 to 18:0 is relatively insensitive (Jaworski et al., 1974).

Cerulenin is thought to bind covalently to the target enzyme (D'Agnolo et al.; 1973), whereas the inhibition of de novo fatty-acid biosynthesis by thiolactomycin seems to be reversible at least in E. coli (Nishida et al., 1986). Previous work on the inhibition of fatty acid bionsynthesis in higher plants by cerulenin and thiolactomycin mentioned above in general was performed with leaf material and only in part with chloroplasts. Since plastids are the only site of fatty acid biosynthesis in higher plants (Ohlrogge et al., 1979) we studied the effect of the two antibiotics on ^{14}C-acetate incorporation into the total fatty acid fraction of isolated chloroplasts of a mono- and a dicotyledonous plant (oat, spinach) in order to determine the I_{50}-values and effective inhibition concentrations. In the same chloroplast test-system we also established the I_{50}-values of the new grass herbicides (cyclohexanediones and aryloxyphenoxypropionic acids) such as sethoxydim, clethodim, cycloxydim as well as diclofop, fenoxaprop and haloxyfop, which are known to specifically inhibit the plastid-bound acetyl-CoA-carboxylase in the Poaceae family (Burton et al., 1987; Focke & Lichtenthaler, 1987; Kobek et al. 1988a, 1988b; Rendina & Felts, 1988; Secor & Cséke, 1988). The rather low I_{50}-values of the new grass herbicides, which block one of the enzymes of the initial fatty acid biosynthesis sequence, are in contrast to those of the two antibiotics, which apparently affect the same target-enzyme (3-oxoacyl-ACP synthase) of the fatty acid synthetase at a later stage of the biosynthetic sequence. The chemical structure of the two antibiotics and the two major grass herbicides are given in Figure 1.

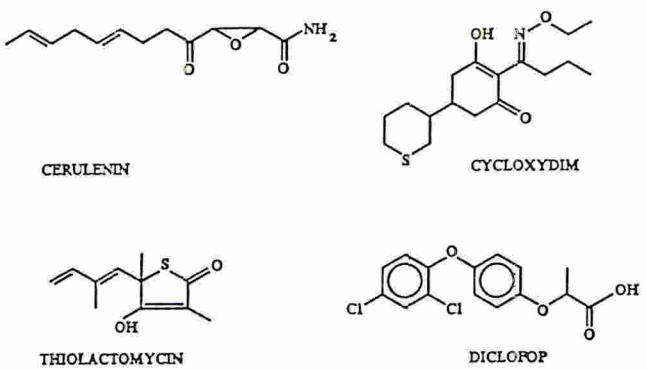

FIGURE 1. Chemical structure of the antibiotics cerulenin and thiolactomycin and of the grass herbicides cycloxydim and diclofop.

MATERIAL AND METHODS

Intact chloroplasts were isolated from seedlings of oat (var. Flämmingnova) and spinach (var. Matador). The plants were cultivated and the chloroplasts were isolated as described previously (Kobek et al., 1988b). The isolated almost fully intact chloroplasts were incubated with 1-[^{14}C]-acetate for 20 min in an iso-osmotic buffer-system as described by Lichtenthaler et al. 1987. The incubation medium (0.5 ml) contained 300 mM sorbitol, 50 mM tricine, 50 mM phosphate (pH 7.9), 30 mM $NaHCO_3$, 20 mM ATP, 0.5 mM Coenzyme A, 0.5 mM $MgCl_2$, 35 µM 1-^{14}C acetate (37kBq per assay) and

chloroplasts with a chlorophyll content of about 50 µg. The incubation was carried out at room temperature and the light intensity during the incubation period was 1400 µmol/m^2/s. After 20 minutes the incubation was stopped with 30 % KOH and the lipids were hydrolysed at 80° C for 90 minutes. After acidification with 6 N H_2SO_4 and 30 % trichloroacetic acid the fatty acids were extracted with petrolether and the radioactivity was measured with a liquid scintillation counter. The average rates of ^{14}C-acetate incorporation into total fatty acids were 9-14 µmol per mg chlorophyll and a 20 min incorporation period for oat and spinach chloroplasts.

RESULTS AND DISCUSSION

The incorporation of ^{14}C-acetate into the total fatty acid fraction of isolated chloroplasts was inhibited by the antibiotics thiolactomycin and cerulenin in a dose-dependent manner with no significant difference for the same antibiotic between chloroplasts from oat (monocotyledon) or spinach seedlings (dicotyledon) (Figure 2). Therefore the fatty-acid synthetases of mono- and dicotyledonous plants are equally sensitive to both antibiotics.

For thiolactomycin the I_{50}-value of the inhibition of ^{14}C-acetate incorporation into fatty acids by isolated chloroplasts was about 4 µM and in the case of cerulenin about 50 µM. Even though both antibiotics seem to affect the same enzyme target, the 3-oxoacyl-ACP synthase (EC 2.3.1.41) as indicated above, thiolactomycin is much more efficient than cerulenin. This inhibition by thiolactomycin is in good agreement with results obtained by other authors with higher plants. In *E. coli* I_{50}-values for cerulenin were found to be 27 µM (Hayashi *et al.*, 1983) and 30 µM (D'Agnolo *et al.*, 1973), whereas for thiolactomycin a I_{50}-value of 2 µM was determined (Hayashi *et al.*, 1983). In higher plants I_{50}-values for plastid/chloroplast preparation hitherto had been given only for thiolactomycin but not for cerulenin. The I_{50}-values for thiolactomycin were 3.8 µM in spinach chloroplasts, 2.8 µM in castor bean plastids and 7.5 µM in avocado plastids (Nishida *et al.*, 1984) and ca. 2 µM in oat leaf sections (Kato *et al.*, 1987); these I_{50}-values are in good agreement with the 4 µM-values for oat and spinach in this investigation. That the I_{50}-values for cerulenin are considerably higher (ca. 50 µM) has the first time been demonstrated for higher plants. In higher plants and in *E. coli* the I_{50}-values for inhibition of fatty acid biosynthesis are 12 to 15 times higher for cerulenin than for thiolactomycin. This indicates that the fatty acid synthetase of *E. coli* and higher plants exhibit a very similar sensitivity towards the two antibiotics.

The degree of inhibition of fatty-acid biosynthesis by cerulenin is dependent on the preincubation period (Figure 3). Inhibition of 70 µM cerulenin increases from 75 % (no preincubation) to 88 % (10 min preincubation) to 93 % (20 min preincubation). The I_{50}-value of cerulenin with respect to the inhibition of de novo fatty-acid biosynthesis consequently decreased after a preincubation with the inhibitor, in oat chloroplasts from 50 µM (no preincubation) to about 12 µM (30 min preincubation) as is indicated in Figure 4.

The enhanced inhibition of de novo fatty-acid synthesis by cerulenin (after preincubation) may be due partly to the fact that cerulenin binds covalently to its target-enzyme, the 3-oxoacyl-ACP-synthase (D'Agnolo *et al.*, 1973). Thus a certain time lag in the inhibiton efficacy of cerulenin could be anticipated. A second reason for the time-delay in the full inhibition effect could be a relatively slow movement of cerulenin through the

chloroplast envelope, which could be slower than for other antibiotics or for particular herbicides such as cycloxydim and diclofop.

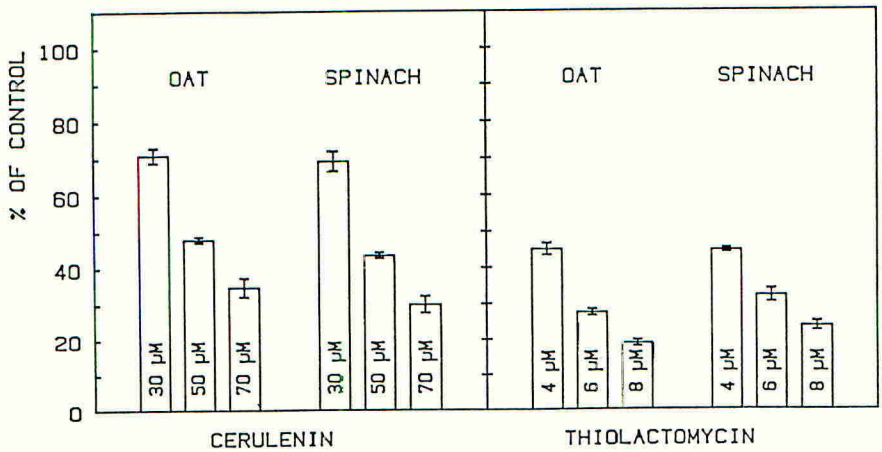

FIGURE 2. The dose-dependent inhibition by cerulenin and thiolactomycin of de novo fatty acid biosynthesis of isolated oat and spinach chloroplasts. Mean of 4 determinations with SD.

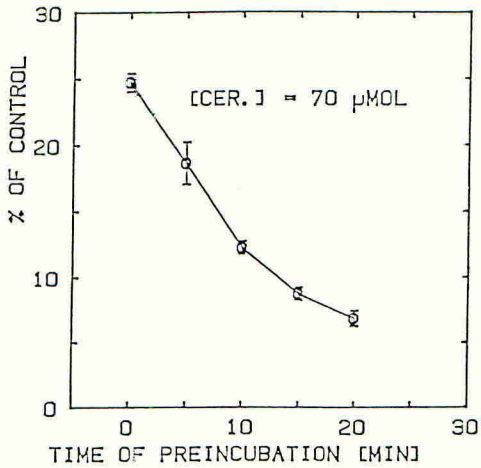

FIGURE 3. Effect of cerulenin on ^{14}C-acetate incorporation in the total fatty acid fraction of isolated oat chloroplasts after different times of chloroplast preincubation with cerulenin (70 µM). Mean of 2 determinations with maximum deviation.

In contrast to cerulenin, thiolactomycin binds reversibly to the presumable sole target 3-oxoacyl-ACP-synthase as has been shown in *E. coli* (Nishida *et al.*, 1986). Preincubation of the chloroplasts with thiolactomycin had little or no effect on the inhibition efficiency and the I_{50}-values for inhibition of de novo fatty acid formation by thiolactomycin. Though cerulenin and thiolactomycin seem to act on the same enzyme of the plastidic

fatty acid synthetase, the effective concentrations and the time-dependent inhibition characteristics are quite different. Only kinetic studies with fatty acid synthetase preparation, which are planned in our laboratory, can give further information on the mode of inhibition and whether both antibiotics bind to the same or a different binding domain of the 3-oxoacyl-ACP synthase. Cerulenin apparently also affects other enzymes of plant metabolism and may have different binding sites in the cell. This could be another reason why a relatively high concentration of cerulenin is required for 50 % inhibition of fatty acid biosynthesis.

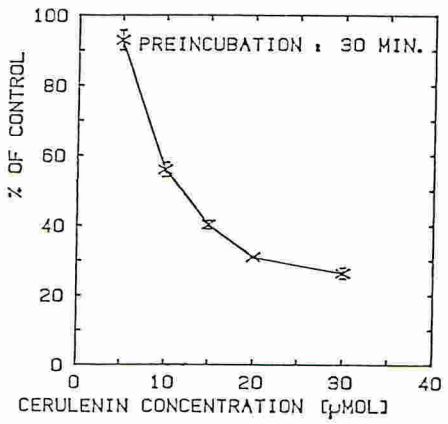

FIGURE 4. Determination of the I_{50}-value for cerulenin against ^{14}C-acetate incorporation after a preincubation period with cerulenin of 30 min in isolated oat chloroplasts. Mean of 3 determinations with SD.

There exist other inhibitors of the de novo fatty acid biosynthesis in isolated chloroplasts: the newly developed selective grass herbicides of the cyclohexanedione type such as cycloxydim, sethoxydim, clethodim and the structurally different aryloxyphenoxy—propionic acid type compounds (diclofop, fenoxaprop, haloxyfop, fluazifop). These graminicides inhibit one of the initial enzymes of fatty acid biosynthesis from acetate, acetyl-CoA carboxylase, which appears to be the key enzyme of fatty acid biosynthesis in chloroplasts (Burton et al., 1987; Focke & Lichtenthaler, 1987; Kobek et al., 1988a, 1988b; Rendina & Felts, 1988; Secor & Cséke, 1988). In the case of the aryloxyphenoxypropionic acid derivatives the herbicides are not applied in the free acid form but as methyl, ethyl or butylesters. The free acids are the physiologically active forms at the target site (Kobek et al., 1988b). After uptake of the esterified herbicides by the plants, in general, by the leaf tissue (post-emergence application), the free acids are formed in the cytoplasm by hydrolysis.

In the same chloroplast test-systems used for the cerulenin and thiolactomycin experiments we also determined the I_{50}-values for several of the new graminicides cyclohexanedione derivatives and aryloxyphenoxy propionic acids including their esters. The I_{50}-values for these selective grass herbicides were much lower than for the two antibiotics described above, e.g. 0.15 µM for cycloxydim and clethodim and 0.1 µM for diclofop and fenoxaprop as the most active inhibitors as compared to 4 and 50 µM for thiolactomycin and cerulenin, respectively (Table 1). The dicotyledonous plants such as spinach, tobacco or pea were tolerant or resistant (Kobek et al., 1988a, b). No inhibition of fatty-acid biosynthesis from acetate by

cyclohexanedione derivatives was observed even at a high concentration of 200 µM of the active ingredients.

In the case of the aryloxyphenoxypropionic acid derivatives spinach and other dicotyledonous plants were in general tolerant or resistant against the free acid forms but showed a very slight sensitivity towards the most effective compounds diclofop and fenoxaprop (Kobek et al., 1988b; Lichtenthaler et al., 1989). The I_{50}-values were, however, found to be above 100 µM (Table 1). The ester derivatives of the aryloxyphenoxy propionic acids exhibited a certain inhibition effect in the sensitive oat plant (I_{50}-values of 10 to 100 µM), which was much lower that that of the free acid forms. It is assumed that this very slight sensitivty of the fatty acid biosynthesis in oat chloroplasts towards these esters may be due to a partial hydrolysis of the ester-form, which may have occurred either before application or was performed by the chloroplast preparation. In the chloroplasts of the dicotyledonous plant spinach, the ester forms did exert no inhibition effects, the I_{50}-values would be far above 100 µM.

TABLE 1. I_{50}-values for the inhibition of ^{14}C-acetate incorporation into the fatty-acid fraction of isolated chloroplasts of a dicotyledonous (spinach) and a monocotyledonous plant (oat) by various antibiotics and herbicides.

	I_{50}-value µM oat	spinach	target with literature reference	
antibiotics:				
thiolactomycin	4	4	3-oxoacyl-ACP-synthase (ACP-acetyl-transferase?)	[1] [1, 2]
cerulenin	50	50	3-oxoacyl-ACP-synthase	[3]
herbicides:				
cyclohexane-1,3-dione derivatives				
cycloxydim	0.15	no inhibition*	acetyl-CoA-carboxylase	[4]
clethodim	0.15	no inhibition*	acetyl-CoA-carboxylase	[4, 5]
sethoxydim	0.5	no inhibition*	acetyl-CoA-carboxylase	[4, 5, 6]
alloxydim	2.0	no inhibition*	acetyl-CoA-carboxylase	[4]
aryloxyphenoxypropionic acid derivatives				
diclofop	0.1	>100	acetyl-CoA-carboxylase	[7]
fenoxaprop	0.1	>100	acetyl-CoA-carboxylase	[7]
haloxyfop	0.3	>100	acetyl-CoA-carboxylase	[6, 7, 8]
fluazifop	3.0	>100	acetyl-CoA-carboxylase	[7]
diclofop-methyl	10	»100	acetyl-CoA-carboxylase	[7]
fenoxaprob-ethyl	10	»100	acetyl-CoA-carboxylase	[7]
haloxyfop-methyl	50	»100	acetyl-CoA-carboxylase	[7]
fluazifop-butyl	100	»100	acetyl-CoA-carboxylase (as free acids)	[7]

* at a concentration of 200 µM

[1] = Nishida et al., 1986; [2] = Focke et al., 1990; [3] = Vance et al., 1973; [4] = Focke et al., 1987; [5] = Rendina & Felts, 1988; [6] = Burton et al., 1987; [7] = Kobek et al., 1988b; [8] = Sekor & Cséke, 1988.

CONCLUSIONS

Graminicides of the cyclohexanedione and aryloxyphenoxy propionic acid types which specifically block the acetyl-CoA carboxylase in the sensitive Poaceae, are more efficient inhibitors of de novo fatty acid biosynthesis in isolated chloroplasts than the antibiotics cerulenin and thiolactomycin, which inhibit at a later stage in the biosynthetic sequence of fatty acid formation from ^{14}C-acetate. A further difference is that the two antibiotics are non-selective inhibitors in monocotyledonous and dicotyledonous plants, whereas the new herbicides appear to be effective only in members of the Poaceae. Synthetic inhibitors of the 3-oxoacyl-ACP synthase or other enzymes of the plant fatty-acid synthetase are unknown. It appears, however, feasible to develop such chemicals which could well be more potent than cerulenin and thiolactomycin.

ACKNOWLEDGEMENTS

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PERTURBATION OF CHLOROPLAST PROTEIN METABOLISM BY THE PLANT GROWTH REGULATOR, CHLORMEQUAT

M.P. PERCIVAL, J.W. GREEN, N.R. BAKER

Wolfson Agrochemical Unit, Department of Biology, University of Essex, Colchester, Essex, CO4 3SQ.

ABSTRACT

A significant effect on chlorophyll fluorescence induction kinetics from developing wheat leaves was observed within 24 hours of treatment with the plant growth regulator chlormequat. The fluorescence response was enhanced, presumably by elevated chlormequat uptake, in treatments formulated with the adjuvant 0.5% v/v LI-700 (acidified soya lecithin), which by itself did not modify fluorescence. The fluorescence response was found to be light and concentration dependant and only partially relieved by simultaneous application of gibberellin.

A decline in the oxygen-evolving capability of the leaves was also observed, but photosystem II electron transport in thylakoids from untreated leaves was unaffected by high concentrations of chlormequat. A reduction in chlorophyll content (15%) was attributable largely to loss of chlorophyll a and the 10% reduction in carotenoid content was predominantly of β-carotene. Electrophoresis of thylakoid polypeptides revealed a decrease of some major proteins, including chlorophyll-containing species associated with photosystem II reaction centres, but the light-harvesting chlorophyll a/b protein was apparently unaffected. A large depletion (51%) of the photosystem II reaction centre D1 protein, as indicated by reduction in ^{14}C-atrazine binding to thylakoid membranes, probably accounted for the fluorescence responses.

The data suggests that chlormequat treatment perturbs protein synthesis in chloroplasts of developing wheat leaves and possible mechanisms are discussed.

INTRODUCTION

The effects and benefits of cereal crop treatment with the plant growth regulator (PGR) chlormequat ((2-chloroethyl)-trimethylammonium chloride) have been well documented (see Herbert, 1982), but field responses can be extremely variable (Woolley, 1981; Harris, 1984). The reliability of field performance may be improved by formulation with adjuvants since uptake and translocation of the PGR is inefficient and environmentally (particularly temperature) sensitive (Lord and Wheeler, 1979; Hunt and Baker, 1983).

We have demonstrated that the most efficacious chlormequat adjuvant formulations can be rapidly selected by monitoring changes in chlorophyll fluorescence emission resulting from chlormequat uptake by leaves (Percival and Baker, 1989). In this paper we present preliminary results

of investigations into the causes of the large, and somewhat unexpected, perturbation of chlorophyll fluorescence emission from developing wheat leaves treated with chlormequat.

MATERIALS AND METHODS

Wheat (cv. Broom) seedlings were grown in controlled environment cabinets at 15°C at constant humidity (70%) under a mean photosynthetically-active photon flux density (ppfd) of 250umol/m^2/s with a 16h photoperiod. Eleven day old seedlings (2 leaf stage), selected for uniformity, were used in all experiments. Seedlings were treated by total immersion of aerial tissue for 5s in chlormequat (Cycocel, BASF) formulated as described in the legends. All subsequent measurements and thylakoid preparations were made from a 3cm leaf section of the youngest leaf located 9cm from the base.

Chlorophyll fluorescence emission from leaves was monitored and quantitated essentially as previously described (Habash et al., 1985; Percival and Baker, 1985 α 1989). Oxygen evolution was monitored using a ppfd of 600umol/m^2/s (saturating) in a Clark O_2 electrode leaf disc chamber (Hansatech) as described by Ireland et al., 1986. Pigments were extracted and analysed using h.p.l.c. by the method of Val et al., 1986.

Thylakoid membranes were prepared (Leegood and Walker, 1979) and photosystem II electron transport assayed by the photoreduction of oxidised phenylene diamine (PD_{ox}) (Saha et al., 1971). Thylakoids were also used in ^{14}C-atrazine binding studies (Paterson and Arntzen, 1982) and for identification of membrane polypeptides visualised by Coomassie blue after separation by SDS-polyacrylamide gel electrophoresis (PAGE), essentially as previously described (Percival et al., 1986).

RESULTS

The increasing perturbation of chlorophyll fluorescence from leaves treated with increasing chlormequat concentrations (Table 1) suggested that the extent of the response was determined by relative uptake of the PGR and that photosynthetic membrane function was being increasingly perturbed. The effect was greatly inhibited by post-treatment dark incubation but inhibited to a much lesser extent by inclusion of gibberellin in the treatment (Table 1).

Table 2 demonstrates that the oxygen-evolving capability of the leaves is also affected by chlormequat treatments. The decline in oxygen evolution follows the reduction in fluorescence (Table 1) suggesting that photosynthetic electron transport was perturbed probably by malfunction of photosystem II.

Pigment analysis (Table 3) from treated leaves revealed a 15% reduction of total chlorophyll most of which (11%) was accounted for by lower chlorophyll a content. Changes in carotenoid content were also observed in treated tissue (Table 3) and reduced concentrations of both lutein (8%) and carotene (22%) were detected. The diminished levels of pigment may have resulted from inhibition of synthesis and/or enhanced degradation. However, it would seem unlikely that inhibition of synthesis

TABLE 1. Changes in a chlorophyll fluorescence emission induction parameter (Fx/Fp) from wheat leaves 24h after treatments with chlormequat formulated with the adjuvant LI-700 (0.5% v/v).See Habash et al. (1985) for full details of Fx/Fp.

Chlormequat (mg/ml)		Fluorescence (% control) (S.E.M.)	
0	(untreated)	100.0	(0.9)
0	(+ adjuvant)	98.8	(2.0)
2.01		98.5	(3.5)
4.02		62.2	(6.6)
8.04		14.1	(0.9)
8.04	(+ 5mM Gibberellin)	28.8	(3.5)
8.04	(dark)	80.1	(4.2)

TABLE 2. Changes in oxygen evolving capacity of untreated wheat leaves and wheat leaves 24h after treatments with chlormequat formulated with the adjuvant LI-700 (0.5% v/v).

Chlormequat (mg/ml)		O_2 evolution ($umol/cm^2/h$)
0	(untreated)	4.97
2.01		5.29
4.02		1.80
8.04		0.00

alone could account for the selective nature of the pigment loss which appears to be indicative of breakdown of membrane components, particularly photosystem complexes.

Chlormequat had no effect on photoreduction of PD_{ox} in thylakoids isolated from untreated wheat leaf tissue indicating that the PGR had no direct inhibitory effect on photosystem II photochemistry (Table 4) and that the perturbation of PSII in vivo possibly resulted from effects on synthesis and/or organisation of PSII components.

The SDS-PAGE profile of thylakoid polypeptides of chloroplast membranes isolated from chlormequat-treated wheat leaves shows a depletion in major polypeptides between 30kDa and 67kDa (Fig. 1), some of which may correspond to plastid-encoded chlorophyll-containing protein complexes associated with the photosystem II. However, the nuclear-encoded light-harvesting chlorophyll a/b polypeptides (LHCII) appear to remain unaffected

TABLE 3. Changes in chlorophyll and carotenoid pigment in wheat leaves 24h after treatment with chlormequat (8.04 mg/ml) formulated with the adjuvant LI-700 (0.5% v/v).

Pigment	Concentration (ng/mm^2) (S.E.M.)	
	Untreated	+Chlormequat
Chlorophyll a	63.6 (1.5)	56.5 (1.3)
Chlorophyll b	15.0 (0.7)	14.5 (0.3)
Neoxanthin	2.1 (0.1)	2.0 (0.1)
Violoxanthin	3.8 (0.1)	3.8 (0.1)
Lutein	8.3 (0.2)	7.6 (0.1)
β carotene	3.7 (0.1)	2.9 (0.1)

TABLE 4. Changes in photoreduction of PD_{ox} in thylakoids isolated from untreated wheat leaves in the presence of chlormequat and diuron.

Treatment	O_2 evolution (umol/mg Chl./s) (S.E.M.)
Untreated	59.8 (4.3)
+ 4.30 mg/ml Chlormequat	62.9 (5.2)
+ 0.28 ug/ml Diuron	3.1 (1.3)

by the treatment (Fig. 1). The negligible loss of chlorophyll b 24h after treatment (Table 3) was also indicative of the absence of effect on this polypeptide.

^{14}C-Atrazine binding to thylakoids isolated from leaves 24h after treatment with chlormequat was significantly reduced in chlormequat treatments in the light (Fig.2). Approximately 51% less atrazine was bound to these membranes indicating a severe decrease in the ability of the D1 reaction centre protein of photosystem II complexes to bind plastoquinone, which would occur by either damage to, or depletion of, D1 polypeptides. Dark incubation after treatment with chlormequat resulted in very little alteration to atrazine-binding characteristics (Fig.2) and the effect of chlormequat on PSII photochemistry, as indicated by chlorophyll fluorescence (Table 1), was much reduced (66%) with post treatment dark incubation.

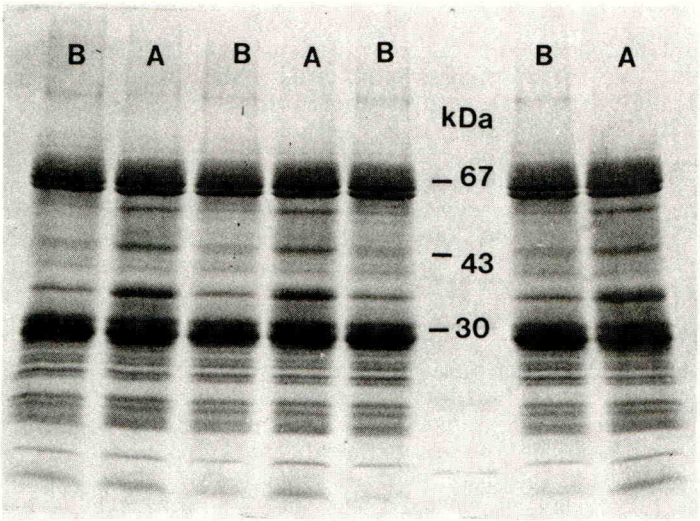

FIGURE 1. SDS-PAGE polypeptide profiles of thylakoids isolated from (A) untreated and (B) chlormequat (8.04 mg/ml in 0.5% v/v LI-700) treated leaves 48h after treatment.

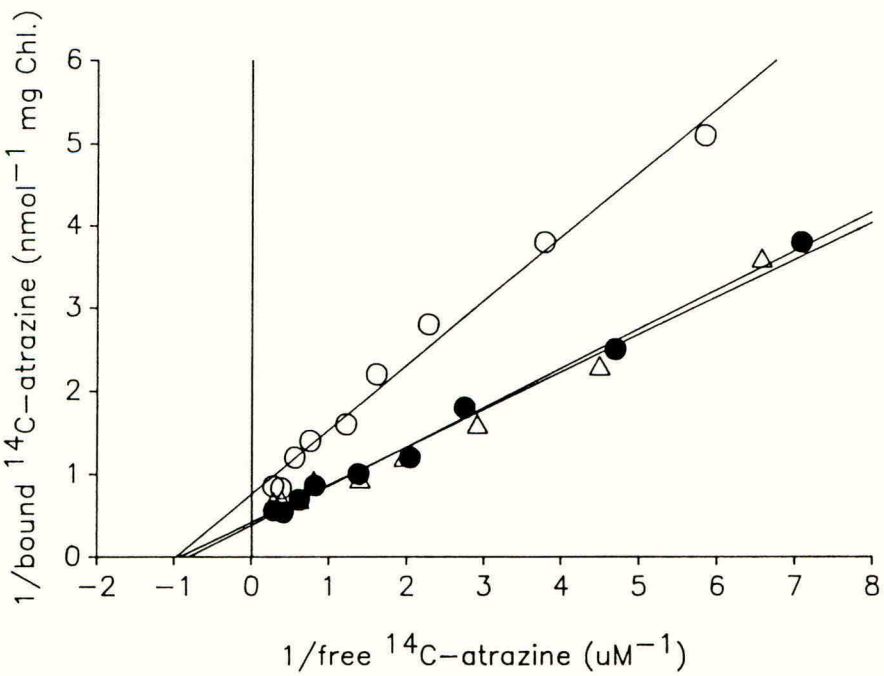

FIGURE 2. ^{14}C-atrazine binding to thylakoids isolated from (△) untreated and chlormequat (8.04 mg/ml in 0.5% v/v LI-700) treated wheat leaves 24h after incubation in the presence (○) or absence (●) of light.

DISCUSSION

The SDS-PAGE polypeptide profiles of thylakoids isolated from chlormequat-treated leaves show significant losses of some major membrane polypeptides including chlorophyll-containing proteins associated with photosytem II. Reduction in ^{14}C-Atrazine binding observed in the same thylakoids also demonstrate that a significant depletion of the PSII reaction centre D1 polypeptide had occured within 24h. Since the D1 polypeptide has a rapid turnover in chloroplast membranes (Mattoo et al., 1984) inhibition of synthesis or processing of D1 protein would probably result in a rapid reduction in the amount of functional polypeptide present.

The chlorophyll fluorescence responses and loss of oxygen-evolving capability observed in chlormequat-treated wheat leaf tissue probably reflect the subsequent effects of loss of D1 function on photosystem II photochemistry. A previous study (Schulz and Grimme, 1985) also attributed the effects of chlormequat on oxygen evolution from wheat leaves 8 days after treatment to modification of chloroplastic functions. The large reduction in fluorescence response seen in dark-incubated treated leaves and the absence of an effect on atrazine-binding in thylakoids isolated from these leaves probably suggest that the inhibitory effects of chlormequat on D1 synthesis were more manifest in the light because of enhanced D1 metabolism.

It has been proposed that chlormequat effects may result from inhibition of gibberellin biosynthesis (Lang, 1970). However, simultameous gibberellin addition with chlormequat only partially alleviated the fluorescence perturbation suggesting that this effect of chlormequat may not specifically relate to gibberellin biosynthesis (Douglas and Paleg, 1974). The chlormequat effects were also observed in chloroplasts where biosynthesis of the terpenoid intermediate kaurene, a major inhibitory site in the gibberellin biosynthetic pathway, probably does not occur and which is also unlikely to be specifically inhibited by chlormequat (see Graebe, 1987).

Chlormequat may act at other unidentified sites, possibly in the terpenoid pathway inhibiting sterol production (Douglas and Paleg, 1974), leading to inhibition of protein biosynthesis, particularly of plastid-encoded thylakoid polypeptides. However, it remains to be seen whether the inhibition is confined to the chloroplast and specific for plastid-encoded proteins and how important these effects are in relation to physiological effects of chlormequat treatment observed in the field.

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INFLUENCE OF HOE 70542 ON THE BEHAVIOUR OF FENOXAPROP-ETHYL IN WHEAT

H. KÖCHER, B. BÜTTNER, E. SCHMIDT, K. LÖTZSCH, A. SCHULZ

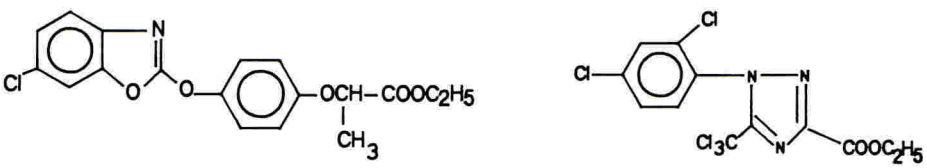
Hoechst AG, Frankfurt (M) 80, Federal Republic of Germany

ABSTRACT

The post-emergence herbicide fenoxaprop-ethyl selectively controls a broad spectrum of grass weeds in dicotyledonous crops and rice, but causes unacceptable phytotoxicity in wheat. Phytotoxic effects of the herbicide in wheat are completely overcome by combination with the novel compound ethyl-1-(2,4-dichlorophenyl)-5-trichloromethyl-(1H)-1,2,4-triazole-3-carboxylate (Hoe 70542). This compound does not influence foliar uptake of the herbicide by wheat plants, nor the degree of inhibition of acetyl-CoA carboxylase, the biochemical site of fenoxaprop action, when tested with chloroplast preparations from wheat. Levels of fenoxaprop-ethyl and of its herbicidally active free acid fenoxaprop in shoot tissue of wheat decrease, however, more rapidly when applied with Hoe 70542, and at the same time formation of presumably inactive degradation products of the herbicide increases.

INTRODUCTION

The post-emergence herbicide fenoxaprop-ethyl (ethyl-2-(4-(6-chloro-2-benzoxazolyloxy)-phenoxy)-propionate) selectively controls a broad spectrum of grass weeds in dicotyledonous crops and rice, but is phytotoxic in wheat. A compound has been discovered (ethyl-1-(2,4-dichlorophenyl)-5-trichloromethyl-(1H)-1,2,4-triazole-3-carboxylate, Hoe 70542; Fig. 1) which provides desirable selectivity of fenoxaprop-ethyl in wheat species without reduction of efficiency against grass weeds. One part per weight of Hoe 70542 is applied in combination with 4 parts per weight of the herbicide (Bieringer et al., 1989).



I II

FIGURE 1 Structure of (I) Fenoxaprop-ethyl and (II) Hoe 70542

Fenoxaprop-ethyl causes rapid cessation of growth and inhibition of lipid biosynthesis of susceptible grass species, followed by destruction of the shoot meristems (Köcher et al., 1982).
After penetration into the leaf it is readily hydrolysed to the free acid fenoxaprop. Studies with chloroplasts isolated from grass species showed that the free acid is a powerful inhibitor of fatty acid biosynthesis de novo (Hoppe & Zacher, 1985) and specifically inhibits acetyl-CoA carboxylase, the first enzyme in this biosynthetic pathway (Kobek et al., 1988).

In a series of experiments we studied the mechanism by which Hoe 70542 modifies the behaviour and physiological effects of fenoxaprop-ethyl and fenoxaprop in wheat. The results are reported in this paper.

MATERIALS AND METHODS

Chemicals

Pure standards of Hoe 70542, fenoxaprop-ethyl, degradation products, and of fenoxaprop-ethyl (chlorophenyl-U-^{14}C), sp. act. 910 MBq/g, were prepared at Hoechst AG. With the exception of the enzyme study, herbicide and Hoe 70542 were applied to plants in the commercial EW (oil in water) formulation, containing 60 g fenoxaprop-ethyl and 15 g Hoe 70542 per liter of product. Spray volumes were equivalent to 400 or 600 l/ha.

Plants

Spring wheat, cv.Schirokko, was cultivated in sandy loam in 9 cm or 13 cm plastic pots (10 plants/pot). For the fenoxaprop-ethyl degradation study plants were cultivated during the growing season under outdoor conditions, and treated in the early tillering stage. For the other experiments the plants were cultivated in the greenhouse at 60 - 70 % r.h. 25 °C/15 °C (day/night) and treated at the 2-to 3-leaf stage.

Analytical procedures

Radioactivity in plant tissue was determined by liquid scintillation counting after combustion in a Tricarb Sample Oxidizer (Packard Instruments). In the study of ^{14}C-fenoxaprop-ethyl metabolism 40 to 60 g shoot material were macerated and extracted with 3 x 200 ml acetonitrile/ water (8/2 v/v). After evaporation of the acetonitrile the aqueous solution was acidified with hydrochloric acid (pH 2) and extracted with 3 x 100 ml ethyl acetate. The extracts were analyzed by HPLC on a SP 8700 (Spectra Physics), equipped with a Spectroflow 757 UV detector (Kratos) and a LB 506 ^{14}C-detector (Berthold). The stationary phase of the column was Hypersil RP 18. Gradient elution was used, beginning with 100 % water (pH 2) and changing to 100 % acetonitrile in 60 min at a flow rate of 1.5 ml/min.

Acetyl-CoA carboxylase

Wheat chloroplasts were isolated from leaves, following standard procedures, and acetyl-CoA carboxylase activity was measured as described by Secor and Cseke (1988).

RESULTS

Plant growth

Daily measurements of leaf length showed that growth of the youngest (3rd) leaf of wheat was strongly inhibited 2 days after treatment with fenoxaprop-ethyl (180 g a. i./ha) alone, whereas after treatment in combination with Hoe 70542 (45 g a. i./ha) leaf growth continued with the same rate as in control plants (Fig. 2).

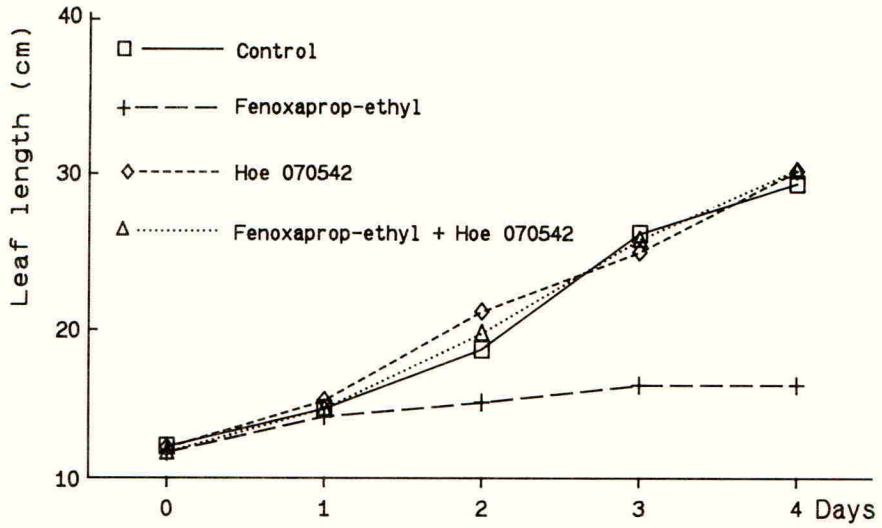


FIGURE 2 Leaf growth of wheat (means from 10 replicates)

Uptake and translocation

Wheat was treated on the adaxial surface of the first leaf with 6 µg ^{14}C-fenoxaprop-ethyl in 10 µl solution, applied by microsyringe in the form of 1 µl-droplets, with or without addition of 1.5 µg nonlabelled Hoe 70542. The rate of foliar uptake was determined after washing the leaf surface for 3 x 5 s with diethylether. The foliar penetration of the herbicide, followed over a period of 72 h, was in no case significantly influenced by Hoe 70542.

Neither total translocation of ^{14}C-labelled material nor specifically the translocation to the shoot base, including the meristems, was significantly influenced by Hoe 70542 at 4 h and 24 h after application. Only after 72 h was the percentage of translocated material lower in presence of Hoe 70542 (Table 1).

TABLE 1 Foliar penetration and translocation of ^{14}C-fenoxaprop-ethyl in wheat.
A) total translocation, B) translocation to shoot base; 8 replicates.

	Time after application (h)	% of applied activity In absence of Hoe 70542	In presence of Hoe 70542
Penetration	4	38.6 ± 3.3	33.9 ± 2.5
	24	53.3 ± 3.2	54.5 ± 3.8
	72	63.8 ± 5.7	66.6 ± 3.9
Translocation	4	A) 0.28 ± 0.08	A) 0.33 ± 0.05
		B) 0.15 ± 0.05	B) 0.18 ± 0.03
	24	A) 1.06 ± 0.16	A) 1.03 ± 0.10
		B) 0.62 ± 0.11	B) 0.62 ± 0.08
	72	A) 2.08 ± 0.30	A) 1.55 ± 0.29
		B) 1.23 ± 0.13	B) 0.74 ± 0.21

Acetyl-CoA carboxylase activity

The activity of acetyl-CoA carboxylase (ACC) in isolated wheat chloroplasts was strongly inhibited by fenoxaprop (IC_{50} = 0.6 µM). When Hoe 70542 was added at the comparatively high concentration of 100 µM to the assay medium, the level of ACC inhibition by fenoxaprop was not altered. The same result was obtained, when instead of Hoe 70542 its corresponding free acid, Hoe 72829, was added to the assay mixture (Table 2).

TABLE 2 Acetyl-CoA carboxylase inhibition in isolated wheat chloroplasts.

Treatment	IC_{50} (µM)
Fenoxaprop	0.6
Fenoxaprop + Hoe 70542 (100 µM)	0.7
Fenoxaprop + Hoe 72829 (100 µM)	0.7
Hoe 70542 (100 µM)	no inhibition
Hoe 72829 (100 µM)	no inhibition

Fenoxaprop-ethyl degradation

Wheat was sprayed with ^{14}C-fenoxaprop-ethyl (180 g a. i./ha) alone or in combination with nonlabelled Hoe 70542 (45 g a. i./ha), and the kinetics of degradation in the shoots analysed over a period of 4 days. Levels of the parent compound as well as of 2-(4-(6-chloro-2-benzoxazolyl-oxy)-phenoxy)-propionic acid (fenoxaprop) declined faster, when the herbicide was applied in combination with Hoe 70542. Levels of the other organo-soluble metabolites - aside from some minor products predominantly the

physiologically inactive 6-chloro-2,3-dihydrobenzoxazol-2-one - were higher in the combination treatment with Hoe 70542 than in the treatment with the herbicide alone, when determined in plant samples taken 2 and 4 hours after treatment. A tendency to the opposite was observed at the later sampling dates. The levels of water-soluble metabolites and of non-extractable ^{14}C-labeled residue, however, were throughout the experiment higher in samples from the combination treatment than from the treatment with ^{14}C-fenoxaprop-ethyl alone (Table 3).

TABLE 3 Degradation of ^{14}C-fenoxaprop-ethyl in wheat A) without, B) with Hoe 70542. (Values are % of total radioactivity.)

		Time after application (h)			
		2	4	27	96
Fenoxaprop-ethyl	A	63.5	52.5	19.7	9.4
	B	46.3	39.8	12.2	4.0
Fenoxaprop	A	27.3	21.4	17.5	11.6
	B	26.6	15.3	11.4	5.0
Other organoso-	A	7.3	19.5	38.8	30.8
luble metabolites	B	21.4	34.4	29.8	22.1
Water-soluble	A	0.5	0.7	10.1	22.0
metabolites	B	0.8	3.0	22.3	32.6
Non-extractable	A	0.7	1.3	6.8	15.3
residue	B	1.5	2.5	14.6	25.3

DISCUSSION

The results of the growth measurements show the rapid and complete protective effect of Hoe 70542 against fenoxaprop-ethyl phytotoxicity in wheat. Such a protective compound could influence the uptake and translocation, or the biochemical target site of the herbicide, or alternatively its degradation in the plant.

It is clear from our data, that Hoe 70542 does not interfere with the foliar uptake of the herbicide. The lower percentage of translocated ^{14}C-labelled material seen 3 days after foliar application of ^{14}C-fenoxaprop-ethyl, is probably a consequence of the modified degradation kinetics of the herbicide in presence of Hoe 70542.

The enzyme test with isolated wheat chloroplasts shows that Hoe 70542 and its free acid (Hoe 72829) do not directly interfere with ACC inhibition by fenoxaprop. Therefore the protective effect cannot be explained by an interaction with the biochemical target site of the herbicide.

The degradation study indicates a more rapid decline in the level of fenoxaprop-ethyl and of its hydrolysis product fenoxaprop in wheat, when applied in combination with Hoe 70542. Fenoxaprop is phytotoxic, and in chloroplasts isolated from grass species, it in fact inhibits ACC much more strongly than the parent compound fenoxaprop-ethyl (Hoppe & Zacher, 1985; Kobek et al., 1988). The results available to date support the hypothesis that Hoe 70542 prevents phytotoxic effects of fenoxaprop-ethyl application in wheat by accelerating the conversion of the herbicide to non-phytotoxic degradation products.

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